Kerala Agricultural University

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SRE II PROJECT

Funded by Vikram Sarabhai Space Centre, Thiruvananthapuram

EFFECT OF MICROGRAVITY AND HIGH ENERGY RADIATION ON GENE EXPRESSION IN RELATION TO GROWTH, YIELD AND QUALITY OF MEDICINAL PLANTS AND VEGETABLES

FINAL REPORT

(April 2009 to November 2015)

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Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram 695522

FINAL REPORT

1. Title of the project : Effect of microgravity and high energy radiation on gene expression in relation to growth, yield and quality of medicinal plants and vegetables

2. Location : Department of Plant Biotechnology
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5. Objectives of the Projects:

1. To study the effect of microgravity and high energy radiation on the quality and quantity of secondary metabolites in medicinal plants and the expression of key genes involved.
2. To study the effect of microgravity and high energy radiations on growth, quality and yield of vegetable crops.

6. Expected outcome

Change in the growth, yield and quality of vegetable crops, which and exploited for crop improvement. Modifications in the quality/quantity of the

secondary metabolites could be utilized for the production of novel metabolites of commercial importance.

7. Details of the work done

Four medicinal plants (*Catharanthus roseus Bacopa monnieri, Plumbago rosea and Indigofera tinctoria*) and two vegetable crops (*Amaranthus tricolor Solanum melongena)* were selected for the study based on their medicinal/ nutritional quality, commercial importance and type of pollination. *Catharanthus roseus, Bacopa monnieri, Plumbago rosea* are of high demand in pharmaceutical industry. *Indigofera tinctoria* is the source of natural dye, indigo. *Amaranthus tricolor* and *Solanum melongena* are popular vegetables with good nutritional and medicinal properties The plant species selected for the study were self pollinated or often self pollinated.

Category	Species	Material used	Component of interest
Medicinal plants	*Bacopa monnieri*	Callus	Bacosides
	Plumbago rosea	Callus	Plumbagin
	Catharantus roseus	Seeds	Vincristine, Vinblastine
	Indigofera tinctoria	Seeds	Indican
Vegetable crops	*Amaranthus tricolor*	Seeds	Growth, yield &quality
	Solanum melongena	Seeds	Growth, yield &quality

Catharanthus roseus

Vincristine
Vinblastine

Indigofera tinctoria

Indican

Bacopa monnieri

Bacoside

Plumbago rosea

Plumbagin

Amaranthus tricolor

Solanum melongena

7.1 Collection of seeds

Seeds of vegetable crops were obtained from the Department of Olericulture, College of Agriculture, Vellayani, where the crops are maintained for the production of breeder seeds. Seeds of *Catharanthus roseus* were obtained from the medicinal plant garden maintained by The Instructional Farm, Vellayani. Seeds of *Indigofera tinctoria* were obtained from Department of Plantation Crops and Spices, College of Horticulture, Vellanikara, Thrissur.

Selection of mother plants for seed collection was based on the phenotypic similarity. Considering the limited time available, seeds were collected from individual plants to minimize plant to plant variation. In all the above crops, a single plant was sufficient enough to obtain the required quantity of seeds for various experiments.

7.2 Analysis of seed quality

Seeds collected from different individual plants were analysed for quality parameters like seed weight and density, imbibition rate, germination percentage, speed of germination, and vigour index for the selection of seed lot for further experiments. The lot having uniformity in the above parameters was selected and stored for further studies. Mother plants for all the species under study were raised and maintained at College of Agriculture, Vellayani.

Scarification of *I. tinctoria* seeds using sand paper enhanced its germination percentage from 10% to 80 %. Likewise, soaking of *C. roseus* seeds in water for 24hrs followed by placing in dark enhanced its germination up to 50%. No such pre-treatments were required for amaranthus and brinjal seeds.

Seed weight, density and imbibition rate

Seeds (100 Nos) from 3 different source plants were analysed for dry weight, density and imbibition rate using the formula given below and the results are shown in Table 1.

Seed density = Seed weight/ seed volume

Imbibition rate = Weight after soaking - Dry weight/Time soaked.

Imbibition rate after 2, 4 and 24 hrs. was observed.

Table 1: Seed quality parameters studied

Species		Seed weight (mg)	Seed density (mg/mm^3)	Imbibition rate (mg/hr)		
				2 h	4 h	24 h
Catharanthus roseus	1	1.40	0.70	3.5	4.3	0.7
	2	1.08	0.54	2.5	3.5	1.2
	3	1.36	0.68	1.5	2.5	0.5
Indigofera tinctoria	1	2.96	0.74	25.0	12.7	2.3
	2	3.00	0.75	26.0	13.7	2.4
	3	3.25	0.81	16.5	8.5	1.7
Amaranthus tricolor	1	0.79	1.04	7.0	5.5	1.9
	2	0.78	1.03	13.5	7.5	1.7
	3	0.85	1.12	10.5	6.2	2.0
Solanum melongena	1	3.72	0.41	80.6	50.6	9.9
	2	3.93	0.44	100.0	50.7	9.8
	3	3.92	0.43	93.0	48.7	8.7

Samples 1, 2 and 3 are the seed samples collected from three different mother plants

Germination percentage, vigour index and speed of germination

100 seeds from 3 different source plants were kept for germination. Seeds were divided into 5 sets, each containing 20 seeds and the various seed quality parameters like germination percentage, vigour index and speed of germination were calculated on the 10th day using the formulae given below:

a. Vigour Index I = Germination Percentage x Mean length of roots and shoots (cm)

b. Vigour Index II = Germination Percentage x Dry weight of seedling (mg)

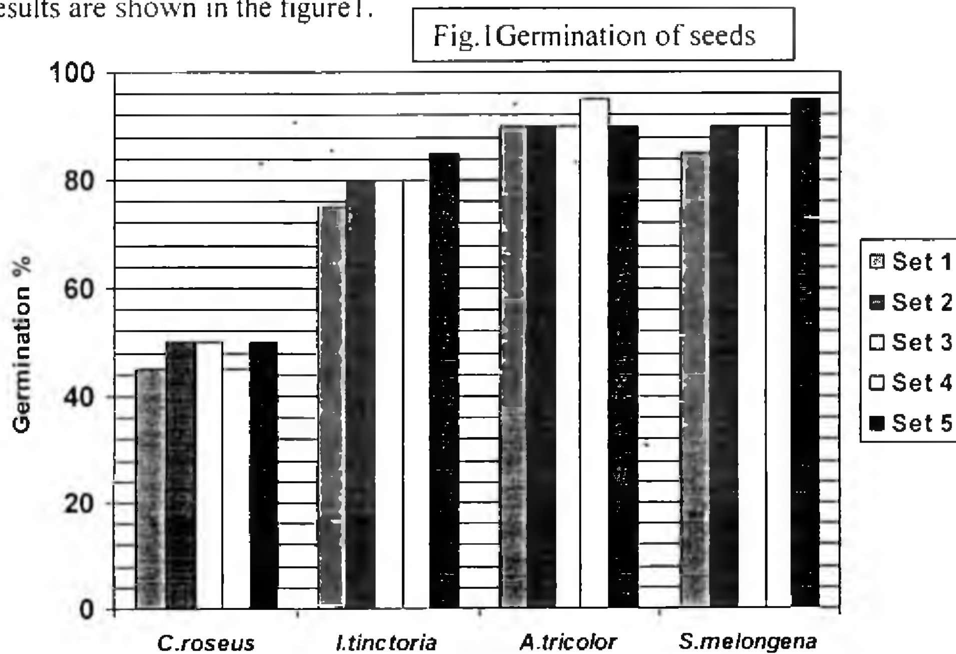
c. Speed of Germination = X_1 / Y_1 + X_2-X_1 / Y_2.................... X_n-$X_{(n-1)}$/ Y_n where X_n is percentage of germination on the nth day, Y_n is no. of days from day of sowing to the nth count. The results are tabulated in Table 2.

Table 2: Germination Studies

Species		Germination % (X)	Mean root+shoot length(cm) (Y)	Dry wt. per seedling (mg) Z)	Vigour Index I (XY)	Vigour Index II (XZ)	Speed of Germination
Catharanthus roseus	1	50	5.75	3.5	287.5	175.0	12.76
	2	45	6.10	3.3	274.5	148.5	10.84
	3	40	5.30	3.2	212.0	128.0	10.29
Indigofera tinctoria	1	75	4.55	2.5	341.3	187.5	29.08
	2	80	4.95	3.0	396.0	240.0	30.08
	3	70	4.35	2.3	304.5	161.0	27.91
Amaranthus tricolor	1	70	4.78	0.6	334.6	42.0	41.67
	2	75	3.97	0.5	297.7	37.5	40.83
	3	90	4.32	0.5	388.8	45	55.83
Solanum melongena	1	85	5.82	2.1	494.5	178.5	13.48
	2	90	5.65	2.8	508.5	252.0	14.11
	3	80	5.45	2.5	436.0	200.0	12.85

The observations showed that set 1 of *C. roseus*, sample 3 of *A. tricolor* and sample 2 of *I. tinctoria* and *S. melongena* were of good quality and were considered for further studies. Germination percentage of selected seed sample varied only up to 5% when 5 sets of 20 seed each (making a total of 100 seeds) were analysed. The results are shown in the figure1.

Fig.1Germination of seeds



Epicuticular wax estimation by quantitative method

Leaf sample of area 10 cm^2 (leaf lamina without midrib)was carefully dipped with the help of forceps into clean pre weighed beaker containing 10 ml chloroform and kept for 10 sec. so that the wax from the sample got extracted. Then the beaker was heated at 50°C to evaporate the chloroform until no trace of chloroform was found. The weight of beaker was noted again. Then by subtracting pre-weight of the beaker from the weight of the beaker with wax, wax content was calculated and expressed in mg/cm^2 (Table 3).

Table 3: Wax content of plants under study

Species	Wax (mg/cm^2)
Catharanthus roseus	0.3
Indigofera *tinctoria*	0.1
Bacopa monnieri	0.2
Plumbago rosea	0.4
Amaranthus tricolor	0.3
Solanum melongena	0.3

Based on these experiments the seed lot showing good quality was selected for further studies.

7.3 Pre-flight ground control experiments

These experiments were planned to study the effect of environmental factors, which were expected inside the recovery capsule, on the plant materials during its stay in the outer space. The technical aspects of the capsule were discussed with the SRE II team members and as per the discussion it was decided to study the effect of a range of temperature and radiation on seed viability. All these experiments were conducted using the seed lot tested to have good quality parameters.

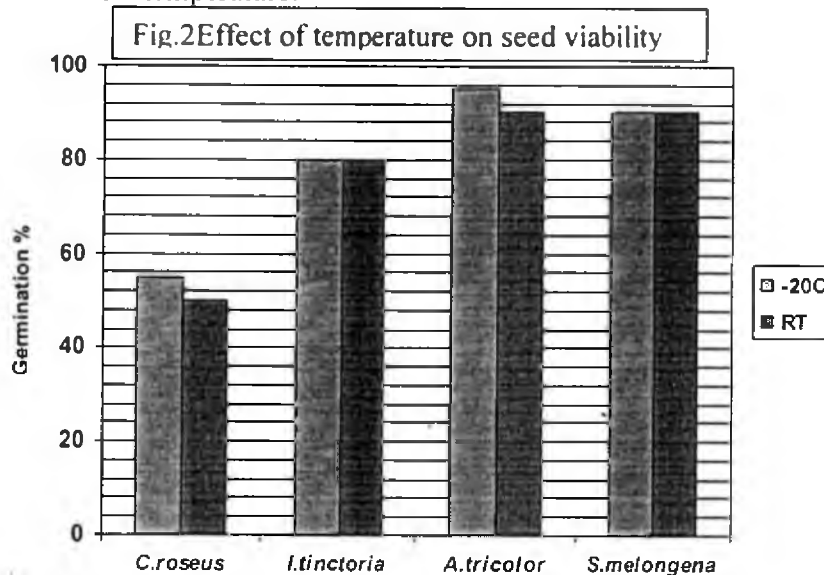
The effect of microgravity on the selected plant materials could not be studied because of lack of facility.

Effect of temperature on seed viability

100 seeds each of *C. roseus*, *I. tinctoria*, A. *tricolor* and *S. melongena* were exposed to -20°C and room temperature for a period of 3 days (the expected exposure

period in the outer space) each and the effect on germination percentage was observed (figure 2). The observations showed that seed viability is not significantly affected by exposure to low temperature.

Fig.2Effect of temperature on seed viability

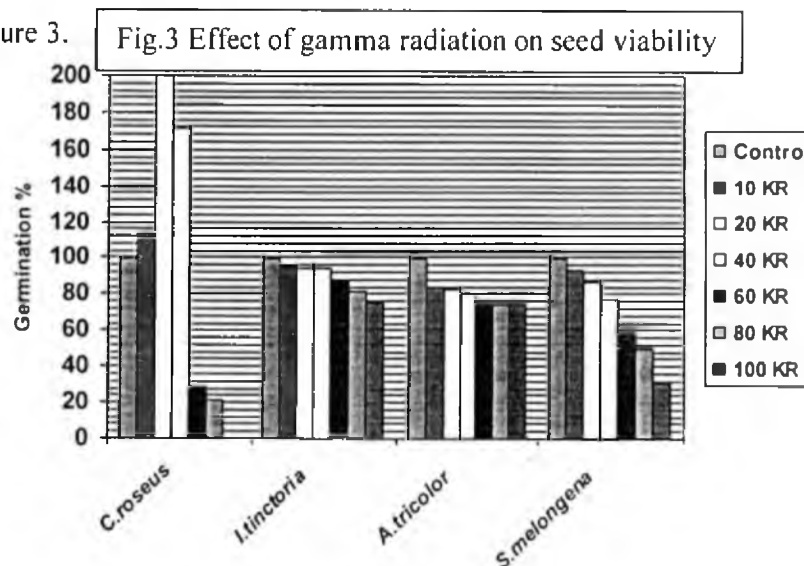


The percentage germination in *C. roseus* was less compared to others in normal conditions also.

Effect of gamma radiation on viability of seeds

Since there were no sources available for different types of high energy radiations, the available source of gamma radiation was used for this study. 100 seeds each of *C. roseus*, *I. tinctoria*, A. *tricolor* and *S. melongena* were exposed to gamma radiation at 0, 10, 20, 40, 60, 80 and 100 kilorad (KR) and their effects on germination percentage and seedling survival was carried out. The germination percentage decreased in all the plants except *C. roseus* under study with increase in radiation dosage. But in the case of *C. roseus*, the germination percentage increased with increase in radiation dosage up to 40 KR, then decreased sharply. Germination percentage in control is taken as 100 and the relative percentages of test samples are given in the figure 3.

Fig.3 Effect of gamma radiation on seed viability



Effect of radiation on growth of medicinal plants and vegetables

The seeds of C. *roseus, I. tinctoria, A. tricolor* and *S. melongena,* irradiated with gamma radiation @ 10, 20, 40, 60, 80 and 100 KR were raised in pots and analyses were carried on growth and yield. Growth parameters like mean shoot length, no. of leaves, time to flowering, days to seed set, no. of fruits if any, and mean fruit weight were measured and the results are shown in Table 4 to 12.

Table 4 Effect of radiation on height (cm) of *C. roseus*

Duration (months)	0 KR (Control)	10 KR	20 KR	40 KR	60 KR	80 KR	100 KR
1	3.16	2.9	4.5	-	-	-	-
2	14.5	13.2	12.3	-	-	-	-
3	36.5	38.0	34.0	-	-	-	-
4	41.5	48.5	42.2	-	-	-	-
5	68.0	65.0	61	-	-	-	-

Table 5 Effect of radiation on height (cm) of *I. tinctoria*

Duration	0 KR	10 KR	20 KR	40 KR	60 KR	80 KR	100 KR
1 month	9.3	6.0	4.0	7.5	5.5	4.0	9.0
2	13.3	13.0	15.0	14.5	-	-	16.0
3	56	50.5	64.0	63.0	-	-	21.0
4	81.5	95.0	105.2	103.0	-	-	64.0
5	134	157	151.0	156.0	-	-	102.0

Table 6 Effect of radiation on height (cm) of *A. tricolor*

Duration (months)	0 KR (Control)	10 KR	20 KR	40 KR	60 KR	80 KR	100 KR
1	5.3	7.7	7.2	3.0	-	-	-
2	36.2	35.0	26.0	19.5	-	-	-
3	67.5	72.0	64.0	-	-	-	-
4	77.0	86.5	83.0	-	-	-	-
5	90.0	131.0	83.0	-	-	-	-

Table 7 Effect of radiation on height (cm) of *S. melongena*

Duration (months)	0 KR (Control)	10 KR	20 KR	40 KR	60 KR	80 KR	100 KR
1	6.7	5.0	5.3	4.6	6.6	6.0	-
2	11.0	9.3	11.0	10.0	10.0	16.0	-
3	31.0	30.0	30.0	43.5	45.0	39.0	-
4	56.0	60.0	62.0	50.0	64.0	60.0	-
5	64.0	76	75.0	60.0	75.5	72.3	-

Table 8 Effect of radiation on leaf number in *C. roseus*

Duration (months)	0 KR (Control)	10 KR	20 KR	40 KR	60 KR	80 KR	100 KR
1	5.3	5.5	5.0	-	-	-	-
2	13.5	9.7	6.5	-	-	-	-
3	44.5	37.0	11.0	-	-	-	-
4	167.0	47.0	25.0	-	-	-	-
5	180.0	70.0	62.0	-	-	-	-

Table 9 Effect of radiation on leaf number in *I. tinctoria*

Duration (months)	0 KR (Control)	10 KR	20 KR	40 KR	60 KR	80 KR	100 KR
1	4.0	7.0	5.5	2.0	-	-	-
2	21.6	19.0	12.0	10.5	-	-	-
3	55.0	60.5	67.0	-	-	-	-
4	80.0	100.0	85.0	-	-	-	-
5	118.0	120.0	90.0	-	-	-	-

Table 10 Effect of radiation on leaf number in *A. tricolor*

Duration (months)	0 KR (Control)	10 KR	20 KR	40 KR	60 KR	80 KR	100 KR
1	24.5	12.0	8.0	17.0	8.0	3.5	14.0
2	64.8	37.5	17.0	37.7	-	-	48.0
3	858.0	120.0	125.0	145.0	-	-	100.0
4	930.0	990.0	1010.0	330.0	-	-	398.0
5	1450.0	1500.0	1610.0	650.0	-	-	650.0

Table 11 Effect of radiation on leaf number in *S. melongena*

Duration (months)	0 KR	10 KR	20 KR	40 KR	60 KR	80 KR	100 KR
1	5.0	4.0	4.3	5.2	4.0	5.5	-
2	5.0	5.0	6.0	6.0	5.0	6.0	-
3	9.5	11.0	11.0	9.5	15.0	11.0	-
4	10.0	20.0	25.0	16.0	21.0	23.0	-
5	18.0	33.0	33.0	20.0	32.0	35.0	-

Table 12 Effect of radiation on yield in *S. melongena*

Parameter	0 KR (Control)	10 KR	20 KR	40 KR	60 KR	80 KR	100 KR
Fruit No./Plant	12	10	11	9	10	-	-
Fruit Wt.	53.7	50.3	41.0	55.1	53.0	-	-

7.4 Experiments using callus cultures of medicinal plants

Callus induction, maintenance and regeneration

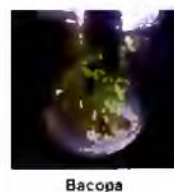
For the two medicinal plants, namely *B. monnieri and P. rosea* callus was induced from leaf explants (Plate 1) on MS media fortified with the growth regulators BA (0.5mgL^{-1}) and 2, 4-D (0.5 mgL^{-1}). Shoot regeneration from callus culture of *B. monnieri* and *P. rosea* were obtained on MS medium supplemented with BA (1.75 mgL^{-1}) and IAA (0.05 mgL^{-1}).

Callus Induction from leaf explants
MS media fortified with BA (0.5ppm) and 2, 4-D (0.5ppm)

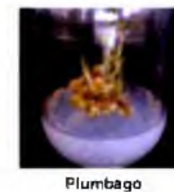
Bacopa

Plumbago

Shoot regeneration from callus culture
MS medium supplemented with BA (1.75ppm) and IAA (0.05ppm)



Bacopa



Plumbago

Media standardized

Bacopa monnieri

Callus induction

0.5 mg/L BAP and 0.5 mg/L 2,4-D.

Callus regeneration

MS + 1.75 mg/L BAP and 0.05 mg/L IAA

Rooting

Basal MS medium

Plumbago rosea

Callus induction

MS+ 0.5 mg/L BAP and 0.5 mg/L 2,4-D.

callus regeneration

MS + 1.75 mg/L BAP and 0.05 mg/L IAA

Rooting

MS+ 0.5 mg/L IAA

Catharanthus roseus

Callus induction

MS + 1 mg/L 2,4-D and 1 mg/L Kinetin

Regeneration

½ MS medium

Rooting

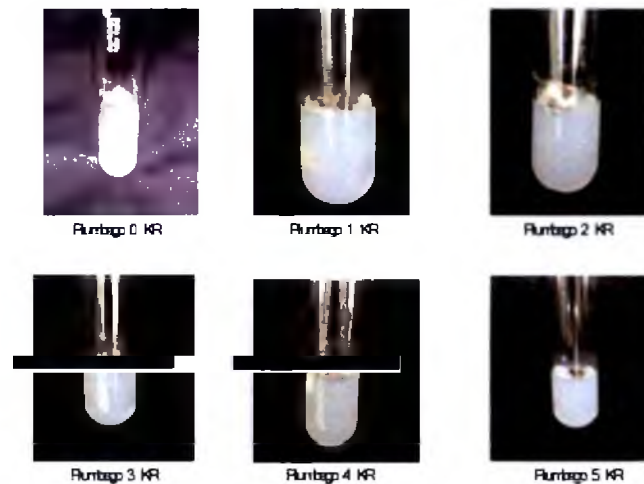
Basal MS medium

Effect of temperature on callus regeneration

Callus cultures were kept at -20^{0}C and room temperature for 3 days and its effect on callus growth and regeneration was studied. Calli were seen to withstand room temperature, but dried at -20^{0}C. But when encapsulated in calcium alginate beads, calli were able to withstand -20^{0}C. Effects of different agar concentration (4, 5 and 6%), limited space on callus growth and regeneration were also studied. The study showed no significant difference in callus growth under these conditions.

Effect of radiation on viability and regeneration of callus

The callus cultures of *Bacopa monnieri* and *Plumbago rosea* were exposed to different doses of gamma radiation (1 to 5 KR). Callus cultures of Bacopa showed Increased proliferation rate irrespective of the radiation dose. Regeneration of the callus not affected. In Plumbago pronounced rooting was observed in 2KR to 4 KR treatments. 5KR was lethal. No regeneration was observed after irradiation.



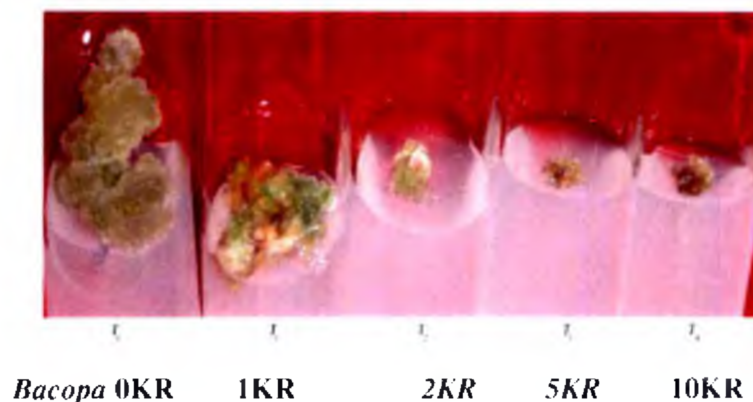


Table 13 Effect of radiation on growth and nature of callus

Species	Radiation (KR)	Fresh wt. (mg)		GI	Nature of callus
		Initial	30 days		
B. monnieri	0	51	394	6.7	G
	1	39	238	5.1	YG
	5	41	193	3.7	YG
	10	30	261	7.7	G
	15	50	150	2.0	YG
	20	37	10	-0.7	D
P. rosea	0	56	463	7.2	W
	1	57	308	4.4	W
	5	58	317	4.5	W
	10	64	304	3.7	BW
	15	40	119	1.9	B
	20	55	61	0.1	D
C. roseus	0	90	781	7.6	Y
	1	93	795	7.5	Y
	5	92	767	7.3	Y
	10	91	716	6.8	Y
	15	94	723	6.7	Y
	20	93	747	7.0	Y

G-Greenish, YG-Yellowish green, D- Dead, W- Whitish, BW- Brownish white, B-Brown, Y-Yellowish, GI- Growth Index

Table 14 Effect of darkness on growth index of callus

Treatment	Mean initial Fresh Weight (mg)	Mean Fresh Weight after 30 d (mg)	GI (Mean±SE)
C. roseus 30°C Light	46.7	353.3	6.57±0.23
C. roseus 30°C Dark	46.7	356.7	6.77±0.42
B. monnieri 30°C Light	28.3	166.7	4.90±0.28
B. Monnieri 30°C Dark	18.3	113.3	5.42±0.38
P. rosea 30C Light	46.7	233.3	4.18±0.36
P. rosea 30°C Dark	31.7	186.7	5.05±0.29

Standardization of medium for maintenance of callus for long duration

As Bacopa callus regenerated within 3 weeks of inoculation, which is not desirable for the onboard studies, various combinations of different plant growth regulators were tried to prolong the cells in callus stage (Table 13). For the callus to be fresh different combinations of media were tried.

Considering the limited space availability in the biopan, different types of containers (microfuge tubes with dome shaped cap and flat cap) were included in the experiments to examine compactness of the callus.

Table 15 Media formulations to prolong the callus stage of Bacopa

Basal Medium	Growth regulators (ppm)	Response (No. of days the cells remain in callus stage)
MS	BA 0.5 + 2,4-D 0.5	~ 30 days, then started drying
MS	BA 0.5+ 2,4-D 0.2	~30 days, then started regeneration
MS	BA 0.2+ NAA0.5	Regeneration within 30 days
MS	BA 0.5 + NAA 0.2	Regeneration within 30 days
MS	BA 0.5 + NAA0.5 + 2,4-D 0.5	~30 days, then started drying
MS	BA 0.5 + NAA 0.2 + 2,4-D 0.2	60 Days as callus

Further studies were conducted to standardize a medium suitable for maintaining the callus for 3 months, expecting the delay in the recovery of the space capsule. Half strength medium with 1.5% sucrose, BA 0.5 mgL^{-1} and 2,4-D mgL^{-1} was found to support the callus upto 3months.

7.5 Biochemical profiling of medicinal plants using HPLC

The secondary metabolites of interest in the medicinal plants under-study were analysed using HPLC method. The procedures mentioned below were adopted for the extraction of secondary metabolites.

Extraction of Vinblastine and Vincristine rich fraction from *Catharanthus roseus*

1 kg of *Catharanthus roseus* powdered plant was percolated overnight with methanol (95 %, 3L x 3). The total methanolic extract was filtered and concentrated at 50 °C *in vacuo* to 500 ml, diluted with water (500 ml), then acidified with 1N sulphuric acid (pH 2). The combined aqueous acidic extract was kept overnight in a refrigerator, and then filtered from resinous bodies. The clear filtrate was allowed to pass through a glass column packed with 500 (g) charcoal (4 cm x 100 cm) previously activated at 120 °C in an oven for 2 hrs. Eluates were taken at different intervals and tested for the presence of alkaloids using Mayer's reagent to be sure that the alkaloidal mixture was completely adsorbed. The column was first washed with distilled water (2 L), then eluted in a gradient manner starting with 30% methanol, followed by 50 %, 70 % and finally 100 % methanol, respectively using 500 ml for each solvent. The eluted fractions; separately; were concentrated in *vacuo* at 50 °C till solvent free. The aqueous solution was adjusted to pH 6.4 using NH_4OH solution (25 %) and then extracted with methylene chloride (500 ml x 5). The combined methylene chloride extract was washed with distilled water till alkali free, dried over anhydrous sodium sulfate and then evaporated *in vacuo* to dryness to give an alkaloidal fraction eluted by 70% methanol (I) to get vincristine and vinblastine rich fraction.

Standard Solutions: 0.5 mg of vincristine (Vc) and 1 mg of vinblastine (Vb) were dissolved; separately; in 1 ml of methylene chloride.

Extraction of Indoxyl β-D-glucoside rich fraction from *Indigofera tinctoria*

Fresh plant materials of *Indigofera tinctoria* Linn. were cut to small pieces, put into cotton bag and soaked in water for 24 h. The bag was transferred into a new chamber and soaked in water for another 24 h. The soaked solutions from twice

sample soaking were combined and twice volumes of $Ca(OH)_2$ solution (pH ~11) were added. After 30 min under air blowing, the extracted indigo was allowed to precipitate overnight. Then, the upper solution was discarded and the precipitated pigment or kram paste was passed through drum dryer to get kram powder. Dried and powdered Kram paste of *I. tinctoria* (0.010 g) was dissolved with 5 ml DMSO. All samples were filtered over a 0.45 mm. membrane filter and analyzed by HPLC.

Extraction of Bacoside rich fraction from *Bacopa monnieri*

The coarse powder of Brahmi (30 g) was taken. The dried plant material was soaked in 300-ml water. After 24 hrs, the water was squeezed out of the plant material. The plant material was percolated with circulating 95% ethanol (200 ml) for three rounds. The residue was extracted again twice using the same procedure. The combined extract was filtrated and dried under reduced pressure. Percentage yield of the extract was calculated using the following equation.

%yield = weight of crude extract extract/ weight of dried plant material x 100

The Liebermann-Burchard test for detection of triterpenes was performed as described by Houghton & Raman (1998). The sample extracts were redissolved in acetic anhydride and treated with a few drops of concentrated sulphuric acid. The change of color was observed.

Extraction of Plumbagin rich fraction from *Plumbago rosea*

Plumbagin is chemically 5-hydroxy-2-methyl-1, 4-naphthoquinone. Roots of *P. rosea* were oven dried at 40^0C and powdered (5 mm of particle diameter). The plant (9.5 g) was extracted with 300 ml of chloroform by extraction in the Soxhlet apparatus for 5h. All the solutions were evaporated to dryness under reduced pressure. The extraction efficiency was defined as follows:

$$\text{Percentage extraction (w/w)} = \frac{\text{Mass of extracts}}{\text{Mass of dried material (roots)}} \times 100$$

Sample preparation: A portion of the crude chloroform extracts (2 g each) was dissolved in ethyl acetate (1 ml).

Standard Solutions: Stock solution of plumbagin was prepared with ethyl acetate in volumetric flask. Known concentration aliquots of plumbagin (0.10; 0.50; 1.00; 1.50; 2.00; to 2.50 mg/ml) were used in order to have a standard curve.

HPLC data are shown below. There was no significant difference in the percentage of the secondary metabolites in the samples analysed.

Table 16 Effect of radiation on secondary metabolite production in *B. monnieri*

Sample Name	% of Bacoside per dry weight
Bacopa Control	0.124
Bacopa 1KR	0.015
Bacopa 2KR	0.007
Bacopa 5KR	0.007
Bacopa 10KR	0.008

Table 17 Effect of radiation on secondary metabolite production in *V. rosea*

Sl. No.	Sample name	% of Vincristine per dry.wt.	% of Vinblastine per dry wt.
1	Vinca Control	0:0011	0.0002
2	Vinca 1KR	0.0008	0.0001
3	Vinca 2KR	0.0013	0.0007
4	Vinca 5KR	0.0018	0.0006
5	Vinca 10KR	0.0012	0.0008

Table 18 Effect of radiation on secondary metabolite production in *P.rosea*

Sl.No.	Sample name	% of Plumbagin per dry wt.
1	*Plumbago rosea* Control	0.03
2	*Plumbago rosea* 1KR	0.01
3	*Plumbago rosea* 2KR	0.02

7.6 Gene expression Studies:

RT-PCR was conducted to study the expression of key genes involved in the biosynthesis of secondary metabolites in medicinal plants. The key genes involved in the biosynthesis of Vicristine and Vinblastine in *C. roseus* are strictosidine synthase (Str), desacetoxyvindoline 4-hydroxylase (D4h) and deacetyl vindoline 4-O-acetyl transferase (Dat). Those involved in indigo production in *I. tinctoria* are Naphthalene 1, 2-dioxygenase and UDP-glucosyltransferase. The key genes in *B. monnieri* for bacoside production being 3-hydroxy3-methyl glutaryl CoA reductase (Hmgr). The critical gene in *P. rosea* is Polyketide synthase (Pks) for plumbagin production. The sequences for primer designing were retrieved From GenBank of NCBI. For some genes the mRNA sequences were available from the same plant concerned. In such cases the sequence was directly used to design primers with Primer 3.0 software. The

primers for the key genes are shown in Table 19. All these primers were checked for amplification using genomic DNA.

RT-PCR Amplified product of RNA samples using amyryn synthase specific primer in *Bacopa monnieri*

RNA was isolated from the *in vitro* piantlets regenerated from irradiated *Bacopa monnieri* calli. The integrity and size distribution of total RNA was determined by running the RNA on 1.4 per cent agarose gel. The isolated RNA

Table 19. Genes selected and the primers designed

Gene	Primer
STR (*Catharanthus roseus*) Forward Reverse	5'CCTTCCTATGCTCCGAATGC3' 5'CCATCGTGCTCTTGAATCTG3'
D4H (*Catharanthus roseus*) Forward Reverse	5'ACTATCAGAAGCTTTGGGGC3' 5'GGGGTACCTCTAATGTTCACCGGAAG3'
DAT (*Catharanthus roseus*) Forward Reverse	5'GAAGTTCGAATTCGTTGCCG3' 5'GCTCTAGAAGTAGCTGTTCGTTCTTCTC A3'
Naphthalene 1,2-dioxygenase (*Indigofera tinctoria*) Forward Reverse	5'ATGGAATGGATCAAGGTTGC3' 5'CTGGGGCGACAACACATAGA3'
UDP-glucosyltransferase (*Indigofera tinctoria*) Forward Reverse	5'CTTCTAGCGCAACGTGGTCT3' 5'ACTGAGCAATCACACCTGGA3'
HMGR (*Bacopa monnieri*) Forward Reverse	5'ACAGAAGGATGTTTAGTGGCTAG 3' 5'CTTGCCATCATTTACAGCCTC C 3'
PKS (*Plumbago rosea*) Forward Reverse	5'ATCTCACGGTGGAGCATACC3' 5'GAAGCATCCCTGCTGGTAGA3'

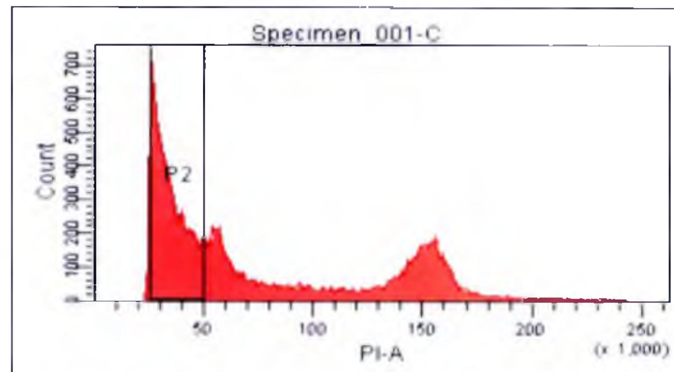
showed two intact bands corresponding to 18S and 28S rRNA with no DNA contamination. The quality of RNA was good as viewed on gel under UV. The purity of samples (A260/A280) was found to be 2.

RT-PCR using primers for gene encoding amyryn synthase produced an amplified product of size near 100-200bp. The expression pattern was different in the samples treated with different doses of gamma radiation. There was an increase in the expression of the gene as evidenced from the thickness of the amplified product. Samples treated with 2KR of gamma radiation showed maximum expression compared to the samples given with other doses, ie. 1 and 5KR.

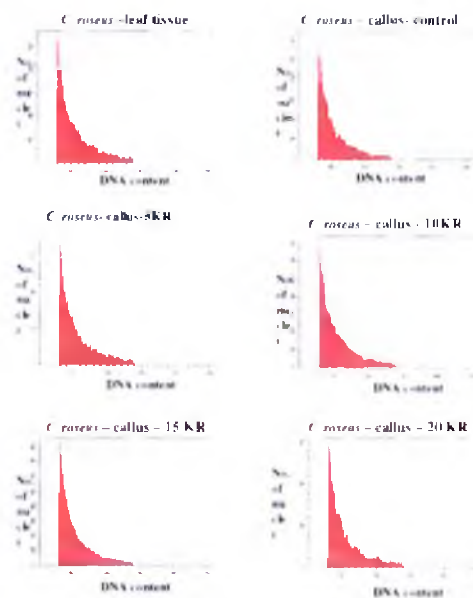
RT-PCR Amplified product of RNA samples using amyryn synthase specific primer in *Bacopa monnieri* (Lane 1: Marker, 2: Control, 3-6: 1KR, 2KR,5KR, 10KR)

7.7 Ploidy analysis using Flow Cytometer

The study on the effect of high energy radiation on ploidy level of the plant samples after exposure to outer space conditions was envisaged in the project. Flow cytometry was the best technique for this. The Flow cytometer facility at Rajiv Gandhi Center for Biotechnology was utilized for the analysis. The procedure was standardized to get sufficient number of nuclei to get enough events in a short time. In each species, 50 mg of young leaf tissue was used for sample preparation. In each case, 1 mL of nuclei isolation buffer solution (0·2 M Tris.HCl, 4 mM $MgCl_2.6H_2O$, 2 mM EDTA $Na_2 \cdot 2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7·5.) was added to a Petri dish containing the plant tissue, which was chopped using a sharp razor blade for approx. 60 s. Leaf tissue from both the sample and DNA reference standard (*Lycopersicum esculentum*) were chopped simultaneously. The resulting homogenate was filtered through a 70-µm nylon cell strainer to remove large debris into polystyrene falcon tubes. Nuclei were stained with 50 µg mL^{-1} propidium iodide. 50 µg mL^{-1} RNase was added to nuclear suspension to prevent staining of double-stranded RNA. The resultant solution were incubated on ice and analysed within 10 min. The results using *Amaranthus tricolor* are shown in figure.



Lycopersicum esculentum (Reference standard)



7.8 **BIOPAN QUALIFICATION TEST FOR SRE-II**

Designing of Biopan

Discussions were made on designing the biopan to carry the study materials. Since two separate studies were included (one to allow the plant material to expose to radiation and the other to block radiation during the revolution of the capsule in the outer orbit) the material for making the biopan, slots to provide space for keeping seeds and callus were important considerations.

Qualification test

The qualification test for Biopan included extreme conditions that can arise in a spacecraft. The tests included Thermal shock test, Thermo vacuum test, Vibration

test, Shock test and Leak tests. The biopans were loaded at the Department of Plant Biotechnology, College of Agriculture, Vellayani on 25.01.2011.

The Biopans (two numbers named ST/1 and ST/2) were sterilized by autoclaving at 121^0C and 1.06 kg/cm2 pressure for 20 minutes. The screws and nuts were sterilized by keeping them in absolute ethanol for 30 minutes. Seeds of *Catharanthus roseus*, *Indigofera tinctoria*, *Amaranthus tricolor* and *Solanum melongena* from Kerala Agricultural University were placed directly into the slots provided in the biopan under sterile conditions inside a Laminar Air Flow chamber (Plate 1). Cushioning over the seeds was provided with sterile non-absorbent cotton.

Seeds of *Oryza sativa*, collected from Pune University were brought in two types of packets (black and white), which were of the same dimensions as that of the slots in the biopan. The boxes were marked "D" (for dry seeds) and "S" (seeds soaked in water). Some of the boxes were fungal contaminated and were discarded. The boxes were kept inside slots in the biopan under sterile conditions.

The calli of *Bacopa monnieri* and *Plumbago rosea* were kept in polypropylene vials containing half strength MS medium supplemented with 2% sucrose and full strength MS medium augmented with 3% sucrose respectively. Two types of vials were used; one screw capped (capacity 2ml) and the other flat capped polypropylene vials (0.2 and 0.5 ml capacity). The vials were wrapped with parafilm and were placed in biopan. The order in which the samples were kept in the Biopan is mentioned below.

Biopan ST/I

<table>
<tr><td>O. sativa SW</td><td>S. melongena</td><td>S. melongena</td><td>-</td><td>-</td><td>-</td><td rowspan="3">B. monnieri callus - 4 vials (0.2ml &.5ml)</td><td rowspan="3">P. rosea callus- 4 vials (0.2ml & 0.5ml)</td></tr>
<tr><td>O. sativa SW</td><td>C. roseus</td><td>I. tinctoria</td><td>A. tricolor</td><td>-</td><td>-</td></tr>
<tr><td>O .sativa SB</td><td>C. roseus</td><td>I. tinctoria</td><td>A. tricolor</td><td>-</td><td>-</td></tr>
</table>

Biopan ST/2

<table>
<tr><td>-</td><td>C. roseus</td><td>I. tinctoria</td><td>A. tricolor</td><td>A. tricolor</td><td>O. sativa DW</td><td rowspan="3">B. monnieri
Callus- screw-capped vial</td><td rowspan="3">P. rosea callus
screw-capped vial</td></tr>
<tr><td>-</td><td>-</td><td>A. tricolor</td><td>C. roseus</td><td>I. tinctoria</td><td>O. sativa SW</td></tr>
<tr><td>I. tinctoria</td><td>-</td><td>S. melongena</td><td>S. melongena</td><td>S. melongena</td><td>O. sativa DB</td></tr>
</table>

Post test analysis for viability of the seeds/ calli

Team members of VSSC brought back the tested samples in biopan on 29.03.2011. The samples were de-loaded from biopan in the Laminar Air Flow chamber for post test analyses. The seed samples were kept for germination along with control (stored at 4^0C). A total of 3 replications, each containing 20-30 seeds were taken for calculating germination percentage.

The mean germination percentage of the tested seed samples of *I. tinctoria*, *A. tricolor* and *S. melongena* was found reduced compared to the control. The results of the germination studies are given in the Table 18 and Plate 2.

In the case of *C. roseus*, the tested seeds did not germinate at all whereas the control seeds showed 45% germination. The storage condition is an important factor affecting the germination. The viability may lose upon storage beyond 6-8 months and also when stored above 4^0C for prolonged period. The time duration between the loading and recovery of the samples was about 2 months.

The tested seeds of *O. sativa* also showed 0% germination (plate 2). The control seeds were not provided. The rice seeds are sensitive to storage conditions and will lose viability when kept beyond 3-4 months under room temperature. The time of collecting the seeds and the storage conditions need to be verified in this case.

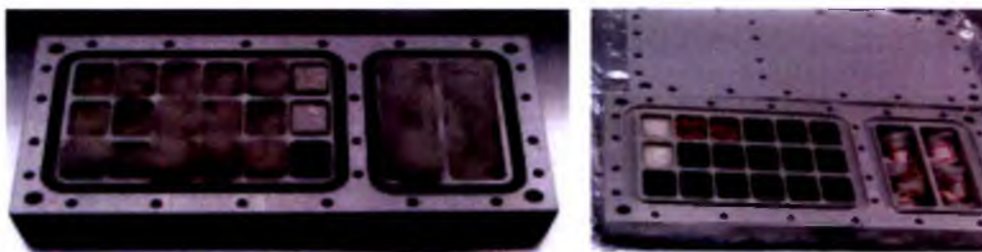
The callus mass taken in screw capped vials were seen scattered and mixed up in the medium. While those kept in flat capped vials were found less disturbed. The calli were transferred to multiplication medium (MS medium supplemented with 0.5 mg/L BAP, 0.2 mg/L NAA and 0.2 mg/L 2,4-D) and incubated at culture conditions (25^0C ± 2^0C and 16 hours photoperiod). The cultures after maintenance in callus multiplication medium for about 50 days are shown in Plate 3. The tested callus cultures showed very slow growth, while the control callus cultures showed 3 times increase in mass. The tested calli were maintained in the multiplication medium for the culture revival.

For tissues culture materials like callus container with flat cap was found suitable, which reduced the scattering of medium. This can be considered for future space biology experiments.

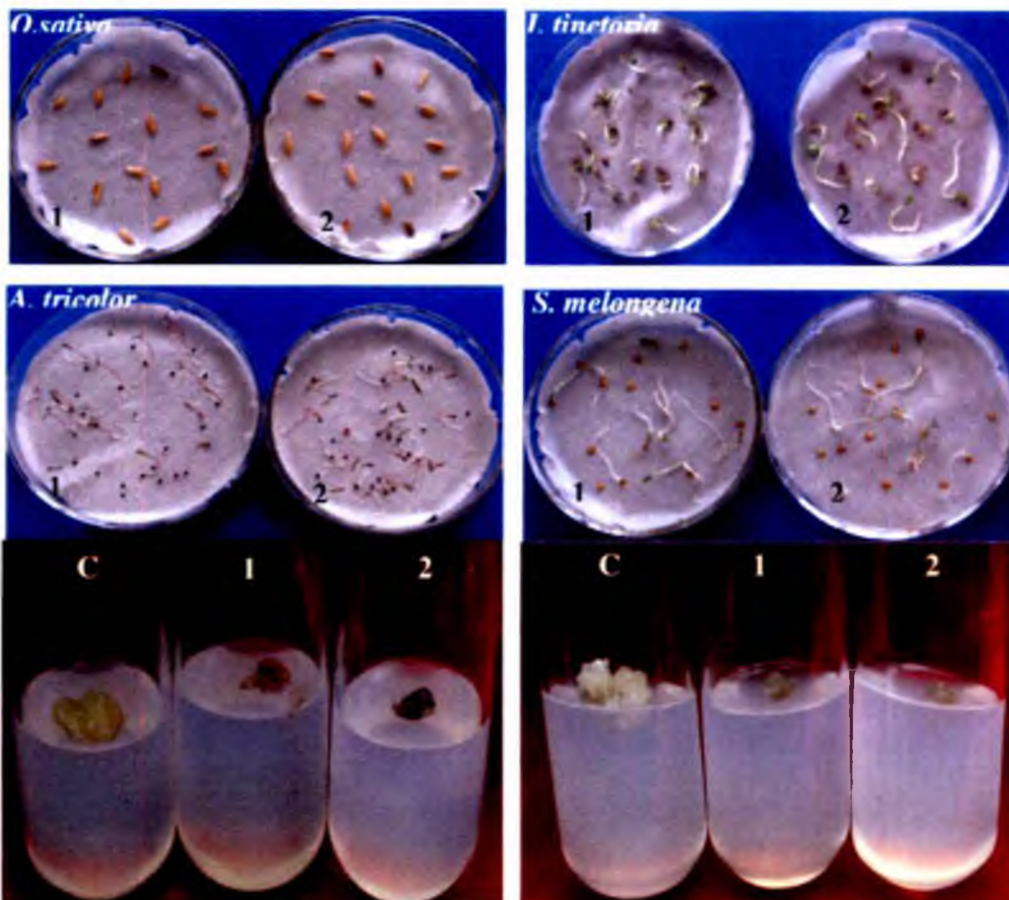
Table 18 Germination of biopan tested seed samples

Set No.	Mean Germination Percentage				
	C. roseus	*I. tinctoria*	*A.tricolor*	*S. melongena*	*O. sativa*
ST/1	0	70	82.5	77.5	0
ST/2	0	95	87.5	75	0
Control	45	95	94	85	-

Biopan loaded with seed and callus samples



Germination studies on seed samples tested in the biopan



B. monnieri ***P. rosea***

Callus cultures of *B. monnieri* and *P. rosea* after biopan test. C-control

- Samples from ST/1 represented as 1 and those in ST/2 as 2

The conditions included in the test did not seriously affect the viability of the seeds and calli, except in the case of *C.roseus,* which need to be verified.

7.9 Summary

The project envisaged to study the effect of microgravity and high energy radiations experienced in the outer space on gene expression related to growth, quality and yield parameters of some medicinal plants and vegetable plant species. The project included preflight onground experiments and post flight experiments, after recovering the samples from the capsule. The project was initiated in April 2009, expecting the launch of SRE II mission in 2009. According to the project, the studies mentioned in the preflight experiments have been completed. These included

(i) selection of plant materials for the study and assessment of quality parameters for including in the preflight and post flight experiments. Seeds of medicinal plants and vegetable species selected were tested for quality parameters. Callus cultures of two medicinal plants (*B. monnieri* and *P. rosea*) were developed and media compositions were standardised for the induction and maitenance and regeneration of calli.

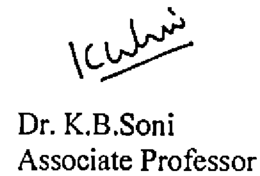
(ii) Studies on the effect of different environmental conditions expected in the recovery capsule , like the effect of radiation, temperature, darkness etc, on the viability, growth and quality parameters of the plant materials and gene expression were carried out.

(iii) Biopan qualification test (Thermal shock test, Thermo vacuum test, Vibration test, Shock test and Leak test) was conducted after laoding the selected plant materials. The flat capped microfuge tubes were found suitable for carrying the callus, as it maintainied the compactness of the cell mass.

(iv) The recovered seed materials were tested for viability and the calli were tested for regeneration. Only *C. roseus* showed variation in viability. The calli retained the regeneration capacity.

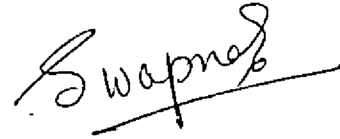
(v) For the post flight studies the different procedures were standardised, including physiological and biochemical analyses, cytological analysis and molecular analysis.

The study generated basic information on the effect of environmental parameters like temperature and gamma radiation on the viabilty of seeds of medicinal plants and vegetable crops selected and the growth and physiological parameters of the plants developed from these seeds. Data on the effect of temperature, gamma radiation on regeneration of callus cultures of medicinal plants and their gene expression in relation to irradation were also evolved.The data generated can be utilized in future space biology programmmes.



Name and signature of Principal Investigator: Dr. K.B.Soni
Associate Professor

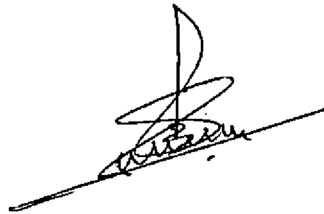
Name and signature of Co-investigators

1. Dr. Swapna Alex, Associate Professor 

2. Dr. Roy Stephen, Associate Professor

3. Dr. R.V.Manju, Associate Professor

Place: Vellayani
Date: 8.2.2016



Signature of Head of Institution

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EXPENDITURE STATEMENT

Head of account	Budget Allocation (Rs. in Lakhs)	Total Expenditure incurred for the project (Rs.)
120 Technical &SS (Salary to RA)	2.16	3,6,2571
300 TA	1.00	2,25,94
153 Contractual services	2.00	59,024
210 Research materials	6.00	4,44,484
220 OE & M	1.0	37,809
840 Other items, including labor charges	2.00	54,019
Total	14.16	9,80,501
Overhead	1.41	1,40,000
Total	**15.57**	**11,21,501**
Fund received from VSSC 1. Letter No. SRE-II/MG/09/11 dated 12.1.2009 2. SRE II/KAU/PD/2 dated 6.9.2010	7.79 4.86	12,65,000
Balance		**Rs. 1,43,499**