CHAPTER 4: RESULTS

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4.1 Plant material

Juvenile, healthy and disease-free plants of *Rheum emodi* were selected and procured from the Pothivasa region in district Rudraprayag, Uttarakhand. The fully grown and seed staged plants were dried, an herbarium was made, and submitted to the Northern Regional Centre of the Botanical Survey of India in Dehradun for species-level identification. **(Fig. 4.1)**. The plants were identified as *Rheum emodi* Wall. of family polygonaceae **(Acc. No. 1145).**

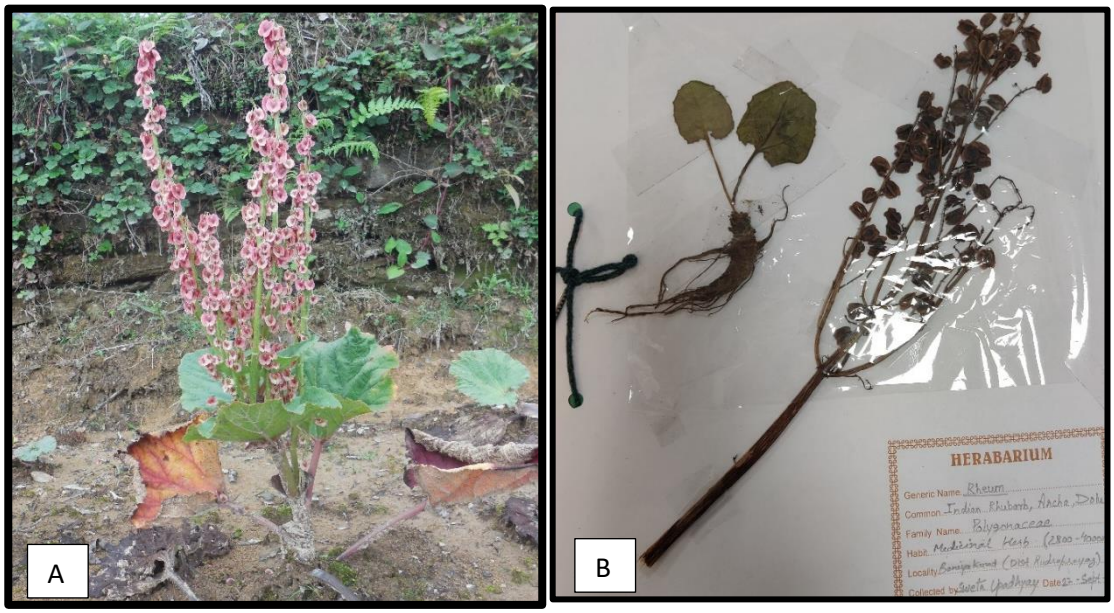

Fig. 4.1: Plant of *Rheum emodi* (A): Flowering stage; (B): Herbarium specimen of *Rheum emodi*

4.2 Sterilization

4.2.1 Explant sterilization

Juvenile *Rheum emodi* plants were washed with Tween 20 and then rinsed properly firstly with tap water and then with double distilled water for quite a few times **(Fig. 4.2)**. 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 **(Fig. 4.3)**. 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 **(Table. 4.1)**. 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.

Fig. 4.2: (A): Procured plant of *Rheum emodi*; (B): *Rheum emodi* plant (Rinsed and washed with Tween 20)

Table: 4.1: Different treatments of sterilizing agent and their responses to explants.
Where, A-J are the treatments with different exposure time of sterilant. Experiments were conducted in triplets.

Treatments	Plates	Contaminations per plate	Contamination %	Germination	Germination %
A	1	5	100	NO	0
	2	4	80	NO	0
	3	5	100	NO	0
B	1	4	80	1	20
	2	3	60	1	20
	3	4	80	NO	0
C	1	2	40	2	40
	2	4	80	NO	0
	3	3	60	NO	0
D	1	1	20	3	60
	2	NO	0	4	80
	3	2	40	2	40
E	1	3	60	2	40
	2	1	20	3	60
	3	2	40	1	20
F	1	NO	0	5	100
	2	NO	0	4	80
	3	1	20	3	60
G	1	1	20	3	60

	2	1	20	2	40
	3	NO	0	2	40
H	1	NO	0	2	40
	2	2	40	1	20
	3	1	20	1	40
I	1	NO	0	2	40
	2	1	20	2	40
	3	NO	0	NO	0
J	1	NO	0	1	20
	2	NO	0	NO	0
	3	NO	0	NO	0

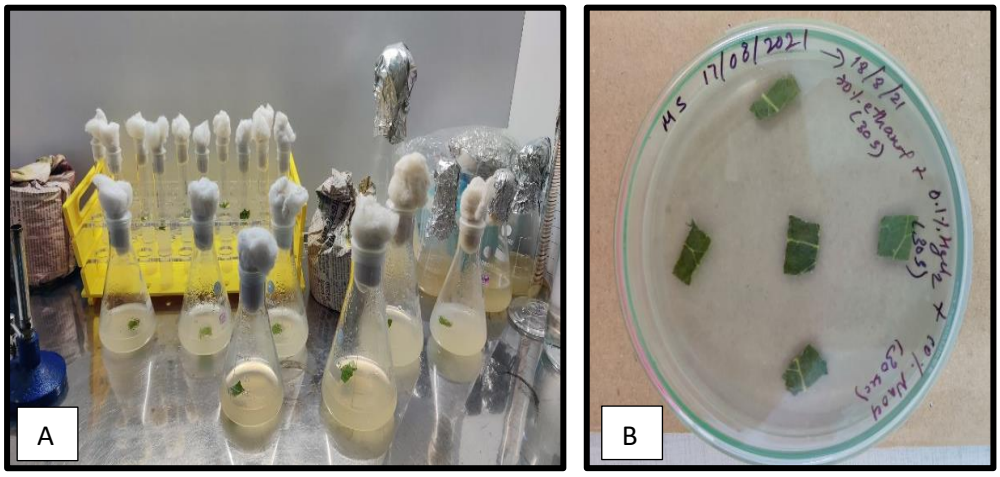

Fig: 4.3: (A), (B): Explant established in inoculating media after sterilization.

4.3 Micropropagation

4.3.1 Inoculation

After complete sterilization and blot dry of explants, leaves; they were selected and cut into pieces of about 2-3 cm and were used for callus initiation. Leaves having midrib were proved to be a better explant for callus initiation. Finally sterilized and selected explants were inoculated to medium and then cultures were kept in culture room under controlled environmental conditions **(Fig. 4.4)**.

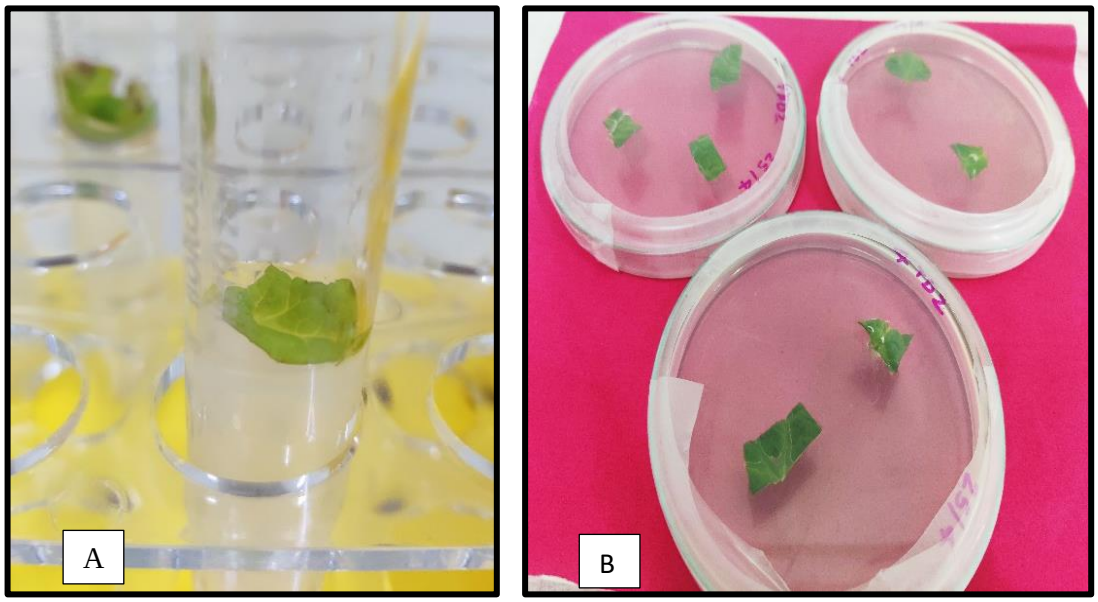

Fig. 4.4: (A), (B): Leaves as an explant inoculated in MS media; used for the callus induction

4.3.2 Callus induction

After the sterilization and inoculation of explant, experiments were conducted for the callus initiation on MS basal media with different auxin and cytokinin concentration. The results depicted that the callus forming response of leaves explants has increased when MS medium supplemented with BAP and 2,4-D **(Table. 4.2)**.

Among the different auxin's concentration tested, callusing was observed and the highest percentage of callus generation (84.44 ± 0.27 %) was observed in MS medium with 8.0 mg/L of 2,4-D in combination with BAP having concentration as 2.5 mg/L. Afterwards about 6 weeks, a fragile yellowish-brown callus was observed **(Fig 4.5)**. Thus, the result showed that the combination of auxin with cytokinin can be proved to be the most appropriate combination of hormones for inducing callus from the leaves explants of *R. emodi*. A high rate of callus induction (80.0 ± 0.47%) was detected from the leaves explants that were cultured on MS medium supplemented with of 6.0 mg/L of 2,4-D with 2.0 mg/L of BAP (cytokinin). Further, the decrease in callusing was observed in the leaves explants that cultured in MS basal medium devoid of any phytohormones. Nature of callus was also observed as fragile, yellowish-brown.

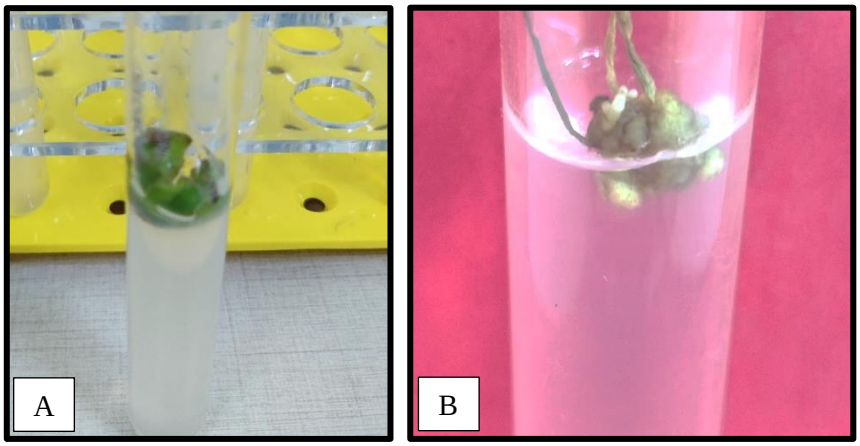

Fig. 4.5: (A) Curling of leaves explant; (B) Fragile yellowish- brown callus from the leaves explant of *Rheum emodi*

Table. 4.2: Effect of different combination and concentration of Auxins (Phytohormones) on the induction of callus from the leaves explant of *Rheum emodi.*

Means of values having the same letters in columns are not significantly different by the Duncan's multiple range tests (P = 0.05). A-T are the treatments of different combination of auxins.

Treatments	Phytohormones (PPM)			% Of Callus induction ± SE	Nature of callus
	2, 4-D	IBA	BAP		
A	2.0	-	1.0	60 ± 0.47^{cdef}	Yellowish brown
B	4.0	-	1.5	71.11 ± 0.54^{fg}	Yellowish brown
C	6.0	-	2.0	80 ± 0.47^{gh}	Friable yellowish
D	8.0	-	2.5	84.44 ± 0.27^{h}	Brownish yellow fragile
E	10.0	-	3.0	71.11 ± 0.27^{gf}	Yellowish brown fragile
F	-	2.0	1.0	53.33 ± 0.47^{bcd}	Fragile Yellow brown
G	-	4.0	1.5	60 ± 0.47^{cdef}	Yellowish-brown
H	-	6.0	2.0	71.11 ± 0.27^{fg}	Yellowish brown fragile
I	-	8.0	2.5	68.89 ± 0.27^{efg}	Yellowish brown fragile
J	-	10.0	3.0	64.44 ± 0.27^{def}	Yellowish brown
K	2.0	-	-	48.88 ± 0.27^{bc}	Yellow-brown fragile
L	4.0	-	-	57.7 ± 0.27^{cde}	Yellowish brown Friable

M	6.0	-	-	60 ± 0.94^{cdef}	Yellowish fragile
N	8.0	-	-	73.33 ± 0.47^{fgh}	Yellowish brown
O	10.0	-	-	64.44 ± 0.27^{def}	Yellowish brown fragile
P	-	2.0	-	31.11 ± 0.72^{a}	Brownish yellow fragile
Q	-	4.0	-	35.55 ± 0.27^{a}	Yellowish brown fragile
R	-	6.0	-	35.55 ± 0.27^{a}	Yellowish brown
S	-	8.0	-	42.22 ± 0.98^{ab}	Brownish fragile
T	-	10.0	-	51.11 ± 0.54^{bc}	Yellowish brown fragile

4.3.3 *In vitro* multiplication of shoots

The *in vitro* shoot multiplication was established by varying the combination and the concentrations of different plant hormones which are as follows:

4.3.3.1 Effect of cytokinins on *In vitro* shoot multiplication

The mother explants' proliferating callus cultures were removed, and they were then subcultured on semisolid MS media for further multiplication of shoots. The media was supplemented with the different cytokinins viz. BAP (6- Benzylaminopurine), TDZ (thidiazuron) and Kn (kinetin). The combination of these phytohormones with varying concentration were used to achieve the maximum shoot multiplication. After 15 days of callusing *in vitro* shoots were noticed. Further sub culturing to 3-4 weeks leads to the multiple shooting. Thidiazuron (TDZ) in the range of 2-8 mg/L were tested and the two other hormones kinetin (Kn) and 6- Benzylaminopurine (BAP) in different concentrations combination were tested; Kn in range of 1.5-3 mg/L and 6-BAP in the range of 2.0-8.0 mg/L. Control was used as hormone- free medium. No regeneration was observed in hormone–free medium.

The best shoot multiplication and sizeable development of *in vitro* shoots were obtained after four to five weeks of culturing on the plant growth hormones as BAP (6.0 mg/L) in combination with Kn (2.5 mg/L) **(Fig. 4.6).** The maximum frequency of adventitious shoot generation was observed as 75.56 ± 0.27% and the most shoots per explant was observed as 3.67 ± 0.27 **(Table. 4.3)** with a mean length of 19.00 ± 1.70 mm. The frequency of shoot regeneration was decreased when treated with TDZ alone. TDZ was found to be a weak cytokinin as compared to BAP and Kn for *invitro* shooting when cultured on MS media. MS media perforated with the phytohormones such as 4.0 mg/L BAP and 2.5 mg/L Kn without TDZ also gave the good number of shoots percentage (71.11 ± 0.27 %) with the mean number of shoots (2.33 ± 0.72) per explant were observed. The mean length of shoots was recorded as 10.33 ± 2.60 mm.

Table. 4.3: Effect of different cytokinins (treatments) on regenerative response of 6-week callus cultures derived from leaves explant of *R emodi*.
According to Duncan's multiple range test, the means of values with the same letter in columns are not statistically different (P = 0.05).

Phytohormones (PPM)			% Of Shoot Regeneration ± SE	Mean No. Of Shoots/Callus ± SE	Mean Shoot Length(mm)± SE
BAP	Kn	TDZ			
2.0	1.5	-	35.55 ± 0.27^{bc}	1.33 ± 0.54^{a}	6.00 ± 2.49^{abc}
4.0	1.5	-	53.33 ± 0.47^{defgh}	2.33 ± 0.27^{ab}	7.67 ± 1.78^{abcd}
6.0	1.5	-	55.56 ± 0.27^{efghi}	2.33 ± 0.27^{ab}	9.33 ± 2.76^{abce}
8.0	1.5	-	51.11 ± 0.27^{cdefgh}	1.33 ± 0.27^{a}	8.67 ± 1.09^{abc}
2.0	2.0	-	53.33 ± 0.82^{defgh}	1.00 ± 0.47^{a}	7.33 ± 3.03^{abc}
4.0	2.0	-	62.22 ± 0.27^{ghij}	1.67 ± 0.72^{ab}	10.33 ± 4.53^{abce}
6.0	2.0	-	66.67 ± 0.47^{hij}	2.00 ± 0.47^{ab}	16.67 ± 0.98^{e}
8.0	2.0	-	55.56 ± 0.27^{efghi}	1.67 ± 0.27^{ab}	9.33 ± 1.44^{abce}
2.0	2.5	-	48.896 ± 0.27^{cdefg}	2.00 ± 0.47^{ab}	10.67 ± 0.54^{abce}
4.0	2.5	-	71.11 ± 0.27^{ij}	2.33 ± 0.72^{ab}	10.33 ± 2.60^{abce}
6.0	2.5	-	75.56 ± 0.27^{j}	3.67 ± 0.27^{b}	19.00 ± 1.70^{e}
8.0	2.5	-	60.00 ± 0.47^{ghi}	2.33 ± 0.72^{ab}	14.00 ± 2.9^{bce}
2.0	3.0	-	44.44 ± 0.27^{bcdef}	1.67 ± 0.72^{ab}	9.00 ± 3.74^{abc}
4.0	3.0	-	46.67 ± 0.47^{bcdefg}	1.33 ± 0.27^{a}	15.33 ± 2.37^{ce}
6.0	3.0	-	48.89 ± 0.54^{cdefg}	2.00 ± 0.82^{ab}	12.33 ± 1.91^{abce}

8.0	3.0	-	42.22 ± 0.72^{bcde}	1.67 ± 0.27^{ab}	12.33 ± 1.19^{abce}
-	-	2.0	20.00 ± 1.25^{a}	1.33 ± 0.54^{a}	5.33 ± 2.37^{ab}
-	-	4.0	37.78 ± 0.72^{bcd}	1.00 ± 0.47^{a}	3.67 ± 1.52^{a}
-	-	6.0	31.11 ± 0.72^{ab}	0.67 ± 0.27^{a}	5.33 ± 2.37^{ab}
-	-	8.0	20.00 ± 1.25^{a}	0.67 ± 0.54^{a}	3.00 ± 2.45^{a}

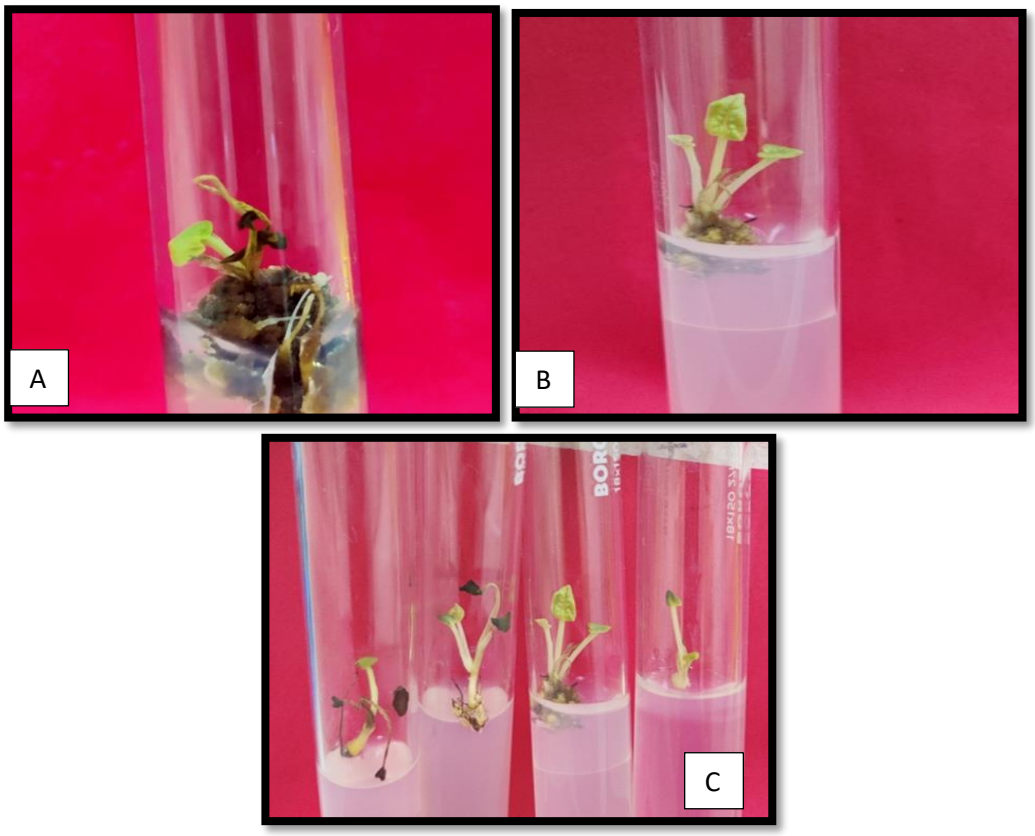

Fig. 4.6: (A), (B), (C): *In vitro* shoot regeneration with sizable development obtained on BAP with combination with kn.

4.3.3.2 Effect of sucrose concentration on *invitro* shoot multiplication.

For the growth and multiplication of *invitro* shoots, sucrose was found to be an important constituent of MS media that provides carbon and energy source. Different concentrations of sucrose ranges from 1-6 % in MS media were tested for *invitro* shoot multiplication with respect to the length measured in mm. At lower concentration of sucrose (1-2 %) 1-2-fold *in vitro* shoot multiplication was recorded with deprived growth of shoots. Increased percentage of sucrose ranges (5-6%) leads to retarded growth of shoots along with the yellowing of leaves and basal callusing of shoots. Sucrose at 3% and 4% in the MS medium gave the best results with 5-6 folds *invitro* shoot multiplication with maximum shoots number of 12.22. A graph represents mean shoot no. where the superscripts (a, b) shows that there is no significantly difference within the values (**Fig. 4.7).** Maximum percentage recorded as 82.22 ± 0.72 with mean number of shoots were recorded as 20.33 ± 0.72 mm **(Table. 4.4).** Experiments were conducted for 3 times; 15 explants were tested for each replication.

Table. 4.4: Effect of different sucrose concentration (treatments) on regenerative response of 6-week callus cultures derived from leaves explant of *R emodi*. According to Duncan's multiple range test, the means of values with the same letter in columns are not statistically different (P = 0.05).

S.no.	Treatments	Mean shoot no.	% Response ± SE	Mean length (mm) ± SE
A	MS + 1-2 %	9.33	62.22 ± 0.55^{a}	17.0 ± 0.48
B	MS + 3-4 %	12.33	82.22 ± 0.72^{b}	20.33 ± 0.72
C	MS + 5-6%	8.0	53.33 ± 0.47^{b}	13.66 ±1.08

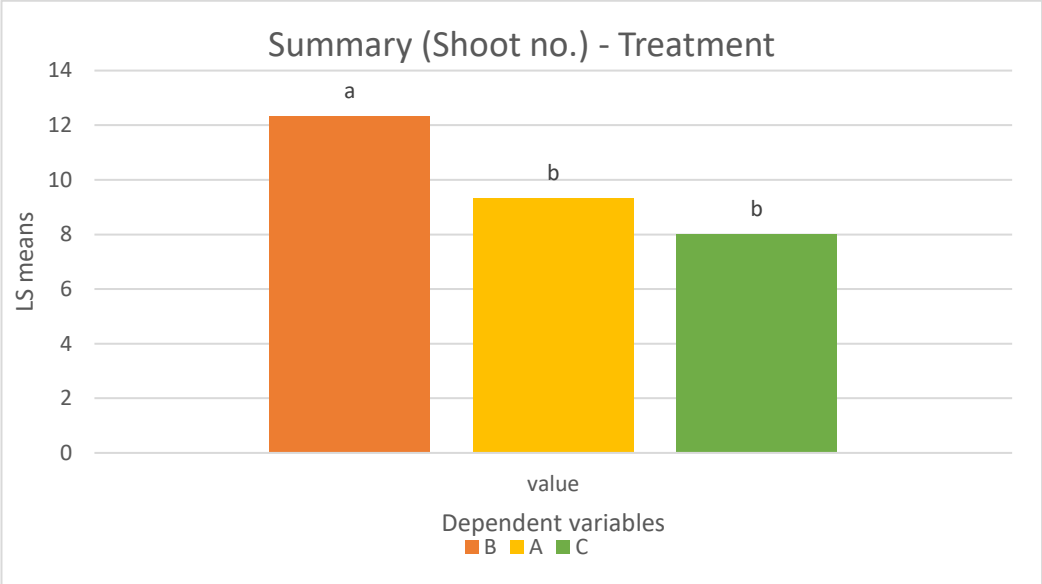

Fig. 4.7: Graph represents mean shoot no. the superscripts (a, b) shows that there is no significantly difference within the values

4.3.4 *In vitro* multiplication of roots

4.3.3.1 Effect of auxins on *in vitro* rooting

Different concentrations and combination of auxins with cytokinin were tested for *in vitro* rooting. Various concentrations of auxins; IBA (Indole- 3- butyric acid) and IAA (Indole-3-acetic acid) in half strength and full-strength MS media were directed to achieve the rooting. Initiation of rooting is an important step for the establishment of the tissue culture raised plantlets in the soil. Shootlets of about 2-3 cm were excised from the shoot cultures and were then transferred onto the rooting media which were hormone free half MS and full MS having the combination of IBA (2.5, 5.0 mg/L) and IAA (2.5, 5.0 mg/L) for about 3 to 4 weeks along with the BAP with a concentration range from 2.0, 4.0 mg/L.

After 15 days, the shooted cultures showing rooting were transferred to fresh media and maintained upto 4 weeks **(Fig. 4.8).** The high frequency of rooting was observed in MS basal medium (full strength) + IAA (5.0 mg/L) in combination with BAP (2.0 mg/L) with the maximum frequency of roots per shoot of 5.0 ± 0.47 with an average root length of 11 ± 1.25 mm **(Table. 4.5).**

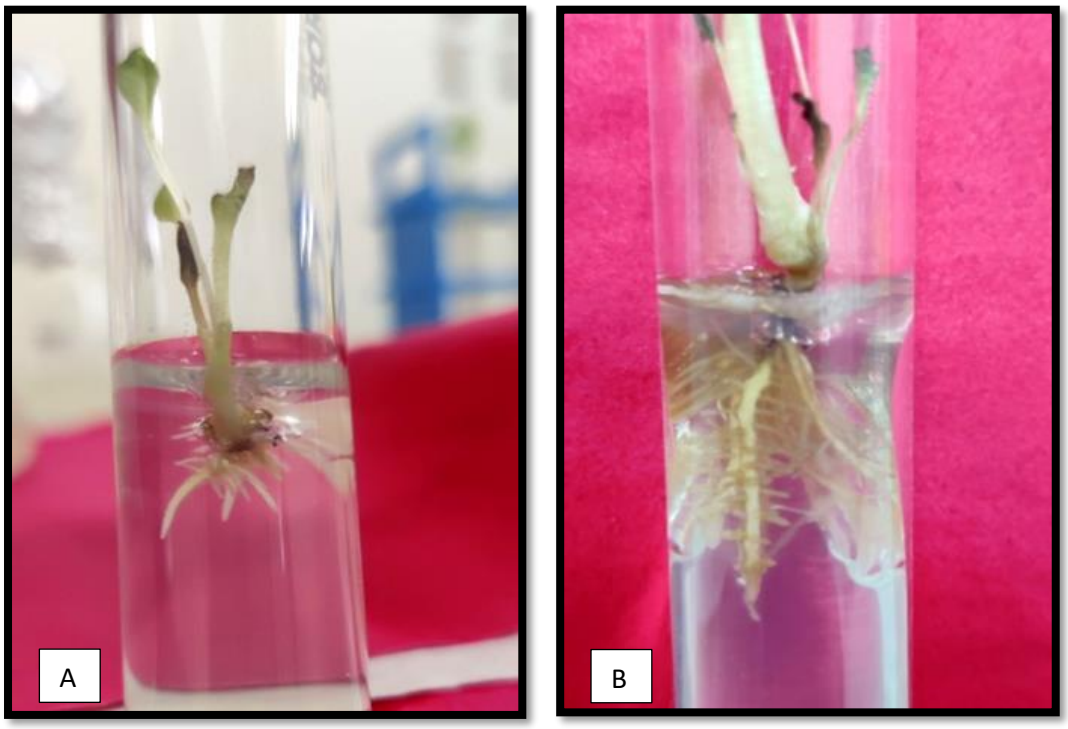

Fig. 4.8: Shooted cultures showing rooting were transferred to MS medium (full strength along with IAA (5.0 mg/L) + BAP (2.0mg/L); (A). Initiation of roots; (B). Roots maintained upto 4 weeks.

Table. 4.5: Effect of different concentration and combination of plant growth regulators (auxins) and strength of MS medium on rooting of adventitious shoots of *Rheum emodi*

Mean ± standard error values followed by the similar superscript letter within the column are not significantly different at level P ≤ 0.05, in accordance to Duncan's multiple range test.

S. no	Treatments (PPM)	No. of roots/ shoot Mean ± SE	Root length(mm) Mean ± SE
A	Half MS	1.33 ± 0.27 ^{a}	7.66 ± 0.54
B	Full MS	1.33 ± 0.27^{a}	9.33 ± 0.98
C	MS+IBA (2.5) + BAP (2)	3.0 ± 0.47^{b}	9.66 ± 0.98
D	MS+IBA (5) + BAP (2)	3.33 ± 0.27^{bc}	10.00 ± 0.47
E	MS+IBA (2.5) + BAP (4)	4.0 ± 0^{bcd}	10.0 ± 0.47
F	MS+IBA (5) + BAP (4)	4.33 ± 0.27 ^{cd}	11.0 ± 0.47
G	MS+IAA (2.5) + BAP (2)	4.33 ± 0.27^{cd}	10.66 ± 0.98
H	MS+IAA (5) + BAP (2)	5.0 ± 0.47^{d}	11.0 ± 1.25
I	MS+IAA (2.5) + BAP (4)	3.0 ± 0.47^{b}	11.33 ± 0.54
J	MS+IAA (5) + BAP (4)	4.33 ± 0.27^{cd}	10.66 ± 0.54

4.4 Hardening and field transfer

Before being transplanted to the field, in vitro rooted shoots must be hardened and acclimated. As the tissue cultured plantlets were heterotrophic in their mode of nutrition; they cannot survive with the environmental conditions without appropriate hardening and acclimatization. The seven weeks old plantlets that were directly

transfer to polybags without acclimatization have very low (5%) survival rate that too when transplanted in rainy season. In contrast, when plantlets were hardened and acclimated before being transferred to the field, a survival rate of 85–90% was achieved.

The rooted plantlets were carefully repositioned to the pots having sterilized soil mixed with the vermiculite in the ratio (1:1) and then the pots were covered with the poly-bags having minute holes and were then kept inside the culture room for acclimatization and *in vitro* hardening for about 15 days. They were then moved and kept back in a mist chamber with a relative humidity of 80 to 90 percent and temperature 25 ± 1°C and then placed in pots with a 1:1 ratio of soil to sand. The plants were moved to open shade house conditions after the mist chamber stage for additional acclimatization and hardening. During hardening the shoots elongated, leaves turned greener and expanded **(Fig. 4.9).** In shade house the plants were further transferred to bigger polybags and thereafter this stage the plants were field planted. 2-4 months old hardened and acclimatized plants were found to be the best for field transplantation.

Fig. 4.9: Hardening of *invitro* regenerated plantlets of *Rheum emodi*; (A) Pots covered with perforated polybags (B) Field transfer of the plantlets from polybags.

4.5 Synthetic seed production

Synthetic seeds are developed as alginate beads encapsulated with a single embryo in a protective coating that supports the mechanised handling. The hydrated encapsulated beads could only be stored by using low temperatures for days or weeks. The ability to prolonged storage of somatic embryos was attained when the dried and moisture content be less than 20%. The common definition of synthetic seed can as encapsulate somatic embryos which can functionally mimic the seeds and able to develop into seedings under the sterile and favourable environment. Differentiation of somatic embryos take place directly in the explant via callus.

4.5.1 Gelling agent for encapsulation

For the encapsulation of somatic embryos, different concentration percentage of sodium alginate (2.5 %, 3%, 4%) (w/v) in the MS basal solution. 5 weeks after the standard subculturing of callus, embryos were isolated by drawing them with suction dropper containing the sodium alginate solution. Foe the ease of capsule formation and low toxicity to the embryo, alginate was chosen as encapsulating agent. During handling, the fragile embryo is kept safe by the encasing gel beads' rigidity. The gel beads may act as a layer of protection and a storage area for nutrients, helping the somatic embryo survive and expand more quickly. The coating material and its concentration is an important limiting factor for the artificial seeds or synthetic seed technology. MS media with 3-4 % of sodium alginate with no agar produced the coat firm enough for handling, circular shaped beads when submerged in $CaCl_2$. The seeds were also able to develop into plantlets considering to this sodium alginate concentration **(Table. 4.6).**

4.5.2 Complexing agent

The somatic embryos encapsulated with the 3% sodium alginate were thrown down to calcium chloride with different concentration (25, 50 and 75 mM) (w/v) solution. Each 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. For the Control test, non-encapsulated embryos were tested. Further both encapsulated and non-encapsulated embryos were cultured in MS basal medium for evaluating their germination potential. 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 or syn seeds to convert to the plantlets **(Table.4.7).**

Table. 4.6: Germination and shooting response of different Na-Ag percentage with $CaCl_2$. Where (-) shows no response; (+) shows slightly responsive; (++) signifies mildly responsive; (+++) shows highly responsive.

S. no	Encapsulation matrix (Na-Ag %)	Treatments	Complexing agent ($CaCl_2$)	Bead appearance	Time/ exposure	Germination	Shooting
1	1-2%	A	25-50 mM	Irregular shaped	20-30 min.	-	-
		B	50-75 mM	Spongy	20-30 min.	-	-
		C	75-100 mM	Soft	20-30 min.	-	-
		D	25-50 mM	Soft and circular	20-30 min.	-	-

2	**2-3%**	**E**	**50-75 mM**	**Circular**	**20-30 min.**	**+ +**	**+ + +**
		F	**75-100 mM**	**Circular**	**20-30 min.**	**+**	**+ +**
3	**3-4%**	**G**	**25-50 mM**	**Circular and hard**	**20-30 min.**	**+**	**+**
		H	**50-75 mM**	**Circular and hard**	**20-30 min.**	**+ + +**	**+ + +**
		I	**75-100 mM**	**Circular and hard**	**20-30 min.**	**+**	**+ +**
4	**4-5%**	**J**	**25-50 mM**	**Hard**	**20-30 min.**	**+**	**+ +**
		K	**50-75 mM**	**Circular and too hard**	**20-30 min.**	**+**	**+ +**
		L	**75-100 mM**	**Too hard**	**20-30 min.**	**-**	**-**

Table 4.7: Effect of different concentrations / treatments of Na- Ag with $CaCl_2$ on Bead texture and their germination.
Treatments (A-L) are the combination of different concentration of sodium alginate with $CaCl_2$

Treatments	Nature/Texture of Beads	Germination	Observation
A	Irregularly Shaped	No Germination	Shoot bud turned brown
B	Irregular shaped	Brittle	Brown to Yellow
C	Too soft to handle	Slow Growth	Brown to Yellow
D	Circular and Soft	Slow Growth	Plantlet formed
E	Circular and firm	Germinated	Plantlet formed
F	Circular	Germinated	Plantlet formed
G	Circular	Slow germination	Plantlet formed
H	Circular/Smooth	Well germinated	Complete plantlet
I	Circular/Smooth	Delay in sprouting	Complete plantlet
J	Slightly Hard	Delay in sprouting	Shoot bud turned yellow
K	Hard	Failed to germinate	Basal callusing
L	Too Hard	Failed to germinate	Basal callusing

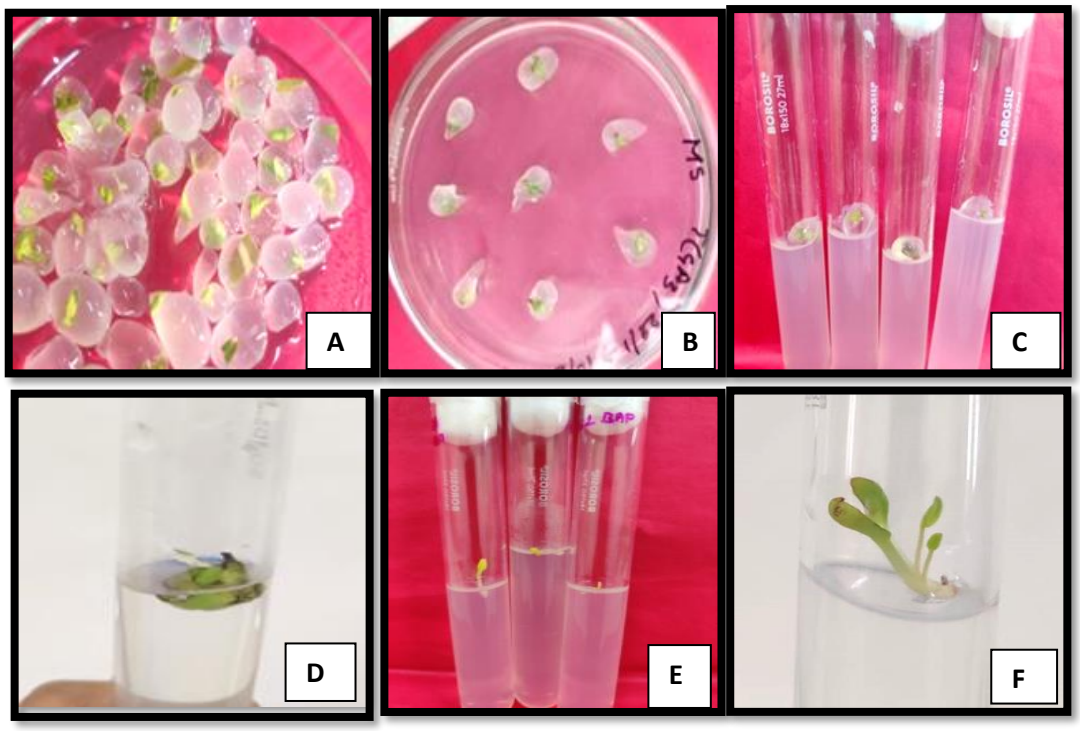

Fig. 4.10: Encapsulation and germination of Somatic embryos with 3% sodium alginate and 50 mM Calcium chloride (A), (B) Somatic embryos' beads prepared by 3% Sodium alginate; (C), (D) Germination of somatic embryos; (E), (F) regeneration in MS + BAP (8.0 mg/L) and Kn (2.5 mg/L).

4.5.3 Germination and survival of encapsulated somatic embryos

The somatic embryos that had been encapsulated in 3% sodium alginate (prepared in MS salt solution) were then placed in 50mM CaCl2 to create the well-shaped beads **(Fig. 4.10)** with an effective coat that would enable them to develop into plantlets. **Table. 4.8** shows the survival and the germination percentage of *R. emodi* capsulated and non- encapsulated somatic embryos. All the concentrations of sodium alginate allowed survival and germination of synthetic seeds but there is reduction of survival % (42.2 ± 0.2) as well as germination % (20 ± 0.4) as concentration of sodium alginate increases upto 4%. The highest germination frequency (33.3 ± 0.4) among the

encapsulated embryos was observed in 3% sodium alginate as capsule matrix. This concentration produced uniform, firm beads as compared to others that lead to the greater frequency of survival and germination.

The survival and germination response of *Rheum emodi* embryos that were enclosed in various sodium alginate concentrations are shown in the **Table. 4.8**. Non encapsulated somatic embryos cultivated in an aseptic, sterile environment served as the control and controlled conditions exhibits the survival percentage as 75.6 % and the germination ratios as 51.1%. All treatments allowed the survival of all beads but Synthetic seeds that were encapsulated in MS salt solution with 2.5% sodium alginate had a survival percentage of just 51.1%, compared to 60.0% for embryos that were encapsulated with sodium alginate of concentration 3%. The germination percentage of somatic embryos encapsulated with the sodium alginate (2.5%) was also reduced to 24.4%. The observed highest germination percentage of encapsulated embryos with 3% sodium alginate was 33.3% when transferred to the pots containing soil: vermiculite (2:1). Low sodium alginate concentrations prevented homogenous or sufficiently solid beads from forming, which lowers the frequency of germination.

Table. 4.8: Growth response of the encapsulated somatic embryos of *R. emodi* in diverse concentrations of the Sodium alginate. The non-encapsulated embryos as control.

[a] Values shows Mean ± Standard Errors of the three repeated experiments, and each experiment conduct with 15 embryos per treatment. According to Turkey's HSD test, within the column, superscript values that are tailed by the same letter are not statistically distinct at the level P=0.05.

Treatment	Capsule matrix	Survival [a] % Mean ± SE	Germination [a] % Mean ± SE
A	Control	75.6 ± 0.9	51.1 ± 0.5
B	MS Salt Solution + 2.5% alginate	51.1 ± 0.7	24.4 ± 0.7^{cd}

C	MS Salt Solution + 3% alginate	60.0 ± 1.2	33.3 ± 0.4^{bd}
D	MS Salt Solution + 4% alginate	42.2 ± 0.2	20.0 ± 0.4^{bc}

4.5.4 Storage experiment of encapsulated embryos

Encapsulated embryos were stored to 30 days or 60 days to test their germination rate. After storage at the temperature 4°C, the survival rate and the germination rate of the encapsulated embryos had a reduction from the 46% to 27% when compared to encapsulated embryos with no storage period. Table compares both the germination and the survival rate of naked as well as the encapsulated embryos. Observed values of survival and germination frequencies are obtained after the storage of 20 days to 30 days after the 5 weeks culture. The storage signifies the reduction in both the survival and germination percentage of naked and the encapsulated embryos in comparison to the embryos which have not been stored. The germination percentage of encapsulated embryos after the storage of 30 days (22.2%) was lower than the storage of 20 days (44.4%) **(Table 4.9).** Plantlets germinates through this were morphological identical to the mother plant and further cultured in *invitro* condition.

Table. 4.9: The survival and germination rate/frequencies of naked (control) and the encapsulated embryos after culturing for 5 weeks.

***Mentioned values are the Mean ± standard error of the separate three experiments; each of which consist of 30 embryos per treatment.**
[a]F – test: ***P= 0.0001
[b]Differences between the Means when subjected to Tukey's HSD test range at the 0.5% level.

Storage period(days)	Survival *(%)		Germination * (%)	
	Naked	Encapsulated	Naked	Encapsulated
without storage	57.8 ± 1.45	78.9 ± 1.33	37.8 ± 1.86	66.7 ± 0.58
20 days	46.7 ± 2.08	65.6 ± 0.88	21.1 ± 0.88	44.4 ± 1.67
30 days	27.8 ± 1.67	37.8 ± 0.88	6.7 ± 1.15	22.2 ± 0.88
Source of variance	F- test[a]	HSD test[b]	F- test [a]	HSD test[b]
Storage (A)	6.73***	7.61	11.78***	5.91
Encapsulation (B)	35.63***	4.57	34.28***	4.94
A*B	13.4***	5.43	10.49***	6.42

4.6 Molecular analysis

4.6.1 Isolation of Genomic DNA

PCR compatible DNA was isolated from *Rheum emodi* plants using the CTAB method. The method led to the effective extraction that produced DNA of high quality and quantity that was pure and free from impurities like proteins and RNA. Lysing cells and solubilizing DNA are the major steps in the DNA isolation process, which is followed by chemical removal of macromolecules, protein, lipids and RNA. Organic solvents such as chloroform, proteins are separated from nucleic acids using a mixture of chloroform and isoamyl alcohol, and the denatured proteins are then removed using centrifugation and washing procedures. RNA is removed from the genomic DNA samples by using the RNase A during the isolation procedure. RNase disrupted the phosphodiester bond between the 5'-ribose of nucleotide and the 3'-ribose of phosphate group. Among the regenerated plants of *Rheum emodi*, nine plants were randomly selected to isolate their genomic DNA. Subsequently genomic DNA was also isolated from the mother plant. Further these were compared using molecular markers. Isolated DNA was stored in –20 °C after dissolved in 200 µL TE buffer for further analysis.

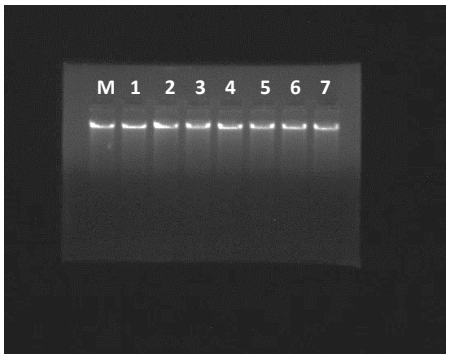

Fig. 4.11: Genomic DNA tested in 0.8% agarose gel. Lane M represents mother plant DNA; 1-7 represents the DNA isolated from the randomly selected regenerants of *Rheum emodi*

4.6.2 Qualitative and Quantitative estimation of Genomic DNA

For the qualitative analysis of isolated DNA, 0.8% agarose gel was prepared in 1X TAE buffer and DNA samples were tested by observing sharp bands **(Fig. 4.11)**. Ethidium bromide, an intercalating agent was used as a fluorescent tag. The stained agarose gel showed the presence of intact bands of genomic DNA. DNA sample that is free from any contamination such as phenols, proteins or RNA are said to be pure DNA. Purity was measured by observing the concentration by determining the absorbance using Spectrophotometer (Shimadzu spectrophotometer UV-1800). The purity and quantification of DNA samples was determined by measuring absorbance at A260/A280 nm. The ratio of the absorbance at 260/280 were assumed to be in the range of 1.7-1.9 for good quality and pure genomic DNA. The ratio below 1.8 signifies the contamination of proteins whereas the ratio above 1.8 signifies the presence of RNA contamination. All the DNA samples were further diluted according to the need of further experiments. The quantification analysis was carried out separately for all the selected invitro regenerated plantlets along with the mother plant. Results **(Table. 4.10)** were in the range of 1.7-1.9 that showed the good quality of genomic DNA that can be used further for assessing the genetic fidelity among the regenerated plantlets.

Table: 4.10: Observations to determine the purity and quantification of DNA samples isolated from randomly selected regenerants of *Rheum emodi*

Randomly selected plants	Absorbance at 260	Absorbance at 280	A260/A280	Concentration of DNA (µg/ml)
M	0.169	0.09	1.88	845
P1	0.11	0.06	1.83	550
P2	0.247	0.14	1.77	1235
P3	0.109	0.06	1.82	545
P4	0.124	0.07	1.77	620
P5	0.340	0.19	1.79	1700
P6	0.2013	0.11	1.83	1006
P7	0.169	0.09	1.88	845
P8	0.224	0.13	1.72	1120
P9	0.163	0.09	1.82	815

4.6.3 Optimization of PCR conditions for RAPD and ISSR analysis.

4.6.3.1 Optimization of genomic DNA concentration

As the PCR based analysis doesn't require a great amount of DNA, thus the concentration of DNA was measured by multiplying the absorbance at 260 with the 50µg/ml and dilution factor **(Table. 4.11)**. Further the PCR amplification was checked by using 10-50 ng/µl for genetic fidelity analysis.

Concentration of DNA(µg/mL) = OD_{260} × 50µg/mL × dilution factor

Where, A_{260} is the maximum absorbance of DNA sample

Conversion Factor A_{260} of 1.0 = 50µg/mL pure dsDNA

Table. 4.11: Concentration of DNA observed in randomly selected *invitro* regenerated plantlets of *Rheum emodi*

Randomly selected *invitro* regenerated plantlets	**Absorbance at 260**	**Concentration of DNA (µg/mL)**
M	0.169	845
P1	0.11	550
P2	0.247	1235
P3	0.109	545
P4	0.124	620
P5	0.340	1700
P6	0.2013	1006
P7	0.169	845
P8	0.224	1120
P9	0.163	815

4.6.3.2 Optimization of Primer concentration

Primer concentration that provides the specific and intense amplification of notable reproducible loci was observed at the concentration of 0.2 µM for the RAPD-PCR reaction. Lower concentration of primer results in low or no amplification and higher concentration contributes to the mispriming amplification. Lower concentration gave the faint bands resulting in low yield and smeared visualization. Thus, concentration of 0.2 µM was used while optimization the further components of RAPD analysis. For ISSR analysis, 0.4 µM concentration of primer was used.

4.6.3.3 Optimization of Taq polymerase

The concentrated stock of 5 U/µL of Taq polymerase was used to optimize the admirable results. From the 10 X concentrated stock, 2.0 µL of 1X working solution was optimized for the excellent results of RAPD and ISSR. The concentration more or less than the 2.0 µL of enzyme results in the smear and hazy background that results in no sharp bands. During the elongation steps of a PCR cycle, a suboptimal concentration of the enzyme (Taq polymerase) might result in inadequate elongation of the primer or an early termination of the synthesis of the PCR product.

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

Free dNTPs are required for DNA synthesis. dNTPs provide the nucleotide for the new strands. The four dNTPs (dATP, dGTP, dCTP, dTTP) are used at equimolar concentration to minimize the misincorporation errors. The 2.5 mM concentration of dNTPs was found to be the optimum concentration for maximum amplification in all RAPD and ISSR analysis that results in the sharp and clear bands. The optimized Mg^{++} concentration was observed at 3.0 mM in the RAPD analysis and 2.0 mM in the ISSR analysis that was needed to maintain the fidelity of taq polymerase. The reduced concentration of Mg^{++} resulted in low yield of PCR product, non-specific

amplification or no amplification of PCR product, while the higher amount of Mg^{++} resulted in the yield of non-specific products and promote the misincorporation.

After optimization of all the reaction components of RAPD **(Table 4.12)** and ISSR **(Table 4.13)** molecular marker, the total reaction volume was made to final volume of 20 µL.

Table. 4.12: Optimized reaction components of RAPD

S. no.	Component	Concentration	1X (µL)
1.	Taq Buffer	10 X	2.0
2.	$MgCl_2$	3mM	1.2
3.	dNTPs	0.2 mM	1.6
4.	Primer	0.2 µM	0.4
5.	Taq Polymerase	1unit/µL	0.18
6.	Template DNA	20 ng/ µL	2.0
7.	DNase free Water	To make up final volume	7.38
Total Reaction Volume			**20 µL**

The PCR amplification cycle consisted of one cycle of denaturation for 5 min at 95°C, 40 cycles of annealing and extension at 94°C for 1 min, and annealing temperatures appropriate to each primer used for 1 min at 72°C. The last extension took place for 8 minutes at 72 °C. The reaction mixture was held at 4°C until it was taken to be stored. The annealing temperature varied depending upon the primer used in every cycle.

Table. 4.13: Optimized reaction components of ISSR

S. no.	Component	Concentration	1X (µL)
1.	Taq Buffer	10 X	2.0
2.	$MgCl_2$	2 mM	0.8
3.	DNTPs	0.2 mM	1.6
4.	Primer	0.4 µM	0.8
5.	Taq Polymerase	0.60 unit/µL	0.15
6.	Template DNA	20 ng/ µL	2.0
7.	DNase free Water	To make up final volume	12.65
Total Reaction Volume			**20 µL**

The amplification cycle was composed of a denaturation cycle lasting 5 minutes at 94 degrees Celsius, followed by 40 cycles each lasting 94 degrees Celsius for 30 seconds, 72 degrees Celsius (extension) for 1 minute, and annealing temperatures appropriate to each primer utilized for 30 seconds. The reaction mixture was held at 4°C until it was taken to be stored.

4.6.3.5 Optimization of annealing temperature

Efficient annealing of oligonucleotide primer to the target sequences of separated DNA strands was found to be at 37°C for most of the RAPD primers **(Table. 4.14)**. After running with different temperature gradient (5°C below and above the T_m), annealing temperature was determined. Similarly, for ISSR primers, different temperature gradients were analysed and annealing temperature was determined for every primer **(Table. 4.15)**. After running repeated cycles that resulted in the

maximum number of PCR amplified products while any changes in the annealing temperature effects the reproducibility of the amplified product.

Table. 4.14: Annealing temperature of different screened RAPD primers

S. no	Primer code	Oligo Name	Sequence (5'- 3')	Tm (ºC)	Experimental Tm (ºC)
1	REi1	**OPP 13**	AAAGTGCCTC	23	No amplification
2	REi2	**OPA 09**	GGGTAACGCC	27	37
3	REi3	**OPB 09**	TGGGGGACTC	27	No amplification
4	REi4	**OPE 06**	AAGACCCCTC	25	37
5	REi5	**OPL 10**	TGGGAGATGG	25	No amplification
6	REi6	**OPL 19**	GAGTGGTGAC	25	37
7	REi7	**OPP 11**	AACGCGTCGG	27	30
8	REi8	**OPP 12**	AAGGGCGAGT	25	37
9	REi9	**OPA 17**	GACCGCTTGT	25	No amplification
10	REi10	**OPA 19**	CAAACGTCGG	25	37
11	REi11	**OPB 04**	GGACTGGAGT	25	37
12	REi12	**OPE 08**	TCACACGGT	25	No amplification
13	REi13	**OPD 04**	TCTGGTGAGG	25	
14	REi14	**OPD 09**	CTCTGGAGAC	25	
15	REi15	**OPP 05**	AGGGGTCTTG	25	

Table. 4.15: Annealing temperature of different screened ISSR primers

S. no	Primer code	Oligo Name	Sequence (5'-3')	Tm (ºC)	Experimental Tm (ºC)
1	IRr1	UBC 825	ACACACACACACACACT	49.3	No amplification
2	IRr2	UBC 836	AGAGAGAGAGAGAGAGCTA	49.3	49.3
3	IRr3	UBC 844	CTCTCTCTCTCTCTCTAGC	49.3	51.7
4	IRr4	UBC 816	CACACACACACACACAT	43.3	No amplification
5	IRr5	UBC 855	ACACACACACACACACCTT	49.7	No amplification
6	IRr6	UBC 834	AGAGAGAGAGAGAGAGCTT	51.1	52.7
7	IRr7	UBC 835	AGAGAGAGAGAGAGAGCTC	51.5	No amplification
8	IRr8	UBC 817	CACACACACACACACAA	52.8	No amplification
9	IRr9	UBC 842	GAGAGAGAGAGAGAGACTG	49.7	52.7
10	IRr10	UBC 811	GAGAGAGAGAGAGAGAC	43.3	43.3

11	IRr11	UBC 807	AGAGAGAGAGAGAGAGT	42.5	43.8
12	IRr12	UBC 808	AGAGAGAGAGAGAGAGC	46.8	47.1
13	IRr13	UBC 809	AGAGAGAGAGAGAGAGG	46.6	48.7
14	IRr14	UBC 868	GAAGAAGAAGAAGAAGAA	47.8	48.7
15	IRr15	UBC 840	GAGAGAGAGAGAGAGACTT	48.2	47.1
16	IRr16	UBC 841	GAGAGAGAGAGAGAGACGACTC	58.1	60.0
17	IRr17	UBC 889	AGTCGTAGTACACACACACACAC	59.2	59.2
18	IRr18	UBC 891	AGTACGAGTTGTGTGTGTGTG	62.7	64.0
19	IRr19	UBC 890	ACGAGTAGGGTGTGTGTGTGTGT	65.6	67.0
20	IRr20	UBC 888	CTGAGTCGTCACACACACACACA	67.6	67.0

4.6.4 Assessing the genetic fidelity through the molecular markers (RAPD and ISSR)

For assessing the clonal or genetic fidelity, nine aseptically regenerated *Rheum emodi* plants were chosen at random, along with a control plant (mother plant), and assessments were made using the RAPD and ISSR molecular markers.

Out of the 15 RAPD primers that were evaluated, seven yielded 32 repeatable, scoreable, and resolvable bands with the base pair length ranged from 300 to 2500 bp & the number of different bands that each primer set intended to achieve ranged from 02 (OPP11) to 07 (OPA19) with an average of 4.5 bands per primer **(Table. 4.16)**. All of the *in vitro* regenerated plants shared a similar DNA banding pattern and consistent across all seven RAPD markers, in respect to the mother plant, indicating the monomorphic pattern. Similarly, among the 20 ISSR marker screened. A total of 66 bands with lengths ranging from 300 to 2700 bp were generated by 15 primers. Each primer set has a variable number of bands that can be scored, ranging from 02 (UBC809) to 10 (UBC840) with an average of 4.4 bands per primer **(Table. 4.17)**. All the 15 ISSR markers shows scorable, resolvable, monomorphic banding pattern. Thus, the current investigation reveals that neither RAPD nor ISSR analysis found any polymorphism, this demonstrates the genetic stability of the plantlets grown through *in vitro* culture. **Fig. 4.12** shows the pattern of monomorphic amplification of *in vitro* generated *Rheum emodi* plantlets obtained with RAPD primer OPE06 and OPL19, and ISSR primer UBC889 and UBC841 **(Fig. 4.13)**.

Table. 4.16: *Rheum emodi* micropropagated plantlets' genetic stability was evaluated using RAPD primers.

Primer Code	Oligo name	Primer Sequence (5´-3´)	No. of total bands	Range of amplification(bp)
REi2	OPA09	GGGTAACGCC	06	300-1300
REi4	OPE06	AAGACCCCTC	05	600-2000
REi6	OPL19	GAGTGGTGAC	03	600-1600
REi7	OPP11	AACGCGTCGG	02	1600-1800

REi8	OPP12	AAGGGCGAGT	06	600-1200
REi10	OPA19	CAAACGTCGG	07	550-3000
REi11	OPB04	GGACTGGAGT	03	1300-2500

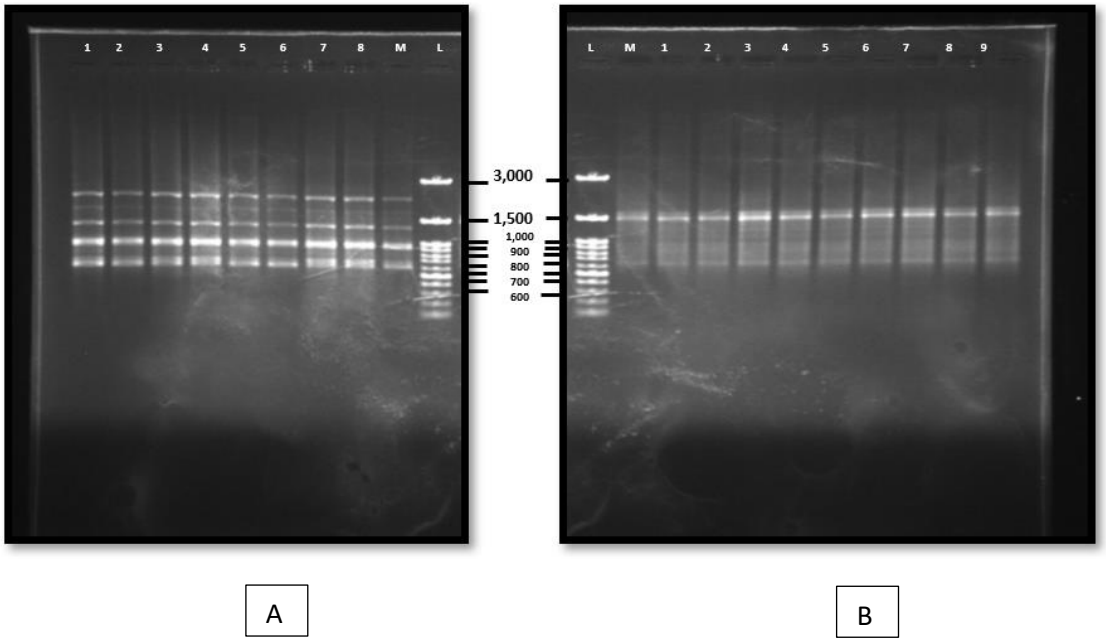

Fig. 4.12: RAPD profiles of the *invitro* regenerated plants and mother plant of *Rheum emodi*. (A): PCR Product obtained by PCR amplification with RAPD-OPE06 (5'-AAGACCCCTC-3'); (B) RAPD-OPL19 (5'-GAGTGGTGAC-3') primer. Lane L signifies 100 bp DNA ladder (Himedia); Lane M shows PCR product of mother plant's genomic DNA; Lane 1-9 shows PCR product of randomly selected micropropagated plants.

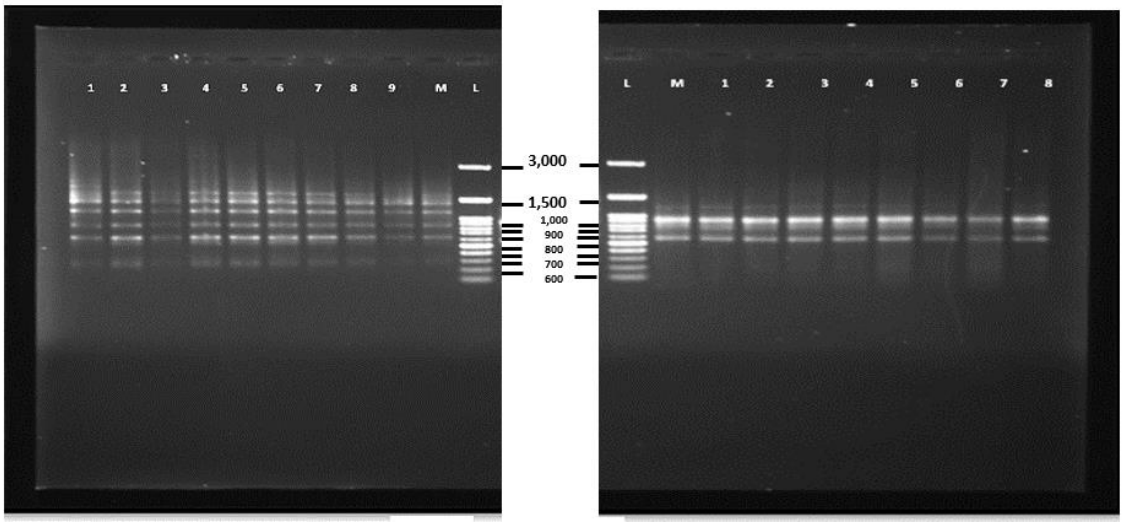

Fig. 4.13: ISSR profiles of the *in vitro* regenerated plants and mother plant of *Rheum emodi*. (A) The results of PCR amplification using ISSR - UBC889 (5'-AGTCGTAGTACACACACACACAC-3'); (B) PCR amplification products obtained with ISSR- UBC841 (5'-GAGAGAGAGAGAGAGACGACTC-3') primer.
Lane L denotes the 100 bp DNA ladder (Himedia); Lane M denotes the mother plant's PCR product; Lane 1-9 denotes PCR product of randomly selected micropropagated plants.

Table. 4.17: *Rheum emodi* micropropagated plantlets' genetic stability was evaluated using ISSR primers.

Primer Code	Oligo name	Primer Sequence (5´-3´)	No. of total bands	Range of amplification (bp)
IRr2	UBC836	AGAGAGAGAGAGAGAGCTA	03	650-1400
IRr3	UBC844	CTCTCTCTCTCTCTCTAGC	01	900
IRr6	UBC834	AGAGAGAGAGAGAGAGCTT	04	500-1200

IRr9	UBC842	GAGAGAGAGAGAGAGACTG	04	650-1500
IRr10	UBC811	GAGAGAGAGAGAGAGAC	06	550-1800
IRr11	UBC807	AGAGAGAGAGAGAGAGT	06	650-1700
IRr12	UBC808	AGAGAGAGAGAGAGAGC	04	1100-2000
IRr13	UBC809	AGAGAGAGAGAGAGAGG	02	700-1500
IRr14	UBC868	GAAGAAGAAGAAGAAGAA	03	550-1700
IRr15	UBC840	GAGAGAGAGAGAGAGACTT	10	550-2700
IRr16	UBC841	GAGAGAGAGAGAGAGACGACTC	04	600-1200
IRr17	UBC889	AGTCGTAGTACACACACACACAC	06	300-1600
IRr18	UBC891	AGTACGAGTTGTGTGTGTGTGTG	03	350-800
IRr19	UBC890	ACGAGTAGGGTGTGTGTGTGTGT	04	450-1600
IRr20	UBC888	CTGAGTCGTCACACACACACACA	06	350-2000