M. P. BIOTECHNOLOGY COUNCIL RESEARCH PROJECT

FINAL TECHNICAL REPORT (2010-2013)

Ref. No.	:	110/P-5/2010
Date of sanction	:	03.03.2010 to 02.03.2013
Title of the Project	:	Studies on i*n vitro* clonal propagation of anti-diabetic tree Marorphali (*Helicteres isora*) and evaluation of genetic fidelity through RAPD analysis.
Commencement of Project	:	01.08.2010
Duration of Project	:	3 years
Principal investigator	:	Prof. Y. K. Bansal Department of Biological Science R. D. University, Jabalpur
Co-Investigator	:	Nil
Total amount sanctioned	:	Rs. 5,52,000/-
Grant received in 1st year	:	Rs. 2,28,000/-
Grant received in 2nd year	:	Rs. 1,62,000/-
Grant received in 3rd year	:	Rs. 1,62,000/-

Details of Expenditure:-

S.No	Item	1st Year		2nd Year		3rd Year	
		Amount Sanctioned	**Amount Utilized**	**Amount Sanctioned**	**Amount Utilized**	**Amount Sanctioned**	**Amount Utilized**
A.	**Non Recurring**						
	Castor Rack	66,000	66,000	-	-	-	-
B.	**Recurring**						
1	Fellowship	72,000	72,000	72,000	72,000	72,000	72,000
2	Consumables	60,000	59,946	60,000	60,000	60,000	59,826
3	Travel	5,000	5,000	5,000	5,000	5,000	1,500
4	Other Cost	25,000	25,000	25,000	25,000	25,000	25,000
	Total	**2,28,000**	**2,28,000**	**1,62,000**	**1,62,000**	**1,62,000**	**1,58,326/-**

9. Whether there is any deviation from the purpose for which: Grant was released. If so detail of amount to be given: NA

OBJECTIVES OF THE PROJECT

1. Collection of elite plant material.
2. Axillary bud/apical bud elongation under different nutritional, media & PGR conditions.
3. Adventitious shoot morphogenesis from juvenile as well as mature explants.
4. Induction of somatic embryogenesis.
5. Shoot elongation (steps 2&3) & development of shoot buds.
6. Development of somatic embryos (step 4).
7. Rooting of regenerated shoots & whole plant formation.
8. Hardening and acclimatization.
9. RAPD analysis of *in vitro* regenerated plants.

Give details of the activities carried out during the research tenure:

Details of individual activities:

DESCRIPTION OF *HELICTERES ISORA* L.

Kingdom : Plantae

Phylum : Magnoliophyta

Class : Magnoliopsida

Order : Malvales

Family : Sterculiaceae

Subfamily : Helicteroideae

Genus : *Helicteres*

Species : *isora*

Helicteres isora belonging to the family Sterculiaceae is one of the economically important medicinal plants. It is a deciduous shrub or tree, 4-5m tall with gray bark. The plant generally occurs throughout India to Nepal & Sri Lanka. It flowers between July & September & fruiting occurs between Oct-March.

Its root and bark are expectorant, demulcent, constipant & lactifuge & is also used in colic, scabies, emphysema, gastropathy, diarrohoea & dysentery (Pohocha & Grampurohit 2001) and also used in curing snake bite. The root extract has also been found to be very effective in controlling diabetes (Jagadhesan *et al.* 2001), (Kumar *et al.* 2006), lowering cholesterol & reducing blood glucose levels (Venkatesh *et al.* 2007).

Due to habitat loss and deforestation in recent years its availability is declining rapidly (Yakandawala *et al*. 2006). The difficulty in propagating this plant through seeds is yet another factor responsible for its infrequent occurrence. There is an urgent need to save this plant by propagating it in large numbers and one of the method by which it can be achieved is through tissue culture.

Tissue culture work on this plant would enable the continued availability of such an enormously important medicinal tree in form of seedlings in nurseries for ready use wherever required. RAPD analysis of the *in vitro* raised plantlets will also provide an insight into the genetic makeup of this plant and will also help to ascertain any somaclonal variation if any in the *in vitro* raised plantlets as compared to the nature grown plants.

MATERIALS AND METHODS

Survey was carried out in different forests and urban areas around Jabalpur. Superior plants were selected on the basis of certain attributes like plant height, width of the trunk, dense branching, etc. Plants growing in natural habitats grew up-to an average height of 3-4 meters, within a period of 2-3 years. Young plants attained a trunk width of 3-4 inches within one year itself and by 3 years it attained a width of 9-10 inches. The plant flowered between June-September and produced pods between October-December. Pods were collected and its average length and weight of 5 pods were recorded. Mature pods were collected from the superior trees and were later dried and germinated during the months of July-September.

The experimental material of Marorphali (*Helicteres isora* L.) was obtained from the wild in and around Jabalpur (23°9’38″ North latitude and 79°56′19″ East longitude). Mature explants were collected from the nature grown plants. Juvenile explants were procured from seedlings obtained from germinated seeds. Mature seeds were collected from wild in the months of Oct-March and were germinated between the months of June-Sept. The seeds along with its coat were soaked for 2-3 days in water. The water was drained and seeds along with its seed coat were dried for a day. It was observed that the twisted capsule unwound itself and all its seeds were shed naturally.

The seeds were then again soaked for a day in water. Finally the seeds were transferred to soil for germination. The seeds germinated after 6-7 days but only 3-5% of germinated seeds formed healthy seedling after 8-10 days. Apical buds from the seedlings were used as explants for various treatments (Figs. 6-9).

The seedling derived explants viz. apical buds of 3-4 mm were first cleaned with a soft brush and then thoroughly washed under running tap water for 1-2 hrs. They were moistened with 1% labolene solution (5 min) followed by tap water wash. The explants were transferred to the laminar air flow and rinsed with 70% ethanol for 60 sec after which they were washed with autoclaved distilled water 2-3 times. Finally the explants were surface sterilized with 0.1% mercuric chloride for 60 secs. The explants were subsequently washed with autoclaved distilled water for 2-3 times. The explants were dried on sterile filter paper and inoculated onto MS (Murashige and Skoog's medium) supplemented with different concentrations (0.1, 0.5, 1.0, 5.0 mg/l) of cytokinins (BAP and KN alone and in combination), auxins (2,4-D, IAA, IBA, NAA) for determining the role of best possible PGR for initiation of morphogenetic response. The pH of the media was adjusted between 5.6-5.8 before adding agar (0.8%). The media was sterilized at 1.054 kg/cm^2 pressure for 15 min by autoclaving. Cultures were incubated at 25±2°C at photoperiodic cycle of 16 hr light (approx. 1500 lux) and 8 hr dark. All cultures were sub-cultured on fresh medium at every four week interval. 12 explants were taken for each treatment and the procedure was repeated at least thrice.

MOLECULAR ANALYSIS

The regenerated plants from different sources were assessed for its genetic integrity using marker (RAPD). The DNA was isolated from nature grown, direct(ly) and indirect(ly) regenerated plantlets by CTAB method (Murray and Thompson 1980). The isolated DNA was subsequently analysed for its purity by spectrophotometric analysis and qualitative analysis was confirmed by gel electrophoresis. Finally RAPD amplification of the all the samples were performed to screen for possible polymorphism among them.

REAGENTS REQUIRED FOR DNA ISOLATION

EXTRACTION BUFFER:

〉 **Stock Solution:**

– 1M Tris-HCl (pH 8.0)
– 0.5M EDTA (pH 8.0)
– 3M NaCl (pH 8.0)
– 2% (w/v) CTAB (Cetyltrimethylammonium bromide)
– 0.2% (v/v) β-mercaptoethanol
– 1% (w/v) PVP (Polyvinylpyrrolidone)

} Extraction Buffer

〉 2mM Sodium acetate (pH 5.0) (pH adjusted with glacial acetic acid)
〉 Tris saturated Phenol: Chloroform: Isoamyl alcohol (25:24:1)
〉 Chloroform: Isoamyl alcohol (24:1)
〉 Absolute Isopropanol
〉 Absolute Ethanol
〉 70% Ethanol
〉 RNAse A in TE (10mg/ml)
〉 TE Buffer
〉 Liquid nitrogen

Preparation of stock solutions required for DNA extraction

1. **100ml 1M Tris-HCl (pH 8.0)**

   Tris is an abbreviation of the organic compound Tris hydroxymethyl aminomethane with a molecular formula $(HOCH2)_3CNH_2$. The molecular weight of Tris salt is 121.14. For preparing 100ml of 1M Tris-HCl solution 12.114gm Tris was weighed and dissolved in 80ml of double distilled water in a conical flask. The pH of the solution was adjusted to 8.0 by drop wise addition of conc. HCl. After pH adjustment, the solution was made up to 100ml with double distilled water and autoclaved.

2. **50ml 0.5M EDTA (pH 8.0)**

   EDTA stands for ethylenediaminetetracetic acid having the molecular formula $[(HO_2CCH_2)2NCH_2CH_2N(CH_2CO_2H)]$. The molecular weight of EDTA is 292.25. For preparing 50ml of 0.5M EDTA solution 7.306 gm EDTA was weighed and dissolved in 30ml of double distilled water in a conical flask. The pH of the solution was adjusted to

8.0 by drop wise addition of 10N NaOH. After pH adjustment, the solution was made up to 50ml with double distilled water and autoclaved.

3. **50 ml 3M NaCl**

   Molecular weight of NaCl is 58.44. 8.7gm NaCl was weighed and dissolved in 50 ml double distilled water and autoclaved.

4. **50ml 2mM Sodium acetate (pH 5.0)**

   Molecular formula of sodium acetate is $C_2H_3NaO_2$ having a molecular weight of 82.0. 8.2mg of sodium acetate was weighed and dissolved in 30ml double distilled water in a conical flask. The pH of the solution was adjusted to 5.0 by drop wise addition of glacial acetic acid. After pH adjustment, the solution was made up to 50ml with double distilled water and autoclaved.

5. **Preparation of TE buffer**

   Required amount of TE buffer was prepared with 10mM Tris-HCl (pH 8.0) and 1mM EDTA (pH 8.0).

Working Concentration of Extraction Buffer:

100mM Tris HCl, 25mM EDTA, 1.5M NaCl, 2% CTAB, 0.2% (v/v) β-mercaptoethanol (added immediately before use) 1% (w/v) PVP (added immediately before use).

Measured amount of stock solutions were transferred to a measuring cylinder and the final volume was made to 50ml with sterile double distilled water. The extraction buffer was made fresh before use.

Preparation of Tris saturated phenol:

1. Solid phenol crystals was taken in a measuring cylinder wrapped with aluminium foil and kept on a water bath (65°C) for melting the crystals.
2. Measured volume of phenol (100 ml) was transferred to an amber coloured bottle.
3. To the phenol, 99ml of 1M Tris-HCl (pH 8.0) was added and kept on a magnetic stirrer for 1 hr.
4. Upper Tris-HCl layer was removed with a sterile glass pipette.
5. In a separate flask 50 ml of DD water was taken, of which few ml was poured into the bottle and stirred for another 10-15min.

6. Upper Tris-HCl layer was pipetted out again and few ml of DD water was poured into the bottle. (This procedure was repeated to replace 50ml Tris-HCl solution with DD water).
7. In another sterilized conical flask 100ml of DD water was mixed with 1ml of 1M Tris-HCl and 200µl of 0.5M EDTA.
8. This solution was poured into the amber coloured bottle having phenol.
9. A pinch of 8-hydroxyquinoline was added in the solution.
10. The solution was kept for overnight stirring on a magnetic stirrer.
11. The solution was kept at 4°C in a bottle wrapped with black paper and kept in dark.

Preparation of Phenol-Chloroform-Isoamyl alcohol solution (25:24:1)

For 100ml of solution, 50ml of Tris saturated phenol was transferred in a brown bottle. To it 48ml of chloroform and 2ml of isoamyl alcohol was mixed. Solution was stored at 4°C.

Preparation of Chloroform-Isoamyl alcohol solution (24:1)

For 100ml of solution, 98ml of Tris chloroform and 2ml of isoamyl alcohol was transferred to a brown bottle and mixed. Solution was stored at 4°C.

Preparation of Rnase A Solution

Reagents required for RNase A buffer:

Stock Conc	Conc Required
500 mM NaCl	15mM
1 M Tris Cl	10 mM
Double distilled water	

100mg Rnase A was dissolved in few ml of RNase A (SRL, India) and made up to 10 ml. It was stored at -20°C.

Equipments required:

Cooling Centrifuge, water bath, mortar pestle (pre-chilled), 10ml polypropylene centrifuge tube, 1.5 ml eppendorf tube, pipettemann, pipette tips (1ml, 200µl, 10µl), UV-Spectrophotometer and sterilized hand gloves.

Sterilization of Equipments:

Mortar pestle, measuring cylinder, glass pipette, eppendorf tubes and pipette tips (1ml, 200µl, 10µl) were autoclaved at 15lb/inch^2, 121°C for 15mins.

DNA EXTRACTION PROTOCOL:

Extraction:

1. Approx. 1g of fresh leaf was ground to a fine powder with liquid nitrogen in a mortar pestle.
2. The powder was transferred to an autoclaved 10ml polypropylene tube and mixed with extraction buffer.
3. The mixture was incubated at (65°C) for 1 hour with intermittent shaking.
4. An equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed by gentle inversions for about a minute.
5. It was centrifuged at 15000rpm for 10 minutes at 4°C.
6. The upper aqueous phase was transferred to a fresh polypropylene tube and 2/3 volumes of ice-cold isopropanaol was added and mixed by gentle inversion for 2 minutes.
7. DNA was precipitated by incubating for overnight at -20°C.
8. It was centrifuged at 5000 rpm for 10 minutes at 4°C.
9. Supernatant was discarded and the pellet was washed with 500µl 70% chilled ethanol.
10. It was centrifuged at 10000rpm for 10 minutes at 4°C. This step was repeated 3-4 times.
11. The pellet was air-dried at room temperature. The pellet was re-suspended in 100 µl TE buffer.

Purification:

1. To the TE (Step 11 of DNA Extraction) 10µl of DNase free RNase A (10mg/ml) was added and incubated at 37°C for 1 hr.
2. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed by gentle inversions for 2 minutes.
3. It was centrifuged at 10000rpm for 10 minutes at 4°C.
4. The upper phase was transferred to a fresh eppendorf tube and the extraction was repeated twice.
5. To the supernatant, 1/10 volume of 3M sodium acetate and 2/3 volume of chilled ethanol was added and mixed by gentle inversion. It was incubated at -20°C for 20 minutes to precipitate DNA.
6. It was centrifuged at 10000rpm for 10 minutes at 4°C.
7. The pellet was washed with 1ml chilled 70% ethanol, thrice.
8. The pellet was air dried and stored in 100 µl TE buffer and stored at -20°C.

Characterization of Isolated DNA:

Quantification: Isolated DNA was quantified using a UV-spectrophotometer. 2990µl TE was taken in a quartz cuvette and 10µl DNA solution was added to it. The optical density (absorbance

A) was taken at 260nm (A_{260}) and 280nm (A_{280}). The amount of DNA present in the solution was calculated from absorption at 260nm (A_{260}) and the purity of DNA was calculated by ($A_{260/280}$) (Sambrook *et al*. 2001). For an ideal DNA preparation the $A_{260/280}$ ratio should be ≥1.8.

Formula for calculating the DNA concentration:

DNA Conc. = Spectral reading (A_{260}) X 50µg/ml X Dilution factor

(For double stranded DNA, O.D. corresponds to 1.0 containing 50µg/ml of DNA.)

Dilution factor = $\frac{\text{2990µl TE buffer + 10 µl DNA}}{\text{50µl}}$

Qualitative analysis of isolated DNA:

The quality of the extracted DNA was analysed by running the DNA in 0.8% agarose gel.

Running buffer (1X TBE)= Stock solution of 1L 10X TBE buffer contains 54g Tris base, 27.5g boric acid and 20 ml 0.5 M EDTA. The pH was adjusted with conc. HCl to 8.0 and final volume was made up to the required amount with distilled water. The buffer was autoclaved and stored at room temperature.

For preparation of 1L of 1X TBE, 100 ml of 10X TBE was mixed with 900 ml of distilled water.

Gel loading dye

Pre mixed 6X Gel loading dye (Genei Pvt Ltd.) was used at 1X conc. for loading of DNA samples.

Gel staining dye

Ethidium bromide (EB) solution (HiMedia, India) at a concentration of 10mg/ml was used. It was stored in dark at 4°C.

Gel casting

The isolated DNA was analysed by running in 0.8% agarose gel in 1X TBE running buffer. For preparing the gel the required amount of agarose was weighed and dissolved in required amount of 1X TBE buffer. The agarose was melted in a microwave oven. The gel tray was kept in the casting tray and the comb was properly placed. When the agarose gel was down to about 55°C, ethidium bromide (EB) was added to a final concentration of 0.5µg/ml. The gel solution was then mixed thoroughly by a gentle swirl and the molten gel was poured immediately. The whole

gel was kept undisturbed until the gel solidified. The gel tray was removed from the casting tray and the comb was removed carefully upwards and the gel was placed in the gel running tank.

Gel electrophoresis

The gel running tank was filled with 1X TBE buffer so as to immerse the gel completely. 5µl DNA was mixed with 5µl bromophenol blue dye. This 10µl mix was loaded on each well. The gel was run at a voltage of 50V till the dye travels out of the well and then it was run on a constant voltage of 100V till the dye front reaches ¾ distance on the gel. The gel was examined under ultraviolet transilluminator and photographed using Gel Documentation system.

PCR Amplification

Reagents required: PCR reaction for RAPD was carried out using the following list of reagents:

1. **Assay Buffer (10X):** 100mM Tris-HCl (pH 9.0), 500mM KCl and 0.01% gelatin (w/v), without MgCl2 was diluted to 1X during PCR mix preparation.
2. **dNTP mix**: dNTP was supplied as 10mM stock. It was diluted to 2mM as working standard with PCR grade water.
3. **$MgCl_2$**: 25mM $MgCl_2$ stock solution was supplied which was diluted to 1.5mM as working standard with PCR grade water.
4. **Taq DNA polymerase**: Taq DNA polymerase of 5U/µl strength without gelatine was used at a conc of 1U/ µl in all PCR reactions.
5. **Primers**: All the primers (oligos) for RAPD were supplied as lyophilized powder. The primer hydration was performed by adding PCR grade water so as to make a 100pmol/µl conc.
6. **PCR grade water**

Equipments required: Thermocycler, PCR tubes, pippetmann, pipette tips, eppendorf tubes.

RAPD-PCR Reaction

RAPD amplification was performed with extracted and purified genomic DNA from nature grown plant, direct (clonal) propagated plant and indirect (adventitious origin) plants of *Helicteres isora*. Each RAPD reaction mixture (25µl) contained 20ng purified genomic DNA (template), 2.5µl of 1X PCR Assay buffer, 0.5µl of 10mM dNTP mix, 1.5µl of 25mM MgCl2, 1.5U Taq DNA polymerase (5U/µl) and 0.75µl of 10µM/L primer stock (final conc. 0.5µM/L). The final volume of 25µl was made up with PCR grade water.

PCR Cycle conditions:

94°C	**94°C**	**38°C**	**72°C**	**94°C**	**45°C**	**72°C**	**72°C**	**4°C**
5 min	45 sec	1 min	1.5 min	45 sec	1 min	1 min	10 min	∞
	10 cycles			35 cycles				

Analysis of PCR Products by agarose gel electrophoresis

PCR amplified products were visualized by running the PCR products in 1.8% agarose gel in 1X TBE running buffer. A low range DNA ruler containing fragments (3000, 2500, 2000, 1500, 1000, 600, 300, 200, 100bp) was used as known molecular weight marker. Rest of the gel electrophoresis was carried out as described earlier.

Data Scoring and statistical treatments:

Reproducible, clear and unambiguous amplified fragments of RAPD analysis were selected and scored for data analysis. Each scorable band was treated as marker. As RAPD is a dominant marker system, binary scoring of the gel was performed. RAPD amplification fragments between 100-3000bp indicated presence (1) or absence (0) of band irrespective of band intensity, since each PCR product of identical molecular weight was supposed to represent a single locus. The data analysis was based on Jaccard's coefficient for binary data via XLSTSAT software package ver. 2009.06.01. Analysis utilized the Sequential Agglomerative Hierarchical Non-overlapping (SAHN) clustering programme of XLSTAT to construct an UPGMA (Unweighted Pair Group Method of Arithmetic Mean) phenogram. A phenogram for RAPD data was generated. Similarity coefficient generated from Jaccard's coefficient was used to construct squared data matrix with Principle Component Analysis (PCA) via. XLSTAT.

RESULTS

1. To survey, identify and collect elite plus tree material from different forest/urban sites within and around Jabalpur.

In the present study a survey was carried out in and around Jabalpur for the selection of superior phenotypes of *Helicteres isora*. The superior plants were selected with certain attributes like plant height, width of the trunk, dense branching, etc (Table- 1). The trunk of superior trees of *Helicteres isora* was straight, cylindrical and smooth. The plants studied from several habitats showed emergence of several branches from the base of the plant. The plants growing in natural conditions grew upto an average height of 6-7 feet. The leaves were broad with serrated margins and flowers red in colour with reflexed petals. Flowering took place during July- September and

the fruiting occurred in the months of October to December. The leaf shedding started from early January and persists till late March (Figs. 1-5).

Table-1 Description of individual *Helicteres isora* L. trees studied in different areas of Jabalpur (M.P.)

S.No	Plant height (in Ft)	Diameter at base (In Inches)	Crown	Avg. length of 5 pods	Wt of 5 pods
1.	4	3.5	Narrow	3.5	3.15
2.	3.5	3	Narrow	3.5	3.45
3.	3	4	Narrow	4.0	3.23
4.	7	4	Narrow	0	0
5.	6	4.5	Narrow	3.8	3.725
6.	4.5	3	Narrow	5.0	3.76
7.	8	3.5	Narrow	No pod	No pod
8.	7.5	3.25	Narrow	No pod	No pod
9.	9	4.25	Narrow	No pod	No pod
10.	8	4.5	Narrow	No pod	No pod
11.	6	5	Narrow	No pod	No pod
12.	4	2.5	Narrow	No pod	No pod
13.	4	3	Narrow	No pod	No pod
14.	4	3	Narrow	No pod	No pod
15.	5	3.5	Narrow	No pod	No pod
16.	6	4	Narrow	No pod	No pod
17.	5	4	Narrow	No pod	No pod
18.	5.5	3	Narrow	No pod	No pod
19.	5.5	3.25	Narrow	No pod	No pod
20.	5.5	3.5	Narrow	No pod	No pod
21.	5	3	Narrow	No pod	No pod
22.	7	5	Narrow	No pod	No pod
23.	4.5	3.5	Narrow	No pod	No pod
24.	4.5	3	Narrow	No pod	No pod
25.	3.5	4.5	Moderate	No pod	No pod
26.	3.75	5	Moderate	No pod	No pod
27.	4	4.5	Moderate	No pod	No pod
28.	6	4	Moderate	No pod	No pod
29.	5.5	3.5	Narrow	3.0	3.98
30.	5	3.5	Narrow	4.0	4.67
31.	4	4	Moderate	3.0	4.258
32.	3.5	4	Moderate	5.0	3.572
33.	4	3	Moderate	No pod	No pod
34.	4	5	Moderate	No pod	No pod
35.	4	4	Moderate	No pod	No pod

36.	5	3	Moderate	No pod	No pod
37.	3.5	4	Moderate	No pod	No pod
38.	4	3.5	Narrow	No pod	No pod
39.	3.5	3	Narrow	No pod	No pod
40.	8	4	Narrow	No pod	No pod
41.	4.5	4	Narrow	3.8	4.71
42.	5	4	Narrow	4.0	4.44

S.No	BM	FSI (%)	MSN	MSL (in cm)
43.	4	4	Moderate	4.0	4.055
44.	4	3.5	Moderate	4.0	3.15
45.	3	3.5	Narrow	3.8	3.45
46.	3.5	4	Narrow	No pod	No pod
47.	3.5	4	Narrow	No pod	No pod
48.	4	4	Narrow	No pod	No pod
49.	3	4	Moderate	No pod	No pod
50.	6	4	Moderate	No pod	No pod
51.	7	4.5	Moderate	No pod	No pod
52.	5	5	Moderate	No pod	No pod
53.	4	4.5	Moderate	No pod	No pod
54.	8	4.5	Moderate	No pod	No pod
55.	5	6.5	Moderate	4.0	4.01
56.	5	6.5	Moderate	4.0	4.305
57.	5	3	Moderate	5.0	3.23
58.	10	6.5	Moderate	3.8	4.8
59.	11	6.5	Moderate	4.2	3.98
60.	10	6	Moderate	4.8	4.59
61.	**15**	**8.5**	**Dense**	**5.6**	**3.57**
62.	**14**	**7**	**Dense**	**5.8**	**4.79**
63.	9	5	Moderate	5.0	4.52
64.	10	5	Moderate	5.8	3.51
65.	10	5	Moderate	4.8	3.44
66.	**15**	**17.5**	**Dense**	**6.2**	**5.204**
Avg	**5.9**	**4.35**		**4.21**	**3.61**

Different types of morphogenetic responses were observed in presence of cytokinins and auxins. MS medium devoid of PGR's i.e. Basal Medium (BM) was used as control (Table 2). The shoot regeneration on basal medium was poor and the plants exhibited poor elongation response.

Table-2 Effect of Basal Medium (BM) on shoot differentiation in apical bud explants of *Helicteres isora*

1	Control	58.33	0.66	0.99

FSI- Frequency of shoot initiation
MSN- Mean shoot number
MSL- Mean shoot length

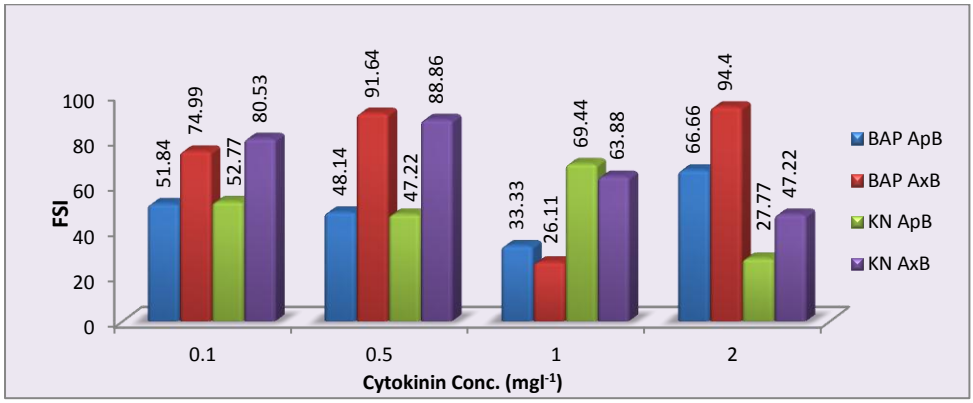
2. Axillary bud/apical bud elongation under different nutritional, media & PGR conditions

2a. Effect of cytokinin (BAP & KN) alone:

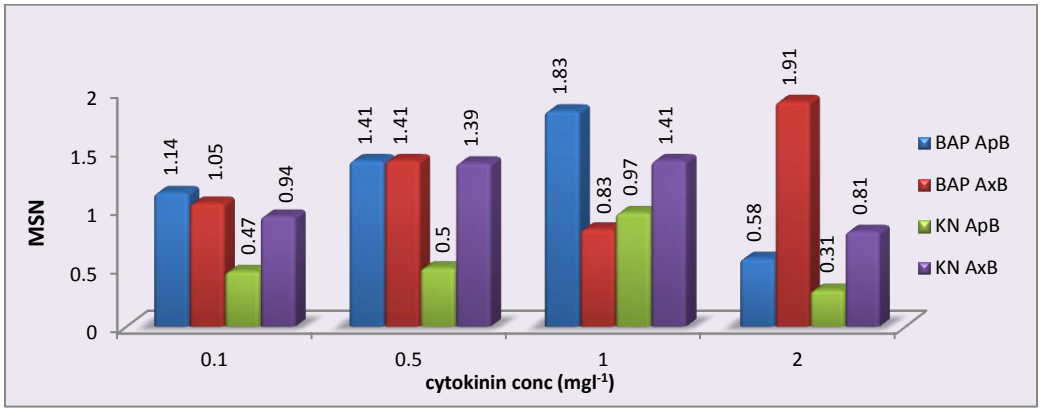
The effect of two cytokinins viz. BAP and KN at four concentrations, two explants viz. apical bud and axillary bud and their interactions was studied on the frequency of shoot initiation (FSI), mean shoot number (MSN) and mean shoot length (MSL) on **Murashige and Skoog's Medium (MSM)**.

Maximum FSI of 94.4 and MSN of 1.91 were obtained on BAP (2 mgl^{-1}) from AxB explant respectively. Shoot initiation started within 3-4 days from ApB explants and within 5-6 days from AxB explants. Shoots bore healthy leaves with predominantly 2 shoots being produced from AxB but only a single shoot from ApB explants. Also the maximum MSL of 1.35 was observed on KN (1mgl^{-1}) from ApB explants. Medium supplemented with KN produced single shoot only (Graphs 1-3; Figs. 10-22)

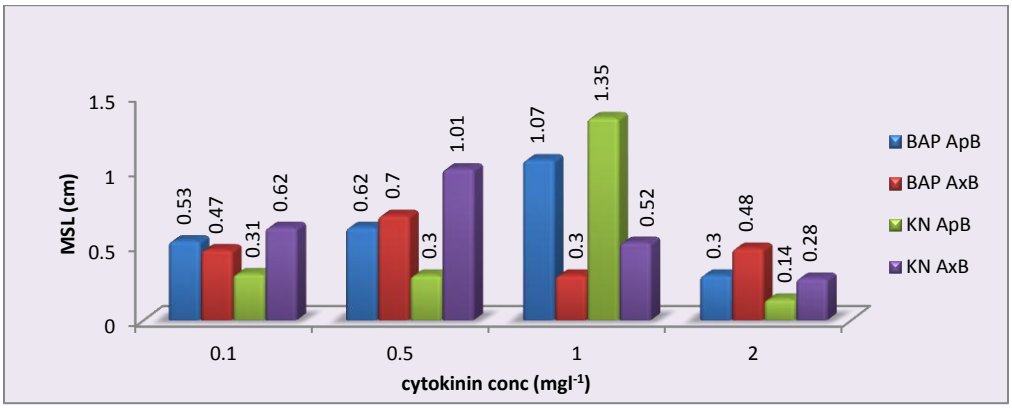
Graph 1. Effect of cytokinins alone (BAP & KN) on frequency of shoot initiation (FSI) in *Helicteres isora* L. on MS medium (MSM)



Graph 2 Effect of cytokinins on Mean shoot number (MSN) in *Helicteres isora* L. on MS medium (MSM)



Graph 3 Effect of cytokinins on Mean shoot length (MSL) in *Helicteres isora* L. on MS medium (MSM)



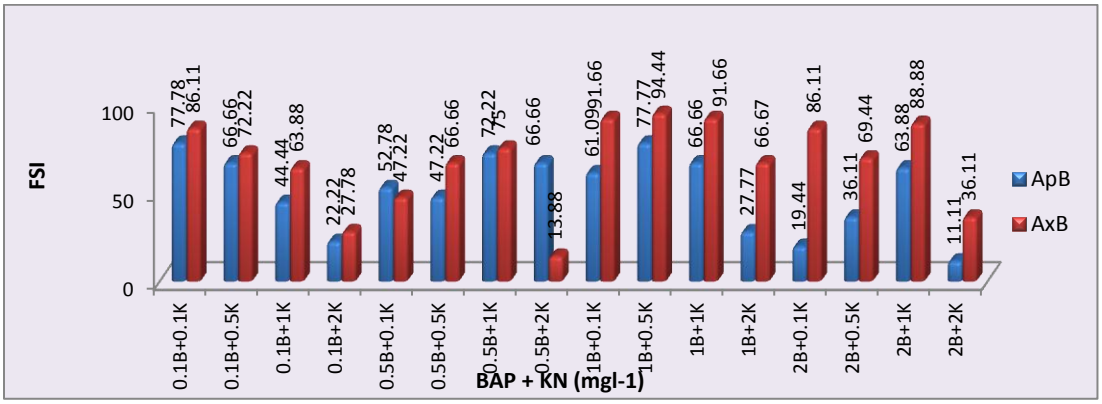
Callus formation was observed at the base of regenerated shoots within 3 weeks under all cytokinin treatments. Friable to compact green callus was obtained on BAP supplemented medium, while creamish brown basal callus with occasional rooting was observed under KN supplemented medium.

2b. Effect of cytokinin (BAP+KN) combination:

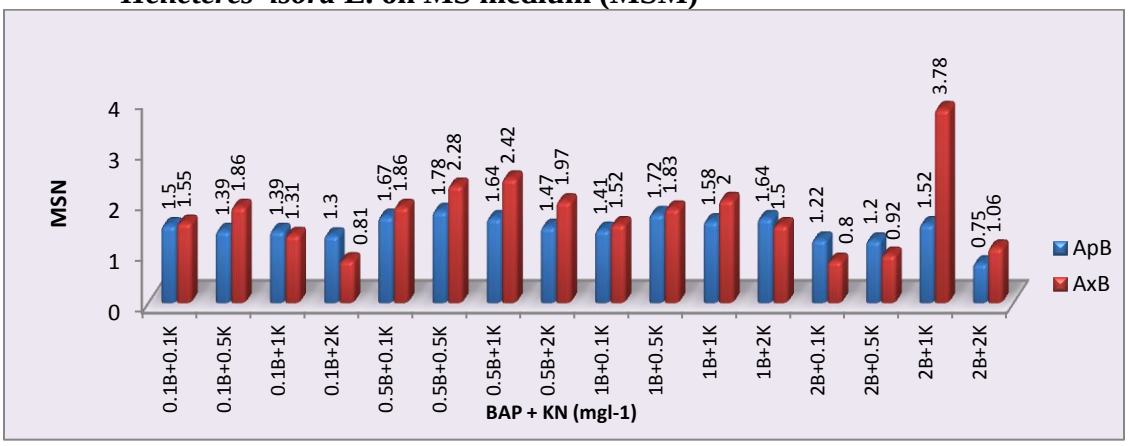
Cytokinin combination was significant for FSI, MSN and MSL. As compared to the cytokinin alone treatments the combination of cytokinins had a remarkable effect on all the three parameters. Shoot initiation started within 2-3 days from ApB and within 4-5 days from AxB explants respectively. Multiple shoots (1-2) sprouted from AxB present on each node. Maximum FSI of 94.44 was observed from AxB explants on BAP ($1mgl^{-1}$) + KN ($0.5mgl^{-1}$). Maximum shoot number of 3.78 was obtained from AxB explants on BAP ($2mgl^{-1}$) + KN ($1mgl^{-1}$). At this conc. the multiple shoots produced had small lengths with short internodes. Maximum MSL of

3.04 was, however, obtained with ApB explants on BAP (0.5mgl^{-1}) + KN (1mgl^{-1}) Graphs 4-6; Figs. 23-34).

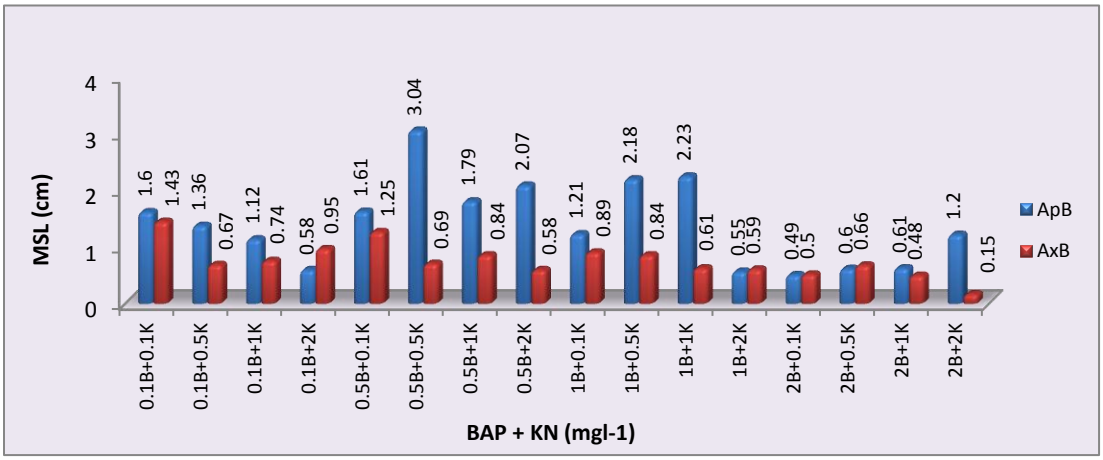
Graph 4 Effect of cytokinin combination (BAP+KN) on Frequency of shoot initiation (FSI) in *Helicteres isora* L. on MS medium (MSM)



Graph 5 Effect of cytokinin combination (BAP+KN) on Mean shoot number (MSN) in *Helicteres isora* L. on MS medium (MSM)



Graph 6 Effect of cytokinin combination (BAP+KN) on Mean shoot length (MSL) in *Helicteres isora* L. on MS medium (MSM)



2c. Effect of auxins alone:

This experiment was conducted to study the effect of different auxins on the two explants viz. ApB and AxB. None of the three auxins studied induced regeneration and instead led to variable degree of callusing. Callusing was observed on all the concentrations with degree of callusing increasing with the concentration of auxin (Table 3; Figs. 35-43).

2,4-D

Lower to moderate concs. of 2,4-D supplemented medium produced high degree of callusing. The callus produced was soft, sticky and light cream coloured. High conc of auxin produced dark brown calli. However, none of the calli turned out to be organogenetic.

NAA

Low to high concs. of NAA induced callusing. The callus produced was compact, creamish green coloured. None of the calli showed organogenesis at any concentration.

Table 3. Effect of Auxins on degree and type of callus from various explants in *Helicteres isora* L. on MS medium (MSM)

Auxin (mgl^{-1})		ApB		AxB	
		DC	Texture	DC	Texture
2,4-D	0.1	++	S,C	++	S,C
	0.5	++	S,C	+++	S,C
	1.0	+++	S,C	+++	S,C
	5.0	+++	S,B	+++	S,B
NAA	0.1	++	Fr, B	+	Fr, G
	0.5	+++	Fr, YG	++	H, YG
	1.0	+++	Fr, YG	+++	H, YG
	5.0	+++	H, B	+++	H, B
IBA	0.1	+	Fr, Y	+	Fr, CB
	0.5	++	Fr, YB	++	Fr, YG
	1.0	+++	H, G	+++	H, YB
	5.0	++	H, B	+++	H, B
IAA	0.1	+	H, Y	+	H, CB
	0.5	++	H, YG	++	H, YG
	1.0	+++	H, YB	+++	H, YG
	5.0	+++	H, YB	+++	H, B

S C= Soft Creamy	S B=Soft Brown	Fr B=Friable Brown	Fr G=Friable Green
Fr Y=Friable Yellow	Fr Y G=Friable Yellow Green	Fr C B=Friable Creamy Brown	H G=Hard Green
H Y G=Hard Yellow Green	H Y B=Hard Yellow Brown	H C B= Hard Creamy Brown	H B=Hard Brown

IBA

Lower to higher concentrations of IBA produced variable degrees and types of calli. Soft, light creamish brown calli were produced at lower concentrations while dark brown calli were observed at higher concentrations.

IAA

Different concentrations produced different types of calli. Low ($0.1mgl^{1}$) concentrations of IAA produced soft, friable, creamish green calli while higher ($>1mgl^{-1}$) concentrations produced hard, light to dark brown calli. No organogenesis was observed at any of the concentrations studied.

3. Adventitious shoot morphogenesis from juvenile as well as mature explants.

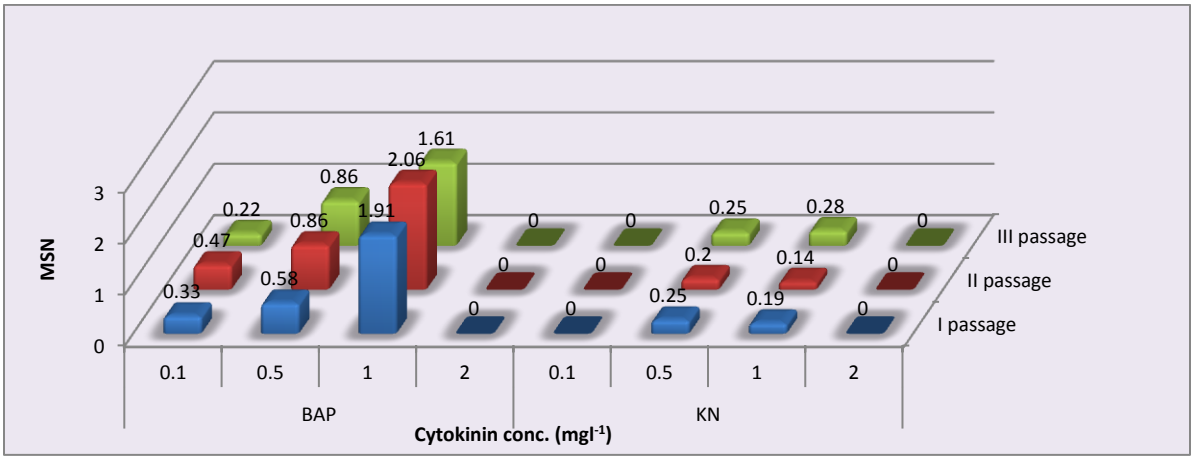
3a. Effect of cytokinin (BAP & KN) alone on indirect organogenesis:

Experiments were carried out to study the effect of four concentrations (0.1, 0.5, 1.0, $2.0mgl^{-1}$) of cytokinins BAP and KN (alone) on the adventitious organogenesis from callus. The shoots obtained were subjected to elongation and rooting. The remaining calli were sub-cultured on to the same medium in further subculture passages.

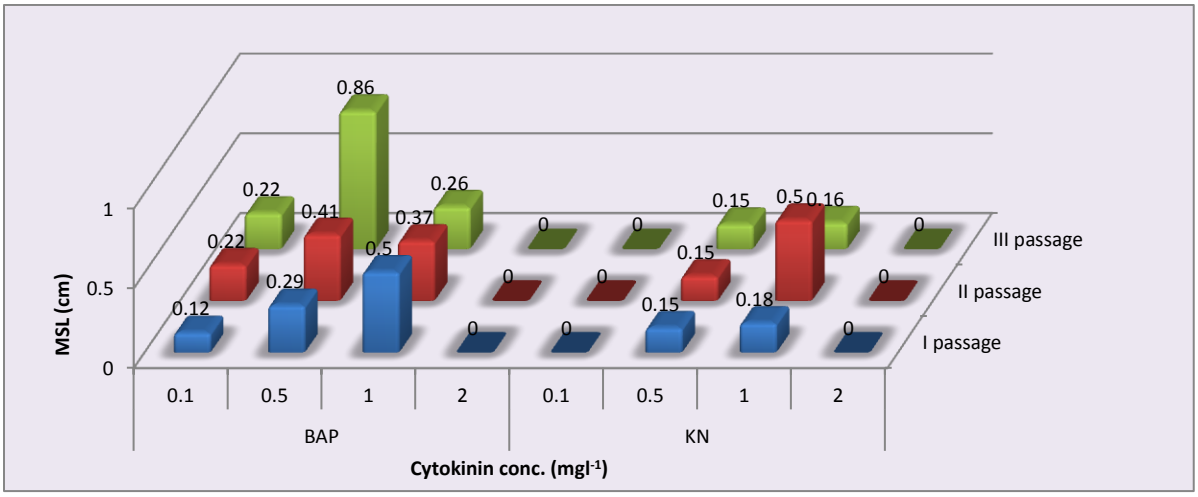
Adventitious organogenesis was rather low in the treatments involving cytokinins alone. Among the 4 concs. of BAP tested, BAP ($1mgl^{-1}$) favoured the initiation of adventitious shoot buds. The shoot primordia were formed on the periphery of loose, friable yellow green calli after 3-4 weeks of culture. The organogenetic calli on which the shoot primordiums were formed showed the presence of stellate hairs, a morphological feature associated with this plant. Maximum MSN of 1.86 was observed on BAP ($1mgl^{-1}$) while lower conc. of BAP ($0.5mgl^{-1}$) resulted in higher MSL (0.522). High conc. of BAP ($2mgl^{-1}$) formed hard green-brown callus which failed to show any regeneration.

Very poor adventitious shoot induction pattern was observed on KN supplemented medium. Maximum mean shoot number and MSL were observed on KN (0.5mgl^{-1}). Neither lower nor higher concs. of KN supported any regeneration (Graphs 7,8; Figs. 44-51).

Graph 7 Effect of cytokinins (alone) & subculture passages on Mean shoot number (MSN) (indirect) in *Helicteres isora* L. on MS medium (MSM)



Graph 8 Effect of cytokinins (alone) & subculture passages on Mean shoot length (MSL) (indirect) in *Helicteres isora* L. on MS medium (MSM)

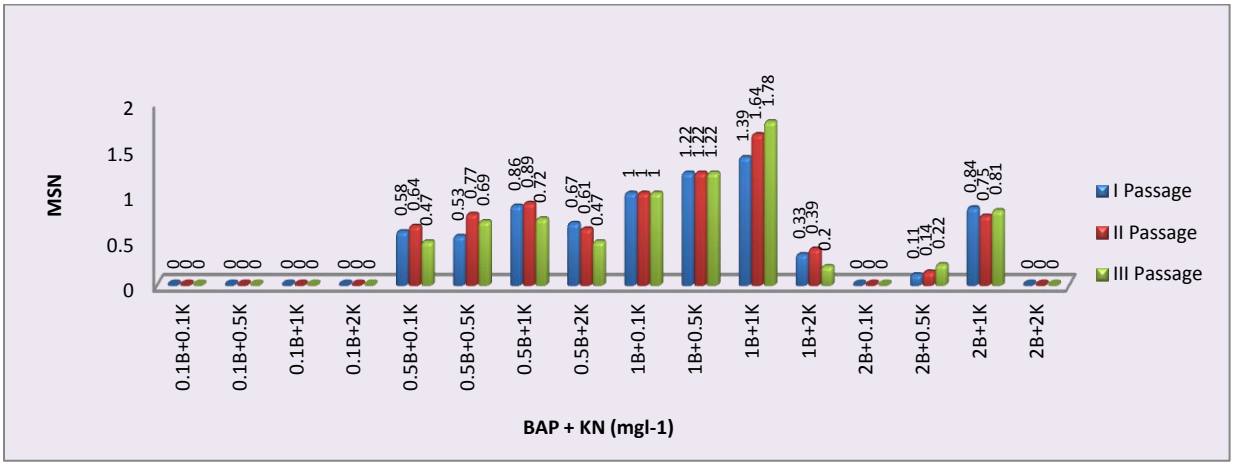


3b. Effect of cytokinin (BAP+KN) combination on indirect organogenesis:

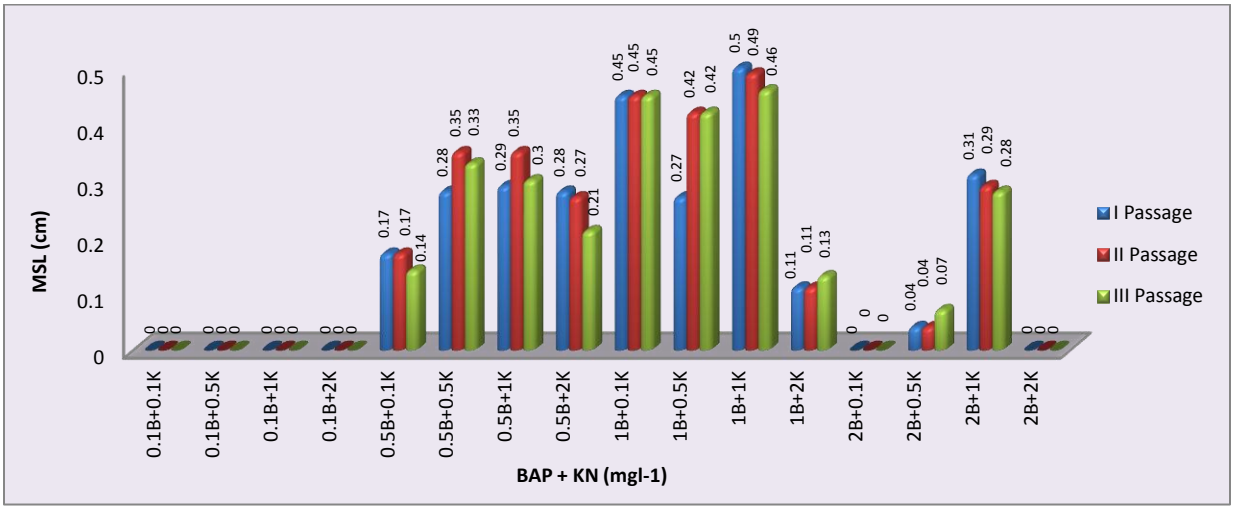
This experiment was carried out to study the effect of cytokinin combination (BAP+KN) on indirect shoot multiplication and elongation from callus. A combination of lower concentrations of BAP and KN failed to initiate any organogenesis. Organogenesis was observed from friable yellow green calli.

Maximum shoot number (1.78) was obtained on BAP (1 mgl^{-1}) + KN (1 mgl^{-1}) in the III passage. It was 1.08 fold higher than in the II passage and 1.28 fold higher than in the I passage. Infrequent shoot bud formation was observed which failed to multiply and the organogenetic potential seemed to decline after II subculture. MSL, however, remained unaffected by the effect of subculture passages (.Calli obtained on lower and higher conc. of BAP and KN failed to produce any shoot (Graphs 9,10; Figs. 52-54).

Graph 9 Effect of BAP and KN (combination), subculture passages and their interaction on Mean shoot number (MSN) (indirect) in *Helicteres isora* L.



Graph 10 Effect of BAP and KN (combination), subculture passages and their interaction on Mean shoot length (MSL) (indirect) in *Helicteres isora* L.



4. Induction of somatic embryogenesis:

Table 4. Effect of 2, 4-D on immature seeds of *Helicteres isora* for induction of somatic embryogenesis

2-4, D (µM)	FC	DC	Texture
0.45	65.33±0.53	++	Soft, friable, green/cream colour
2.25	76.00±0.46	+++	Soft, granular, brown
4.54	82.67±0.70	++++	Soft, light green
22.7	54.66±0.26	++	Sticky, light brown

FC=Frequency of callusing; DC= Degree of callusing

Immature fruits of *Helicteres isora* were inoculated on MS medium supplemented with different concentrations of auxin 2, 4-D (0.45 to 22.7 µM). The immature seeds were given pulse treatment by treating them for 1 week, 2 week and 3 week respectively on 2,4-D. After the stipulated interval of the pulse treatment the calli was inoculated on basal medium (Table 3), (Plate 1). In some concentrations of 2,4 –D (0.45 to 4.54 µM) early pro-embryonic masses were observed when calli from 2 weeks old cultures were transferred to basal medium (BM). However, they did not form proper somatic embryos till now. The calli remained non regenerative and in some cases turned brown (Table 4; Figs. 55-60).

4. Shoot elongation (steps 2&3) & development of shoot buds.

Owing to the significantly higher values of MSN observed on BAP ($2mgl^{-1}$) + KN ($1mgl^{-1}$), this medium was marked as **Selected Medium (SM)**. Further, experiments were, therefore, carried to study the shoot multiplication and development on **SM** in combination with various additives like $AgNO_3$, CM and PG on two explants ApB and AxB.

5a. Effect of Selected Medium (SM) + Additives on direct organogenesis:

- ***Effect of SMI + $AgNO_3$***

An increment was observed in all the regeneration parameters viz. FSI, MSN and MSL from both the explants compared to the control. Maximum FSI of 94.44 and MSN of 4.14 were obtained from AxB on SM + $AgNO_3$ ($1mgl^{-1}$) with a maximum of 9 shoots proliferating per explant within 20-25 days. The shoots were initiated better with green and healthy leaves within 2-3 days from AxB explants. Formation of shoots from ApB explant was less than 2. On the other hand maximum mean shoot length of 1.04 (1.93 fold) was obtained from ApB explants on SM + $AgNO_3$ ($2mgl^{-1}$). $AgNO_3$ supplementation in the medium also resulted in significant reduction of the basal callus formation in both the explants (Graphs 11-13; Figs. 61-72).

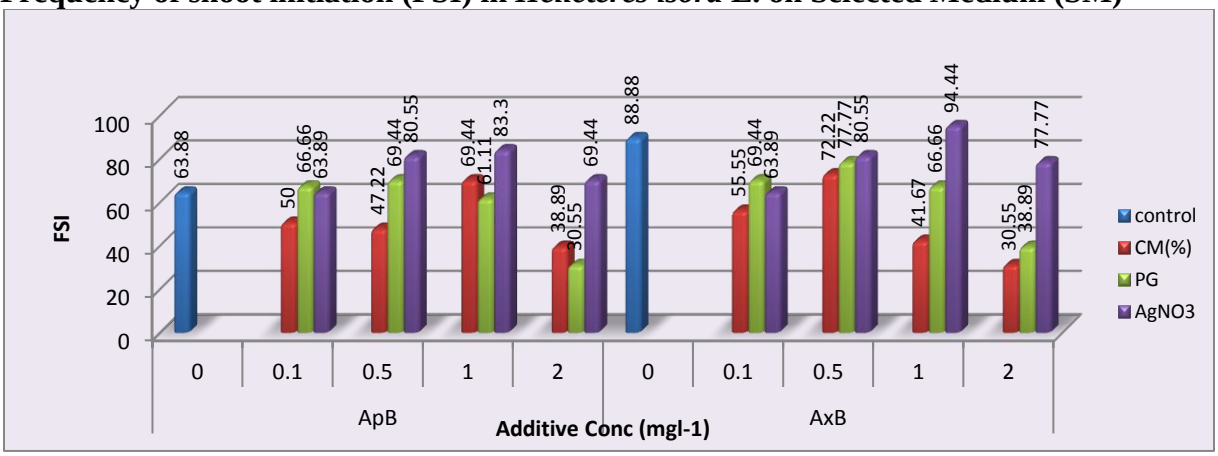
- ***Effect of SM + CM***

Coconut milk (CM) produced a very poor response both in terms of both shoot formation and their elongation. Mostly single shoots were produced and the explants showed a tendency of extensive basal callusing. Besides, browning of the leaf edges and hyperhydricity was also observed after 2 weeks of culture. Effect on FSI, MSN and MSL was significantly lower at all concentrations of CM as compared to the control treatments (Graphs 11-13; Figs. 73-76 & 84-86).

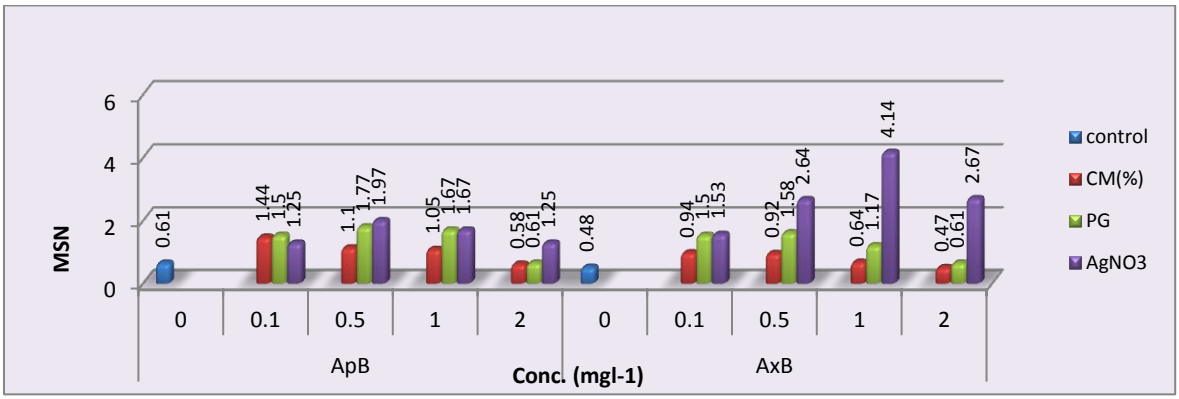
- ***Effect of SM + PG***

Phloroglucinol (PG) too exhibited a poor shoot formation and elongation pattern as compared to the control treatments. Single shoots were obtained predominantly with basal callus at higher concs. Effect on FSI, MSN and MSL was found meagre compared to control at all concentrations of PG. The callus produced was soft, light brown colour and largely non-regenerative (Graphs 11-13; Figs. 77-83).

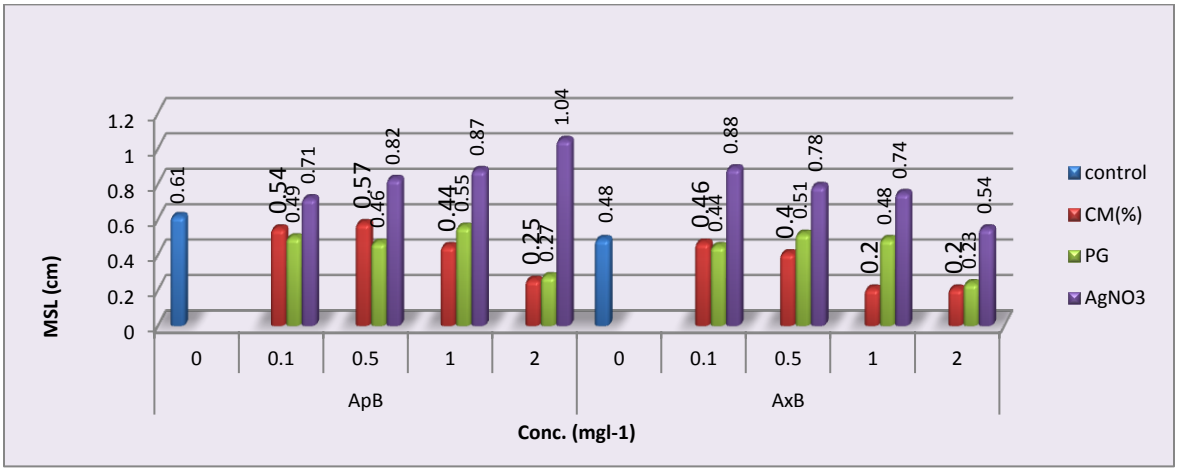
Graph 11 Comparative effect of different additives CM, PG & AgNO3 & explants on Frequency of shoot initiation (FSI) in *Helicteres isora* L. on Selected Medium (SM)



Graph 12 Comparative effect of different additives CM, PG & AgNO3 & explants on Mean shoot number (MSN) in *Helicteres isora* L. on Selected Medium (SM)



Graph 13 Comparative effect of different additives CM, PG & AgNO3 & explants on Mean shoot length (MSL) in *Helicteres isora* L. on Selected Medium (SM)



On the basis of earlier experiments, optimum indirect organogenesis was observed on BAP (1mgl^{-1}), therefore, this concentration was marked as **Selected Medium Indirect (SMI)**. Further experiments were carried out to study the effect of **SMI** in combination with different auxins (IAA, IBA and NAA) and additives like CM, CH and $AgNO_3$ for adventitious organogenesis (indirect) from callus.

5b. Effect of SMI + auxins on indirect organogenesis

This experiment was designed to study the effect of 3 concs. (0.1, 0.5, 1mgl^{-1}) of three auxins (IAA, NAA and IBA) in SMI for adventitious organogenesis (indirect) from callus. Effect of auxin was found unfavourable for both shoot formation and elongation. IAA and IBA produced pale brown calli while NAA produced greenish calli which failed to convert into organogenetic ones. The calli produced remained non-organogenetic (Table 4; Figs. 87-95).

Auxins (mgl^{-1})	**IBA**						**NAA**						**IAA**					
	Subculture passages																	
	I		**II**		**III**		**I**		**II**		**III**		**I**		**II**		**III**	
	DC	**TC**	**DC**	**TC**	**DC**	**TC**	**DC**	**TC**	**DC**	**TC**	**DC**	**TC**	**DC**	**TC**	**DC**	**TC**	**DC**	**TC**
0.1	++	S,C	+++	S,C	+++	S,C	++	Y,G	++	S,B	+++	S,B	+++	S,C	+++	S,C	+++	S,C

0.5	+++	S,C	+++	H,C	+++	H,C	+++	Y,G	++	S,B	+++	S,B	+++	S,C	+++	S,C	+++	S,C
1.0	+++	H,C	+++	H,C	+++	H,C	+++	S,B	+++	S,B	+++	S,B	+++	S,C	+++	S,C	+++	S,C

DC= Degree of Callusing, TC= Type of Callus

S C= Soft Creamy HC=Hard creamy YG=Yellow green S B= Soft brown

5c. Effect of Selected Medium Indirect (SMI) + Additives on indirect organogenesis from callus:

- ***Effect of SMI + CM***

Maximum MSN (2.22) was obtained on SMI+ CM (1%) in II passage. The enhancement over control was observed in II passage (1.08 fold increase) and III passage (1.22 fold increase). Overall MSN was 5.69 fold higher than the minimum value obtained in this interaction. On the other hand, maximum MSL was observed on the control treatments as compared to CM supplemented medium. Lower and higher concentrations of CM were not favourable for either MSN or MSL (Graph 14,15; Figs. 96-98).

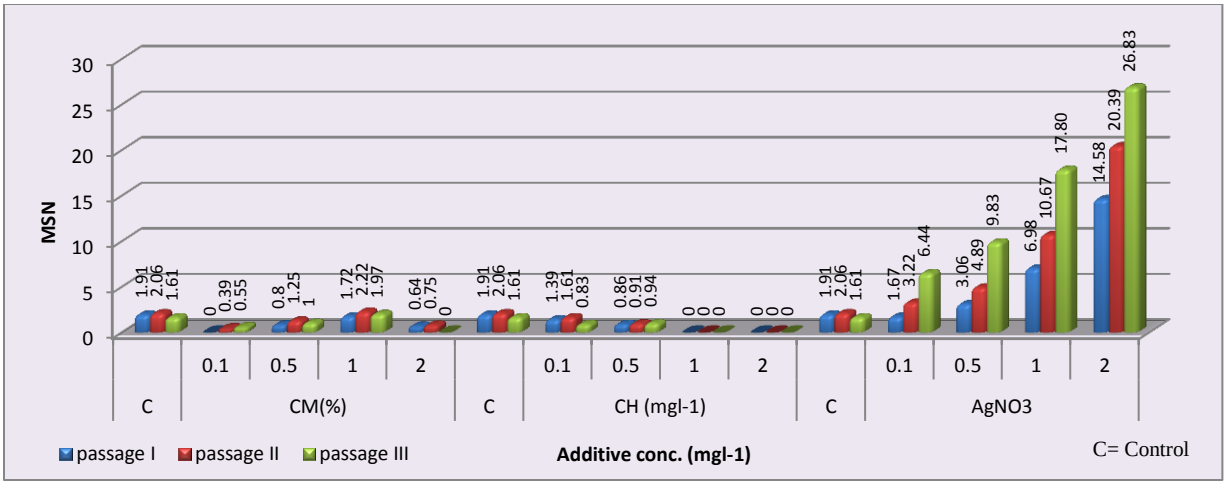
- ***Effect of SMI + CH***

CH exhibited a poor response both in terms of shoot regeneration and elongation of regenerated shoots. MSN and MSL were lower than the control (SMI). No regeneration was observed in experiments employing more than $0.5mgl^{-1}$ CH. The callus produced had low tendency of regeneration with maximum MSN on SMI + CH ($0.1mgl^{-1}$). MSL on the contrary was not found significant for this interaction ((Graph 14,15; Figs. 99-101).

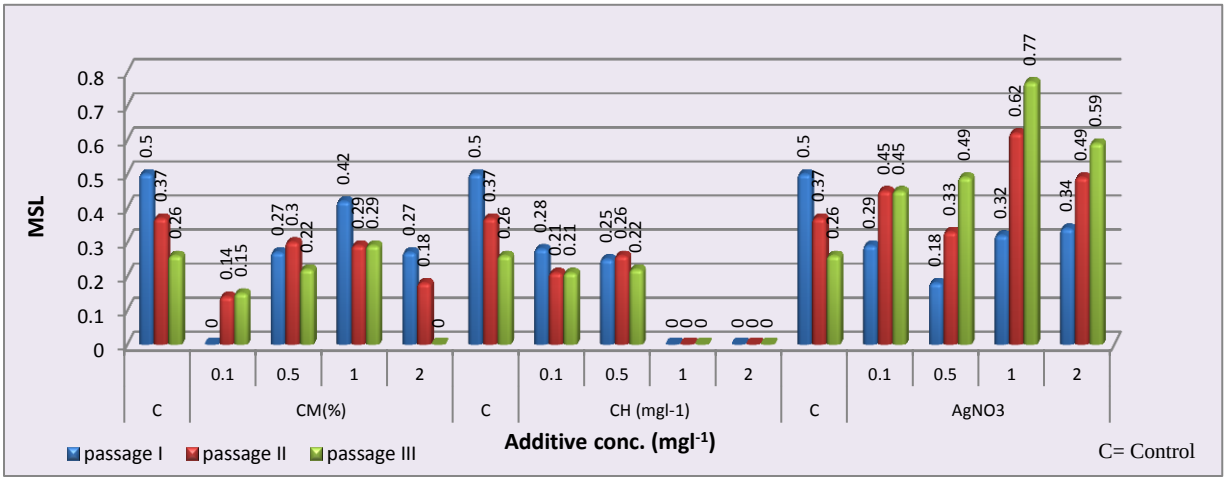
- ***Effect of SMI + $AgNO_3$***

The enhanced effect of silver nitrate which was evident in experiments involving direct organogenesis was also observed in indirect organogenesis. The shoot primordia formed on SMI led to induction of new shoot buds when sub-cultured on $AgNO_3$ supplemented SMI. Maximum shoot number was observed on SMI + $AgNO_3$ ($2mgl^{-1}$) in the III passage. Innumerable shiny, green shoot buds were observed on yellow green calli, which eventually produced healthy shoots and leaves. Maximum MSL of 0.77 was observed in III passage on SMI + $AgNO_3$ ($1mgl^{-1}$) which was 4.32 fold higher than the minimum value recorded (Graph 14,15; Figs. 102-109).

Graph 14 Effect of additives (CM, CH & $AgNO_3$), subculture passages and their interaction on Mean shoot number (MSN) (Indirect) in *Helicteres isora* L. on Selective Medium Indirect (SMI)



Graph 15 Effect of additives (CM, CH & $AgNO_3$), subculture passages and their interaction on Mean shoot length (MSL) (Indirect) in *Helicteres isora* L. on Selective Medium Indirect (SMI)



6. Rooting of regenerated shoots & whole plant formation

This experiment was designed to study the effect of 3 different concs. (0.1, 0.5 and 1mgl^{-1}) of auxins (IAA, NAA, IBA) on root induction and elongation. Half strength MS medium (liquid) was supplemented with different concs. of auxins. It was observed that rooting on half strength MS medium (solid) resulted in excessive basal callus irrespective of the auxin used. While rooting of the shoots on liquid medium with filter paper supports showed little or no callusing. The elongated shoots obtained from different PGR treatments and subculture passages were pooled together and subjected to rooting. All auxins initiated rooting but the rooting patterns were different under different auxins and concentrations. Further elongation of the shoots was also observed on all rooting media.

FRI

Maximum frequency of root initiation (58.32) was observed on NAA supplemented MS medium. It was 1.28 fold higher than IBA and 1.09 fold higher than IAA fortified medium. Interaction was significant for root initiation. Maximum FRI of 91.64 was obtained on IBA ($0.5mgl^{-1}$). It was 6.6 fold higher than the minimum value obtained. Only the optimum conc. of IBA ($0.5mgl^{-1}$) was favourable for root initiation and long, white, slender roots were formed on this concentration. IAA produced very sparse rooting with basal callus formation, yellowing of leaf while NAA produced fleshy roots (Graph 16).

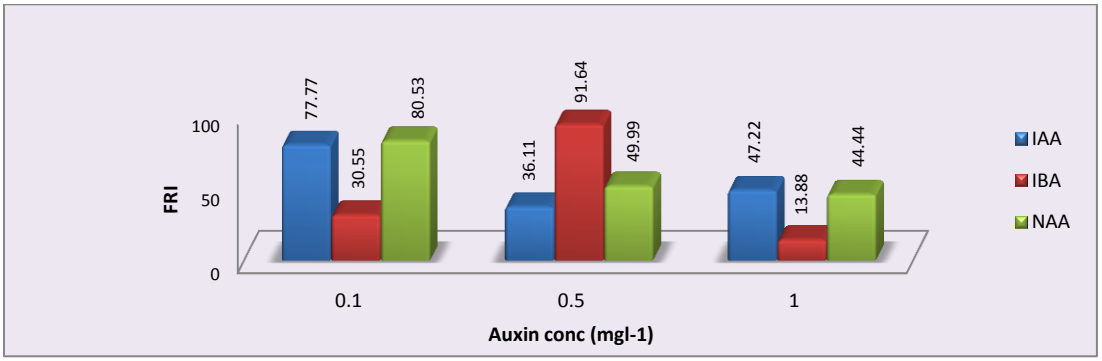
MRN

Maximum roots (9.69) were produced on NAA supplemented medium. It was 2.88 fold higher than IAA and 2.10 fold higher than IBA. Maximum mean root number (15.97) was observed on NAA ($1mgl^{-1}$) with a maximum of 28 roots per shoot. It was 11.29 fold higher than the lowest value obtained. NAA produced white, thick and fleshy roots (Graph 17).

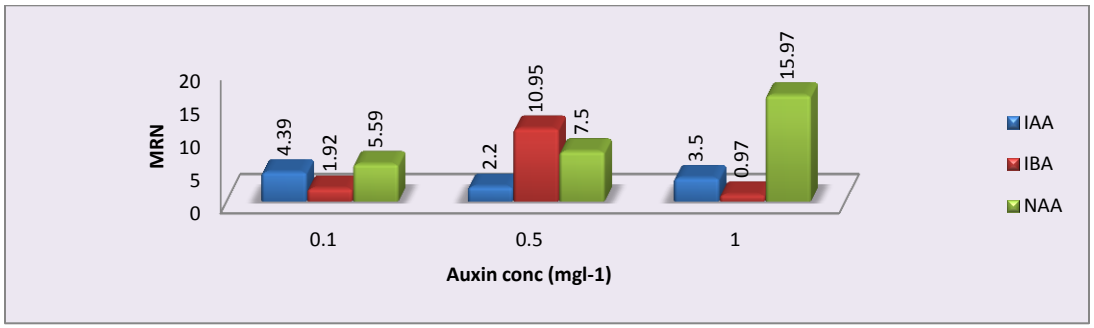
MRL

IBA was better than IAA or NAA for effect on MRL. Maximum MRL (2.65) observed on IBA supplemented medium was 2.51 fold higher than IAA and 1.27 fold higher than NAA respectively. Highest mean root length of 5.76cm was observed on IBA ($0.5mgl^{-1}$). It was 19 fold higher than the minimum value observed. The roots obtained were white, long and branched (Graph 18, Figs. 110-119).

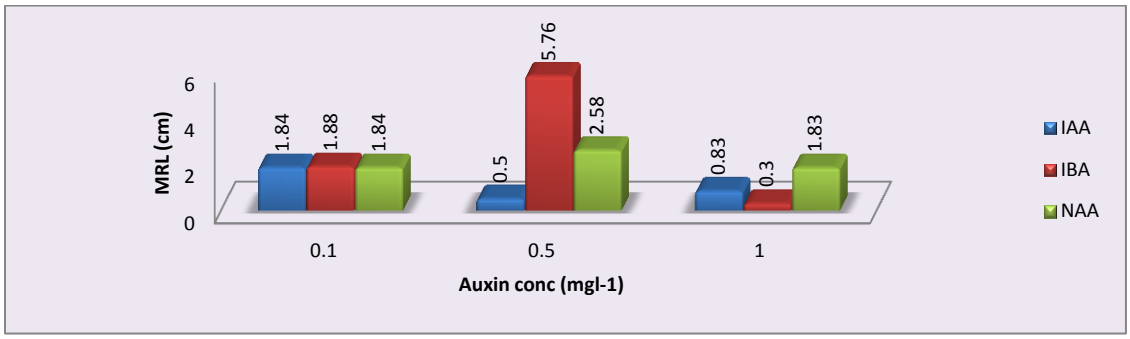
Graph 16 Effect of different auxins on Frequency of root initiation (FRI) in *Helicteres isora* L. on MS medium (MSM)



Graph 17 Effect of different auxins on Mean root number (MRN) in *Helicteres isora* L. on MS medium (MSM)



Graph 18 Effect of different auxins on Mean root length (MRL) in *Helicteres isora* L. on MS medium (MSM)



7. Hardening and Acclimatization

Plantlets with well developed shoot and root systems were transferred to soil: sand: farmyard manure: vermiculite (1:1:1:1). The plantlets were covered with transparent polythenes without any perforation for first 2 weeks to maintain high humidity. After two weeks, polythene bags were perforated with small holes. After another 2 weeks, the perforation was increased and after 4 weeks, the bags were removed for 10-15 min every day, gradually the exposure time was increased and finally bags were completely removed. After 2 months the plantlets were transferred to bigger pots and transferred to field. By this method, about 65-70% plantlets survived (Figs. 120-125).

Characterization of Isolated DNA

Quantification: DNA was extracted from nature grown plant (HA), direct (clonal) propagated plant (HB) and indirect (adventitious origin) plants (HC) of *Helicteres isora*. Optical density (OD) of individual samples was taken with the help of UV-VIS spectrophotometer. DNA

concentrations and yield was calculated using the formula and quality assessment was performed by agarose gel electrophoresis (Table 6; Fig. 126).

Table 6: DNA quantification of the nature grown plant (HA), direct regenerated propagated plant (HB) and indirect (adventitious origin) plants (HC) of *Helicteres isora* L.

Sample	OD_{260}	OD_{280}	DNA Conc. ($ugml^{-1}$)	DF	DNA purity
HA	0.571	0.319	2855	100	1.78
HB	0.660	0.357	3300	100	1.85
HC	0.465	0.257	2325	100	1.81

RAPD-PCR analysis

The aim of RAPD analysis was to assess genetic nature of *Helicteres isora* parents (nature grown plant) (HA) as well as *in vitro* regenerated plantlets viz. from direct regeneration (HB) and indirect regeneration (from callus) (HC).

DNA fingerprinting profiles of the parent plant HA and *in vitro* direct regenerated plant (HB) and indirect regenerated plant from callus (HC) were generated employing 10 primers of which 4 primers (OPA3, OPA5, OPA6, OPA8) generated distinct and reproducible amplified fragments (Figs. 127-130). Each primer produced a unique set of amplified products and these fragments were characterized based on their sizes, ranging from approx. 120-1500kb.

Comparison between the nature grown plants and *in vitro* direct regenerated plants shows that the smallest size of the scorable fragment was 120kb, amplified with the primer OPA8, whereas, the largest fragment was 1200kb generated by primer OPA6. A total of 49 amplified loci per primer was detected between HA and HB. Among these 30 loci were monomorphic among the two samples while 19 loci were polymorphic with an average of 38.77% polymorphism. The number of amplified fragments varied from 11 (in primer OPA5) to 13 (in primer OPA3 and OPA6). Out of 4 primers, OPA8 generated maximum polymorphism i.e. 50% and OPA3 generated minimum (23.07%) polymorphism among HA and HB (Table 7; Figs. 127-130).

Table 7: Comparative fingerprint analysis between nature grown (HA) and micropropagated plants (direct) (HB) of *Helicteres isora* L.

Primer code	Primer sequence	No. of RAPD products (bands)			Fragment size scored
		Total bands	Monomorphic bands	Polymorphic bands	
OPA3	5'- AGT CAG CCA C- 3'	13	10	3	180-800

OPA5	5’-AGG GGT CTT G- 3’	11	6	5	200-780
OPA6	5’-GGT CCC TGA C-3’	13	8	5	190-1200
OPA8	5’-GTG ACG TAG G- 3’	12	6	6	120-800
	Total	**49**	**30**	**19**	

Table 8: Comparative fingerprint analysis between nature grown (HA) and micropropagated plants (indirect) (HC) of *Helicteres isora* L.

Primer code	Primer sequence	No. of RAPD products (bands)			Fragment size scored
		Total bands	Monomorphic bands	Polymorphic bands	
OPA3	5'- AGT CAG CCA C- 3'	15	9	6	180-1200
OPA5	5’-AGG GGT CTT G- 3’	16	5	11	200-1200
OPA6	5’-GGT CCC TGA C-3’	13	8	5	190-1500
OPA8	5’-GTG ACG TAG G- 3’	14	5	9	220-1000
	Total	**58**	**27**	**31**	

Comparison between the nature grown plants and indirect regenerated plants (from calli) shows that the smallest size of the scorable fragment was 180kb, amplified with the primer OPA3, whereas, the largest fragment was 1500kb generated by primer OPA6. A total of 58 amplified loci per primer was detected between HA and HB. Among these 27 loci were monomorphic among the two samples while 31 loci were polymorphic with an average of 53.44% polymorphism. The number of amplified fragments varied from 16 (in primer OPA5) to 13 (in primer OPA6). Out of 4 primers, OPA5 generated maximum polymorphism i.e. 68.75% and OPA6 generated the minimum (38.46%) polymorphism among HA and HC (Table 8; Figs. 127-130).

Table 9: Similarity matrix computed with Jaccard’s Coefficient

	HA	HB	HC	HAR	HBR	HCR
HA	1	0.394	0.286	1.000	0.394	0.286
HB		1	0.400	0.394	1.000	0.400
HC			1	0.286	0.400	1.000
HAR				1	0.394	0.286
HBR					1	0.400
HCR						1

Note: HAR, HBR and HCR are the repeat of samples HA, HB and HC which were considered for the statistical compilation

The Jaccard's similarity co-efficient between the genomes of parent plant and those of *in vitro* regenerated (direct) plant was 0.394, whereas between the parent plant and indirect regenerated plant was 0.286 (Table 9). This analysis indicated that genetic variation was higher in indirect regenerants than the direct regenerated plants.

Dendogram 1 Phylogenetic Tree Analysis: Phenogram