CHAPTER 5: DISCUSSION

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One of the endangered species, *Rheum* species, have less satisfying and successful reproduction using conventional or natural techniques. As a result, mass multiplication to fill the gap created by environmental change and a lack of planting material can be effectively replaced by the *in vitro* production of *Rheum* plants.

In the present study, in spite of the fact that the sterilization of the explant was carried out carefully, a high contamination percentage was observed in the initial stage as less exposure or no sterilant contribute to the more contamination and no germination. Three sterilant viz. $HgCl_2$ (Mercuric Chloride), 70% Ethanol, NaOCl (Sodium Hypochlorite) combination (treatment A to J) were used to sterilize the explant properly. Treated explants were then inoculated in media. After observing the inoculated explants for 15 days for growth and contamination, it was found that the treatment F that is Mercuric chloride (1 min.), 70% Ethanol (1 min.) and Sodium hypochlorite (30 secs) Proves to be the best combination for 100% germination and no contamination. Less exposure or no sterilant contribute to the more contamination and no germination while the increasing time contributes to the reduced contamination but also effects the growth as they prove lethal to the leaves/explant.

For the induction of callus, different auxin concentration was tested, from which the best percentage of callus production was seen in MS (Murashige and Skoog) media perforated with 2,4-D (2,4-dichlorophenoxyacetic acid) along with BAP (6-Benzylaminopurine). The results demonstrated that this hormone combination was the most effective for producing callus from *Rheum emodi* leaves explants. For the callus induction, different auxin concentration was tested, from which the highest percentage of callus formation (84.44 %) was observed in MS (Murashige and Skoog) medium perforated with 8 mg/L of 2,4-D (2,4-dichlorophenoxyacetic acid) along with BAP (6- Benzylaminopurine) having concentration of 2.5 mg/L. After 6 weeks,

a fragile yellowish-brown callus was observed. Further, the decrease in callusing was observed in the leaves explants that cultured in MS basal medium devoid of any phytohormones. Different previous studies shows that the plant growth regulators have various effects on proliferation and plant development. The callus induction growth trend observed in *Rheum emodi* plant propagation resembles that previously described in investigations on other *Rheum* species.[12]

After sub culturing to 4-5 weeks, it was observed that the best *in vitro* shoot multiplication with sizable growth was obtained on the BAP (6 mg/L) in combination with Kinetin (2.5 mg/L). Three well-known cytokinin i.e., BAP, TDZ and Kn were tested to have a maximum multiplication rate in combination and alone.

Bakhtiar and coworker also tested the different cytokinins for the induction of multiple shooting in medicinal plant. [158] The maximum percentage of adventitious shoot generation was observed as 75.56 ± 0.27% and the highest number of shoots per explant was observed as 3.67 ± 0.27 with a mean length of 19.00 ± 1.70 mm. The frequency of shoot regeneration was decreased when treated with TDZ (Thidiazuron) alone. TDZ was found to be a weak cytokinin as compared to BAP and Kn for *in vitro* shooting when cultured on MS media. Sucrose at 3% and 4% in the MS medium gave the best results with 5-6 folds *invitro* shoot multiplication with maximum shoot number of 12.22 and maximum percentage recorded as 82.22 ± 0.72 with mean number of shoots as 20.33 ± 0.72mm. Past studies shows that the BAP alone and with the combination with the other cytokinin proves to be a better combination for the multiplication of shoots grown in MS media.[76] After 15 days, the shooted cultures that showed signs of rooting were transferred to new media and kept for up to 4 weeks. With a maximum frequency of roots per shoot of 5.0 ± 0.47 and an average root length of 11 ± 1.25mm, the high frequency of rooting was seen in MS basal medium (full strength) with IAA (Indole-3-acetic acid) of concentration of 5 mg/L in combination with BAP (2 mg/L).

For artificial or synthetic seed production, the MS media with 3-4 % of sodium alginate that were treated with the 50 mM $CaCl_2$ produced the circular shaped, coat firm enough for handling beads. This concentration of calcium chloride with 3%

sodium alginate allowed the artificial seeds to convert to the plantlets. Many previous studies shows that the 3% sodium alginate proves to the best concentration for producing firm, circular and suitably handed beads.[104] Previous studies were reported that show that the leaf explants were proved to be the better explant for inducing the somatic embryos.

Alone TDZ can be used for inducing the embryogenesis and somatic embryos. For the encapsulation of somatic embryos, the maximum conversion rate was seen in embryos that had been enclosed in 3% sodium alginate and then exposed for 30 minutes to calcium chloride at a concentration of 50 mM.[99] Similarly, Redenbaugh and coworkers reported the different concentration of sodium alginate that were needed for encapsulation.[165] Alike studies have been reported for the plants such as alfalfa and celery that suggest the maximum regeneration frequencies of the plantlets having optimal sodium alginate concentration as 3.2%.[166] Present study shows that the propagules have proliferative potential with survival % as 60.0 ± 1.2 on basal MS media with 3% alginate.

Encapsulated embryos were stored to 30 days or 60 days to test their survival and germination rate. The survival and germination rates of the encapsulated embryos dropped from 46% to 27% after being stored at 4°C when compared to encapsulated embryos that had no storage. The survival and germination rates of naked and encapsulated embryos are compared in experiments. Observed values of survival and germination frequencies are obtained after the storage of 20 days to 30 days after the 5 weeks of culture. The storage signifies the reduction in both the survival as well as the germination frequencies of naked and the encapsulated embryos in comparison to the embryos which have not been stored. The factor like oxygen shortage in the gel beads and its quick drying may be the cause of the fall in the germination percentage of the preserved encapsulation embryos.[99] The germination rate of encapsulated embryos as 44.4% is reduced to 22.3% when storage time was increased from 20 days to 30 days. Plantlets germinates through this were morphological identical to the mother plant and further cultured in *invitro* condition. This method is useful for those plants in which production of seeds is very less; viability of seeds is low or the seeds

are very expensive. These approaches could be used in the future for mass propagation of *Rheum emodi*. More studies and experiments should be performed for improved and better output. Development of medicinal plant sector should be done and dissemination of synthetic seeds should be promoted.

In the Present study two types of molecular markers have been employed to detect any occurrence of somaclonal variations. Between the mother plant and the invitro-raised plantlets, RAPD and ISSR analysis was studied to confirms genetic homogeneity of *in vitro* raised plantlets. The assessment of somaclonal variation or the genetic fidelity in regenerated plantlets is extremely important when the desired end product is true-to-type plants because genetic variation may arise in regenerants due to many factors related with the *in vitro* growing circumstances. PCR based markers like RAPD/ISSR involves *in-vitro* amplification of particular sequences by using thermostable DNA polymerase enzyme and specific oligonucleotide primers. To evaluate the genetic integrity of the regenerants, molecular markers like RAPD and ISSR are used as these markers use only a tiny amount of DNA sample, don't entail any radioactive labels, are quick, easy to handle, cost-effective, and very reliable procedures. The somaclonal variation in many therapeutic plants has been successfully detected using these markers. Previous studies suggested these molecular markers for examine the genetic stability and proved to be a better and cost-effective method to indicate good genetic fidelity among the regenerants.[167]

In the present study, the randomly selected regenerants of *Rheum emodi* were subjected to genetic fidelity analyses to ascertain the *in vitro* regenerants' true-to-type characteristics in with regard to the mother plant. For ascertaining the clonal fidelity, total genomic DNA was extracted from the regenerants and the mother plant by CTAB method. PCR components and conditions are optimized simultaneously and then 20 ISSR markers and 15 RAPD markers were assayed.

From the fifteen RAPD and twenty ISSR markers, Seven RAPD and fifteen ISSR primer sets produce 98 different amplicons that gives rise to the distinct, clear, monomorphic patterns among all the randomly selected regenerated plants that were examined. The results demonstrate that there is no polymorphism between the *in vitro*

raised plantlets and the mother plant in the RAPD and ISSR analysis. All of the markers produce monomorphic bands, and no variation was found among the micropropagated plants, which supports the genetic homogeneity of *in vitro* raised plantlets.

Identical research was also reported by Kumar and coworkers that suggest that the molecular markers such as RAPD and ISSR are suitable and appropriate for assessing the genetic fidelity among the *in vitro* regenerants.[111] Our findings imply that the *Rheum emodi* grown *in vitro* is a dependable alternative for producing a significant number of true-to-type regenerants.

These molecular markers based on DNA have served as adaptable tools in different field of biology. These markers could be proved to be an ideal tool for schedule analysis of genetic stability within the micropropagated plantlets prior to commercialization. As per our knowledge, this could be the primary report in state Uttarakhand that shows the employ of molecular markers to conduct the clonal fidelity of the regenerated plantlets of *Rheum emodi* by using RAPD and ISSR molecular markers. These molecular markers based on DNA have served as adaptable tools in different field of biology. These markers could be proved to be an ideal tool for schedule analysis of genetic stability within the micropropagated plantlets prior to commercialization.