CHAPTER 2: REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

India because of its diverse agriculture-based climate conditions and the regional landscape, has been considered as the pivot of the many medicinal herbs. India is recognized as the 12th mega diversity nation among the world's counting. India's rich vegetation constitutes more than 8000 plants of herbal species which is half of total percentage of flowering species and around 70% of the herbal wealth of India is widespread across the western ghat's tropical forests. Around 1800 species were reported to be used in the classic medicinal system of which ayurvedic medicine system uses around 1200 species.

Alone the Himalayan region of India holds on to half of the flowering species, of which about 30% plant species are now endemic. Uttarakhand, considered as the herbal state, harbours about 5000 species of plants among which, 1/3rd species are considered as herbal or medicinal plant species which contributes to the medicinal uses.[35] The evolving field of herbal medicine contributes in the area of nation's economic development. It has been reported that, about 95% of the herbs that are used in the medicinal industries were collected from their natural habitat. With the increase in the overconsumption of herbal plants, extension of cities or urban areas, establishment of new industries in the rural areas, deforestation, depletion of plant genetic resources was reported. This will eventually lead to the extinction or the vulnerable condition of the many important herbal species in the Indian Himalayan region.[36]

The herbal or medicinal plants form the basis of modern herbal or medicinal system that comprises the Unani, Chinese and Ayurvedic. One of the plant family is Polygonaceae which comprises of several species that have the great medicinal qualities and is well known in many medicinal systems. The Asian species of the polygonaceae family also shows many medicinal or herbal uses. *Rumex nepalensis,* one of the species in polygonaceae has great medicinal properties. This plant is mainly

used in the conditions like headache, dislocated bones, inflammation, stomach related issues.[37]

Different useful bioactive components such as anthraquinone, napthalenes, the flavonoids and some phenolic compounds were reported which contributes for the general uses in traditional medicine systems.[38]

2.1 Rheum plant

One of the Pharmaceutically important herbs is *Rheum emodi*. It is commonly known as Rhubarb or Dolu. This plant is considered as an important raw material in many pharmaceutical industries as well as cosmetic industries. Rhubarb firstly originates in the mountains of Northern west provinces of Tibet and China and were cultivated for its therapeutic properties for more than 5000 years. This herb with a property as purgative and stomachic were first mentioned and listed in Chinese herbal Pen-King.[39] After this, rhubarb found its way to the westward via Russia and Turkey and then was first recognized in England and quickly made its way into the kitchen because of its tart flavors and then frequently used in jams, jellies and desserts.[40]

Rheum, an endangered species in the family polygonaceae, is a robust and perennial herb of state Uttarakhand. It has a wide range of medicinal properties such as antioxidants, antimicrobial, anticancerous and wound healing ability. Because of these properties, *Rheum* is known by the name 'wondrous drug' and often used in many traditional systems of medicines.[41] *Rheum* is distributed within the tropical and sub-tropical areas of the world. This genus is mainly present in the Asian countries such as China, Nepal, Tibet, Korea, Russia, Bhutan and India (Kashmir to Assam).[42]

Rheum emodi contains secondary metabolites that have medicinal importance. The compounds present in the *Rheum* species are emodin, rhein, aloe emodin, chrysophanol, and physicon, rhaponticin, stilbene glycosides, tannins, gallic acid, catechins, cinnaminic acid etc. Presence of these active components contributes in the dealing with health issues as well as in treatment of various diseases.[43]

The rhubarb leaves could be poisonous or toxic too, as its leaves contains oxalates in high concentration, as compared to its roots and petioles. A concentration of about 5gm is considered as minimum lethal dose for an adult.[44]

Rheum plant has a height ranging from 1.5 to 3.0 m, with broad leaves and thick stem. Stem is hard, green in colour and leaves are large radical, broadly ovate having 30-45 cm diameter. This perennial herb has small flowers of colour dark purple to pale red with 3 mm diameter in auxiliary panicles.

Inflorescence is upright branched and of about 0.6 to 0.9 m long, leafy with the straight stem branches. The fruits of *Rheum* are ovoid and oblong in shape and about 1 to 2 cm in size arose apex and purple in colour with cordiform base. Seeds are winged in outer appearance and can be collected in the month of August–September when they change their colour to dark brown. Potential of seed production may vary from plant to plant; it can be from 300 seeds to 950 seeds per plant.[45] For *Rheum* species, as the propagation mean, both the vegetative as well as seeds are used. In vegetative means, top rhizomes segments are preferred but the problem associated with this mean is that it is specific to season. Seeds have a very low survival rate and poor germination rate.[46] Other problems that are associated with the seeds are their poor viability, age factor, high dormancy, increased susceptibility to infection and low germination rate. Even the one-year aged seeds show very low germination.[47] Plants that are raised through seedlings require four to five years for maturity; however, two or three years are sufficient if plants were raised through rhizome segments. After one year, flowering takes place in the month of May-June and follows with the fruiting in the month of September-October. Plants attain maturity after four growing seasons and may be harvested after fruiting. Six to seven-year-old plants were mainly used for obtaining raw material used as medicines or by pharma industries.[48]

Rheum emodi extracts (methanolic, ethanolic, aqueous etc.) exhibits many pharmacological properties **(Table. 2.1)** such as anticancerous activity, antimicrobial activity, antidiabetic activity, nephroprotective activity and can cure many gastro related problems, dermatological disorders.[49]

Table. 2.1: *Rheum emodi* extracts and their pharmacological properties

Extract Used	Properties	Compound	Inferences	Reference
Methanolic and Aqueous extract of rhizomes	Anticancer activity	Aloe-emodin	Study conducted in the 2 different human cell lines viz "Hep G2" & the "Hep 3B". The "aloe-emodin" compound in *Rheum* supress the proliferation of cell and thus induce apoptosis by different antiproliferating mechanism	**50** **5**
Ethanolic extract of rhizome	Antioxidant, antidyslipidemic effect	Emodin, Emodin 8-O-β- D-glucopyranoside, Chrysophanol, Chrysophanol 8-O-β-D-glucopyranoside.	In the introductory screening, rhizome extract showed significant activity in rats that were treated with Triton. These compounds reduced the level of plasma-lipid.	**51**

Ethanolic (75%) Extract	Antidiabetic activity	-	Extract administration to the rats for about 30 days results in the decreased activities of fructose-1,6 bisphosphatase, glucose-6-phosphatase, aldolase, 6-disphosphatase, & an elevation in the action of enzymes such as phosphoglucoisomerase and hexokinase in tissues.	**52**
Rhizomes of *Rheum emodi*	Antifungal property	Physcion	Exhibit antifungal activity against the fungus *Aspergillus fumigates, Cryptococcus neoformans, Candida albicans* and *Trichophyton mentagrophytes*	**5**
Ethanol and Benzene extracts of *Rheum emodi*	Antimicrobial activity	-	Inhibits the bacteria *Helicobacter pylori*; even at very low concentration, infection was cleared within seven days. *H pylori* did not acquire resistance even after 10 consecutive passages.	**53**
Aqueous or methanolic extracts of rhizomes	Antioxidant property and cytotoxic activities	-	The extracts prove to hold good number of phenolic compounds that have effectively considerable the positive correlation with the free radicals like OH and DPPH, Fe3+ reductions, the scavenging efficacies, and the LPI percentages.	**54**

Ethanolic extracts of *R. emodi* rhizomes	Hepatoprotective property	-	A study is conducted in Wister rats against the induced liver damage. With the oral administration of rhubarb, normal and original liver shape was restored.	**55**
Aqueous and methanolic extract	Hepatoprotective property	-	Study confirmed that the extracts of *Rheum emodi* works as the hepatoprotective against the liver injury caused by paracetamol medicine in albino rats.	**56**
Alcoholic extract of *Rheum emodi*	Nephroprotective significance	Tannins	Evaluations were done on tested and control rats, by checking the stages and levels of the urea, creatinine, and the N in the serum. *Rheum* extract gave nephroprotective effect on proximal segments i.e., S1, S2 and S3.	**57**
Solvent fraction of Ethyl acetate	Antioxidant activity	Myricetin, Myricitrin and Myricetin 3-galloyl rhamnoside.	Antioxidant activity was determined by using the assay such as 'DPPH' and the 'ABTS+'. Further results were obtainable into IC50 values which extended from 21.52-2448.79 µg/mL (DPPH) and 90.25- 1718.05 µg/mL (ABTS+)	**58**

2.2 In vitro studies

Biotechnological upgrading of medicinal, edible and ornamental plants is considered since the last fifty years, significant research has been conducted all around the world. One method for meeting the objectives of sustainable propagation of conventional plants with commercial value is *in vitro* culture with an aim to save endangered plant species, and for improving the quantity and quality of such plants. Green leafy vegetables are significant considering all of their possible health benefits from a pharmacological and nutritional perspective. This group of plants has received the great attention through the practice of somatic embryogenesis for the long-term cultivation, mass multiplication techniques for effective *in vitro* regeneration, and callus suspension cultures to boost the yield of secondary compounds of high value.

Haberlandt, who focused on the cultivation of single cells, introduced the idea of plant tissue culture in 1902 for the first time. However, Gautheret in 1939, who invented a method for the *in vitro* development of carrot tissues, documented the true procedure. However, it wasn't until Murashige and Skoog's discovery of synthetic nutrient media in 1962 which made plant tissue culture a significant advancement that eventually led to commercial scale tissue culture. Since then, several different plant species have had tissue-culture procedures developed and established. Micropropagation is a vital tool for both fundamental and practical research, as well as for commercial use.[59]

With numerous pharmacological activities and continuously increasing demand of *Rheum emodi* plants as raw material for drug preparation, this species is now facing problems such as species extinction from natural habitat, lack in vegetative part as planting material. This led to the loss or decreased in the genetic diversity of plant.[60] Amongst all the methods of propagation in *Rheum emodi* plants, vegetative propagation is supposed to be the most common and usual method of propagation as it generates the homogenous offspring. Due to a scarcity of planting material, plant division makes it nearly impossible to extend desirable species under challenging environmental circumstances. The *Rheum emodi* is not recommended either via generative propagation or seed propagation methods since their seeds have a short lifespan, even one year old is not fully potential to sprout, even death of seedlings at

two- leaved stage. Seed propagation method is also very slow and is very much hampered by the environmental conditions.[61]

On the basis of these conditions, *in vitro* regeneration could be proved to be a very effective substitute as the initial material or the explant for *in vitro* propagation can be a very small plant part as few centimeters or even less, that could result in abundant clones in shorter period of time. As the vegetative mode of propagation could not meet the essential marketable demands, so to overcome with this problem, the biotechnological approaches are recommendable. Plant tissue culture techniques such as callus and cell suspension culture, organ culture have been recognized for the production of the significant and important secondary metabolites, for the conservation, production and isolation of active components and rapid propagation of the herbal plants that are medicinally important.[62] Techniques for biotechnological propagation which are based on the tissue culture techniques, production of secondary metabolites and the *in vitro* conservation of pharmaceutically significant species of plants, has been practiced from the past 2 to 3 decades. Many medicinal plant species are continuously disappearing, especially those that are well known in the pharmaceutical sectors, as the natural habitat of herbal plants is under stress due to overharvesting in order to supply the demand for plant-based herbal medicines.
To overcome this, many medicinally significant trees were effectively propagated *in vitro* through the different modes of regenerating techniques such as adventitious organogenesis, somatic embryogenesis and auxillary shoot proliferation.[63] In order to increase the production of identical plant material to the mother plant, in vitro regeneration and propagation of these medicinally significant plants and vegetation would become necessary and a dependable procedure.[64,65]

Some scientific reports have been published which shows that the different species of rhubarb can be propagated through different explant but with the possibility of the somaclonal or genetic variation among the regenerants, which concludes that the genetic homogeneity of the mother plant and the regenerants should be tested.[66,67] They introduce the successful *in vitro* propagation of the two common species of *Rheum* such as *Rheum raponticum* and *Rheum rhabarbarum*. As these are regenerated through tissue culture, that shows the risk of genetic variability between them, that

leads to the somaclonal variation. However, in many cases, plants' somaclonal variations can cause genetic changes that may be advantageous for plant breeding or for the crop improvement but in the micropropagated plants, it is important to maintain the genetic homogeneity or genetic fidelity thus avoiding any somaclonal variations.[68]

During the past studies, the somatic embryogenesis was induced in the family polygonaceae, especially in the species belonging to genus *Rumex* and *Fagopyrum*. Through the competent tissues, somatic embryogenesis was induced in the species *R. acetosella* and *F. esculentum.* Embryogenic structures were initiated by the embryos of buckwheat and the fragments of micropropagated *Rumex* in the presence of elevated cytokinin to auxin ratio. If the minimal requirements were met, the *R. acetosella* have the embryogenic capability in all the implied genotypes, such as the aspects for preventing the growth from being stopped at two points: first, when organogenesis changes to embryogenesis and second, when the development of the embryo moves past the torpedo stage thanks to the inclusion of GA_3 and slip of IAA/BAP.

The consistency of previously mentioned responses advises that the initial explants of *Rumex acetosella* could provide the model system for the upcoming researches on the somatic embryogenesis.

The species' genetic and phenotypic stability seems to be quite high as well as a remarkable aptitude for micropropagation from the apical meristem. The vegetative cloning of the species is needed and can easily be achieved through the shoot cultures.[69]

Kakarla and co-workers studied the process of regeneration aseptically and the somatic embryogenesis in *Rumex vesicarius* plants in the family polygonaceae. They used the leaf as explant to induce the callus and high occurrence of somatic embryogenesis was observed in the SH (Schenk and Hildebrandt) media perforated with the 2,4-D (2.5 mg/L) along with the Kinetin (0.5 mg/L). Well-developed embryos generate the 80% shoots on the growth media supplemented with the BA (2.0 mg/L) along with 2,4-D (0.5 mg/L). further the regenerated shoots were transferred to the rooting media as SH media perforated with the IBA (1.0 mg/L). Survival rate after the

hardening were recorded as 65-70%. The phenotypic characters as plants or the flowers were reported similar to the mother plants.[70]

Fagopyrum esculentum, another species of family polygonaceae was studied and a protocol for indirect somatic embryogenesis and plant regeneration was developed by using the hypocotyl as explant. The initial callus of hypocotyl was induced on the MS medium perforated with the 2,4-D (2.0 mg/L) with 6-BA (1.5 mg/L) when cultured for the 30 days. Yellowish white fragile calli was obtained after the transferring to the MS media supplemented with the TDZ (0.5 mg/L) with the 6-BA (1.0 mg/L). The cultures were two to three times subcultured for the 50-60 days breaks. Afterward the somatic embryos germinated from the embryonic callus when subcultured in the MS medium containing TDZ (0.5 mg/L) and 6-BA (1.0 mg/L) for the 20 days. Maximum percentage of the plantlets resulting from the somatic embryos were recorded as 75 %.[71]

Different studies were conducted for the micropropagation of many medicinal plants for the preservation, conservation and good quality, improve the production of aromatic sap. Micropropagation starts by inducing the callus in the juvenile leaves which is followed by the subculturing to attain the regeneration of further shoots and the roots. Different plant growth hormones in different combination were used to provide the desired nutrients for the development and growth of callus, shoots and the roots. For the micropropagation of plant *Styrax benzoin*, the combination of plant growth hormones such as NAA and BAP were proved to be the best matched. Shoots were regenerated with the hormonal combination NAA (0.5mg/L) and BAP (3.0 mg/L), highest root regeneration were observed in NAA (3.0 mg/L) without the growth regulator BAP.[72]

A recent study by Singh and coworkers suggested that the mid rib explants produced a high rate of callus induction in *Rheum* plants (100%) and then in leaf explant (97.67 ± 1.20%).[73] Leaf explant had the more percentage of observed shoot induction as compared to callus. Regenerated shoots showed about 70% rooting response with healthy long roots.

Another study by Tuncer in 2021 concludes the protocol for the callus proliferation in *Rheum ribes L.* under the *in vitro* and aseptic conditions.[74] In the study, they use plant growth hormones such as TDZ and NAA. The most effective hormone combination (TDZ+NAA) was found to be more successful. The MS medium treated with TDZ (2.0 mg/L) and NAA (0.2 mg/L) produced a prominent callus. A study by Tuncer, was conducted for determining the *in vitro* germination and the appearance of rhubarb seeds groups; one with the seed tips cut off and another group with the seed tips.[75] These groups were germinated in different nutrient media combinations such as Gamborg (B5), MS media, White (WH), SH media (Schenk and Hildebrandt media) perforated with or without GA_3 plant growth regulator. Their results suggested that the applications of the seed tip cut are more effective in the term of emergence and germination parameters with the less severe physiological dormancy.

Different studies were conducted for the micropropagation of different species of *Rheum*. Researchers propagate a rare *Rheum* species, *Rheum coreanum Nakai* of North Korea, by using the rhizome as explant.[76] MS media perforated with the plant growth hormone (2,4-D) for the Callus induction. For further development of shoots, roots and plantlets, MS medium supplemented with the combination of different plant growth hormones such as BAP, IBA and NAA was used. Further the 100% acclimatization was achieved in green house and yields of crude drug as compared to those obtained through vegetative propagation, raw medicinal compounds have larger levels. The study may offer chances to reduce extinction hazards and a powerful callus proliferation system for large-scale, rapid resource growth.

Another study conducted by Malik and coworkers showed regeneration of *Rheum* in the MS media perforated with 10.0 µM of 6-BA (6-Benzylaminopurine) along with the 5 µM IBA (Indole-3-butyric acid).[77] Study suggested that the shoot buds were directly emerged without from the leaf explant going in the callus phase. The regenerated plantlets were further relocated to the greenhouse, but they recorded a very low survival percentage in the transferred plants.

An efficient micropropagation system for threatened medicinal plant *Coleus forskohlii* was developed by using leaf explant and nodal explants. Multiple combinations of

phytohormones, including auxin and cytokinin, which were added to MS medium. For the leaf explant, a combination of MS medium perforated with BA (2.0 µM) and NAA (0.1 µM) proves to be the most effective media as it produced 35.0 ± 1.2 shoots per explant with a shoot length of 5.4 ± 0.5 cm.[78] For the direct regeneration using nodal explants of *Coleus forskohlii*, MS medium perforated with BA (5.0 µM) was found as the most approachable medium for the multiplication and development of shoots.[79] Subsequently for better shoot multiplication, combination of auxin was used with the optimal concentration of BA. The medium containing MS media with BA (5.0 µM) and NAA (1.0 µM) was proved to be the ideal for differentiation and development of about 18.80 ± 0.1 shoots per explant.

Another study is conducted in regeneration of *Rheum* which explains the standardization of successful and reproducible protocol using various explants such as leaves, shoots, seeds and rhizomes. These explants were then cultured on plant growth media, MS (Murashige and Skoog) media with different combination and concentration of phytohormones such as BAP (6- Benzylaminopurine), 2,4-D (2,4-dichlorophenoxyacetic acid), IAA (indole-3-acetic acid), TDZ (Thidiazuron) and Zeatin. The most effective combination and the concentration for the regeneration was MS media with BAP (15µM) and IBA (15µM).[80]

For the enhanced shoot multiplication in *Rumex vesicarius*, (a branched succulent herb belongs to the polygonaceae family), the pre-treated seeds show the 95% of germination on MS media. The induction of *in vitro* shoots from the "shoot tip" explant was maximum in MS media perforated with the 2mg/L Kn. Shoot elongation was best in 2mg/l IBA and *in vitro* rooting were efficiently grown in 1% activated charcoal in medium within 3-4 weeks.[81]

Another species of *Rheum* in which *in vitro* regeneration system was achieved is, *Rheum spiciforme royle*. When transferred from soil to half strength MS media, the seed germination exhibits a spectacular rise which was perforated with different concentration of Gibberellic acid, potassium nitrate and calcium chloride. Leaf explants responded best with 2.0 µM 2,4-D and 6-BA (6- Benzylaminopurine) of the four types of explants used to induce callusing. Nodal segmentation derived brown

calluses with significantly high regeneration with the plant growth regulators combination of BA, Kn with GA_3. The leaf explants that germinated at various phytohormone concentrations such as 25.0 µM BA, 2.0 µM GA_3, 1.0 µM NAA, 50.0 µM glutamine and adenine sulfate also showed direct somatic embryogenesis. Further multiple shoot induction and shoot elongation were observed. Later the plantlets having roots which was developed in MS media (half strength) with the phytohormone NAA were hardened and afterward planted successfully to the field.[82]

2.3 Synthetic seed/ Artificial seed

Synthetic seeds are fundamentally the synthetic analogues of true seeds; they are produced by artificially encapsulating the plant parts or propagules such as shoot buds, shoot tips, nodal and leaf segments, somatic embryos or any other tissue that could readily to rejuvenate into a complete plantlet. Synthetic seeds are also called as syn seeds or somatic seeds or artificial seeds.[83] Synthetic seed technology has transformed the concept of plant mass multiplication. The first synthetic seed-based plant propagation was proposed by Murashige in 1977 who presumed the encapsulation of single somatic embryos.[84]

A variety of biotechnological methods or techniques have been developed to conserve various plant species. One of these methods, the syn seed technology, is one of the plant biotechnology's fastest-growing subfields.

The synthetic seed methods provide the production of large amount of identical embryos from the micro-propagules such as embryogenic calli, axillary shoot buds, and apical shoot tips. From the last some twenty years, thoroughly research attempt was made on syn seed production and development in different plant species. In spite of such researches, the practical application of this technique is still comprehended due to the limitation such as error maintenance, special skills required, facilities required are costly, embryo protection from contaminations, development, production, subsequently transformation of micropropagules into the plantlets, maturation under the *in vitro* conditions.[85]

Numerous types of decorative and therapeutic plants have successfully used synthetic seeds. One of the major advantages of this technology is its great capability in preserving the somatic embryos, callus, and the other important tissue of germplasm. Encapsulation plays the substantial role in the propagules' quick and efficient transportation. Production of artificial or synthetic seed in plants species are not dependent of season, accelerates seedless, ploidy plants and ornamental plants multiplication, offers high scale production of micropropagules that are of high quality. Beside these, synthetic seed technology also shares some limitation and factors that control the success of its development and germination, which includes embryos (matured and quality proved), calcium chloride concentration, sodium alginate concentration, storage and time of hardening and concentration of PGRs (plant growth regulators). Encapsulation technology is proving to be a burgeoning field that will create new opportunities for growing, preserving, and transporting priceless plant material for scientific research in the areas of floriculture and pharmaceutical industries.[86]

For the plant propagation and germplasm conservation, encapsulation technology or synthetic seed production proves to be a forthcoming powerful tool. The need for switching to the way of synthetic seed technology was the actuality of cost-effective mass propagation of the leading or elite plant genotypes. The present advances in the artificial seed technology have served in many ways as this is an alternative tool for large scale production or micropropagation and conversion to plantlets.[87]

Synthetic seeds were also known to provide an easy and approachable regeneration methods in difficult to propagate species. *Gossypium hirsutum's* axillary buds were enclosed or encapsulated to create the artificial seeds. in the alginate-calcium beads. Obtained beads were firm, uniform, easy to handle by using a MS solution containing 3% sodium alginate and 1% chitosan and further solidified with the 200 mM $CaCl_2$ and 0.02% NaOH. About 95% of germination rate was observed.[88] Therefore, this synthetic seed technique provides a way of storing and overcoming the various problems related with the long-distance transport and exchange of different elite and endangered germplasm.

The synthetic seed has been applied to many species of plants belonging to the angiosperms. Encapsulating protocols for many plants species were already optimized, but their commercial or profitable production of the artificial or synthetic seeds was limited due to many problems like nondevelopment or asynchronous of somatic embryos, low production of plantlets from the somatic embryos and the limited production of mature somatic embryos. In several studies, artificial seeds were developed by encapsulating the propagules results from micropropagation protocols, thus propagating systems would be one of the initial requirements for synthetic seed development.[89] It is anticipated that the exchange of these as plant material among the laboratories, cities, across or within countries are the more convenient and economical way along with the less quarantine limitations. For the artificial seed production, the *in vitro* derived shoot tips which were then encapsulated through alginate are preferred over the somatic embryos. This process proves to be effectual technique for the germplasm conservation, conservation of RET plants and medicinal plants, clonal propagation and the exchange of valued plants' genetic resources. In some plants, the encapsulation of shoot tips conservation in cryovial without any medium are also reported, they offer great scope and thus to be applied to other plants.[90]

Originally in artificial seed or synthetic seed technology, many researchers studied the various materials and substances that were used for the purpose of encapsulation matrix gel for example sodium alginate, potassium starch, sodium pectate, potassium alginate, carboxy methyl cellulose, carrageenan, agar, agarose, gelatin, acrylamide, nitrocellulose etc.[85,25,83,89,91]

Within these, sodium alginate is the most successful and widely accepted hydrogel as encapsulation matrix because of its non-toxic nature, moderate viscosity, quick gelation, biological compatibility and cost-effective qualities.[92,93,94,95]

Many previous studies shows that the many medicinally significant plants were preserved by the advancement of artificial seed technology. A study by the Faisal and coworker reported the plant species member of family Asclepiadaceae, *Tylophora indica's* in which effective plant regeneration was achieved by the encapsulated shoot tips and somatic embryos.[96]

Another study concluded that the shoot tips of the species such as *C. spiralis* and *C. pusilla* showed the highest fraction of shoot sprouting when the shoot tips were encapsulated with sodium alginate of concentration 3% along with the MS media and plant growth regulators.[97] Among all the encapsulated explants the nodal explant proves to be better than shoot tip, although shoot tips sprout frequently but they develop fewer shoots. These validated processes for encapsulating embryos as artificial seeds could be used for the other species of *Ceropegia* to propagate these plant species by *in vitro* techniques in large numbers.

Onishi and coworkers works on production of encapsulating unit for the scaling up transplant production by using the somatic embryos of carrot and celery.[98] Improved quality of somatic embryos showed the 80% conversion without being encapsulated and without any nutrient source in green-house. By synthesizing self-breaking gel beads, they created artificial endosperm, and by encapsulating the carrot embryos, they sustained the release microcapsule as artificial endosperm. They also developed a production system for the synthetic seeds in which about 80,000 beads could produce per day. Results showed that the conversion rate of the encapsulated carrot embryos is about 52% after sowing on a moist soil in green house.

A scalable method for effective direct somatic embryogenesis of *Paulownia elongata* was developed from the explant leaf and internodal. The somatic embryos were later enclosed to create artificial seeds. Different plant growth regulators were used during the study such as BAP, IAA, Kinetin, TDZ. These plant growth hormones were tested alone and in combination for the capability to stimulate somatic embryogenesis. The Murashige and Skoog medium perforated with the 3% sucrose and 10 mg/L TDZ had the maximum somatic embryo induction percentage.

For the production of artificial or synthetic seeds, the somatic embryos that were taken directly from the plant's leaf explants were employed by encapsulating them in liquid nutrient medium having the diverse concentration of sodium alginate and then dipped into the solution of 50 mM $CaCl_2$ for 30 minutes. Sodium alginate having 3% concentration provide the uniform and circular beads having survival percentage as 73% and germination frequency as 53%. Storage experiments showed the survival and

the germination frequencies of embryos (encapsulated) were augmented after storing them at 4°C temperature.[99]

Likewise, organogenesis and somatic embryogenesis was observed in another critically endangered medicinal plant *Arnebia euchrona* of Himalayan region. Callus from the leaf explants shows the organogenesis as 12.2 shoots per culture in the presence of IBA (1.0 µM) with 6-BA (2.5 µM) and somatic embryogenesis as 16.3 embryos per culture when MS media was perforated with the IBA (2.5 µM) along with 6-BA (2.5 µM). Regenerated shoots showed best rooting in the half strength MS media perforated with the IBA (2.0 µM). By encasing the embryos in the cotyledonary stage for 25 minutes with 3% sodium alginate and 100 mM calcium nitrate, synthetic seeds were produced. They showed the germination (60%) in MS medium. The rooted shoots showed a survival rate of 72% in the field after hardening.[100]

Cheruvathur and his collegues developed a procedure for the production of synthetic seed and *in vitro* propagation of *Hemidesmus indicus* commonly recognized as Indian sarsaparilla, a medicinal plant. They used the nodal cuttings to obtained the callus on MS media when supplemented with the IBA (3.0 µM). By subculturing the callus on the nutrient media that is MS medium (half strength) perforated with IBA (2 µM), the maximum frequency of somatic embryogenesis, 92%, was noted. For the synthetic seeds production, collected somatic embryos were suspended in MS medium, which contains 3% sodium alginate, before being dipped into a calcium chloride solution with a 75 mM concentration. The syn seeds were then successfully germinate on the medium even after the storage of 120 days at 4°C. about 92% success rate was observed ain plantlets that were transferred to the field.[101]

Similarly artificial seeds were successful in endangered plant species known as Malabar-river lily, which is a natural source of galanthamine. Priyadharshini and her coworkers suggested the *in vitro* conservation strategies of this plant species through the production of somatic embryos and then synthetic seeds. MS medium perforated by the auxin and 2,4 D, induces the maximum fraction of the somatic embryos at various stages with an average of 54 somatic embryos. The synthetic seeds were produced by encapsulate somatic embryos with the 2% sodium alginate and 100mM

calcium chloride which further resulted in development of gentle beads with the 93% of germination potential. The combination of BAP and IAA on the germination of somatic embryos were found to be more significant than the BAP and NAA combination. Maximum 12.8 shoots per synthetic seed were regenerated on MS medium perforated with 2.0 mg/L BAP and 0.5 mg/L IAA.[102]

Several researches were reported based on the usage of synthetic seeds for the micropropagation and conservation of many elite and endangered germplasm of diverse plant species that consist of wide range of medicinal plants such as *Ananas comosus* [103]; *Withania somnifera* [104]; *Punica granatum* [105]; *Rauvolfia serpentina* [106]; *Cineraria maritime* [107]; *Spilanthes mauritiana* [108]; *Simmondsia chinensis* [109]; *Cucumis sativus* [110]; *Clitoria ternatea* [111]; *Mentha arvensis* [112]; *Catharanthus roseus* [113]; *Dendrobium nobile* [114]; *Rauvolfia tetraphylla* [115]; *Terminalia arjuna*[116]; *Sterculia urens* [117]; *Salvia splendens* [118]; *Mondia whitei* [119]; *Tecomella undulata* [120]; *Withania coagulans* [121]; *Althaea officinalis.* [122]

The somatic embryogenesis and the production of synthetic seed was studied in one of the species in family polygonaceae, *Rumex vesicarius L*., a medicinal plant. Several explants of the plants were tested for the callus induction on the growth media (MS medium) with the different concentration of plant growth hormones. On MS media that had been perforated with 2 mg/L of BAP and 0.5 mg/L NAA, an embryogenic callus was produced. Somatic embryos were obtained after sub culturing of callus in MS medium supplemented with the 2,4-D (2.0 mg/L) with 40% of coconut water. Obtained somatic embryos were stored in two different temperature that is 4°C (cold) and in room temperature for the 60 days. Multiple shooting was induced by the callus when cultured in the MS medium supplemented with the 4.0 mg/L of BAP with 1.0 mg/L NAA. With further transfer to the half strength MS medium with 1.0 mg/L NAA induced the rooting in shootlets. Stored encapsulated somatic embryos were further cultured on MS media with BAP (2.0mg/L) and NAA (0.5mg/L) for regeneration. Cold storage synthetic seeds showed the high viability as compared to those stored in room temperature.[123]

2.4 The Genetic Assessment of '*In vitro* raised' plants

Plants' *in vitro* propagation process is hindered from scaling up in large part by the development of somaclonal variants, so a quality check for the genetic similarity of progeny becomes essential. Traditionally morphological description, field assessment, genetic analysis, physiological supervisions were used to detect any type of variation among regenerants and mother plant. However, in order to identify and record the clonal fidelity of tissue cultured plants, molecular markers have currently replaced the conventional approaches. The molecular markers are also used to distinguish the variations in micropropagated plants at their genetic level. Medicinal plants, in reference to the herbal medicinal system, have been exploited or utilized from a long period of time because of increased nutritional, medicinal or commercial demand. This results in the declining in many important species. *In vitro* cultivation is one of the solutions for fulfilling the huge demand in short period of time on a large scale. The drawback with *in vitro* regeneration is micromutation, that lead to the somaclonal variation in the regenerants. It is important to maintain the genetic homogeneity to yield true-to-type plants. Aiming the same purposes, different molecular markers like RAPD and ISSR are used for assessing the clonal fidelity of the regenerants as these markers are cost effective, fast and reliable.[124]

Micropropagation techniques were considered as an important method for the in-situ conservation of many endangered or rare plant species and that could only have done when the genetic fidelity among the regenerants were maintained. One of the crucial prerequisites for micropropagation is the genetic stability of the true to type plants. However, tissue cultured plantlets induced somaclonal variation more often, and thus this is responsible for one of the limitations in plant tissue culture.[125] The occurrence of somaclonal variation can be due to multiple reasons including type of explant, explant source and its ploidy level, method of propagation, culture and media conditions, sub culturing time interval, the concentration and type of plant growth hormones.[126]

Molecular markers such as RAPD and the ISSR are the PCR (Polymerase chain reaction) based techniques that are enormously useful in assessing the clonal fidelity or genetic stability in the *In vitro* regenerated plantlets of numerous plant species.[127,128] These molecular marker-based techniques are fast, simple, cost effective, reliable. These techniques require a very less amount of template DNA as well as they also do not require any former information of sequence to design the primer. As uniformity of the tissue cultured plants has to be maintained for the quality of germplasm, thus the analysis through the RAPD and ISSR techniques are adopted for evaluating the genetic homogeneity. Initially RAPD was defined and describes by William.[129] It is a PCR-based marker that amplifies DNA fragments using a single, arbitrary primer made up of 10 bases. The basic principle behind the technique is that when the small quantity of genomic DNA is subjected to PCR, there is a very high likelihood of locating priming sites for random decamers with inverted orientation that are close enough to be amplified by PCR, in complete genomes. This method amplifies the variable length intervening sequences between these priming sites, resulting in multiple bands for every primer that can be separated by agarose gel electrophoresis. The key benefit of this method is that it may be used in any species and doesn't require any prior genomic knowledge.

Mass multiplication through callus is generally considered as the most unreliable for the clonal propagation whereas those plants generated by the somatic embryos or by the branching of axillary buds is considered to be uniform by genetically.[130,131]

Therefore, in respect to assess the somaclonal variation in the regenerated or micropropagated plantlets, evaluation of the genetic make-up and stability of the *in vitro* produced plantlets has become crucial. PCR based DNA markers play an important role to assess the genetic stability or clonal fidelity and thus can be used as valuable tool for the same.[132,133,134]

Many studies and researches proved that the one of the most useful tools is the practice of molecular markers for studying the relationships between genotypes, genetic fidelity and genetic diversity between the plant species. *Plumbago zeylanica* were micropropagated using the nodal segment as explant. Further the genetic integrity was

checked or evaluated through the RAPD-PCR. In order to evaluate the genetic fidelity, genomic DNA from regenerants and the mother plant was amplified using 20 arbitrary decamer. All of the micropropagated plants' RAPD profiles were monomorphic and resembled those of their mother plants. There was no evidence of polymorphism in the micropropagated plants.[135]

For estimating the genetic similarity, diversity, variations and the population structure of different plants, molecular markers are proficiently use. Among all the molecular markers that were used for assessing the genetic fidelity, ISSR markers are most common. The polymerase chain reaction is primed using ISSR markers, which are short sequence repeat motifs, in order to amplify the areas between nearby microsatellites that are oriented in the opposite direction. Because ISSR markers are longer than RAPD markers, they are better able to detect somaclonal variation, with higher levels of sensitivity, stringency, and reproducibility.

There are various factors that cause the variation among the tissue cultured plants. Pradhan and coworkers studied the genetic variation among the regenerants and mother plants of *Cymbidium aloifolium* using the molecular markers RAPD (Random amplified polymorphic DNA) and the ISSR (Inter simple sequence repeats). They also developed the sodium alginate coated synthetic seeds of these plants. During the study, 5 ISSR and 10 RAPD were used to amplify the isolated DNA. While 5 ISSR amplified a total of 99 loci, 9 RAPD primers amplified a total of 256 loci. Low polymorphism was evident in the combined RAPD and ISSR molecular marker data. For determining the genetic fidelity or clonal fidelity in *Cymbidium aloifolium*, both markers found to be equally effective.[136]

For screening genetic integrity of Jojoba plants (*Simmodsia chinesis*) that are propagated through the axillary bud multiplication from the nodal segments, two different molecular markers viz RAPD (random amplified polymorphic DNA) and the ISSR (Inter simple sequence repeats) were used. The *in vitro* cultures were subcultured further upto 12 *in vitro* cultures. 48 primers were used in this investigation, and 24 RAPD primers and 13 ISSR primers combined to yield a total of 191 clear, distinct, and repeatable amplicons. RAPD primers produced 126 bands whereas ISSR produce

clearly 65 bands. All the selected micropropagated plants have monomorphic amplified products were similar to their mother plant. Their research suggested that the safe approach for propagating jojoba plants and true-to-type plants is through axillary bud multiplication.[137]

A study indicates that callus-mediated organogenesis results in *in vitro* regeneration in *Abutilon indicum* plants. Highest callus proliferation was seen in MS medium supplemented with the 2,4-D (4.52 µM) and BA (8.88 µM). further sub culture on MS medium perforated with NAA (2.68 µM) with BA (8.88 µM) and Adenine sulphate (543 µM) revealed the highest number of multiple shooting and their elongation. The well-developed shoots were further developed rooting in MS medium IBA (7.38 µM). The regenerated plantlets resembled their mother plant morphologically exactly. Using the ISSR molecular marker, the genetic homogeneity of the regenerated plantlets was also established.[138]

Similarly, there are number of past studies that shows that the ISSR and RAPD markers could be used to identify genetic integrity in several crops, including *Brassica oleracea* [139]; *Prunus dulcis* [140] and *Swertia chirayita* [141]; *Dioscorea bulbifera* [142]; Banana [143] and *Saccharum officinarum* [144]; *Saussurea involucrate* [145]; *Clerodendyum serratum* [146]; *Dendrocalamus hamiltonii* [147]; *Cymbopogon martini* [148]; *Capparis deciduas* [149]; *Nothapodytes foetida* [150] and *Nardostachys jatamansi*.[151]

Genetic variation may arise in the tissue cultured plantlets due to several factors linked with the regeneration conditions. Thus, the assessment of the genetic diversity become one of the essential factors if true to type plantlets or regenerants are looked-for. RAPD and ISSR are the common and best suited molecular markers to evaluate the genetic fidelity among the medicinal plants. Using 11 ISSR (Inter Simple Sequence Repeats) and 12 RAPD (Random Amplified Polymorphic DNA) primers, the genetic stability of *Dendrobium chrysotoxum* regenerants was examined. The research showed that the mother plants and *in vitro* clones had a significant degree of genetic monomorphism.[152]

For large scale propagation, the micropropagation protocol for *R. rhabarbarum* was developed by the Clapa and co-workers. A DNA marker system that evaluates whether

the regenerated plantlets are true to type was used. They recommend that the *in vitro* proliferation of *Rheum rhabarbarum* possibly be considered as an alternate to provide the vital sources of the bioactive compounds, that possibly will be utilized later in several pharmacological researches. Additionally, high-quality planting materials are also available for use in agriculture.[12] During the study, the regenerated plantlets were subjected to genetic fidelity analysis, thus SRAP marker system was used for the same. Similar methods were used previously by numerous investigators.[153,154] Visual represented of obtained bands does not show any polymorphism or variation among the regenerated plantlets and the mother plant.