CHAPTER 3: MATERIALS AND METHODS

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3.1 Materials

3.1.1 Explant Material

Juvenile plants of *Rheum emodi* were sampled and procured from the Pothivasa region (2200 m above sea level; between 30° 28´ N latitude and 79° 16´ E longitude; District Rudraprayag of Garhwal Himalayan region, state Uttarakhand during the month of July **(Fig. 3.1).** The region has mountainous climate, with annually maximum temperature reaching upto 35℃ in the month of May-June and the minimum temperature may be recorded as less than 0℃ during the wintertime, with infrequent snowfall. The annual precipitation ranges from 345 to 459.5 cm, with the monsoon season (August to September) seeing the heaviest amounts. The soil is acidic in nature (pH 4.67- 5.01), slight black and sandy loam in texture with a rich content of organic carbon and moisture.[155] Leaves were taken from juvenile, healthy plants and were used for callus initiation and proliferation, while the flowering plants were dried, an herbarium made, and it was then submitted to the department of Botanical Survey of India, Northern region in Dehradun, Uttarakhand for species-level identification.

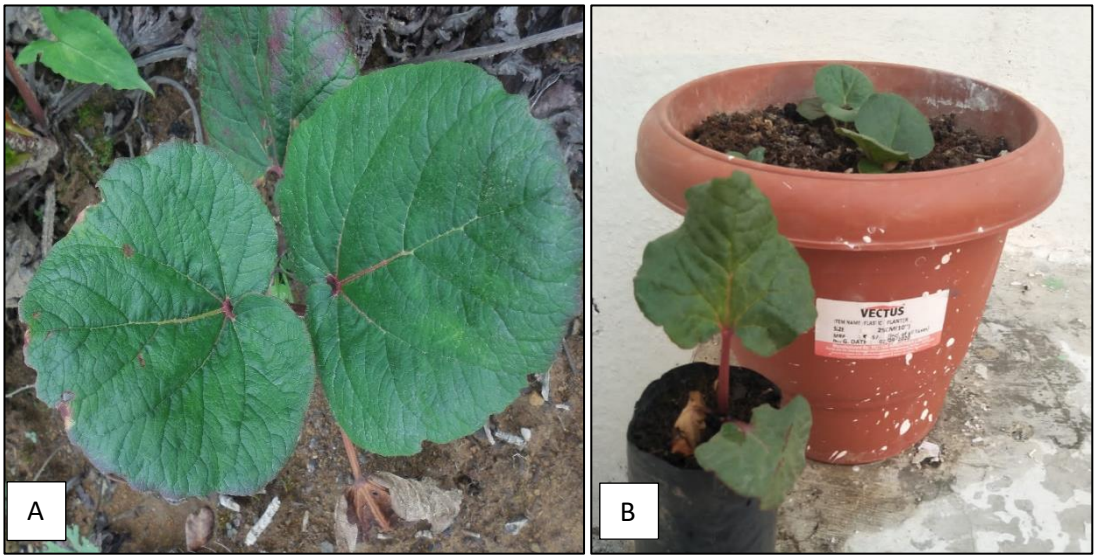

Fig. 3.1: (A) (B): Plants of *Rheum emodi* procured from the Pothivasa region

3.1.2 Chemicals and Reagents

Chemicals and the required reagents employed during the study are of standard analytical grade chemicals of Hi-Media laboratory, India; Bangalore Genei, India; Sigma chemical.

For tissue culture, all majors and minor salts of Murashige and Skoog medium that were used are of Hi-Media laboratory, India; Vitamins used were of Hi-Media laboratory and Sigma chemical company; plant growth regulators, sucrose, agar and clarigel used are of Hi-media laboratory, India. For the production of synthetic seeds, sodium alginate and calcium chloride used which were of Hi-Media laboratory.

For molecular biology study, PCR components, primers and all the reagents that were used are of Bangalore Genei and Sigma company.

3.1.3 Glasswares

For tissue culture studies, glasswares were of Borosil make. Flasks (150 mL, 250 mL), cultures tubes (25 x 150 mm and 25 x 200 mm), reagent bottle with screw cap (1000 mL), petri- plates (100 x 15 mm), conical flask (1000, 2000 mL) was used for media preparation. All the glassware used were cleaned with mild detergent and diluted 0.1 N HCl, and then were repeatedly washed for few times with distilled water and oven dried at 60°C. All the Petri plates and Culture vessels were washed, autoclaved for 20 min at 121°C, 15 psi prior to use.

3.1.4 Plasticwares

For the tissue culture purposes, new range of Phyta jars (370 mL) were used for regular culturing for ease of handing and unbreakable purpose, Phyta jars used were of Hi-Media; Syringe filters (0.22 µm) were of Hi-Media all the tips (2-200 µL) and Eppendorf tubes (2 mL) used in molecular biology experiments were of Tarson products limited.

3.2 METHODS

3.2.1 Sterilization

The important step in tissue culturing procedure is sterilizing of explant for establishing aseptic culture as the explant is the primary source. All the vessels, glasswares, and equipments used in culturing are autoclaved and then dry sterilized in hot air oven. Prepared media were sterilized in the autoclave while the growth regulators were filter sterilized prior to use. For contamination free working area/environment, fumigation with formalin and potassium permanganate were proved as promising method and could lead to contamination free plant tissue culture process.[156]

3.2.1.1 Explant Sterilization

Explants collected from field grown source were cleaned with detergent Tween 20 (4-5 drops) and then washed carefully with the tap water, then multiple times with the double distilled water, and then were sterilized by the $HgCl_2$ (Mercuric chloride), 70% ethanol, and NaOCl (Sodium hypochlorite). Tween 20, a mild non-ionic surfactant, functions as a wetting agent, dispersant, and solubilizer due to its hydrophilic nature. When used in washing procedures, Plant epidermis has trichomes, which can trap both microorganisms and air, tween 20 moistens the plant surface and repel the air. Different sterilization time were checked for the proper sterilization of explants. Explants were finally washed three or four times with double distilled water to eliminate the residues of sterilant. Juvenile leaves were excised from the *Rheum emodi* plants.[169] The plants of *Rheum emodi* were procured from Pothivasa, District Rudraprayag, Uttarakhand.

Phase I describes by the process occurred outside the laminar air flow while the Phase II denotes the procedure occurred inside the laminar air flow; are the two parts of the sterilizing procedure **(Fig. 3.2)** for explant (*Rheum emodi).* Three different types of sterilizing agents, such as mercuric chloride ($HgCl_2$), 70% ethanol, and sodium

hypochlorite (NaOCl) in combination were used varying their concentration and time of exposure are used for sterilization.[168] (**Table. 3.1)**

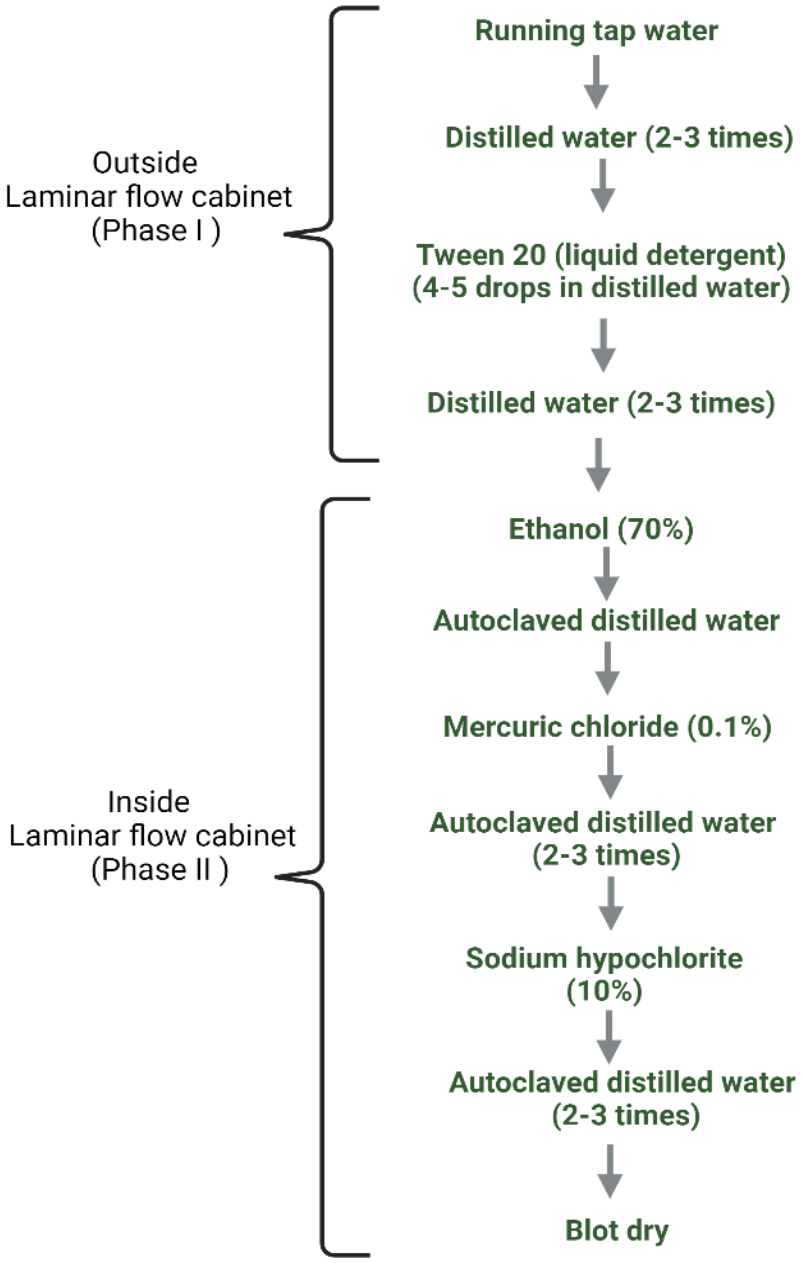

Fig. 3.2: Two phase sterilization procedure of explant: Phase I that is Outside the laminar flow cabinet and the Phase II that is Inside the laminar flow cabinet

Table. 3.1: Treatment and exposure time of sterilizing agent used for explant sterilization.
Where, (s) denotes seconds; (m) denotes minutes

Treatments	Ethanol (70%)	$HgCl_2$ (0.1%)	NaOCl (10%)
A	30 s	No HgCl2	No NaOCl
B	30 s	30 s	30 s
C	1 m	30 s	30 s
D	1.5 m	30 s	1 m
E	30 s	1 m	30 s
F	1 m	1 m	30 s
G	1.5 m	1 m	1 m
H	30 s	1.5 m	30 s
I	1 m	1.5 m	30 s
J	1.5 m	1.5 m	1 m

3.2.2 Media preparation

Stocks solutions of all the major, minor, organic components, iron and vitamin components of the MS media [157] were used and prepared in autoclaved distilled water **(Table. 3.2).** Amber-coloured bottles were used to store iron stocks. All stocks were autoclaved first, then maintained in a 4°C refrigerator. The required volume of double distilled water and the desired volume of stock solution were used to make the medium, agar or clerigel was used as gelling agent in the 0.75% concentration. Sucrose was added as a sole source of carbohydrates. pH was maintained at 5.85. Medium was

added to culture tubes and flasks in amounts of around 15-20 mL. Each culture vessel had a non-absorbent cotton plug inside, which was then wrapped with aluminium foil. After that, the media was autoclaved for sterilization at 121 °C at 15 psi pressure. Plant growth regulators in the necessary quantity were added to all media and kept at 4°C for storage.

All hormones used were prepared from stocks of 1.0 mg/mL strength. Heat labile hormone stocks were filter sterilized by Millipore filter (0.22 µm) and other hormones were sterilized by autoclaving. NaOH was used as a solvent to dissolve the hormones. Then, these hormones were stored in freeze in 4^{o}C.

3.2.3 Inoculation

The procedure for inoculating explants was carried out inside the laminar airflow cabinet under sterile environment. The bench of laminar was thoroughly wiped with ethanol swabbed cotton. Prior to inoculation, the laminar air flow hood underwent a 30-minute UV sterilization process. All the required paraphernalia such as scalpel, forceps, whatman filter paper were pre autoclaved; forceps and scalpel were dipped in 70% ethanol followed by red hot flaming and cooling and sterilized prior to work. The mouth of all the culture vessels and petri plates were also flame sterilized. Leaves of juvenile, healthy *Rheum emodi* served as explants. The explants (leaves having midrib) were then cut into pieces of about 2-3 cm sized and were used for the callus initiation. Explants were sterilized with sterilizing agents followed by washing three time with the autoclaved distilled water. Finally sterilized and selected explants were inoculated to medium and then cultures were kept under controlled environment in the culture room.

Table. 3.2: MS medium: Stock solutions and their compositions.

MS* - Murashige and Skoog (optimized concentration)

Stock Strength	Components	Composition (g/L) MS*
Stock 1 (10X) Macronutrients	NH_4NO_3 $CaCl_2.2H_2O$ $MgSO_4.7H_2O$ KH_2PO_4 KNO_3	16.5 4.4 3.7 1.7 19
Stock 2 (100 X) Micronutrient	KI H_3BO_3 $ZnSO_4.7H_2O$ $MnSO_4.7H_20$	0.0415 0.31 0.43 0.84
Stock 3 (100X) Micronutrient	$NaMoO_4.2H_2O$ $CuSO_4.5H_2O$ $CoCl_2.6H_2O$	0.012 0.00125 0.00125
Stock 4 (100X) Iron	$FeSO_4.7H_2O$ Na_2EDTA	1.39 1.86
Stock 5 (100x) Vitamins	Nicotinic acid Pyridine HCl Thiamine HCl Myoinositol	0.05 0.05 0.05 5
Carbohydrate source	Sucrose	30
Gelling agent	Agar	7.5

3.3 Micropropagation

3.3.1 Optimization of callus induction

Micropropagation proved to be an appropriate method for the development and multiplication of the plants, meanwhile this enables the generation of vast numbers of identical seedlings in a relatively short amount of time that are true to type to the mother plant. Micropropagation started as the callus induction in juvenile leaves followed by the frequent subculturing to obtained the shoots and roots, is a quick method of propagation and enables high multiplication rate. Young, juvenile leaves were selected because of their capability to regenerate cultures with comparatively a high callus growth. The combination of different phytohormones in the media affects the development and growth of the callus, shootlets and roots.[72]

Further regenerated propagules were efficiently utilized to transform into complete plantlets under *in vitro* conditions. MS medium with cytokinin as 6-Benzylaminopurine (6-BAP) in the range of 1.0-3.0 mg/L and auxin such as Indole-3-butyric acid (IBA) and 2, 4-dichlorophenoxyacecetic acid (2, 4-D) in the range of 2.0-10 mg/L were tested for best callus induction. Media devoid of growth regulators were used as control. Experiments were conducted for 3 times, where a total of 15 explants were used for each replication. In current study, somatic embryos were also observed from the callus.

3.3.2 *In vitro* multiplication of shoots

The *in vitro* shoot multiplication was studied by varying concentrations and combinations of different phytohormones which are as follows:

3.3.2.1 Cytokinins effect on *in vitro* multiplication of shoot

The callus was subcultured once in 2 weeks before it showed the signs of shoot emergence. Different natured Leaf-induced friable callus turned green and compact through the regular cultivation. Different sets of experiments were performed to observe the effect of varying concentrations and combinations of cytokinin suitable

for optimum *in vitro* shoot multiplication. Three well-known cytokinin i.e., BAP (6-Benzylaminopurine), TDZ (Thidiazuron) and Kn (Kinetin) were tested to have a maximum multiplication rate in combination and alone.[158] TDZ in the range of 2.0 mg/L to 8.0 mg/L and the two other hormones Kn and BAP in combination were tested; Kn in range of 1.5-3.0 mg/L and 6-BAP in the range of 2.0-8.0 mg/L.

No regeneration was observed in hormone–free medium. 15 explants were tested and experiment were performed three times.

3.3.2.2 Effect of concentration of sucrose on *in vitro* multiplication of shoots

As the carbohydrate source in the medium, the primary source of energy for quick *in vitro* shoot proliferation is sucrose. Hence effect of sucrose, in form of varied concentrations, was studied for in vitro shoot multiplication. Different sets of experiments were conducted to know the best shoot multiplication by varying the concentration from 1% to 6%.[159]

3.3.3 Optimization of *In vitro* rooting

One of the key steps in the generation of tissue cultured plants is the establishment of rooting *in vitro* grown shoots. Thus, a large number of experiments were conducted towards *in vitro* rooting of these shoots. Prior to rooting the *in vitro* raised shoots was elongated on hormone free half-strength MS medium. Bunch of 4 propagules was used for rooting purpose and these shoots were cultured in a tube (25×150 mm and 25×200 mm) for *in vitro* rooting. The average number of roots produced and the average length of the roots were recorded as the rooting response. Variance was calculated as standard error to each observation.

3.3.3.1 Effect of auxins on *In vitro* rooting of shootlets

The numerous concentrations of phytohormone auxins (IAA, IBA) in half strength and full-strength MS media were tested for *in vitro* rooting. Different concentration and combination of auxins with cytokinin were tested. From the shoot cultures, shootlets (2–3 cm) were removed and placed on rooting media (hormone free half MS or full MS) with the combination of IBA (2.5 mg/L and 5.0 mg/L) and IAA (2.5 mg/L and 5.0 mg/L) for 3 to 4 weeks along with the cytokinin (BAP) with a concentration tested was 2.0 and 4.0 mg/L. After 15 days, the shooted cultures that showed signs of roots were moved to new media and kept for up to 4 weeks.

3.3.4 Cultured Conditions

In the culture room, a relative humidity of 70–75 percent was maintained while all the cultures were cultured at a temperature of 23 ± 2°C. During the study, all cultures were kept under illumination of 16/8 hrs (light/dark) photoperiods with light intensity of 50-60 umol m^{-2} s^{-1} which was obtained with white cool fluorescent tube lights of 40 watts (Phillips, India). Embryogenic and callus cultures were maintained in darkness in culture room of controlled conditions of temperature and relative humidity.[160]

3.3.5 Statistical Observation and Analysis

Data has been collected to determine the impact of several media components and hormone responses on the morphogenic status of the mentioned plant species, and analysis was done using the CRD design of experiments. Experiment was performed in triplets and each replicate comprises of ten explants in each repetition. Collected data were calculated statistically by the ANOVA (Analysis of Variance) for analysing the significance of differences observed in the regenerants of *Rheum emodi.* The critical difference at 5% and the standard error both referenced the degree of variance. A significance level of 0.05% was noted. According to the test i.e., Duncan's multiple range the means of every treatment was compared at P = 0.05. The observed values shown are mean ± S.E.

3.4 Hardening and acclimatization

The plantlets were successfully uprooted, rinsed once or twice with the distilled autoclaved water to get rid of the last basal callus and adhering media formerly transferring them to a pot containing sterilized autoclaved soil mixed with vermiculite in 1:1 ratio. The pots were kept inside a culture room for two weeks to harden, covered with perforated polybags and then to greenhouse. After the 4 weeks of culture, observation was recorded based on rooting response percentage, average number of roots per plantlet and the mean root length.

Before being transplanted into the field, *in vitro* produced plantlets need to be hardened and acclimated. Due to their heterotrophic approach of nutrition, tissue grown plantlets are unable to withstand environmental conditions without adequate hardening and acclimation. For further hardening these plantlets were transferred to autoclaved soil rite in bottles and kept in controlled environmental conditions. After that, they were put into polybags with soil and sand and sustained in a mist chamber with a relative humidity of 80–90% and a temperature of 28 ± 2°C. The perforated polyethene covers were then removed when the new pair of leaves started emerging on the shoots and then hardened plantlets were transferred to the soil in pots.

3.5 Synthetic seed production

The plant propagation material that is artificially enclosed is called synthetic seeds. The shoot buds, cell aggregates, somatic embryos, or any other tissue that can act as a seed for propagation can all be considered to be part of the plant material. The differentiation, organogenesis of the somatic embryos, either directly from the explant or from the callus, is depends upon the composition of culture medium, type of explant, time period between subculturing. Generally, in the process, two-staged culture is involved. First is the induction of proembryonic masses, followed by an initial exposure of tissues to the auxin phytohormone such as 2,4- D. Subsequently the proembryonic cultures are transferred to the medium with modified composition with low auxin level or with reduced concentration, so that the somatic embryos can fully

differentiated.[161] The general procedure for syn seed development is the selection of juvenile leaves/ explant followed by the establishment of somatic embryogenesis; after the selection of mature embryos, they were encapsulated by the appropriate gelling agent **(Fig. 3.3).**

3.5.1 Encapsulation of somatic embryos

The major steps for producing Syn seeds or artificial seeds were the selection and sterilization of explant, preparation of MS medium, alginate (%) and calcium chloride solution (mM), encapsulation of explant in sodium alginate along with calcium chloride, incubate and washed them with distilled water, dried and germinate.[86]

3.5.2 Gelling agent for encapsulation

For encapsulating the somatic embryos, we tested different concentration of sodium alginate such as 2.5 %, 3% and 4% (W/V) in the MS basal solution. After the 5 weeks of standard subculturing of callus, we isolated embryos of about 1-2 mm sized by drawing them with suction dropper containing the sodium alginate solution. The synthetic seeds' coatings must not harm or be harmful to the embryo, be gentle enough to protect the embryo while allowing germination, and be appropriate for handling, storage, transportation, and planting. Another significant limiting element for synthetic or artificial seeds is the coating concentration.

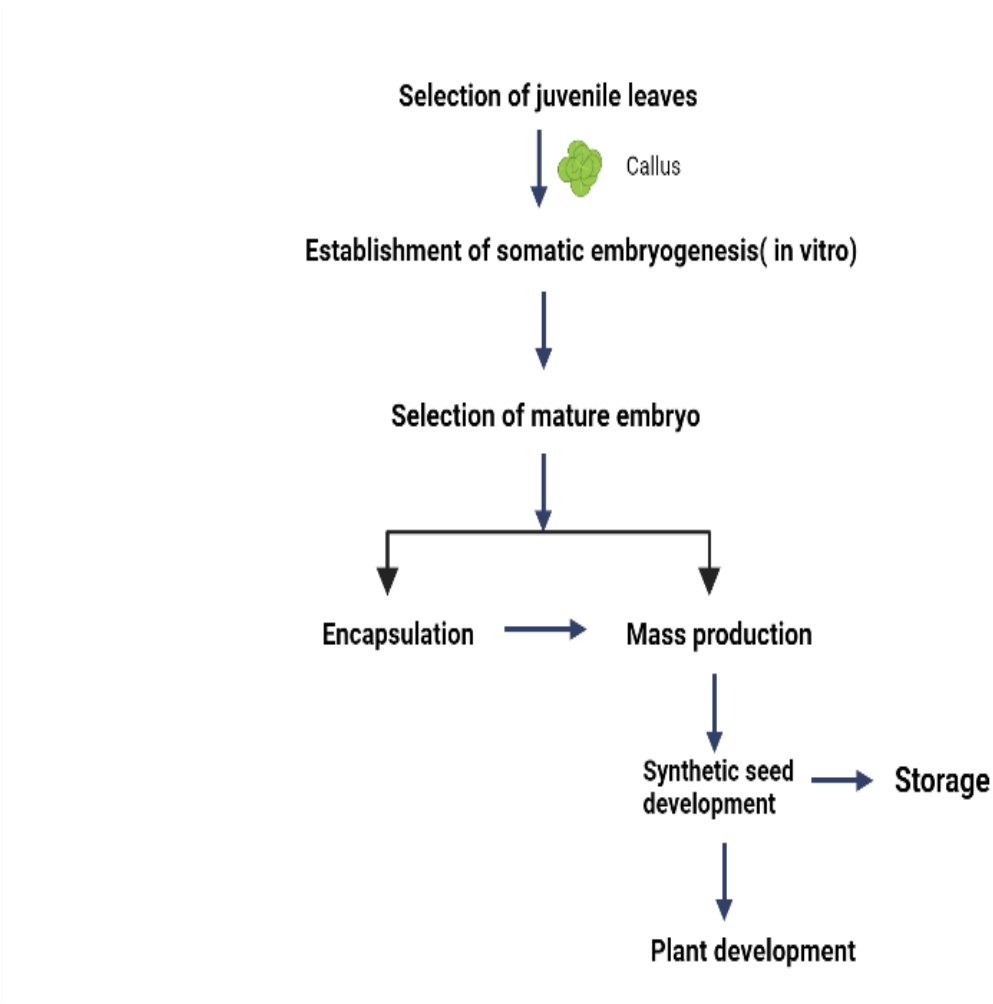

Fig. 3.3: Schematic diagram for production of artificial seed.

3.5.3 Complexing agent

Somatic embryos encapsulated with gelling agent were thrown down to calcium chloride (25, 50 and 75 mM) (w/v) solution. Individual drop with a single embryo was allowed in $CaCl_2$ with the exposure time of 20-30 minutes. After incubation, the beads were finally recovered by pouring off the calcium chloride and washed thrice with the MS basal media containing 3% sucrose.[162] Control here was referred to as non-encapsulated embryos. Further both encapsulated and capsulated embryos were cultured in MS basal medium for evaluating their germination potential.

3.5.4 Storage experiment for encapsulated somatic embryos

The impact of storage on survival and germination percentage of somatic embryos were estimated by either a direct transfer of encapsulated and naked (non-encapsulated) embryos to the growing medium or they were kept in petriplates and left for 20 and 30 days in the cold storage (4°C) for further observation.[99] Following storage, they were cultured in MS medium for observing their survival percentage and germination potential were recorded after 5 weeks.

3.6 Statistical Analysis

To examine and evaluate the data, analysis of variance was used. Tukey's HSD test (Honestly significant difference) having ($p < 0.05$) was used for comparison between the means of each treatment. was compared. The values shown are Mean ± S.E. In each replication of the experiment, a minimum of three triplets were utilized for each treatment, and at least ten explants were employed in each duplicate. Data collected was examined further using ANOVA (analysis of variance) to check whether the values are significant or not.

3.7 Molecular Analysis

3.7.1 Reagents and Chemicals

- Extraction buffer: 2.0 mM EDTA, 100 mM Tris-HCl (pH 8), 2% CTAB (Hexadecyltrimethyl-ammonium bromide) (w/v), 1.4 M NaCl, 2% BME (β-mercaptoethanol) (v/v) was added in the extraction buffer immediately before preheating
- Isopropanol (chilled)
- Chloroform-isoamyl alcohol (24:1 v/v)
- 70% ethanol (stored in -20°C)
- TE buffer (storage buffer): 10 mM Tris-Cl buffer of pH 8.0 and 1.0 mM EDTA of pH 8.0.

3.7.2 Extraction of total genomic DNA

Several protocols have been reported for isolating the plant genomic DNA with the primary objective as development of quick, consistent and inexpensive protocol. However, the basic principle of all extraction procedures is to disruption of the plant nuclear membrane, cell wall, cell membrane, and in order to release the genomic DNA. into the extraction buffer tailed by the precipitation of DNA while making sure that all impurities, including polysaccharides, protein, phenols, lipids, and other secondary metabolites, are removed using enzymatic or chemical processes. The most frequently used detergents are SDS (sodium dodecyl sulfate) or CTAB (cetyl trimethyl ammonium bromide) which disrupt the cell wall whereas the BME (β-mercaptoethanol) which is a reducing agent breaks the disulfide bonds, BME aids in denaturing the proteins and eliminates the polyphenols and tannins that are present in the crude extract. EDTA used to chelates the Mg^{++} ions and keep the DNA intact. Tris and NaCl in buffer worked to maintain the pH and precipitate the DNA respectively. Additionally, the contaminants are removed by washing with chloroform: isoamylalcohol (24:1), in which isoamylalcohol functions as an antifoaming agent,

whereas chloroform aids in binding to complexed polysaccharides and proteins. Finally, washing with chilled alcohol precipitate the nucleic acid.

The total genomic DNA was extracted to analyze the variation among the clonal stock raised during different steps of micropropagation viz. shoot multiplication, *in vitro* rooting and plantlets acclimatization. Fresh leaves of *Rheum emodi* from sample site Pothivasa, Rudraprayag, Uttarakhand was collected. For assessing the genetic stability of *in vitro* raised clonal stock, the micropropagated plants were randomly selected from each set of experiment. Nine samples per experiment were sampled and were then used for isolating total genomic DNA and for further conduction of PCR studies. DNA was prepared from leaves according to the method described by the earlier researchers.[163]

3.7.2.1 DNA extraction protocol

The protocol of DNA extraction was: Preheat CTAB extraction buffer; Then, grind the leaves into a fine powder using cooled mortar pestle in liquid nitrogen (-196°C) using a buffer containing 3% β-mercaptoethanol (v/v) at 60°C in a water bath and then transferred it into the preheated extraction buffer in centrifuge tube. With intermittent shaking, incubate the contents at 60°C for 30 minutes. After complete lysis of tissues, centrifuge at 15,000 rpm for 10 minutes after adding an equal measure of chloroform: isoamyl alcohol (24:1). Pipette out the clear aqueous phase with caution, then add RNase A to remove the RNA. Then kept the obtained aqueous phase overnight after adding the isopropanol to precipitate DNA from the aqueous layer **(Fig. 3.4)**. Wash down the pellet with the 70% ethanol (200 μL) and then dry and stored at -20°C after adding TE buffer (storage buffer).

Some chief instructions which are followed throughout the DNA isolation process were: grind should be fine; propanol and other chemicals should be chilled. Pellets shouldn't be over-dried as its difficult to dissolve excessively dried pellet, DNA is extremely prone to fragmentation, so invert slowly during the mixing stage.

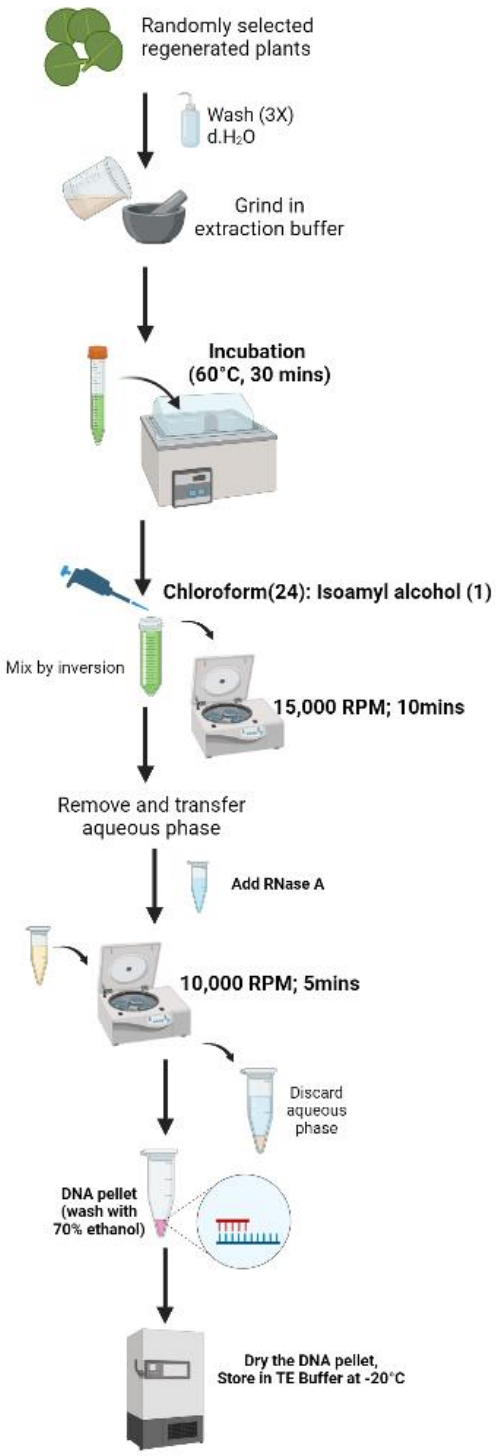

Fig. 3.4: Diagrammatic representation of the plant genomic DNA extraction protocol

3.7.3 Qualitative estimation and quantification of genomic DNA

Integrity of isolated DNA was confirmed by the gel electrophoresis unit, with the agarose gel of concentration 0.8% (w/v) prepared in the 1X TAE buffer (stock solution; 5X(1L): 0.5 M EDTA, 24.2 gm Tris base and 5.71 mL Glacial acetic acid, pH- 8.0). Agarose gel stained with Ethidium bromide (EtBr) were used for visualizing DNA under Gel Documentation system (UVP Gel- Doc, Analytik Jena). The purity of DNA sample (contamination by protein, RNA, phenols etc) can be measured by determining the amount of UV radiation which is absorbed by the bases present in the aliquot of sample. Thus, the quantification of DNA (260/280) was measured by BioSpec-nano UV-VIS Spectrophotometer (Shimadzu). The isolated DNA was diluted up to different concentration according to PCR analysis and then finally stored at -20°C.[164]

3.7.4 Optimization of PCR conditions for RAPD and ISSR analysis.

3.7.4.1 Optimization of Genomic DNA concentration

PCR based analysis does not require a large amount of DNA, thus the optimization of concentration of Genomic DNA was done for obtaining best amplification results at minimum DNA concentration. Amplification was checked using 10-50 ng/µL for molecular markers analysis. The concentration of DNA was optimized in order to get the maximum product yield of the amplified fragment.

3.7.4.2 Optimization of primer concentration

Lower primer concentration can result in low or no amplification while the higher concentration of primer contributes to non-specific and mispriming amplification. Optimization was done in order to amplification of multiple targeted locations. Concentration was varied from 0.1 µM- 10 µM for all the PCR analysis.

3.7.4.3 Optimization of Taq polymerase

The polymerase enzyme (Taq DNA polymerase) is a thermostable, recombinant DNA polymerase of concentration 5 U/μL. Optimization was done after running different sets of PCR conditions with 0.1, 0.2, 0.3 μL corresponding to 1 U of Taq polymerase enzyme reaction mixture for all the PCR analysis.

3.7.4.4 Optimization of dNTPs and Mg^{++} ions

Concentrations of dNTPs was optimized using various concentrations such as 2.0 mM, 2.5 mM and 3.0 mM. $MgCl_2$ concentration was also optimized by varying 2.5 mM, 3mM, 5mM concentrations. By stabilizing the duplex strand, high concentrations of Mg++ may be able to prevent the DNA template from becoming completely denaturized.

3.7.4.5 Optimization of annealing temperature

Efficient binding of primer with strands of DNA was optimized by checking the amplification results at different temperatures as 31°C to 38°C for RAPD, and 43°C to 67°C for ISSR analysis. The optimum temperature resulted in the amplification of a multiple band in RAPD and ISSR for all the DNA samples. The differences in the annealing temperature results in no binding and thus gives non-specific PCR products. Because low annealing temperature results in non-specific binding and high annealing temperature hinders the primers from binding to the template optimally.

3.7.4.6 Visualization of amplification products

All the samples of *Rheum emodi* were amplified by using RAPD and ISSR markers. These samples were then visualized on the agarose gel (1%) prepared by using 1X TAE buffer stained with ethidium bromide under UV radiation by gel documentation system (UVP Gel-Doc, Analytik Jena) and documented for further analysis

3.7.5 RAPD and ISSR ANALYSIS

The Random amplified polymorphic DNA is the method or technique that uses a single primer with any nucleotide sequence to find polymorphisms in the nucleotide sequence of DNA. In this, a single primer anneals to the template DNA's complementary strands at two different locations, if these priming sites are present within an amplifiable range of each other, then a discrete DNA product is formed through the thermocyclic amplification. The technique is effective for detecting nucleotide sequence variability among individuals because each primer guides the amplification of several distinct loci on the genome. The 15 RAPD primers **(Table. 3.3)** were initially screened to amplify the genomic DNA of mother plant and the regenerated plantlets by using standard PCR.

Table. 3.3: RAPD primers for the initial screening to amplify the genomic DNA of *Rheum emodi*

S. no	Coding	Oligo Name	Tm (ºC)	Sequence (5'-3')
1	REi1	**OPP 13**	23	AAAGTGCCTC
2	REi2	**OPA 09**	27	GGGTAACGCC
3	REi3	**OPB 09**	27	TGGGGGACTC
4	REi4	**OPE 06**	25	AAGACCCCTC
5	REi5	**OPL 10**	25	TGGGAGATGG
6	REi6	**OPL 19**	25	GAGTGGTGAC
7	REi7	**OPP 11**	27	AACGCGTCGG
8	REi8	**OPP 12**	25	AAGGGCGAGT
9	REi9	**OPA 17**	25	GACCGCTTGT

10	REi10	**OPA 19**	25	CAAACGTCGG
11	REi11	**OPB 04**	25	GGACTGGAGT
12	REi12	**OPE 08**	25	TCACACGGT
13	REi13	**OPD 04**	25	TCTGGTGAGG
14	REi14	**OPD 09**	25	CTCTGGAGAC
15	REi15	**OPP 05**	25	AGGGGTCTTG

Similarly, 20 ISSR primers **(Table. 3.4)** were initially screened for tissue cultured plantlets and mother plants of *R. emodi*, to amplify the inter- repeat regions in the genomic DNA using standard PCR.

Table. 3.4: ISSR primers screened to amplify the inter-repeat regions in the genomic DNA of *Rheum emodi*

S. no	Coding	Oligo Name	Tm (ºC)	Sequence (5'-3')
1	IR 1	**UBC 825**	49.3	ACACACACACACACACT
2	IR 2	**UBC 836**	49.3	AGAGAGAGAGAGAGAGCTA
3	IR 3	**UBC 844**	49.3	CTCTCTCTCTCTCTCTAGC
4	IR 4	**UBC 816**	43.3	CACACACACACACACAT
5	IR 5	**UBC 855**	49.7	ACACACACACACACACCTT
6	IR 6	**UBC 834**	51.1	AGAGAGAGAGAGAGAGCTT

7	IR 7	**UBC 835**	51.5	AGAGAGAGAGAGAGAGCTC
8	IR 8	**UBC 817**	52.8	CACACACACACACACAA
9	IR 9	**UBC 842**	49.7	GAGAGAGAGAGAGAGACTG
10	IR 10	**UBC 811**	43.3	GAGAGAGAGAGAGAGAC
11	IR 11	**UBC 807**	42.5	AGAGAGAGAGAGAGAGT
12	IR 12	**UBC 808**	46.8	AGAGAGAGAGAGAGAGC
13	IR 13	**UBC 809**	46.6	AGAGAGAGAGAGAGAGG
14	IR 14	**UBC 868**	47.8	GAAGAAGAAGAAGAAGAA
15	IR 15	**UBC 840**	48.2	GAGAGAGAGAGAGAGACTT
16	IR 16	**UBC 841**	58.1	GAGAGAGAGAGAGAGACGACTC
17	IR 17	**UBC 889**	59.2	AGTCGTAGTACACACACACACAC
18	IR 18	**UBC 891**	62.7	AGTACGAGTTGTGTGTGTGTG
19	IR 19	**UBC 890**	65.6	ACGAGTAGGGTGTGTGTGTGTGT
20	IR 20	**UBC 888**	67.6	CTGAGTCGTCACACACACACACA