Final Report of Major Research Project

"*In vitro* and *in vivo* studies on micropropagation, transformation and camptothecin in *Nothapodytes foetida* and *Ophiorhiza species*"

UGC reference F.No.37-261/2009 (SR) dated 12/1/2010

Submitted to

University Grant Commission

Bahadurshah Zafar Marg

New Delhi-110002

By

Principal Investigator: Jitender Singh Laura

Department of Environmental Science

Maharshi Dayanand University

Rohtak, Haryana

FINAL REPORT OF THE WORK DONE ON THE MAJOR RESEARCH PROJECT

1. Project report No.: **Final**

2. UGC Reference No. **37-261/2009 (SR) dated 12/1/2010**

3. Period of report: from **1/2/2010 to 31/1/2013**

4. Title of research project: **In vivo and in vitro studies on micropropogation and camtothecin in "*Nothapodytes foetida* and *Ophiorrhiza species*".**

5. (a) Name of Principal Investigator: **Dr Jitender Singh Laura**

(b) Department and University where work was undertaken.

Department of Environmental Science

Maharshi Daynand Univrsity

Rohtak, Haryana – 124001

6. Effective date of starting of the project: **15/3/2010**

7. Grant approved and expenditure incurred during the period of report:

a. Total amount approved **Rs. 13,25,136**

b. Total expenditure **Rs. 13,18,875**

The investigator is highly thankful to the University Grant Commission Bahadurshah Zafar Marg New Delhi for providing the necessary financial assistance for undertaking and completion of the major research project.

Jitender Singh Laura
Principal Investigator

CONTENTS

Section I

Introduction and objectives

Camptothecin is in high demand worldwide with the pharmaceutical industries due to its anti cancer properties. *Nothapodytes foetida* (Grah.) Mabb. (Syn. *N. nimmoniana, Mappia foetida*), is a tree species found in Western Ghats along with various species of Ophiorrhiza are convenient source for large-scale production of camptothecin (CPT) used in anticancer drug formulation. Furthermore, the global market of camptothecin is growing day by day and it has no synthetic source. *Camptotheca acuminata* and *N. nimmoniana* are the plant sources most widely for large scale production of CPT. The supply of wood chips from bark and roots of *Nothapodytes foetida* there is a short fall lof 50% than the current demand. As CPT accumulates in stem and root bark of *N. foetida,* whole tree is cut to generate biomass for extraction. In Indian market, the current demand for its biomass is 500-700 metric tons. In Maharashtra, overexploitation and habitat destruction for raw material has led to population decline by 50-80 % in last decade (CAMP, 2001). Due to exploitation of these voluble bio resources, efficient methods for rapid propagation of these plant species is highly desirable for conservation and also meet the increasing demand for camptothecin.

Vegetative propagation of the tree through cuttings is also not successful. In addition to this, *in vivo* vegetative propagation techniques are also time consuming and season dependent (Kaushik *et al.,* 2011). Seeds of *Nothapodytes foetida* lose viability quickly and have poor germination capacity (Sharma *et al.,* 2000). *In vitro* production of elite plant and valuable secondary metabolites is considered to be a suitable alternative, in comparison to field production of plants. To ensure sustainable supply of camptothecin, it is important to domesticate and cultivate *Nothapodytes foetida*. Due to exploitation of this plant species, efficient methods for rapid propagation of *N. foetida* are needed. Genetic transformation of plants using *A. rhizogenes,* is an important alternative for the production of secondary metabolites (Giri and Narasu, 2000).

Hairy roots have been reported to yield higher amounts of secondary metabolites than cell suspension cultures and in some cases, intact plant roots (Allan *et al.*, 2002). Hairy roots have been researched for the synthesis of root derived phytochemicals (Hamill and Rhoder, 1993). As it has been reported that maximum concentration of CPT is found in the roots of *N. foetida*, therefore hairy root culture could be a better option for the large scale production of CPT (Namdeo *et al.*, 2012).

Keeping in view, the endangered status of the plant, the obstacles associated with its propagation, and huge global demand of camptothecin, the present study was undertaken with the following objectives:

1. To establish healthy and sterile cultures for future *in vitro* studies and large scale multiplication.
2. To standardized technique of *Agrobacterium rhizogenes* mediated transformation for inducing hairy roots to enhance the camptothecin content.
3. Assessment of differentiated and undifferentiated cultures for camptothecin (CPT) production and check for possible correlation between organization and secondary metabolites production.
4. To standardize the technique for raising liquid cultures from hairy roots obtained by using various explants directly or by infection with *agrobacterium rhizogenes* and analysis of these cultures for Camptothecin (CPT).
5. To estimate the content of Camptothecin (CPT) using different methods of extraction especially quantitatively in the *in vivo* & *in vitro* generated hairy roots.
6. To compare the camptothecin content in transformed and untransformed seedling roots and shoots.

Section II

Report of work done

The present investigation entitled "***In vitro* and *in vivo* studies on micropropagation, transformation and camptothecin in *Nothapodytes foetida* and *Ophiorhiza* species"** was carried out in Tissue Culture Lab of the Department of Environmental Science, Maharshi Dayanand University, Rohtak. The materials and methods used during the study are summarized here.

2.1. Materials

2.1.1 Plant Material:

1. Plant material -Seeds and plants of *N. foetida* were collected from surrounding forests of Dapoli with collaboration from Dr Narkhade from the Forestry Department of Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. Maharashtra. Seeds were picked in the month of January 2010 and received in February 2010. The seeds of *N. foetida* were sown in the Experimental herbal garden of MDU, Rohtak for raising the seedlings. The seeds were also cultured *in vitro* for raising aseptic seedlings. Plant material was kept in the screen house of Herbal garden M.D.University Rohtak. Plants of Ophiorrhiza species were collected from the forests with collaboration from Tropical Botanical Garden and Research Institute (TBGRI), Kerela.

2.1.2. Bacterial strain

In total five strains of *Agrobacterium rhizogenes* were used for hairy root induction in *Nothapodytes foetida.* Two *Agrobacterium rhizogenes* strains: MTCC 532 & MTCC 2364 were procured from IMTECH Chandigarh in the form of freeze dried cultures and one strain: NCIM 5240 was procured from NCIM Pune and two other strains i.e. A4 & LBA 9402 were obtained from PBSM Lab, NABTD, BARC.

2.1.3. Chemicals and reagents

All chemicals of analytical grades were used for the media preparation as well for the extraction process. The chemicals viz. growth hormones, vitamins, myo-inositol, glycine, chelating agents (EDTA), sucrose, agar-agar, antibiotics etc. were obtained from Hi-media Chemical Company, India, Titan Biotech Limited and Sigma-Aldrich Co. India.

2.1.4. Glass wares and culture vessels

Glass wares which were used during investigation were of borosil quality, obtained from Glacier Glass Works, Haryana, India, Laboratory Glassware Co., Riviera Glass Pvt. Ltd. And Borosil glass work limited. Test tubes (25mm x 150mm), petri plates (85mm x 15mm, 50 mm x 17mm), both wide mouth and narrow mouth Erlenmeyer flasks (150ml, 250ml, 500ml and 1000ml), beakers (250ml, 500ml and 1000ml) were used. Some plastic wares like beakers, disposable and sterile Petri dishes, centrifuge tubes, eppendrof tubes etc. used during the experimentation were obtained from Hi-media Laboratories and Tarsons products Pvt. Ltd.

2.2. Method

2.2.1. Preparation of stock solutions

For all the *in vitro* tissue culture experiments, MS (Murashige and Skoog, 1962) media was used. Table. 3.1 presents the comparison of the medium and amount of nutrients required to prepare 1 L medium. Macronutrients and micronutrients stock solutions were prepared separately at 10 and 100 times of their final concentration in the medium respectively. Vitamins and iron stock solutions were made at concentration of 100 times dilution to that of the concentration in final medium. Na_2 EDTA and $FeSO_4$ solution for making iron stock solution. The accurately weighed ingredients for each constituent was dissolved in distilled water and stored for future use.

All the plant growth regulators were prepared at concentration of 1mg/ml. auxins used in the present study such as indole-3-acetic acid (IAA) (50mg), 1-Naphthaleneacetic acid (NAA) (50mg) and Indole-3-butyric acid (IBA) (50mg) were dissolved separately in 0.1 N NaOH and volume make up was done with distilled water upto 50 ml. similarly, cytokinins such as Kinetin (50mg), 6-Benzylaminopurin (BAP) (50mg), Thidiazuron (TDZ) (50mg) and Gibberellic acid (GA3) (50mg) were dissolved separately in 0.1 N NaOH, diluted with distilled water and made

up to 50 ml of stock solution. From this every 1 ml has 1.0 mg/l of hormone in the stock solution. All the stock solutions were stored at 4°C to prevent the growth of bacteria and algae.

2.2.2. Preparation of nutrient medium

The following procedure was followed to prepare 1 liter of MS culture medium:

1. 100 ml of each macronutrients stock solution and 10 ml of each micronutrients, vitamins and iron stock solutions were added in a 1 liter measuring cylinder.
2. Depending upon medium (rooting and shooting), required amount of the plant growth regulator's stock solutions were added.
3. 30 g sucrose was weighed and dissolved in distilled water with the help of magnetic stirrer and added directly to the medium.
4. The final volume was made to 1 liter by adding required amount of distilled water.
5. Then the pH of the medium was adjusted to 5.8 with 0.1 NaOH or 0.1N HCl.
6. 8 gm of agar-agar was added to the prepared medium and this content was heated in the microwave oven until clear solution was obtained.
7. 50 ml of the media was poured in 250 ml of the wide mouth conical flasks and 10 ml medium in 25 ml of test tubes and these culture vessels were capped with non-absorbent cotton wrapped in single layer of muslin cloth.
8. Culture vessels containing the medium were sterilized by autoclaving at 121°C for 15 minutes. This prepared media was kept at room temperature for further use.

2.2.3. Sterilization of equipments and glass wares

All the glass wares viz. pipettes; beakers, flasks, measuring cylinders, test tubes, petri dishes etc. were dipped in soap solution for 30 minutes. After 30 minutes the glass wares were washed thoroughly in tap water. Therefore, glass wares were rinsed with distilled water and kept for drying at room temperature or were dried in hot air oven at 50-60°C for 24 hours. The glass wares other than the culture vessels like pipettes and petri dishes were wrapped in filter paper and then sterilized in autoclavable poly-propylene bags. Autoclaving was carried out at 121°C and 15 psi for 15 minutes. The forceps, scalpels, scissors used during the experiment, were dipped in spirit in a jar under the laminar air flow chamber and were frequently sterilized on the flame every time before use.

2.2.4. Sterilization of culture media

The prepared MS medium with various concentrations of growth hormones was dispensed in culture vessels. These culture vessels were then plugged with cotton and autoclaved at 121°C at 15 psi for 20 minutes. After autoclaving, the culture tubes were left undisturbed until the medium was solidified. Then culture tubes were transferred to the growth chamber after inoculation.

2.2.5. Sterilization of workspace and Inoculation

Before starting inoculation, all the required equipments and materials (sterilized forceps, petriplates, sterile blade, sterile distilled water and spirit lamp) were shifted to laminar air flow chamber and were surface sterilized under UV light for 30 minutes. After 30 minutes of exposur e to the UV light, the laminar air flow was switched on and the working floor of the laminar hood was surface sterilized by thorough cleaning with spirit. Before starting the inoculation, hands were cleaned with spirit. The inoculation was carried out in the vicinity of the flame to avoid contamination. The sterilized explants were placed on the medium at centre of culture tubes or culture vessels.

2.2.6. Calculations

Number of roots per explants and number of shoots per explants were observed and shoot length and root length were also calculated.

The experimental results were calculated as follows:

$$\text{Percentage of response} = \frac{\text{No.of explants responded}}{\text{Total no.of explants inoculated}} \text{ X } 100$$

Table.2.1. Composition of MS medium (Murashige and Skoog, 1962)

Essential element	**MS concentration mg/L**	**Volume of stock solution taken for one liter medium**	**Concentration used for stock solution preparation (gm/L)**
Macro elements			10X
NH_4NO_3	1650		16.5

KNO_3	1900	100 ml	19.0
$CaCl_2 2H_2O$	440		4.4
$MgSO_4 7H_2O$	370		3.7
KH_2PO_4	170		1.7
Micro elements		10 ml	100X
KI	0.83		0.83
H_3BO_3	6.2		6.2
$MnSO_4$	22.3		22.3
$ZnSO_4$	8.6		8.6
$Na_2MoO_4 2H_2O$	0.25		0.25
$CuSO_4 5H_2O$	0.025		0.025
Iron source		10 ml	100X
$FeSO_4 7H_2O$	27.8		0.278
$Na_2EDTA\text{-}2H_2O$	37.3		0.373
Organic supplement		2.5 ml	100X
Mynoinositol	100		100
Nicotinic acid	0.5		0.5
Pyridoxine-HCl	0.5		0.5
Thiamine-HCl	0.1		0.5
Glycine	2		2
Carbon source			
Sucrose	30000	30 gm	
Agar	8000	8 gm	

2.2.7. Bacterial media

Two strains i.e. MTCC 2364 and MTCC 532 were obtained in the form of freeze dried cultures. These were rehydrated and revived according to the instructions in appropriate medium. Other strains were obtained in the form of slants. These strains were grown routinely from single colonies in liquid YEB medium. Following medium were used for culturing the different bacterial strains:

Table.2.2. Various *A. rhizogenes* medium with composition

Bacterial strain	Components	Concentration (gm/L)
MTCC 2364	**Xanthomonas medium**	
	Galactose	20
	Yeast extract	10
	$CaCO_3$	20
	Agar	20
MTCC 532	**Nutrient agar medium**	
	Beef extract	1
	Yeast extract	2
	Peptone	5
	NaCl	5
	Agar	15
NCIM 5240	**Yeast extract Medium**	
	Beef extract	5
	Yeast extract	1
	Peptone	5
	NaCl	5
	Agar	2

A4 and LBA 9402	Yeast Mannitol Medium	
	$K2HPO_4$	0.5
	$MgSO_4.7H_2O$	2
	Yeast extract	0.4
	Mannitol	10
	Sodium chloride	0.4
	Agar	15

2.3. Seed germination

2.3.1. Viability test

Viability of seeds was assessed with tetrazolium (TZ) test using fresh seeds, one year and two year old seeds stored in two storage conditions. Seeds were pre-conditioned with distilled water for 24 hours at a temperature of 30°C. After 24 hours the seeds were cut longitudinally and then treated with 1.0% 2,3,5-triphenyl tetrazolium chloride (TTC) for next 24 hours at 30°C in dark conditions, according to seed viability testing procedure (ISTA, 2003). After incubation period, TTC solution was discarded and seeds were placed on filter paper for capturing image. Red color of the seeds was considered as viable whereas the unaffected seeds or white colored seeds were considered as non-viable. Number of viable seeds divided by the total number of seeds analyzed gave the percentage of viability.

2.3.2. Seed sterilization

The seed coats were removed with the help of surgical blades. The seeds of each category (with seed coat and without seed coat) were sterilized by washing thoroughly with few drops of soap solution. This was followed by washing under running tap water so that the dust and other particulate matter on the surface of the seeds were removed effectively. Then the seeds were washed 3-4 times in distilled water. The washed seeds were transferred to inoculation chamber and surface sterilized with 0.1% mercuric chloride solution and sodium hypochlorite solution for 3-10 minutes in combination with 70% ethanol. Then the solution was removed and the seeds

were washed 3-4 times in sterilized distilled water so that the traces of mercuric chloride and sodium hypochlorite were removed. The excess of water was removed and the seeds were transferred to petri dish containing sterile filter paper. These sterilized seeds were used for inoculation. These seeds after surface sterilization were transferred to culture vessels with different supporting media.

2.3.3. Effect of supporting media

Seed germination was carried out using different supporting media i.e. agar gel in full strength MS medium and distilled water gelled with agar, filter paper bridge in liquid MS medium and sterile distilled water, cotton disc and filter paper disc.

2.3.3. Seed germination

For seed germination a series of experiments were conducted as discussed below:

2.3.3.1. Effect of storage condition on seed viability

Effect of two temperatures conditions i.e. room temperature and refrigerated (4°C); on the enhancement of the seed germination was studied. Initially seeds stored at room temperature were germinated and when these seeds failed to germinate, then the seeds stored at 4°C were germinated on paper bridge. After some time low temperature stored seeds showed low germinations. Then they were treated with different soaking treatments for breaking dormancy.

2.3.3.2. Effect of soaking seed treatment for breaking dormancy

Seeds of *N. foetida* were treated with various soaking treatments for breaking the seed dormancy. These treatments include:

1. KNO_3 (0.2, 0.3 and 0.4%)- 24hr
2. Water-24 hr
3. GA_3 (100, 150 and 200 ppm)

2.4. *In vitro* Shooting

2.4.1. Effect of different plant growth regulators (PGRs) on shoot multiplication

Different PGRs i.e. BAP (0.5-3.0 mg/L), TDZ (0.25-2.0 mg/L), KIN (0.5-2.5 mg/L) were used to study their effect on multiplication of shoots. Shoots were taken from 2 months old seedlings and were inoculated in the culture vessels containing MS medium containing different PGRs for shoot multiplication. Explants were placed properly in contact with the medium and were sub cultured regularly after 25-30 days.

2.4.2. Effect of different explants type on shoot multiplication

To study the effect of explants type, different explants like nodal segments, shoot tip, leaves, roots and cotyledons were used for the *in vitro* shoot multiplication. MS medium supplemented with 0.5 mg/L TDZ was used to induce shoots on various explants as it was found to be most appropriate plant growth medium.

2.4.3. Effect of sucrose concentration

Different sucrose concentration (0, 10, 20, 30 and 40 g/L) was supplemented in MS medium with 0.5 mg/L TDZ.

2.4.4. Shoot elongation

The multiple shoots derived from different explants (nodal segment, shoot tip and leaf explants) were excised and sub cultured on shoot elongation medium augmented with different concentrations of PGRs. Different concentration of GA3 (0.5-2.0 mg/L) in combination with BAP (2.0 mg/L), KIN (0.5 mg/L) and Glycine (1.5 mg/L) MS medium was used for the elongation of the shoots. Further subculture was done by subsequent transfer to fresh medium of the same composition at fort nightly intervals for sixty days total duration.

2.5. *In vitro* Rooting

Shoots derived from the previous experiments were used as the starting material for the initiation of the rooting in *N. foetida.*

2.5.1. Optimization of MS media strength and PGRs concentration for maximum root induction

Rooting was induced under *in vitro* conditions. 1.5-2.0 cm long shoots with 2-3 leaves derived from previous shoot cultures were transferred to MS full, half and One-fourth strength media containing IBA (3.0 mg/L) in combinations with BAP (0.5-2.0 mg/L) for *in vitro* root induction. Full strength MS basal medium was taken as control.

2.5.2. Response of shoots derived from different explants on rooting

Shoots derived from different explants (nodal segment, shoot tip and leaves) were rooted in MS media supplemented with IBA (3mg/L) and BAP (0.5-2.0 mg/L) for efficient rooting.

2.5.3. Effect of agar concentration

Different agar concentrations (0.2-1%) were evaluated for the proper rooting of the explants on MS medium supplemented with 3mg/L IBA + 2mg/L BAP.

2.5.4. Effect of activated charcoal (AC) on rooting

To overcome the problem of callusing and shoot tip necrosis in plantlets different concentration of activated charcoal (100-500 mg/L) were added in MS media (full strength and half strength) with optimized concentration of PGRs.

2.6. Hardening and Acclimatization

Plantlets with well developed roots were removed from the flasks and washed with sterilized distilled water to remove adhering medium. Subsequently, plantlets were transferred to glass beakers containing soilrite (Soilrite is mixture of Irish peat moss and perlite in 3:1 ratio) and and moisture was retained by covering with another beaker. Plantlets were maintained in controlled environment (Temperature: 26±2° C, light: 16 hrs light and 8 hrs dark) for 20 days. Thereafter, plantlets were shifted to green house in pots containing soil and manure in 1:2 ratios in green house of herbal garden at Maharshi Dayanand University Rohtak.

2.7. *Agrobacterium* mediated genetic transformation

2.7.1. Plant material and explants preparation

In vitro grown seedlings were used as the explant (leaves, nodal segment, petiole and radical) for the induction of hairy roots. The explants were excised and used for transformation studies.

2.7.2. Bacterial strains

Following *Agrobacterium rhizogenes* strains were used for transformation:

Table.2.3. Bacterial strains used for infection

S.No.	Bacterial strains	Growth medium
1	*Agrobacterium rhizogenes* NCIM *5140*	**YEB**
2	*Agrobacterium rhizogenes* A4	**YMB**
3	*Agrobacterium rhizogenes* MTCC 532	**YEB**
4	*Agrobacterium rhizogenes* MTCC 2364	**Xanthomonas**
5	*Agrobacterium rhizogenes* LBA 9204	**YMB**

2.7.2. Culture conditions and media

After the induction of hairy roots in all the experiments, transformation frequency was calculated using following formula:

$$\text{Transformation frequency} = \frac{\text{Number of explants forming hairy roots}}{\text{Total number of explants taken}} \text{ X } 100$$

Co-cultivation medium: MS basal medium without PGRs

Growth medium: MS basal medium with antibiotics

Proliferation medium: MS basal liquid medium without PGRs

2.7.3. Transformation

2.7.4.1. Preliminary transformation experiments

For the preliminary studies standard protocol given in the literature was followed. Standard protocol is discussed as follows:

1. First of all bacterial culture for all the five strains were initiated on the respective medium. Single colony of *A. rhizogenes* was picked from 18 hrs old culture and was inoculated in suitable liquid bacterial medium (shaker incubate at 28°C(200rpm) overnight).
2. After this, aliquot of the culture was taken at different time interval and optical density (OD) at 600 nm was taken (0.6, 0.8, 1, 1.2, 1.4, 1.6). Different concentrations of *Agrobacterium rhizogenes* were identified for the different strains of *Agrobacterium rhizogenes* used in the study.
3. On the day of experiment bacterial cultures were centrifuged at 6000 x g for 8 minutes and the pellet was resuspended in liquid plant MS medium.
4. Explants were taken from *in vitro grown* seedlings (leaves, petiole, nodal segment, radical) in small pieces of about 1-1.5 cm.
5. Two methods were employed for the infection. Explants were either pricked with needle of a syringe containing *Agrobacterium rhizogenes* culture or cut and submerged in the bacterial suspension (10-15 explants/5ml of media) for 5-35 minutes of infection time.
6. After the completion of infection period, explants were blotted dried on sterile filter paper to remove excess of the bacterial suspension.
7. Then the explants were transferred to MS basal media without PGRs for co-cultivation (24-96 hours).
8. Temperature for co-cultivation was varied from 18°C to 26°C.
9. After co-cultivation, the infected explants were washed three times with sterile distilled water, followed by washing with antibiotic solution (50mg/L) with vigorous stirring using sterile forceps and then blotted dry with sterile filter paper.

10. Further explants were transferred to MS medium without PGRs and supplemented with different concentration and type of antibiotics.
11. Subsequent sub culturing of the infected explants was done under same incubation conditions on solid medium containing different concentration and type of antibiotics to prevent the growth of bacteria.

The hairy root induction protocol needs optimization for getting positive results. Thus, different parameters, which affect the infection probability, were optimized. After selection of explants, these were transformed with all the available strains of *A. rhizogenes* then, concentration of acetosyringone was optimized with above optimized factors. Exposure time of bacterial culture with wound was then optimized.

2.7.4.2. Optimization of various parameters for efficient production of hairy roots

A number of factors like method of wounding, type of explants, co-cultivation time and infection medium are responsible for successful transformation, which ultimately affects the hairy root induction. These factors were studied and optimized according to the results obtained after preliminary experiments.

2.7.4.2.1. Effect of infection time and co-cultivation period on transformation frequency

Wounded explants were exposed to bacterial suspension for different time intervals (15, 20 and 25 minutes) before they were placed on the co-cultivation media. After exposure to bacterial suspension, explants were co-cultivated for different time periods of 24, 48 and 72 hours on MS basal medium.

2.7.4.2.2. Effect of co-cultivation temperature on hairy root induction

Effect of co-cultivation temperature (20, 22, 24, 26 and 28^{0}C.) on transformation frequency was studied.

2.7.4.2.3. Effect of addition of acetosyringone

In order to increase successful infection, co-cultivation medium was supplemented with different concentrations (100, 150 and 200 µM) of acetosyringone .

2.7.4.2.4. Potential of bacterial strain and bacterial cell density on transformation.

Effect of different bacterial strains at different cell density (0.6-1.2 OD at 600 nm) on transformation frequency was studied.

2.7.4.2.5. Effect of explants on hairy root induction

Various types of explants (leaf, radical, nodal segment and petiole) were used for transformation by three different strains of *A. rhizogenes* (A4, LBA9204 and MTCC532).

2.7.4.2.6. Effect of method of making wound on transformation frequency:

(a) Cutting: Explants were wounded by cutting using a sterilized scalpel blade under the laminar hood.

(b) Pricking: Wound in all the explants were made by pricking with sterilized syringe containing adequate activated bacterial suspension.

2.7.4.2.7. Effect of antibiotic type and concentration on recurrence of *A. rhizogenes* after co-cultivation

In order to determine the effective antibiotic which is capable of suppressing the growth of *A. rhizogenes* after co-cultivation, two antibiotics were tested. These include carbencillin and cefotaxime, alone (250, 500 and 750 mg/L) or in combination (250+250, 500+500 and 750+750 mg/L).

2.7.4.3. Development of hairy root culture in liquid medium

After the proper growth on semi-solid agar supplemented medium, roots were transferred to 250 ml Erlenmeyer flasks containing 50 ml MS liquid medium. Hairy roots in liquid medium, were kept rotating on a gyratory shaker at 60 RPM under 16/8 h light/dark regime. Temperature was maintained at 26±1 °C and initial pH of the medium was set at 5.8 after 25 days hairy roots were harvested for the generation of biomass (DW, gm) and subsequent production of camptothecin (mg/L).

2.8. Polymerase chain reaction (PCR)

DNA isolation from plant and plasmid DNA for PCR analysis was carried out using following procedures:

2.8.1. Plant genomic DNA isolation

Plant DNA was isolated using CTAB method.

Requirements:

(a) Plant sample (untransformed root and hairy root), liquid nitrogen, sterile pestle and mortar, centrifuge, water bath, sterile micro centrifuge tubes (2ml), sterile micro tips and micropipette.

(b) Stock solutions

(i) 1.0 M Tris-HCl

Dissolve 60.57g TrisHCl in distilled water, adjust pH-8.0 with conc. HCl and raise the volume up to 500 ml. Autoclave and store at room temperature.

(ii) 0.5 M Na_2EDTA

Dissolve 93.06 g EDTA in distilled water, adjust the pH 8.0 and raise the volume upto 500 ml. Autoclave and store at room temperature.

(iii) 5.0 M NaCl

Dissolve 146.1 g NaCl in distilled water, raise the volume upto 500 ml. Autoclave store at room temperature.

(iv) 10 % CTAB (Cetyl trimethyl-ammonium bromide)

(v) B-mercaptoethanol

(vi) Chloroform: Isoamyl alcohol solution (24:1)

(vii) 3.0 M Sodium acetate solution

Dissolve 204.12 g sodium acetate in distilled water, adjust pH to 5.2 and raise the volume upto 500 ml, autoclave and store at room temperature.

(viii) Alcohol

(ix) RNase A (10 mg/ml)

(c) Working solution- Preparation of 50 ml DNA extraction buffer

(i)	1.0 M Tris-HCl	5.0 ml
(ii)	0.5 M Na_2EDTA	2.0 ml
(iii)	5.0 M NaCl	14 ml
(iv)	10% CTAB	10 ml
(v)	B-mercaptoethanol	0.1 ml
(vi)	D-H_2O	19 ml

Procedure: DNA isolation

(a) 100 mg sample (fresh roots and hairy roots) was taken and grinded to fine powder with liquid nitrogen, transferred to autoclaved 2 ml micro centrifuge tubes.

(b) 1 ml of pre-heated (65°C) DNA extraction buffer was added in each tube, vortexed and incubated in water bath at 65°C for 1 hour by mixing 3-4 times in between.

(c) Samples were centrifuged for 5 minutes at 12000 rpm. Supernatant was transferred to new tubes and 5 µl RNAase A solution was added and it was kept for incubation at 32°C for 20 minutes.

(d) Samples were kept at room temperature for 10 minutes and then 667 µl ($2/3^{rd}$ vol.) of chloroform: isoamylalcohol (24:1) was added, vortexed for 5 sec then centrifuged for 1 minute.

(e) Upper aqueous layer was transferred carefully to new tube. This was repeated until upper phase was clear.

(f) $2/3^{rd}$ vol. of ice cold isopropanol was added and mixed gently and were kept on ice for 10 minutes to precipitate DNA.

(g) Then it was centrifuged at 12000 rpm for 15 minutes. Supernatant was discarded and the pellet was washed with 500 µl ice cold 70% ethanol twice.

(h) Ethanol was removed and the pellet was dried. The DNA pellet was dissolved in 20 µl of TE buffer.

2.8.2. Plasmid DNA isolation from *A. rhizogenes*

Plasmid DNA was isolated using Plasmid Mini Kit (Sure spin).

Requirements

Incubator, waterbath, culture flasks, centrifuge, micropipette, 2 ml centrifuge tubes, collection tubes, plasmid column, microtips.

Reagents: Resuspension buffer, Lysis Buffer, Neutralization buffer, Wash buffer, wash buffer (concentrate), Elution buffer, RNase A and Ethanol

Procedure

(a) 5-10 ml of the saturated *Agrobacterium rhizogenes* culture was centrifuged at 11000 for 30 seconds. Supernatant was directed.

(b) Pelleted bacterial cells were resuspended in 500 µl resuspension buffer and transferred to microcentrifuge tube. RNase A solution was added to this resuspension buffer.

(c) 500 µl of lysis buffer was added to it and was mixed thoroughly by inverting the tube 5-7 times. Lysis reaction was not allowed to proceed more than 5 minutes.

(d) 600 µl of neutralization buffer was added, mixed immediately and thoroughly by inverting the tubes 5-7 times. Solution became cloudy.

(e) Then it was centrifuged for 10 minutes at 11000 rpm. A compact white pellet and clear supernatant was obtained.

(f) Plasmid column was placed in a collection tube and supernatant from above step was decanted in it. It was centrifuged for 1 minute at 11000 x g. The flow through was discarded and the column was placed back into the collection tube.

(g) Now the column was washed with 500 µl wash buffer and centrifuged for 30 seconds. Then wash buffer supplemented with ethanol was added and centrifuged at 11000 x g for 1 minute. Flow –through was discarded and column was placed back into the empty collection tube.

(h) For the drying of the silica membrane, it was centrifuged for 2 minutes at 11000 xg and collection tube was discarded.

(i) For the elution of the DNA, plasmid column was placed in 1.5 ml microcentrifuge tube and 50 µl of the elution buffer preheated to 70°C was added and was incubated for 2 min at 70°C. Then centriguged for 1 minute at 11000 x g.

2.8.3. PCR analysis

2.8.3.1. Primer used

Rol B gene: Forward-5'-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3'

Reverse-5'-TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3'

2.8.3.2. PCR condition

The PCR reactions and DNA amplifications were carried out according to the procedure given by Pal *et al*., 2013. The PCR reactions were carried out in total 25 µl volume and consisted of 3 µl of DNA, 1 µl DNTPs, 2 µl $MgCl_2$, 5 µl PCR Buffer, 1 µl each 10 µM forward and revearse primers. 0.2 µl of Taq DNA polymerase. DNA amplifications were performed in a thermal cycler (Agilent SureCycler 8800, Agilen Technologies) using the programme: initial tempelate denaturation at 95 °C for 2.5 minutes, followed by annealing at 55°C for 1 minute, extension at 72°C for 3 minutes, for the first cycle. This first cycle was followed by 33 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 30 seconds and extension at 72°C for 1.5 minutes. The final cycle was extended with an additional step at 72 °C for 5 min prior to hold at 4 °C.(Pal *et al*., 2013).

The amplified products were separated by electrophoresis on 1 % agarose gels stained with 0·5 mg L^{-1} ethidium bromide in 0·5X TAE (Tris-acetate EDTA buffer). The gel was photographed using gel documentation system (Bio- RAD Gel Doc XR^{+}).

2.9. CPT estimation and analysis

2.9.1. Plant materials and reagents-

In vitro generated plant material viz. leaves, shoots, roots and hairy roots along with seed and seed coat were used. All chemicals (methanol, acetonitrile and distilled water were of HPLC grade) were of analytical grades. Camptothecin was obtained from Sigma (India).

2.9.2. Camptothecin extraction

All the samples including plant samples, seed coat, seeds and hairy roots of *N.foetida* were dried at 50°C for 48 hours in an oven and were grinded using pestle and mortar to obtain fine powder. The powdered was stored in sealed polyethylene bags and used for further extraction using different extraction techniques. Procedure of different extraction techniques are discussed below.

2.9.2.1. Stirring extraction

For stirring extraction 1 gm of the plant material was taken in a 250 ml beaker with 25 ml of methanol and stirred on a magnetic stirrer with heater. The stirring was carried out at approximately 120 rpm at 70°C for 1 hour. After one hour the extract obtained was cooled and centrifuged at 6000 rpm. The subsequent was used for the estimation of CPT using HPLC. The extract was passed through 0.2 µm nylon syringe filter before injecting into the HPLC.

2.9.2.2. Soxhlet extraction

1 gm powdered plant material was taken in filter paper thimble and was inserted in the soxhlet extractor of 20 ml capacity. This extractor was fitted with 50 ml round bottom flask containing 30 ml of 90% methanol. The solvent was heated and refluxed for 2 hours at 65°C.

2.9.2.3. Microwave extraction

1 gm powdered plant material was taken in 25 ml volumetric flask containing 5 ml of 90% methanol. This flask was exposed for 3-4 seconds in microwave oven at full power (800 W), so that the material is not allowed to super boil. Then the flask is taken out and cooled in a water bath for 30 seconds. Again the same steps were repeated to get a total exposure time of 60 seconds.

2.9.3. Calibration of HPLC

For making standard stock solution, 5mg of camptothecin was dissolved in 2 ml DMSO and volume made up to 25 ml with methanol in a volumetric flask. Dilutions were made to get concentration of 5, 10, 20 and 40 µg/ml. Standard solutions filtered through 0.2 um filters before injection. It was observed that the area under the peak doubled with each increment in CPT

concentration. Retention time of CPT was 6.2 minutes. This method is sensitive and accurate with good reproducibility. The results of the three injections from the same samples at the four concentrations (5 µg/ml-40µg/ml) showed similar retention time. The analytical operation can be completed in 15 min.

2.9.4. Camptothecin estimation

The HPLC analysis of camptothecin were carried out on a Younglin Instrument Korea HPLC (model Acme 9000) equipped with a 20 µl injection loop and a double-wavelength detector (Model No. UV730D). Data collection and integration were accomplished using Autochrom 3000 software. Separation was performed on a Promocil C18 column (250 mm x 4.6 mm i.d., Agela Technologies, USA). The camptothecin was determined by using acetonitrile and distilled water (40:60, v/v) as mobile phase. The flow rate was 1 ml/min and the elution was monitored at 254 nm.

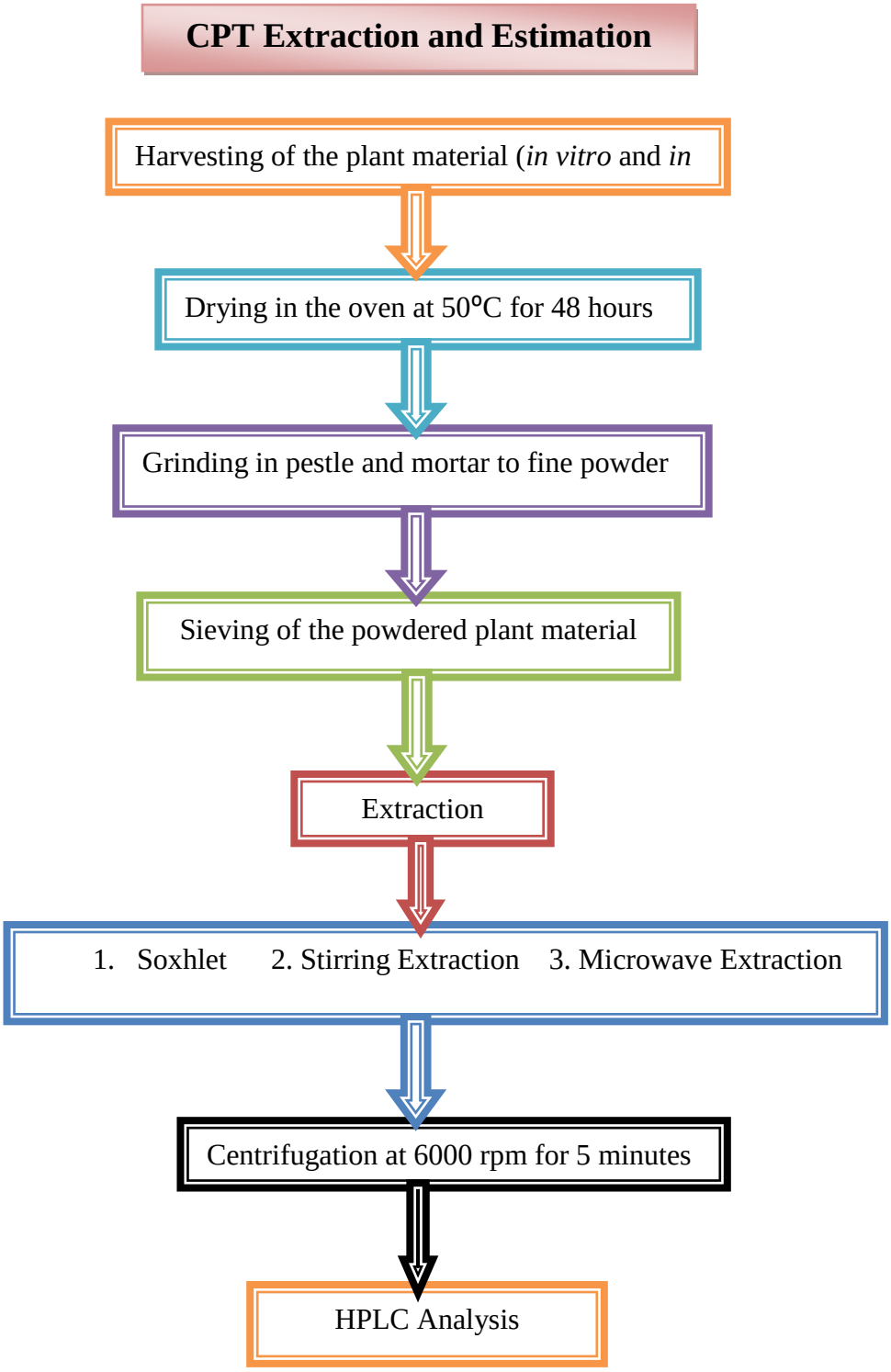


Figure. 5. Flow chart showing the Camptothecin extraction and estimation procedure

Section III

RESULTS AND DISCUSSION

3.1. *In vitro* seed germination

3.1.1. Viability test of seeds stored at low temperature and room temperature

Viability of seeds was determined by tetrazolium (TZ) test which is biochemical method for evaluation of seed viability. The vital strain 2, 3,5-triphenyl tetrazolium chloride, produces a red colored insoluble compound named triphenyl formazon due to the enzymatic dehydrogenation process in the living tissues (Franca-Neto, 1999). Due to this the viable embryos within the seeds give red color and non viable seeds remain unaffected. However the test can be affected by the given conditions such as, the presence of fungi, which can affect the germination test results (Franca-Neto, 1999).

In the present study, the fresh seeds of *N. foetida* showed 90.7% of viability which implies that seeds were having high vigour before storage. However, after one year of storage of seeds at room temperature the viability decreased to 44%, which shows that the viability decreased after storage of seeds. Whereas, the seeds stored at low temperature conditions showed a viability of 73.3 %, even after a year. After two years of storage, the seeds stored at low temperature showed 61% viability, whereas the seeds stored at room temperature didn't showed any viability (Table 3.1).

The TTC- test is usually used to check the viability of the seeds of many plant species. For instance, *Rubia fruticosa* Ait Orchids grasses and *Cucumis anguria* . (Marrero *et al.*, 2007)

Table 3.1. Viability test of seeds stored at low temperature and room temperature

Duration of storage	**Storage conditions**	
	Room temperature	**4 ^{o}C**
	Viability (%)	

Fresh seeds	90.7± 1.2 [a]	-
1 year old seeds	44.0± 2.1 [b]	72.3 ± 1.5 [a]
2 year old seeds	0	61.0 ± 2.3 [b]

The mean value of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

3.1.2. Effect of various sterilizing treatments on seed germination and contamination

The initial and most important step in plant tissue culture is sterilization of explants because this step ensures the further success of the cultures. It has been reported that in appropriate amount of sterilants used during sterilization have lethal effect in cell division and it ultimately restricts the growth and development of explant. Thus, appropriate concentration, combination and duration of exposure of sterilant play an important role *in vitro* propagation. And if the concentration and exposure time goes beyond the optimum level, it may lead to the loss of explants (Danso *et al*., 2011).

For the sterilization experiments seeds with seed coat and without seed coat were used. Prior to sterilization, seeds were washed with soap solution and under running tap water for 5 minutes. Effect of two sterilizing agents ($HgCl_2$ (0.1%) and NaOCl (2%)) and their duration of exposure (3-10 minutes) were studied during the present investigation. Various treatments are presented in (Table. 3.1). Sterilized distilled water was used as a control and under control conditions 100% contamination was observed.

However, there was no contamination in the treatments where seeds were subjected to 70% ethanol for 1 minute followed by 2% NaOCl for 5, 8 and 10 minutes. The best combination of sterilizing agents was found to be 70% ethanol for 1 minute followed by 2% NaOCl for 5 minutes; because this treatment did not inhibit the seed germination and maximum germination (76% was obtained). Whereas, in other treatments where contamination was found to be zero, seed germination was inhibited due to longer duration (8 and 10 minutes) of exposure of NaOCl.

In present investigation results showed that NaOCl with ethanol significantly reduced the contamination as well as has no inhibitory effect on the seed germination. It was found that surface sterilization carried out using $HgCl_2$ and ethanol was not enough to disinfect the seeds as contamination was recorded in cultures and hence poor germination as well.

The microbial effectiveness of NaOCl is based on its high pH (12.5) due to OH^- ions, which interferes with cytoplasmic membrane and act on essential bacterial enzymatic sites (Esterella *et al*., 1995). Therefore, sodium hypochlorite is considered as a very effective microbe killer and is used frequently for surface sterilization of plants in plant tissue culture (Nakagawara *et al*., 1998; Talei *et al*., 2011). However, the concentration and exposure of NaOCl varies species to species (Aasim *et al*., 2013). The use of sodium hypochlorite for surface sterilization of plant has been widely reported (Miche and Balandreau, 2001; Vejsadova, 2006; Maina *et al*., 2010; Colgecen *et al.*, 2011; Morla *et al*., 2010; Sathyagowri and Seran, 2011).

Table. 3.2. Effect of various sterilizing treatments on seed germination and contamination

Treatments	Sterilizing agent with time duration	% Contamination	% Germination
Control	Sterilized distilled water	100±00	0
1	Ethanol(70% v/v) for 1 min + $HgCl_2$ (0.1% w/v) for 3 min	67.3 ±1.5 [b]	29±2.0 [f]
2	Ethanol(70%) for 1 min + $HgCl_2$ (0.1%) for 5 min	54.3±2.2 [c]	34.7±0.9 [e]
3	Ethanol(70%) for 1 min + $HgCl_2$ (0.1%) for 8 min	33.0±1.5 [d]	46.0±2.1 [d]
4	Ethanol(70%) for 1 min + $HgCl_2$ (0.1%) for 10 min	26.7± 3.0 [e]	62.0±1.7 [b]
5	Ethanol(70%) for 1 min + NaOCl (2% v/v) for 3 min	6.3±0.9 [f]	51.0±1.5 [c]
6	Ethanol(70%) for 1 min + NaOCl (2%) for 5 min	0	78.3±1.5 [a]

7	Ethanol(70%) for 1 min + NaOCl (2%) for 8 min	0	63.0±1.7 [b]
8	Ethanol(70%) for 1 min + Na OCl (2%) for 10 min	0	53.7±0.9 [c]

The mean value of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

3.1.3. Effect of different supporting media on seed germination

The nature of the support provided to the seeds was found to be a critical factor for successful seed germination. Four supporting medium were employed i.e. cotton disc, filter paper disc, agar and paper bridge. The emergence of radical was considered as the initiation of the germination. Out of these, paper bridge method gave maximum i.e. 82.33% germination in seeds without seed coat and 52.67% in the seeds with seed coat. Distilled water gelled with agar gave a germination of 61% in seeds without seed coat and 37.67% in seeds with seed coat. Other supporting media like filter disc and cotton disc gave significantly low percent germination in both seeds with seed coat and without seed coat.

It is speculated that the excess phenolics released by seeds may not diffuse rapidly on agar-solidified medium and that the resulting local concentration of phenolics around the seeds might be toxic. The germination method using filter-paper bridges proved to be a better one, probably because the rapid diffusion of phenolics in liquid medium prevented toxic levels from being reached. In paper bridge method, there is a constant excess of nutrients, which is limited in semi-solid medium. Moreover, the scarcity of water is there in other treatments, which is pre-requisite for germination.

The results also indicated that in paper bridge method high moisture was maintained inside the test tube. This high humidity may be congenial for the germination of seeds in this taxon (Tejavati *et al*., 2011). This clearly indicates that the seeds of *N. foetida* require high moisture and water content for the process of germination.

Paper bridge method with sterile distilled water has beneficial effect on seed germination. The results obtained on seed germination of *Macrotyloma uniflorum* also support the present results

(Tejavathi *et al*., 2011). Easy accessibility of culture medium components leads to effective hydration of the seeds so that there is a proper germination in *Acacia nilotica* L. (Dhabhai *et al.,* 2010), *Triticum aestivum* (Bildar *et al.,* 2011), *Zea mays* (Bildar *et al*., 2012). Seed germination and seedling growth was increased when paper bridge with distilled water was used *in Viola wittrockiana* (Bildar *et a*l., 2014).

Table. 3.3. Effect of different supporting media on seed germination

Treatment	With seed coat		Without seed coat	
	% Germination	Emergence time days	% Germination	Emergence time days
Agar with distilled water	37.7±1.8[b]	17	61.3±2.4[c]	11
Agar with MS media	30.0±4.6[b]	19	57.0±2.3[c]	14
Paper bridge With distilled water	52.6±2.3[a]	12	82.3±2.0[a]	4
Paper bridge With liquid MS media	47.3±2.9[a]	15	72.3±2.2[d]	6
Filter paper disc	15.3±2.3[c]	19	24.3±1.7[d]	16
Cotton disc	17.3±1.5[c]	18	29.7±2.3[d]	15

The mean value of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

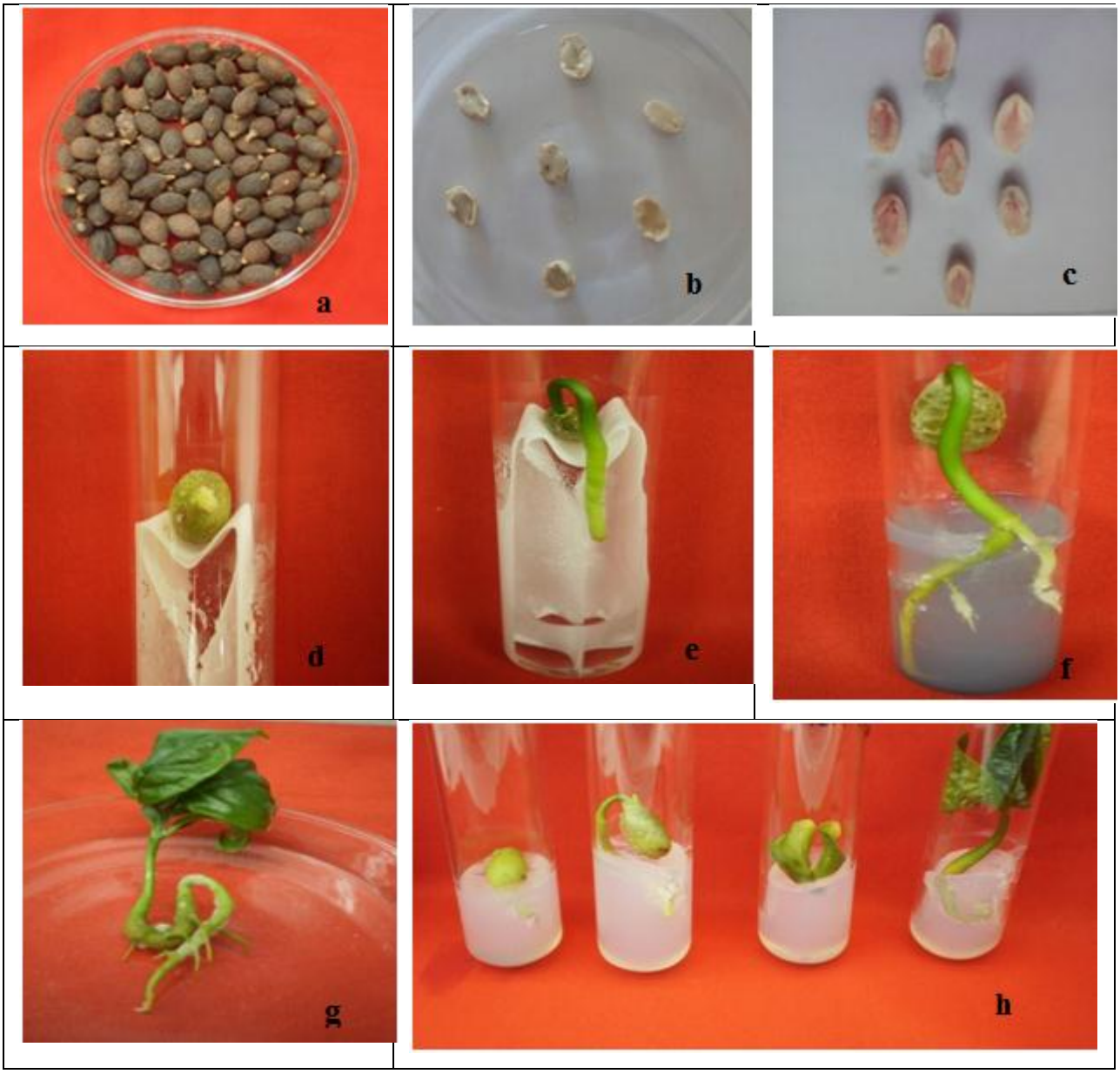


Plate 2. Viability test and germination behavior of *N. foetida* (a) Seeds (b) Tetrazolium test showing non-viable seeds (c) Viable seeds (d-f) Radicle emergence of seed on paper bridge (g) Fully grown seedling after 45 days (h) Various stages of seed germination on semi-solid MS medium

3.1.4. Effect of duration of storage and storage conditions on seed germination of *Nothapodytes foetida* during different months

In vitro seed germination experiments were carried out using paper bridge method with sterile distilled water, as it gave the maximum seed germination. After detailed studies, it was found that the *in vitro* seed germination was 84.66% in the seeds without seed coat and 56% in the seeds with seed coat after 3 months of storage at room temperature. After 8 months of storage, seed germination was found to be zero in case of seeds with seed coat whereas in seeds without seed coat, it dropped to 8 % after 10 months of storage at room temperature.

After 10 months of storage, refrigerated seeds were germinated and germination was found to be 76.3% in seeds without seed coat which decreased to 24% after 21 months of storage at 4° C. Moreover, with the passage of time duration of initiation of seed germination was found to increase in subsequent months.

The storage time and temperature conditions affect the germination rate due to change in physiology of seeds or dormancy of seeds. The changes in temperature and humidity can result in low seed vigour, low germination and reduced survival of seedlings (Bewley *et al*., 1994; Begnami *et al*., 1996). These conditions are believed to affect protein metabolism (Bewley *et al*., 1994) and cause a reduction of biochemical activity of seed (Begnami *et al*., 1996).

The germination percentages of soybean seeds stored at 5° C was significantly more than the seeds stored at 25° C (Singh *et al*., 2008). In another study the storage life of soybean was found to be increased by lowering temperature and seed moisture content during storage . In *Swertia chirayita* high seed germination, low mean germination time, and low rate of fall in seed germination percentage in seeds stored at 4° C over different storage period were recorded (Pradhan *et al*., 2012).

Previously,it was reported that the seeds of *N. foetida* loose viability after 2-3 months and failed to germinate after sometime (Tejavathi *et al*., 2011). In present investigation increased seed germination was observed on paper bridge method. Moreover the viability of the seeds was further increased by storing at 4° C.

Results showed that seed germination decreased with the increase in storage time. The viability can be increased with low temperature storage of the seeds of *N. foetida*. Above 50% seed germination even after 12 months of storage at 4°C, suggests that this storage temperature is the most appropriate storage condition for long term storage of seeds.

Table. 3.4. Effect of duration of storage and storage conditions on seed germination of *Nothapodytes foetida* during different months

Time of storage In months	Seeds stored at room temperature			Seeds stored at 4 °C		
	Percent Germination			Percent Germination		
	With seed coat	Without seed coat	Initiation of germination (days)	With seed coat	Without seed coat	Initiation of germination (days)
3	56.0±3.2 [a]	84.6±2.0 [a]	5			
4	42.0±2.9 [b]	73.00±2.3[b]	8			
5	33.0±2.5 [c]	63.7±1.2[b]	10			
6	24.7±1.2 [d]	61.6±2.3 [b]	12			
7	13.0±2.5 [e]	45.0±3.1 [c]	12			
8	6.6±0.9 [f]	26.0±1.7 [d]	15			
9	-	14.3±1.8 [d]	15			
10	-	8.0±1.5 [d]	16	-	76.3±1.2[a]	5
11	-	0.00		-	72.3±1.7 [a b]	5
12	-	-		-	69.0±1.7 [a b c]	6
13	-	-		-	64.6±2.0[b c]	5
14	-	-		-	61.7±2.2 [c]	6

15	-	-		-	59.0±2.1 [c]	5
16	-	-		-	60.7±2.0 [c]	7
17	-	-		-	59.3±2.7 [c]	7
18	-	-		-	49.6±3.5 [d]	6
19	-	-		-	44.6±1.7 [d e]	7
20	-	-		-	40.3±1.5 [e]	6
21	-	-		-	24.0±1.0 [f]	8
22				-	-	

The mean value of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

3.1.5. Effect of soaking treatments for breaking dormancy

The effect of gibberelic acid (GA_3) and potassium nitrate (KNO_3) were conducted to determine the seed germination response for *N. foetida*. An experiment was conducted with three treatments including: different concentrations of GA_3 (100, 150 and200 ppm), KNO_3 (0.2, 0.3 and 0.4%) and distilled water for breaking seed dormancy and promoting seed germination.

Maximum percent germination (68.3%) was obtained with 0.3% KNO_3. When the concentration was varied to 0.2% and 0.4%, percent germination was decreased to 64% and 55.6% respectively. When the seeds were soaked in distilled water 37.7% seeds were germinated (Table 3.5).

Priming is known to activate internal metabolism required for furthering the seed germination process (Basra *et al*., 2005). KNO_3 is most widely used chemical for promoting germination. Effects of seed priming with KNO_3 have been previously studied in other plant species (Amjad *et al*., 2007; Shim *et al.,* 2008; Eskandari *et al.,* 2011). Nitrates clearly stimulate the germination dormant seeds (Alboresi *et al*., 2005).

Seed germination of *Paspalum vaginatum* was improved by soaking in 0.5% KNO_3 solution for two days (Sang *et al*., 2008). Similarly, in maize seed priming with KNO_3 resulted in better seedling establishment .

In case of GA_3 seeds showed 63.3% germination at a concentration of 150 ppm. When the concentration of GA_3 was varied to 100 and 200 ppm, percent germination (53% and 55% respectively) was lowered significantly. Breakdown of endosperm is generally assumed to be an essential process of initiation of germination. A biochemical reaction in endosperm of cereal grains is known to be enhanced by GA_3 i.e. synthesis of hydrolases (Kolumbina *et al*., 2006).

Table. 3.5. Effect of soaking treatments for breaking dormancy

Treatment		**Percent germination**	**Initiation of germination (days)**
Control		0	0
KNO_3 (24 hr)	0.2%	64.0±2.1 [a]	5
	0.3%	68.3±1.5 [a]	5
	0.4%	55.6±2.0 [b]	6
Water (24 hr)		37.7±2.0 [c]	7
GA_3 (1 hr)	100 ppm	53.0±1.5 [b]	6
	150 ppm	63.3±3.2 [a]	5
	200 ppm	55.0±3.2 [b]	6

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level

3.2. *In vitro* shooting

A series of experiments were conducted for standardization of the shoot multiplication step.

3.2.1. Effect of different phytohormones on shoot multiplication

In vitro propagation is one of the key approaches for proliferation of medicinal plants in large scale. Preliminary work was done to assess the effect of various phytohormones on shoot multiplication, number of shoots and nature of response. Generally it is considered necessary to include cytokinins in the medium for proper shoot multiplication (Sutter *et al*., 1996; Dobranszki *et al*., 2010). Multiple shoots were induced on MS media supplemented with BAP, Kinetin TDZ and their combinations. The number of shoot primodia arising from the explants differed significantly with different plant growth regulators and their combinations.

Maximum shoot multiplication of 77.6% was obtained on MS medium supplemented with 0.5 mg/L TDZ with average number of 15.33 shoots per explant (Table 3.6). But further increment in the TDZ concentration in medium, decreased percent shoot multiplication and stunted growth of shoots was obtained.

TDZ is among the most active cytokinin and it induces greater *in vitro* shoot multiplication than other cytokinins in many plant species (Khawar *et al*., 2004). Differential response was obtained in different plant species when TDZ was used as PGR. It has been reported that TDZ induced better shoot regeneration than BAP in peanut (Victor *et al*., 1999: Gairi and Rashid, 2004). Whereas, in some species such as *Tectona grandis* it was found to induce callus .

In case of Kinetin maximum shoot multiplication (52.67%) and maximum number of shoots (7.3) was obtained with a concentration of 1.5 mg/L. when MS medium was supplemented with BAP, best results (67.3%) and maximum shoots (8.6 per explant) was obtained at a concentration of 2.0 mg/L. Further, when combination of TDZ (1 mg/L) and BAP(2mg/L) was used, 70.3% shoot multiplication was observed. Difference in internal growth regulator content of each species has been suggested to be the reason for difference in *in vitro* response.

Table 3.6. Effect of different phytohormones on shoot multiplication

Conc. Of PGRs mg/L			Observations		
KIN	**TDZ**	**BAP**	**Shoot multiplication (%)**	**No. of shoots/ explants**	**Nature of response**
Control	-	-	16.66±2.03	1.33±0.33	Stunted growth
0.5			40.67±2.33 ^{a}	3.67±0.33 ^{a}	Stunted growth
1.0			47.66±2.027 ^{b}	5±0.57 $^{a\,b}$	Stunted growth
1.5			52.67±3.48 $^{b\,c}$	7.33±0.66 ^{c}	Stunted shoots
2.0			42.66±0.88 $^{a\,b\,d}$	5.667±0.88$^{a\,c}$	Callusing
2.5			59±2 $^{b\,c}$	5.33±0.66 $^{a\,c}$	Callusing
	0.25		64.33±1.76 $^{a\,b\,c}$	9±0.58 $^{d\,c}$	Stunted shoots
	0.5		71.66±2.60 ^{a}	15.33±2.60	Numerous shoot primodia
	1.0		63.66±3.48 $^{a\,c}$	11±3.48 ^{d}	Greenish small leaves
	1.5		60±2.31 $^{b\,c}$	7±0.57 $^{b\,c\,e}$	Yellowish green leaves, thin shoots
	2.0		52±1.53 $^{b\,i}$	7±1 $^{c\,b}$	Yellowish green, stunt growth
		0.5	48.66±2.33 $^{b\,d}$	8.33±0.67 $^{c\,f}$	Dark green shoots
		2.0	69.33±2.02 ^{a}	8.67±0.66 $^{c\,f}$	Elongated shoots
		3.0	56.33±2.60 ^{c}	7.66±1.20 ^{c}	Shoots with yellowish green leaves
	0.5	1.0	45.33±2.33 $^{a\,b}$	5.33±0.88 $^{a\,b\,c\,e}$	Stunted shoot
	0.5	2.0	68±1.52 $^{a\,b}$	5.33±0.66 $^{a\,c}$	Nodular callus
	1.0	1.0	70.33±1.45 ^{a}	10.33±0.88 $^{d\,f}$	Large leaves

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level

Plate.3. Shoot multiplication in *N. foetida* with different plant hormones. (a-b)Induction of multiple shoots in MS media supplemented with 0.5 mg/L TDZ after 10 days and 25 days. (c) Stunted shoots at a higher concentration of TDZ. (d-e) Multiple shoots induction in MS media supplemented with 1.0 mg/LBAP + 1.0 mg/L TDZ after 10 days and 30 days.

3.2.2. Effect of different explant type on shoot multiplication

The choice of explant is very important for propagation through tissue culture techniques. Different parts of shoot i.e. apical, middle and basal, have been found to have different response

for shoot multiplication in many species (Tyagi *et al*., 2013). Therefore, different explants like nodal segment shoot tip, leaves, roots and cotyledons were used for in vitro shoot multiplication of *N. foetida.* MS medium supplemented with 0.5mg/L TDZ was used to induce shoots on various explants as it was found to be the most appropriate plant growth medium.

During the first week of culture all the explants get bulged and in the second week of culture, shoot primodia appeared. The nodal segment and shoot tip produced shoot primodia and subsequent shoots in third week whereas, roots and leaf explant differentiated the shoots in the fourth week of culture.

Out of the various explants, shoot tip gave a maximum shoot multiplication of 75% with average number of 16.6 shoots per explant. This was followed by nodal segment with a shoot multiplication of 69.7% and average numbers of shoots were 13.3 per explant, which was at par with the results obtained with shoot tip. Root and leaf explants gave a shoot multiplication of 62% and 55.6% respectively. Minimum multiplication of shoots (48%) was observed in the cotyledons (Table. 3.7).

Shoot tip explants performed better by giving higher shoot multiplication and also better growth of shoot primodia in comparison to the other explants. This might be associated with relative high level of endogenous growth substances in juvenile tissue as compared to adult tissue. In addition to this, higher level of endogenous growth substances in shoot tip also help in active mitotic cell division which indirectly contribute towards the phenomenon of apical dominance and thereby suppressing the performance of other explants for shoot multiplication (Laura *et al*., 2012). Although, the nodal explants are also growing tissues yet, the synthesis of growth substances might have been low and influence of apical dominance might have prevailed upon after the exogenous addition of PGRs, further resulting into poor multiplication in comparison to the shoot tips.

The shoot tip explants were found to be better for multiple shoot production in many palnt species due to higher cytokinin to auxin ratio. For instance, *Cannabis sativa* (Ren Wang *et al*., 2009); *Boehmeria nivea* (L) *Gaud* (Sut *et al.,* 2004); *Ocimum sanctum* (Girija *et al.,* 2006); *Verbena officinalis* (Turker *et al*., 2010); *Alternanthera sessils* (Wesely *et al*., 2011); *Lippia*

nodiflora (Evelyne *et al*., 2011); *Stevia rebaudiana Bert*. (Das *et al*., 2011); *Solanum nigrum* L. (Kavitha *et al*., 2012) and *Bacopa chamaedryoides* (Haque *et al*., 2013).

Table. 3.7. Effect of different explants on shoot multiplication

Explants	% Shoot multiplication	Shoot number	Duration of shoot initiation (weeks)	Average shoot length (cm)
Nodal segment	69.67±2.60 [a]	13.33±0.33 [a]	3	2.73±0.14 [a b]
Shoot tip	75.00±2.52[a]	16.66±0.88	3-4	3.57±0.23[a]
Leaves	55.66±2.33[b]	12.33±1.45 [a b]	4.5	2.63±0.41 [a b]
Roots	62.00±2.08 [b]	9.66±0.66 [b c]	4-5	2.60±0.32 [a b]
Cotyledons	48.00±1.73	8.67±0.67 [c]	5	1.56±0.23 [b]

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

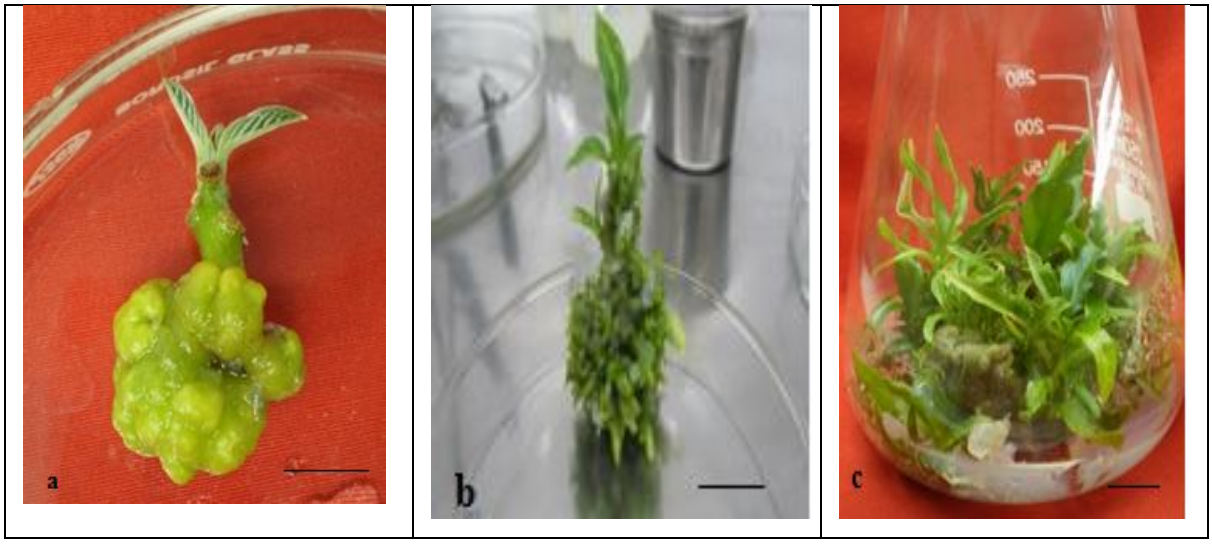


Plate. 4. Shoot multiplication in different explants of *N. foetida*. (a) Swelling in shoot tip explants after 1 week (b) shoot primodia after 2 weeks (c) Healthy shoots after 3 weeks. (d-g) various stages of multiple shoot induction in nodal explants. (h-i) induction of multiple shoots in leaf explants.

3.2.3. Effect of sucrose concentration on shoot multiplication

Generally plant cells and tissues in culture medium are dependent on external carbon for energy. 3% sucrose is normally used in shoot multiplication medium. But in some plant species lower concentration can be used without affecting shoot multiplication, which certainly reduce the cost of plant tissue culture (Karim *et al*., 2007). Therefore, different sucrose concentration (0, 10, 20, 30 and 40 gm/L) was supplemented in MS medium with 0.5 mg/L TDZ.

Results indicate that there was significant difference on shoot multiplication, shoot number and shoot length due to different sucrose concentrations. When the medium was sucrose free, no shoot regeneration was recorded. Shoot multiplication, number of shoots per explant and length

of shoots increased gradually with the increase in sucrose concentration in the medium upto 3% and then decreased. 76.6% shoot multiplication was observed at 3% sucrose level with n average shoot length of 3.4 cm and average 11.6 shoots per explant.

However, an increase in sucrose concentration beyond 3% resulted in decline in shoot multiplication. On the whole varying sucrose concentration did have significant effect on the shoot multiplication and shoot elongation as evident from the significant difference among the treatments. Higher concentration of sucrose in medium increase the osmotic potential and showed inhibitory effect on shoot multiplication (Nowak *et al*., 2004).

It is well known that the carbon source in the culture medium is an essential factor as an energy source as well as for maintaining osmotic potential (Cuenca *et al*., 2000). The sucrose concentration of 3% was found to be optimum in other plant species such as *Acacia Arabica* (Nanda *et al*., 2003); *Tectona grandis* (Tiwari *et al*., 2002) and *Callindra tweedii* (Kumar *et al*., 2002). In contrast, lower sucrose concentration (1.5%) showed best results on shoot multiplication in *Araria elata* (Karim *et al*., 2007).

Table. 3.8. Effect of sucrose concentration on shoot multiplication

Sucrose g/L	Shoot multiplication %	No. of shoots/ explant	Shoot length (cm)
0 (Control)	-	-	0
10	60.3±2.0 ^{c}	7.6±0.3 ^{c}	0.8±0.1 ^{c}
20	71.7±2.0 ^{b}	9.7±0.6 ^{b}	2.1±0.2 ^{b}
30	76.6±1.2 ^{a}	11.6±0.9 ^{a}	3.4±0.2 ^{a}
40	70.5±0.9 ^{b}	9.0±1.2 ^{b}	3.3±0.1 ^{a}

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

Plate. 5. Shoot multiplication at different sucrose concentrations. (a-c) Shoot multiplication in MS medium supplemented with 30 gms sucrose after 1 week, 2 weeks and 3 weeks. (d) Shoot multiplication on MS medium supplemented with 40 gms of sucrose. (e) Shoot multiplication in MS medium supplemented with 10 gms sucrose.

3.2.4. Effect of various PGRs in combination with GA_3 and different explants on shoot elongation.

When the shoots were sub cultured on same shoot multiplication medium, they failed to elongate. Hence, the shoots were transferred to shoot elongation medium containing BAP, KIN and Glycine in combination with various concentrations of GA_3. Table 3.9 describes the performance of different explants (nodal segment, shoot tip and leaf explant) during shoot elongation.

Maximum shoot elongation was reported in shoot tip explants. Previously, BAP was reported to be best for shoot elongation (Rai *et al*., 2002). However, in present investigation BAP was supplemented with different concentrations of GA_3, which was found to be more effective.

BAP at a concentration of 2mg/L + GA_3 favored better elongation of shoots derived from all explants. Maximum shoot elongation response was observed in the shoots regenerated from shoot tip (6.7 cm) explants, followed by nodal segment (5.6 cm) and leaf explant (4.7 cm) respectively, which were significantly different from each other. However, further increase in the concentration of GA_3, decrease in shoot length was observed. A high concentration of cytokinins results in ethylene production that limits the regeneration of shoots and limits the elongation of internodes (Tehrim *et al*., 2013). According to one study, the combination of 1.0 mg/L BAP + 0.1 mg/L GA_3 significantly increased the shoot length

In case of KIN, a combination of 0.5 mg/L KIN + 1.5mg/L GA_3 gave 4.7 cm, 6.2 cm and 3.9 cm shoot elongation in nodal segment, shoot tip and leaf explants respectively. All theexplants responded least to MS medium supplemented with the combination of Glycine and GA_3. Among Glycine supplemented medium, maximum shoot elongation (2.5 cm, 2.9 cm and 1.7cm in nodal segment, shoot tip and leaf explant respectively) was found with 1.5mg/L GA_3 + 1.5mg/L glycine in MS medium.

Explant growth depends on the nutrients and growth regulators added to the culture medium. The combination of various growth regulators and their concentrations significantly influences shoot length due to their effect on cell division and cell expansion (Gordon and Letham, 1975). Therefore, growth regulators are very important for *in vitro* growth, however some internal factors (endogenous level of plant hormones) can modify their activities (Park *et al*., 2001).

GA_3 has been known to elongate the shoots in *Mentha peprita* (Ghanti *et al*., 2004). Where as in apple, BAP was found to be more effective for shoot elongation. However, in *Camellia sinensis* combination of BAP and GA_3 was proved to be better for the elongation of shoots (Gonbad *et al*., 2014).

Table. 3.9. Effect of various PGRs in combination with GA3 and different explants on shoot elongation

PGRs (mg/L)		Shoot elongation (cm)		
BAP	**GA3**	**Nodal explant**	**Shoot tip**	**Leaf explant**
2	0.5	3.23±0.14 b c	3.77±0.15	2.87±0.12 b c
2	1.0	3.6±0.15 a b	4.5±0.17 a	3.1±0.15 b
2	1.5	5.63±0.09	6.77±1.09	4.77±0.12
2	2.0	4.33±0.20	4.33±0.17 a	3.47±0.09 a
KIN				
0.5	0.5	2.43±0.08 d	3.27±0.12 b	2.07±0.15 d
0.5	1.0	3.1±0.06 c	4.4±0.18 a	3.63±0.08 a
0.5	1.5	4.77±0.19	6.26±0.23	3.9±0.12 a
0.5	2.0	3.77±0.09 a	5.43±0.23	2.63±0.14 c
Glycine				
1.5	0.5	1.17±0.08 f	1.67±0.17 c	0.83±0.08 f
1.5	1.0	1.73±0.14 e	2.2±0.17 c	1.1±0.11 f
1.5	1.5	2.46±0.15 d	2.97±0.12 b	1.73±0.15 d e
1.5	2.0	1.37±0.09 e f	1.93±0.08 c	1.53±0.09 e

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

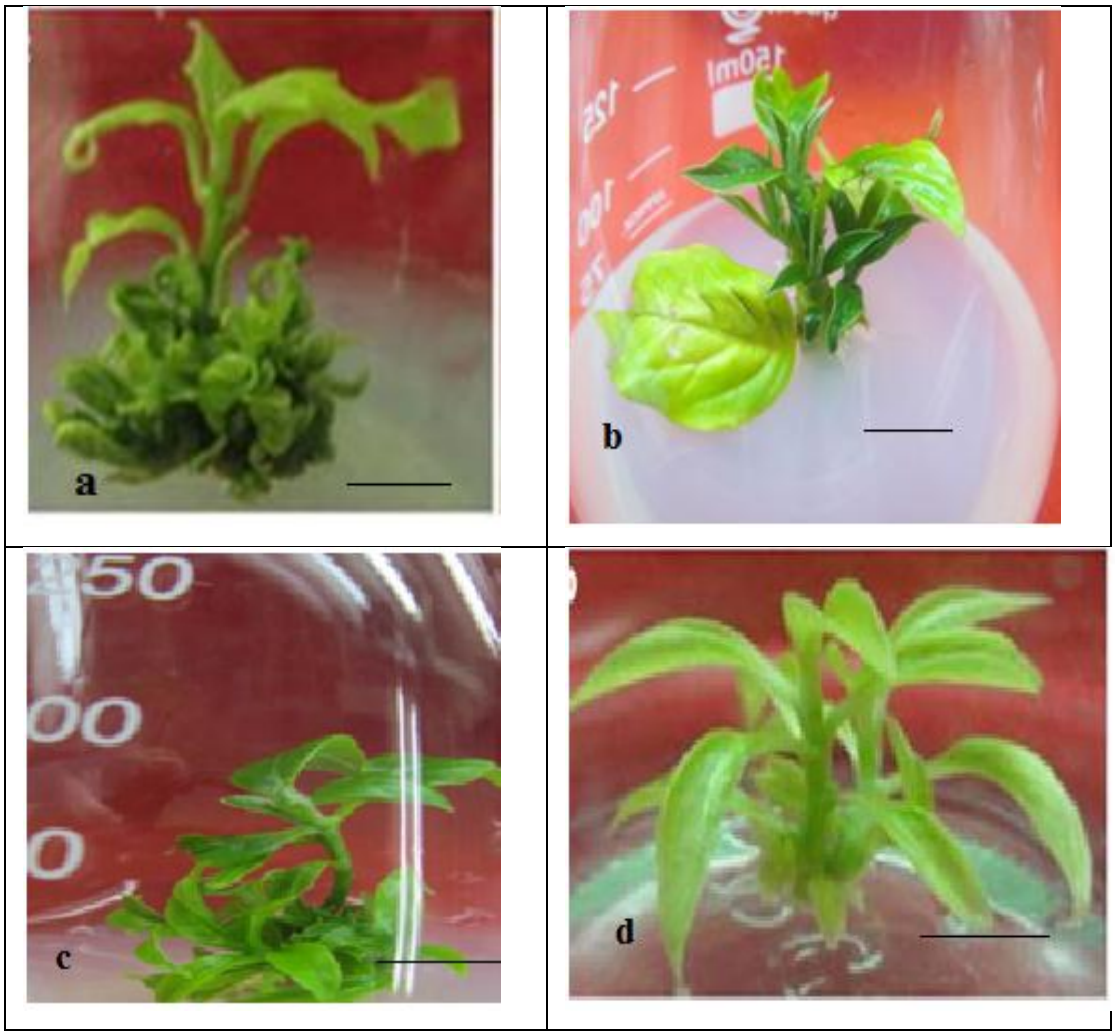


Plate.6. Shoot elongation in *N. foetida*. (a- b) Shoot elongation on MS medium supplemented with 2mg/L BAP + 1.5mg/L GA_3 after 10 days and 20 days respectively. (c) Shoot elongation on MS medium supplemented with 0.5 mg/L KIN + 1.5mg/L GA_3. (d) Shoot elongation on MS medium supplemented with 1.5 mg/L Glycine + 1.5mg/L GA_3.

3.3. *In vitro* rooting

In N. foetida, rooting is difficult and required serious efforts. Three major problems were observed during the rooting phase 1) callusing, 2) shot tip necrosis and 3) leaf shedding. Experiments were conducted to enhance rooting and minimizing callusing and shoot tip necrosis.

3.3.1. Effect of different concentration of MS salts on root induction

Experiment was conducted with three different MS salt concentrations and MS basal medium was taken as control. No rooting was observed in the control. Maximum number of plants responded to ½ strength MS medium supplemented with 2mg/L BAP + 3mg/L IBA with 77 % rooting. This was followed by full strength MS medium supplemented with 2mg/L BAP + 3mg/L IBA with 68 % rooting and least rooting was observed on ¼ strength MS medium supplemented with 2mg/L BAP + 3mg/L IBA with 67% rooting (Table. 3.10.).

The difference was also observed among the treatments with regard to duration of initiation of rooting. In full strength medium, rooting was initiated after 15 days of culture. This was followed by ½ strength MS medium, which took 21 day for root initiation. When ¼ strength MS medium was used, roots initiated after 28 days of culture.

It was speculated that, due to the less availability of nutrients, roots have to spread themselves to get the nutrients. Moreover, lower osmotic potential, caused by the reduction in mineral concentration, could potentially lead to plants forming roots to take in necessary minerals dissolved in the medium (Soheilikhah *et al*., 2010).

It has been reported that IBA supplemented medium exhibited superior rooting than NAA in *Pyrus malus* L.. Similar results were reported in other plant species with the positive effect of IBA on rooting *viz Vigna radiate* (Siddquai, 2006); *Aegle marmelos* (Nayak *et al*., 2007); *Clitoria ternatea* (Barik *et al*., 2007).

Table. 3.10. Effect of different concentration of MS salts on root induction

MS media	BAP mg/L	IBA mg/L	% Response	Duration of initiation (days)	Nature of response
Control(Full strength basal)	-	-	Nil	-	No root formation
Full strength	0.5	3	$66.0 \pm 1.5^{b\,c}$	15	Thick roots

	1	3	60.3±1.5 [c]	15	
	1.5	3	65.7±2.3 [b c]	15	
	2	3	68.0±2.4 [a b c]	15	
½ strength	0.5	3	67.0±2.3 [a b c]	21	Elongated numerous Roots
	1	3	70.7±2.3 [a b]	21	
	1.5	3	71.7±2.4 [a b]	21	
	2	**3**	**77.0±2.7 [a]**	**21**	
¼ strength	0.5	3	60.0±1.2c	28	Roots with callus
	1	3	65.7±1.5 [b c]	28	
	1.5	3	64.7±1.9 [b c]	28	
	2	3	67.7±1.5 [a b c]	28	

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

3.3.2. Response of shoots derived from various explants to rooting

Shoots derived from different explants (shoot tip, nodal segment and leaves) were used to induce rooting on half strength MS medium supplemented with 3mg/L IBA + 2mg/L BAP. Differential response was obtained among the shoots derived from different explants (Table 3.11).

Maximum rooting was observed in shoot tip derived shoots i.e. 86% in MS medium supplemented with 3 mg/L IBA + 2mg/L BAP. After the shoot tip explants, maximum rooting was observed in shots derived from nodal segment (80.3%) in MS medium supplemented with 3 mg/L IBA + 2mg/L BAP . Whereas, the shoots derived from leaf explants, showed significantly low rooting (63%) than other two explants.

In regard to the nature of response, roots were thick and light green in color with an average length of 2.5 cm in case of shoots derived from shoot tip explants. Whereas, in case of nodal segment derived shoots, roots were long and thin with an average length of 1.8 cm. In case of leaf explants derived shoots, small white colored roots were obtained with an average length of 1.1 cm.

Maximum number of roots were obtained from the shoot tip derived shoots (18.3), followed by nodal segment derived shoots (15) and significantly low number of roots were obtained with the leaf explant derived shoots (7.3). Numbers of roots in other two explants were at par with each other.

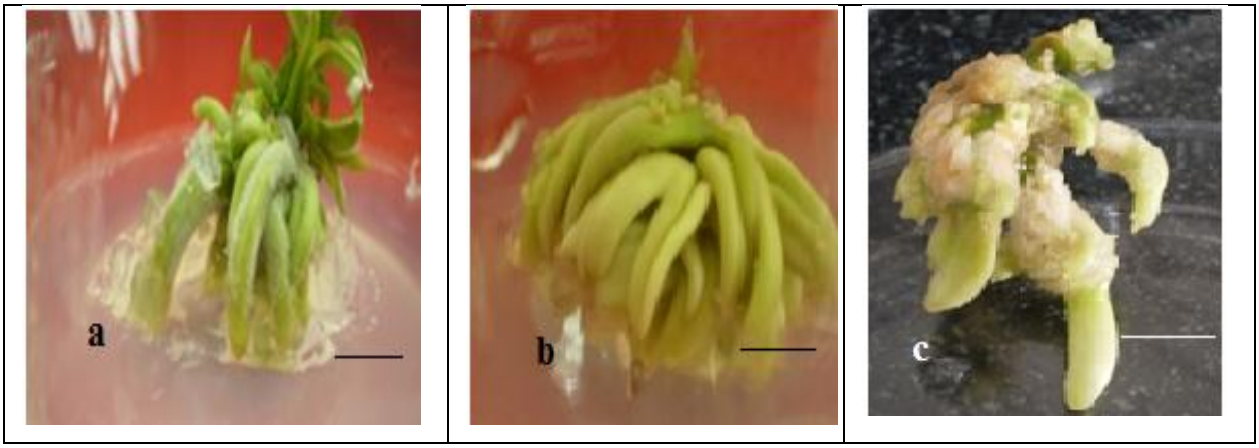
Similarly, shoot tip was found to be superior explant for micropropagation in many plant species, for example *Cannabis sativa* (Ren Wang *et al.*, 2009); *Boehmeria nivea* (Sut *et al*., 2004); *Ocimum sanctum* (Girija *et al*., 2006); *Alternanthera sessils* (Wesely *et al.*, 2011); *Lippia nodiflora* (Evelyne *et al.*, 2011); *Stevia rebudiana* Bert. (Das *et al*., 2011); *Solanum nigrum* (Kavitha *et al.*, 2012) and *Bacopa chamaedryoides* (Haque *et al.*, 2013).

Table. 3.11. **Response of shoots derived from various explants to rooting**

Explants	**IBA mg/L**	**BA mg/L**	**% Response**	**Duration of root initiation (days)**	**No. of roots/explant**	**Average root length (cm)**	**Nature of response**
Nodal segment	3	0.5	70.7±1.5 ^{d}	21	13.7±1.5 ^{b}	1.7±0.5 $^{a\,b\,c}$	Long thin roots
	3	1	73.3±2.3 $^{c\,d}$	21	14.7±0.9 $^{a\,b}$	1.7±0.2 $^{a\,b\,c}$	
	3	2	80.3±0.3 $^{c\,a\,b}$	21	15.0±1.5 $^{a\,b}$	1.8±0.4 $^{a\,b\,c}$	

Shoot tip	3	0.5	78.0±1.5 [b c]	15	15.3±0.9 [a b]	1.9±0.1 [a b c]	Thick light green roots
	3	1	83.3±2.7 [a b]	15	17.3±0.7 [a b]	2.3±0.3 [a b]	
	3	2	86.0±1.4 [a]	15	18.3±0.9 [a]	2.5±0.3 [a]	
Leaves	3	0.5	53.7±1.9 [e]	25	5.7±0.7 [c]	1.0±0.1 [c]	Swelling of explants and small white colored roots
	3	1	48.7±1.5 [e]	25	6.7±0.9 [c]	1.1±0.2 [b c]	
	3	2	63.0±1.2 [d]	25	7.3±0.7 [c]	1.2±0.3 [b c]	

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.



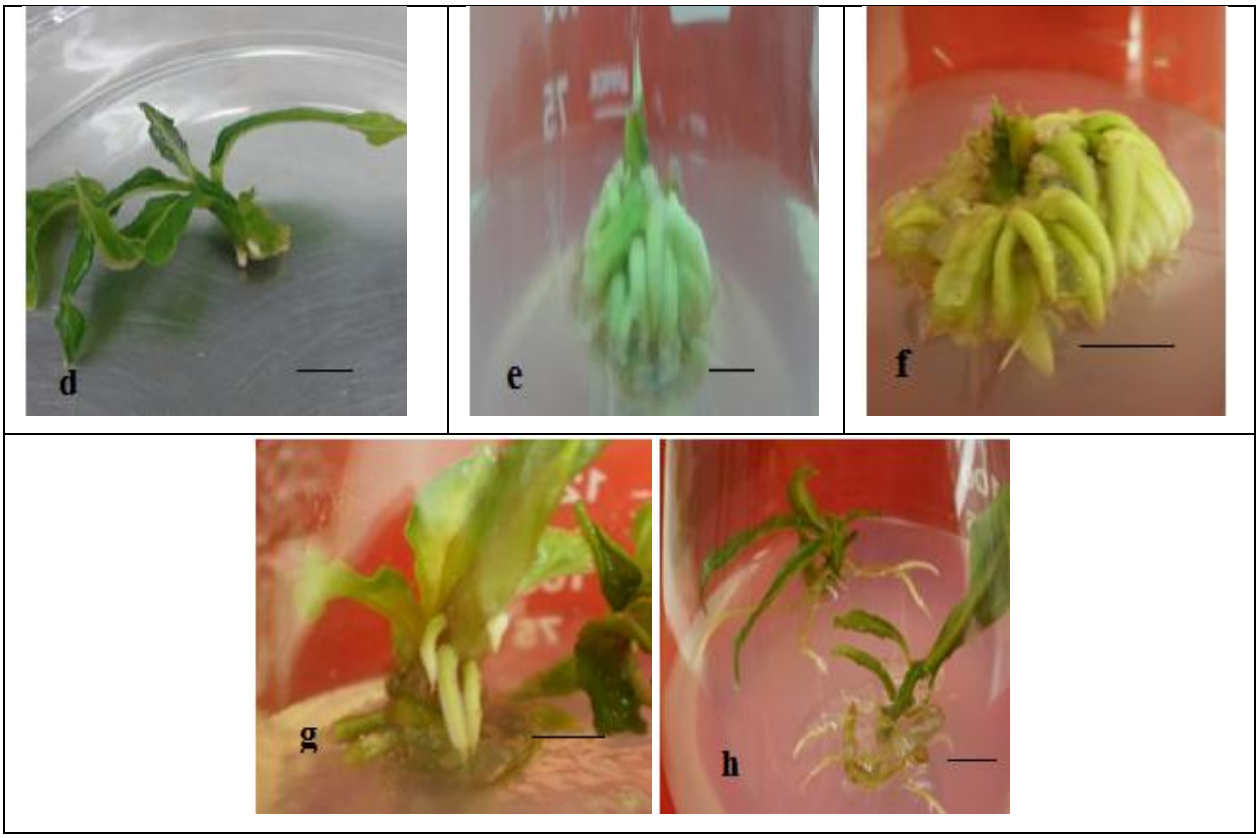


Plate. 7. Root induction in *N. foetida* (a) Root induction on full strength MS media with 1 mg/L BAP + 3mg/L IBA. (b) Root induction on half strength MS media with 2mg/L BAP + 3mg/L IBA. (c) Root induction on one-fourth strength MS medium with 0.5 mg/L BAP + 3g/L IBA (d) Initiation and proliferation (e-f) after 10 and 20 days on MS medium supplemented with 2 mg/L BAP + 2 mg/L IBA in shoot tip derived shoot explant. (g) Root induction on MS medium supplemented with 2 mg/L BAP + 2 mg/L IBA in leaf explant derived shoot. (h)) Root induction on MS medium supplemented with 2 mg/L BAP + 2 mg/L IBA in leaf explant derived shoot.

3.3.3. Effect of different agar concentration on rooting

When the shoots were rooted on standard root induction medium gelled with 0.8 % agar, 79.7% rooting was observed with an average 15.7 roots/explant and 2.4 cm of the root length. However, when the agar concentration was reduced in the medium, rooting was enhanced. At o.6% agar concentration 85.3% rooting was observed with an average 17.7 roots/explant and 3.4 cm of the root length. Medium gelled with 0.4% agar gave maximum rooting of 90% was observed with an

average 19 roots/explant and 4.1 cm of the root length. When the agar concentration was further decreased to 0.2%, decrease in rooting (69.3%) was observed (Table. 3.12).

In vitro rooting was enhanced in reduced agar concentration due to the easy penetration of the roots into the medium. Reduction in agar concentration improved rooting of banana (Ahmed *et al.*, 2014); *Ceropegia thwaitessii* (Selvaraj *et al.*, 2013) and *Pinus roxburghii* (Arvind Arya *et al.*, 2014).

Table. 3.12. Effect of different agar concentration on rooting

Agar concentration (%)	% Rooting	No. of roots/explant	Root length (cm)
0.2	69.3± 0.9 ^{c}	10.3± 0.3 ^{c}	3.2± 0.1 ^{b}
0.4	90.0± 1.7 ^{a}	19.0± 0.6 ^{a}	4.1± 0.2 ^{a}
0.6	85.3± 1.5 ^{a}	17.7± 0.9 $^{a\,b}$	3.4± 0.9 ^{b}
0.8	79.7± 0.9 ^{b}	15.7± 0.8 ^{b}	2.4±0.1 ^{c}
1.0	60.6± 2.3 ^{d}	11.3±0.9 ^{c}	2.0±0.2 ^{c}

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

3.3.4. Effect of addition of Activated Charcoal on rooting

One of the major problems which were accompanied along with rooting was callusing and shoots tip necrosis in all the above experiments which lead to ultimate death of the tissue. Therefore, activated charcoal (AC) was used to check callusing and shoot tip necrosis with improved rooting. Both full strength and half strength MS medium were tested and were supplemented

with 2 mg/L BAP + 3mg/L IBA, as this treatment of plant growth regulators was found to be best for root induction. Different concentration (100, 200, 300, 400 and 500 mg/L) of activated charcoal was added to the medium.

In present investigation, maximum rooting (77.7%) was obtained with half strength MS medium supplemented with 2 mg/L BAP + 3mg/L IBA + 300 mg/L AC, with 16 roots/explant. Among all the concentrations of activated charcoal, 300 mg/L was found to be best in both full strength and half strength MS medium. Moreover, shoot tip necrosis was also decreased in these treatments as compared to other treatments (Table. 3.13).

However, when lower concentrations (100 and 200 mg/L) of AC were used, shoot tip necrosis was found to be 100% and maximum callusing was obtained which adversely affect the plant growth.

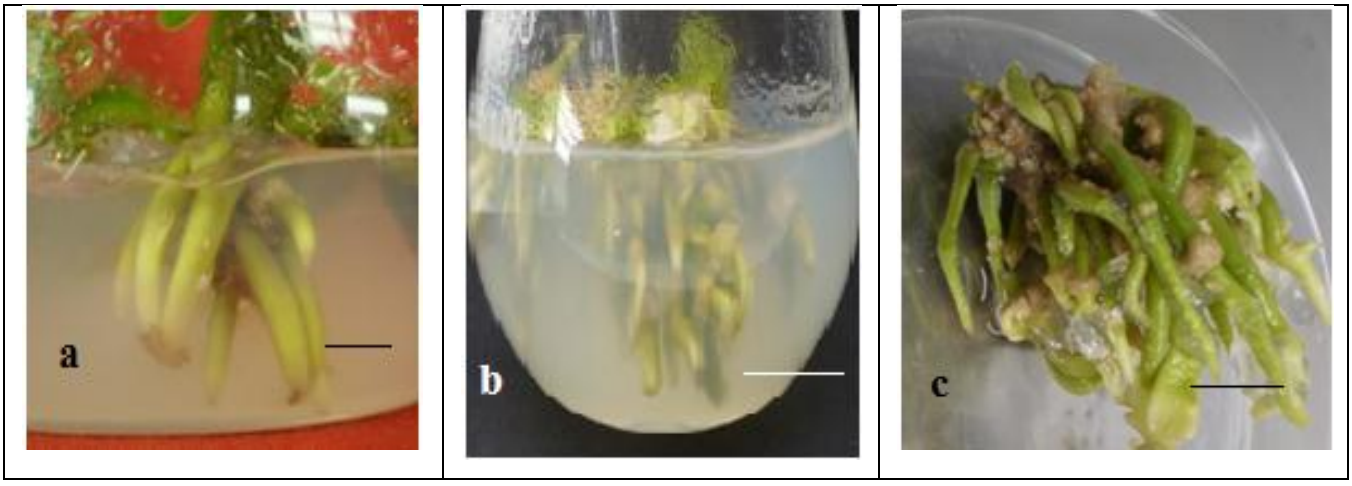
Activated charcoal is often used in plant tissue culture for the adsorption of inhibitory substances in the culture medium which decrease the brown exudates accumulation (Thomas, 2008). It reduces the freely available nutrient as well as light which are helpful in rooting and reducing callus formation (Agarwal *et al.*, 2002). AC has a regulatory effect on *in vitro* rooting in many plant species Number of roots per shoot was increased and positive effect was observed in *Carrizo citrange* (Montoliu *et al.*, 2010); *Myrtus communis* date palm (Abul-soad and Jatoi, 2014); Brahmi with the addition of AC.

Table. 3.13. Effect of addition of Activated Charcoal on rooting

Treatment	Rooting %	No. of roots/ explant	Shoot tip necrosis %	Callusing
MS Basal	0	-	-	-
MS+2mg/L BAP + 3mg/L IBA+100mg/L AC	30.7 ± 4.3^{f}	5.0 ± 1.2^{c}	100	+++
MS+2mg/L BAP + 3mg/LIBA+200mg/L AC	40.7 ± 3.5^{ef}	5.6 ± 1.2^{c}	100	+++

MS+2mg/L BAP + 3mg/L IBA+300mg/L AC	44.0±3.1 [e]	5.3±1.9 [c]	60	+
MS+2mg/L BAP + 3mg/L IBA+400mg/L AC	37.7±1.5 [e f]	6.3±0.9 [c]	80	++
MS+2mg/L BAP + 3mg/L IBA+500mg/L AC	36.0±3.5 [e f]	8.3±0.9 [a c]	85	++
½MS+2mg/L BAP + 3mg/L IBA+100mg/L AC	47.3±1.4 [d e]	11.3±0.9 [a]	100	+++
½MS+2mg/L BAP + 3mg/L IBA+200mg/L AC	56.0±2.6 [c d]	11.0±1.0 [a]	100	+++
½MS+2mg/L BAP + 3mg/LIBA+300mg/L AC	77.7±4.3 [a]	16.0±0.6	50	+
½MS+2mg/L BAP + 3mg/L IBA+400mg/L AC	69.0±3.6 [a b]	12.0±1.2 [a]	65	++
½MS+2mg/L BAP + 3mg/L IBA+500mg/L AC	63.0±1.5 [b c]	10.3±0.7 [a]	70	++

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.



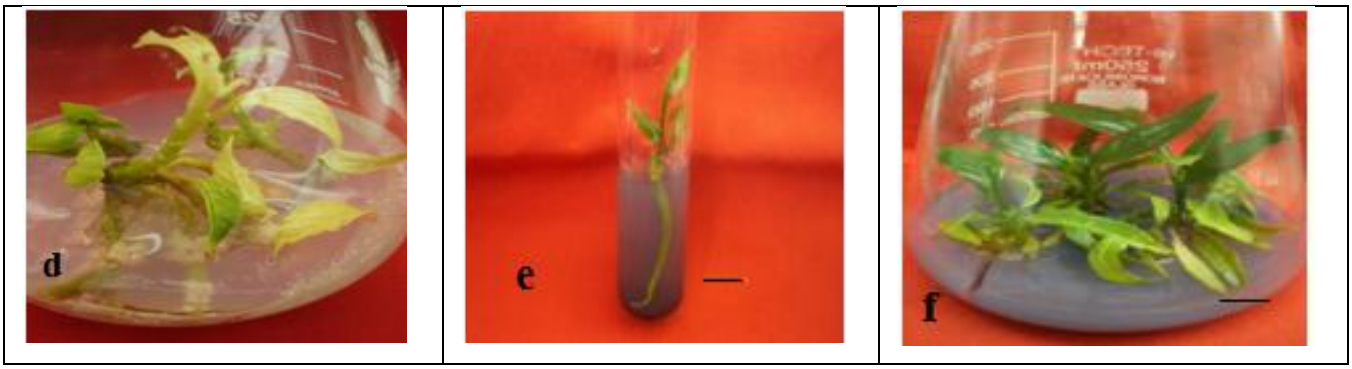


Plate.8. Effect of agar concentration and AC on rooting. (a) Root induction at reduced agar concentration of 0.2 %. (b) Root induction at 0.4 % agar after 10 days. (c) Root proliferation at 0.4 % agar after 20 days. (d) Shoot tip necrosis in *N. foetida* in rooting medium. (e -f) Root induction in ½MS+2mg/L IBA+300mg/L AC.

3.4. Hardening and acclimatization

In the present study, the micropropagated shoots with well developed root system were hardened in glass beakers containing sterilized soilrite and plantlets were maintained under control conditions for 20 days.

During hardening, plantlets were exposed to control conditions from high humidity and low temperature to low humidity to high temperature. The plantlets were covered with beaker to retain humidity. The hardened plantlets were transferred to small pots containing a mixture of soil: manure (1:2) ratio in green house. The plantlets were hardened successfully under controlled conditions with 65% survival rates.

There are various reports where *in vitro* raised plants were acclimatized under controlled conditions such as *Clitoria ternatae* (Singh and Tiwari, 2010), *Gymnema sylvestre* (Thiyagarajan *et al*., 2013), *Sarcostemma acidum* (Rathore *et al.,* 2013) and *Vitex trifolia* (Ahmed *et al.,* 2014).

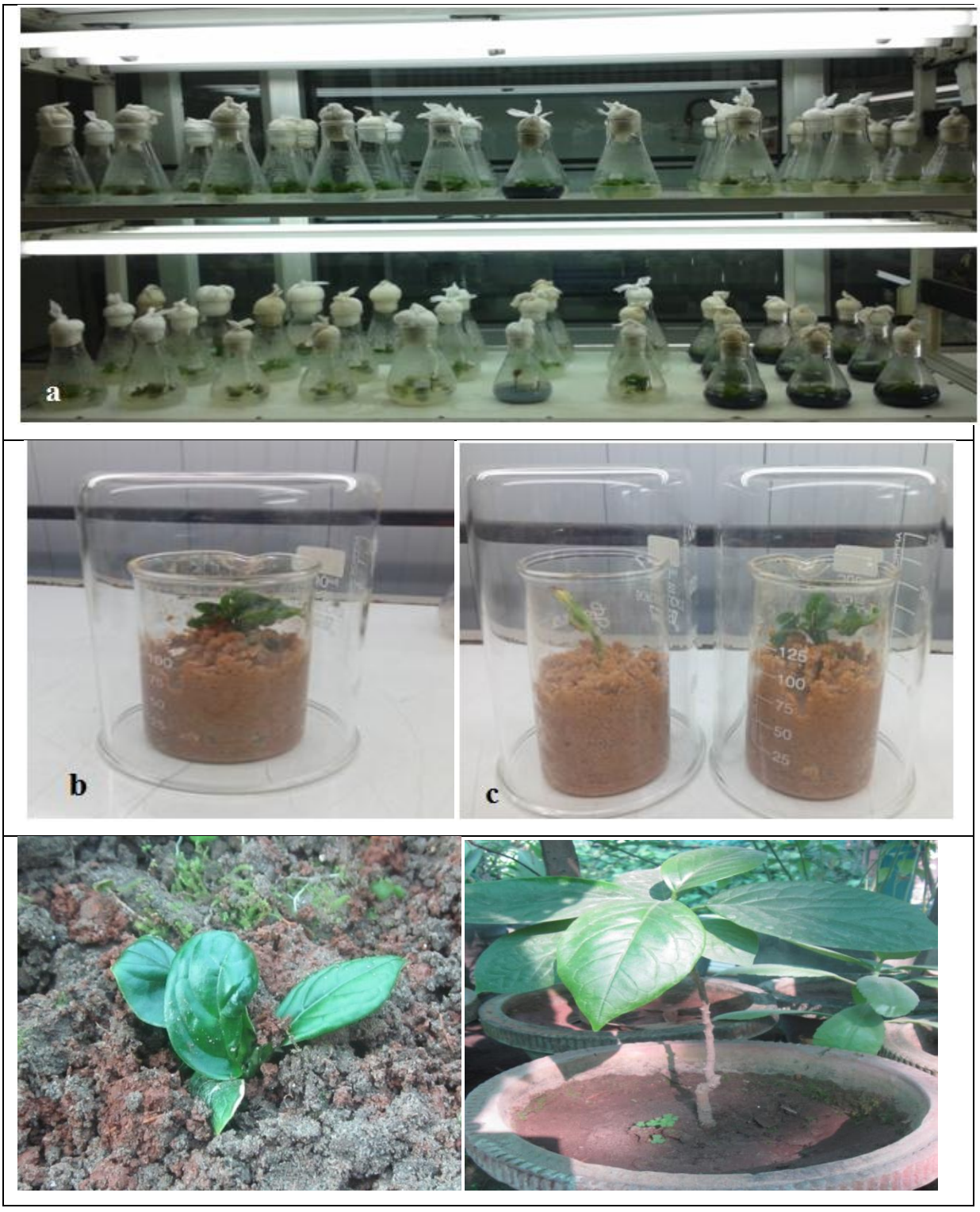


Plate.9. Hardening of micropropagated shoots of *N. foetida*. (a) *In vitro* grown microshoots (b-c) Hardeningof shoots in beakers containing soilrite. (d) Hardening under control condition.

3.5. Hairy root induction

3.5.1. Preliminary studies on hairy root induction in *N. foetida*

Different *Agrobacterium* strains have different susceptibility to induce hairy roots in different plant species. The impact of different factors such as *A. rhizogenes* strains, type of explants, method of making wound, concentration of acetosyringone, exposure time of wounded with bacterial culture (infection time), co-cultivation time, temperature of co-cultivation, affecting hairy root initiation were evaluated in this study. Leaf explant was chosen first among all the explants to transform with various *A. rhizogenes* strains as leaf explant has shown best results in other plant species.

In the explants infected with *A. rhizogenes*, roots emerged directly from the wounded regions. In some explants, swellings were observed along the infected sites and in some cases callus like structure was also observed, from most of which roots also developed. Aerial roots were also obtained due to lack of positive geotropism. These roots displayed plagiotropism (growing parallel to the culture medium).

When the roots were further sub cultured onto fresh medium, most of the rootr showed lateral growth. After 20 days of culture a closely interwoven masses was obtained both over the surface of the medium and over the upper side of the petri dish. Whereas after 25-30 days, hairy roots changed their color from white to brownish white.

The control explants, deprived of bacterial infection, did not produce roots at all. Only, swelling of the explants was observed and prolonged culture lead to browning of the explants and ultimately leading to the death of the explants. The hairy root clones which were detached from the explants and cultured on MS basal medium with antibiotic showed a normal growth.

Initially preliminary studies were carried out following the standard protocol using all the five strains (A4, LBA 9204, MTCC 532, MTCC 2364 and NCIM 5204) of *A. rhizogenes* with leaf as an explant source. Optical density, infection time, c0-cultivation period, co-cultivation temperature, acetosyringone concentration were checked for the induction of hairy roots. Values were not determined due to the overgrowth of the *A. rhizogenes* (Table. 3.14).

Optical density (OD) of 1.0 was found to be optimum, as maximum strains (A4, LBA 9204 and MTCC 532) of *A. rhizogenes* responded to this OD. Two other *A. rhizogenes* strains (MTCC 2364 and NCIM 5204) did not responded to different OD as no hairy roots formation was observed when the explants were infected with these two strains.

Infection times of 5, 10, 15, 20, 25, 30 and 35 minutes were tested for the induction of hairy roots. Again no root formation was observed with MTCC 2364 and NCIM. With infection time of 20 and 25 minutes, all the three strains (A4, LBA 9204 and MTCC 532) produced hairy roots. When the infection time was increased to 30 and 35 minutes, contamination due to overgrowth of *A. rhizogenes* was observed.

To assess the effect of co-cultivation duration on hairy root induction, explants were co-cultivated for 24, 48, 72 and 96 hours with the bacterial suspension. At 48 hours of co-cultivation, three strains (A4, LBA 9204 and MTCC 532) produced hairy roots. MTCC 2364 and NCIM didn't produced hairy roots at all. Overgrowth of A. rhizogenes was observed when the co-cultivation period was extended beyond 72 hours.

Different co-cultivation temperature (18, 20, 22, 26 and 28°C) was checked for the induction of hairy roots. At a temperature of 22°C maximum of 3 strains (A4, LBA 9204 and MTCC 532) induced hairy roots, but other two strains (MTCC 2364 and NCIM) did not responded at all. *A. rhizogenes* strain A4 induced hairy roots in *N. foetida* explants at a wide range of temperature (20-28°C).

According to the preliminary work, it was concluded that only three *A. rhizogenes* strains (A4, LBA 9204 and MTCC 532) induced hairy roots in *N. foetida*. Other two strains (MTCC 2364 and NCIM) did not respond to the transformation experiment. Therefore, for further experimentation on optimization of protocol for efficient production of hairy roots only the three strains (A4, LBA 9204 and MTCC 532) were used.

Table. 3.14. Preliminary studies on hairy root induction in *N. foetida*

Parameters	Strains	MTCC 532	A4	LBA 9204	MTCC 2364	NCIM
Optical density (OD)	0.6	-	+	-	-	-
	0.8	-	+	+	-	-
	1.0	+	+	+	-	-
	1.2	+	+	-	-	-
	1.4	-	-	-	-	-
	1.6	-	-	-	-	-
Infection time (minutes)	5	-	-	-	-	-
	10	-	-	-	-	-
	15	-	+	-	-	-
	20	+	+	+	-	-
	25	+	+	+	-	-
	30	-	-	-	-	-
	35	-	-	-	-	-
Co-cultivation period (hours)	24	-	+	-	-	-
	48	+	+	+	-	-
	72	-	+	+	-	-
	96	-	-	-	-	-
Co-cultivation temperature (^{o}C)	18	-	-	-	-	-
	20	+	+	-	-	-
	22	+	+	+	-	-
	24	-	+	+	-	-
	26	-	+	-	-	-
	28	-	+	-	-	-
Acetosyringone conc. (µM)	0	-	-	-	-	-
	100	+	+	+	+	+
	150	+	+	+	+	+

+ means induction and – means no induction of hairy roots

3.5.2. Optimization of the transformation protocol for efficient production of hairy roots

Optimization of transformation protocol was needed for the efficient production of hairy roots. Based on the results of preliminary experiments, three A. rhizogenes strains were checked for the efficient production of hairy roots by varying various parameters. Co-cultivation time and temperature, different types of explant, concentration of acetosyringone, optical density and lastly method of making wound were optimized.

3.5.2.1. Effect of co-cultivation period and infection time on transformation frequency

Data related to the effect of co-cultivation period and infection time is presented in Table. 3.15. Leaf explant was infected with different *A. rhizogenes* strain for different co-cultivation period (24-48 hours) and different infection time (15-25 minutes), according to the results of preliminary studies.

Results reflect that, infection time of 25 minutes and co-cultivation period of 48 hours was found to be best in all the strains. *A. rhizogenes* strain MTCC 532 when co-cultivated for 48 hours with an infection time of 20 and 25 minutes 39.7% and 60% transformation frequency was recorded respectively.

Significant difference was observed among different infection times in *A. rhizogenes* strain LBA 9402. When the explants were infected for 20 minutes, transformation frequency of 45.3% was observed and when it was increased to 25minutes, transformation frequency was increased to 75.6%. Further increment in co-cultivation period and infection time, caused inhibition of transformation in both the strains.

A.rhizogenes strain A4 showed better and quick response in comparison to other two strains (LBA9402 and MTCC 532). Infection time of 25 minutes and co-cultivation period of 48 hours was again found to be best and gave 80.7% transformation frequency. A4 strain also induced hairy roots at a co-cultivation period of 24 hours with maximum of 62.3% transformation frequency with a infection time of 25 minutes. Similar trend was found in A4 strain as in other two strains regarding the increment of co-cultivation period. Explants did not respond to increase in co-cultivation period to 72 hours.

In brief, it was observed that all the three strains of *A. rhizogenes* induced hairy roots when explants were infected for 25 minutes followed by a co-cultivation period of 48 hours. Uninfected control explants were not able to show any induction of hairy roots, even after 25 days of infection.

Researchers have reported that the effect of infection time on transformation frequency is due to the plant species. Decrease in the transformation frequency at lower infection time and co-cultivation period is due to insufficient time for proper and complete transfer of T-DNA to occur. Whereas, higher periods lead to more than sufficient time, which ultimately cause the bacteria to overgrow and engulf the explants and ultimately to the death of the explant (Kiani *et al.* , 2012).

Infection time of 5 minutes was sufficient for inducing hairy roots in *Linium mucronatum* (Samadi *et al*., 2014) and *Agastache foeniculum* whereas in *Silybum marianum* (Rahnama *et al.,* 2008), *Fagopyrum tataricum*) and *Portulaca oleracea* 20 minutes infection was found to be optimum (Moghadam *et al*., 2014).

In *Cucumbis sativus* 2 days of co-cultivation was required (Selvaraj *et al*., 2010). Co-cultivation period of 48 hours was also found to be effective for *Glycyrhiza glabra* (Mehrotra *et al*., 2008), *Lotus corniculatus* (Jian *et al.*, 2009), *Linium mucronatum* (Samadi *et al*., 2014), *Artemisia annua* (Giri *et al*., 2001), *Artemisia carvifolia* .

Table. 3.15. Effect of co-cultivation period and infection time on transformation frequency

Co-cultivation period (hrs)	**Infection Time (minutes)**	**Transformation frequency (%)**
	MTCC 532	
24	15	0
	20	0
	25	0
48	15	0
	20	39.7 ± 1.5^{g}

	25	60.0±1.7 c [d]
LBA 9402		
24	15	0
	20	0
	25	0
48	15	0
	20	45.3±0.9 [f]
	25	75.7±1.9 [b]
A4		
24	15	49.0±1.2 [f]
	20	56.3±1.2 [d e]
	25	62.3±1.5 [c]
48	15	53.3±1.7 [e]
	20	65.0±1.5 [c]
	25	80.7±1.2 [a]
72	15	-
	20	-
	25	-

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

3.5.2.2. Effect of co-cultivation temperature on hairy root induction

Co-cultivation temperature from 20- 28° C was tested. Differential response was obtained at different temperature irrespective of the strains. In all the A. rhizogenes strains, maximum transformation frequency was obtained at 22° C. in A4 strain 77.6% transformation frequency was reported at 22° C, followed by 69.7% at increased temperature of 24° C and further increase

in temperature leads to further decrease in transformation frequency up tom23.6% at 28°C. At lower temperature also decrease in transformation frequency was observed (Table. 4.16).

In LBA 9402, hairy root were induced at only 22 and 24°C (73.3% and 63.7% respectively). Further variation in co-cultivation temperature gave 0% transformation frequency. Similarly, in case of MTCC 532, hairy root induction was observed only at 20 and 22°C (53% and 63% respectively) and no hairy roots were induced when co-cultivation temperature was further increased.

Co-cultivation temperature of 22°C was found to be optimum in *Lotus corniculatus* (Jian *et al.*, 2009). At higher temperature, reduced functionality of T-DNA transfer machinery has been reported (Fullner *et al.*, 1996), which lead to reduced transformation frequency. Moreover, it has been reported that high temperature leads to reduced level of virulence proteins and hence reduced bacterial virulence (Barcon *et al.*, 2001).

Table. 4.16. Effect of co-cultivation temperature on hairy root induction

Temperature (°C)	Transformation frequency (%)		
	A4	**LBA9402**	**MTCC 532**
20	64.3± 0.7 [c]	0	53.0± 1.0 [b]
22	**77.6± 1.2** [a]	73.3 ± 1.9 [a]	63.0± 1.7 [a]
24	69.7±1.2 [b]	63.7± 1.8 [b]	0
26	55.6±1.0 [d]	0	0
28	23.6±0.8 [e]	0	0

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

3.5.2.3. Effect of addition of Acetosyringone on transformation frequency

The mechanism of transformation depends upon the activity of *A. rhizogenes*, induced by phenolic compounds released from wounded plant such as acetosyringone which can be added to the medium to enhance transformation (Kumar *et al.,* 2006). Results regarding the effect of addition of acetosyringone to the co-cultivation on transformation frequency are presented in table. 3.17.

In the explants infected with A4 strain, when the co-cultivation medium was supplemented with different concentration of acetosyringone, significant difference in transformation frequencies was observed. In the co-cultivation medium supplemented with 150 µM acetosyringone, 82.6% transformation frequency was obtained with leaf explant. Whereas, it was as low as 32% when no acetosyringone was added to the medium. Acetosyringone enhance the rate of transformation of infected explants by the activation of virulence genes (Gelvin, 2003).

Positive effects of acetosyringone was also reported on *A. rhizogenes* mediated transformation of *N. tabaccum* (Kumar *et al*., 2006) *Boerhaavia diffusa* (Sahu *et al.,* 2013), *A. spinosus* (Pal *et al.,* 2013) and *Puraria candollei* (Danphitsanuparn *et al*., 2012) is evident.

Table. 3.17. Effect of addition of Acetosyringone on transformation frequency

Strains	Explants	Acetosyringone concentration µM	Transformation frequency (%)
A4	Leaf	0	32.0±1.5 ^{h}
		100	57.7±1.8 ^{d}
		150	**82.6±1.4** ^{a}
		200	65.7±1.2 ^{c}
	Stem	0	23.3±0.9 ^{i}
		100	51.0±0.6 ^{e}
		150	71.3±0.8 ^{b}
		200	62.0±1.2 $^{c\,d}$
LBA9402	Leaf	0	26.0±1.5 ^{i}

		100	45.0±1.7 [f]
		150	**72.0±1.1 [b]**
		200	59.7±1.7 [d]
	Stem	0	21.3±0.8 [i]
		100	34.6±1.2 [h]
		150	65.7±0.7 [c]
		200	57.6±1.5 [d]
MTCC532	Leaf	0	24.0±2.1 [i]
		100	35.0±1.2 [h]
		150	**61.7±2.2 [c d]**
		200	46.3±0.9 [f]
	Stem	0	17.3±1.5 [j]
		100	24.3±0.7 [i]
		150	53.0±1.0 [e]
		200	40.7±0.3 [g]

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

3.5.2.4. Effect of bacterial strain and bacterial cell density on transformation frequency

Influence of *A. rhizogenes* strain on hairy root induction has been documented earlier in several plant species (Sujatha *et al.*, 2013). In present investigation, among all the three *A. rhizogenes* strains used, A4 was found to be most virulent with respect to transformation frequency and initiation of hairy root formation. A4 strain was followed by LBA 9402 and least transformation frequency was observed in MTCC 532. Low transformation efficiency in strain MTCC 532 has been reported (Tiwari *et al*., 2006). The variation in hairy root induction is attributed to variation in virulence of different *A. rhizogenes* strain (Porter, 1991).

However, no hairy root formation was observed with *A. rhizogenes* strains MTCC 2364 and NCIM. Plasmid harbored by bacterial strains could be one of the reasons of the differences in virulence (Batra *et al.,* 2004; Chaudhuri *et al*., 2005).

This could be explained due to the differential expression of T-DNA genes present in the explants and positional integration effects of the T-DNA in host genome (Cho *et al.,* 1998). Moreover, compatibility between *A. rhizogenes* and host plant tissue, phytohormones production and juvenility of host tissue are contributing factors for efficient production of hairy roots (Huang *et al*., 1991).

The superiority of A4 strain over other strains is due to its wild origin (Bansal *et al*., 2014; Nourozi *et al*., 2014) and has been reported to induce hairy roots in other plant species, for instance *Catharanthus roseus* (Batra *et al*., 2004), *Piccorhiza kurroa* (Verma *et al*., 2007;), *Hyoscymus sp*. (Akramiam *et al.,* 2008), *Clitoria ternatae Monirelica charantia , O. basilicum*. (Swain *et al.,* 2012).

Optical density of 1.0nwas found to be optimum, irrespective of all the A. rhizogenes strains. In A4 strain maximum transformation frequency of 80.6% and 70.3% was obtained with leaf and stem explant respectively. In leaf and stem explants infected with LBA 9402, transformation frequency was found to be 72.3% and 64.3% respectively, at OD=1.0. Whereas, in case of strain MTCC 532, transformation frequency was found to be 60.7% and 49.7% in leaf and stem explants respectively at OD=1.0.

When the optical density of the bacterial suspension was increased or decreased, there was a decrease in the transformation frequency. This is due to the fact that at higher OD values, regeneration and growth of the plant tissue was inhibited because there was a bacterial- induced stress over the explants, and after co-cultivation period the control of bacterial overgrowth also became difficult (Swain *et al*., 2012).

The need of ideal bacterial density also varies with the plant species. Interestingly, it was reported that OD=1.0 was found to be effective in other plant species such as *Vigna radiate* (Jaiwal *et al*., 2001) and *Artemisea carvifolia* .

Table. 3.18. Effect of bacterial cell density on transformation frequency

Strains	Explants	OD	Transformation frequency (%)	Response
A4	Leaf	0.6	30.3±2.0 $^{g h i}$	Creamish long thin roots with huge branching
		0.8	40.3±1.5 $^{f g}$	
		1.0	**80.6±2.3** ^{a}	
		1.2	55.3±0.9 $^{d e}$	
	Stem	0.6	26.3±0.9 $^{h i j}$	
		0.8	33.0±1.5 $^{g h}$	
		1.0	70.3±2.6 $^{b c}$	
		1.2	50.3±2.0 $^{e f}$	
LBA9402	Leaf	0.6	21.0±0.6 $^{h i j}$	
		0.8	30.3±1.5 $^{g h I}$	White roots with large no.of root hairs
		1.0	**72.3±1.5** ^{b}	
		1.2	60.3±2.0 ^{c}	
	Stem	0.6	19.6±2.0 $^{i j}$	
		0.8	39.6±1.5 $^{f g}$	
		1.0	64.3±1.2 $^{b c d}$	
		1.2	44.6±2.0 ^{f}	Hairy roots with less branching with callus formation
MTCC532	Leaf	0.6	19.7±1.5 $^{i j}$	
		0.8	31.0±3.2 $^{g h I}$	
		1.0	**60.7±1.2** $^{c d}$	
		1.2	39.7±2.0 $^{f g}$	
	Stem	0.6	14.7±0.9 ^{j}	
		0.8	22.7±1.5 $^{h I j}$	
		1.0	49.7±1.5 $^{e f}$	
		1.2	31.0±2.1 $^{g h I}$	

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

3.5.2.5. Effect of different explants on hairy root induction

Many factors influence the transformation efficiency, among which the key factor is the choice of suitable explants. Different explants (leaf, petiole, radical and nodal segment) were infected with bacterial suspension at OD=1.0 for 25 minutes and were co-cultivated for 2 days on MS medium supplemented with 150µM acetosyringone. Variation in type of explant affected the transformation frequency significantly. In all the *A. rhizogenes* strains, leaf explant performed better.

In case of *A. rhizogenes* strain A4, maximum transformation frequency of 78% was obtained in leaf explant followed by petiole explant (71.7%), nodal segment (70.7%) and significantly low transformation frequency was observed in radical explant (65.3%).

Same trend was reported in other two strains of *A. rhizogenes*. In LBA 9402, leaf explant responded maximum with 71% transformation frequency, followed by petiole explant (60.7%), nodal segment (64.7%) and least in radical (57%). In MTCC 532, leaf explants gave a transformation frequency of 61% followed by petiole (54.7%), nodal segment (50.7%) and radical (47%).

Higher transformation frequency in leaf explants is related to more competence and sensitivity of leaf explants to *A. rhizogenes*, than other explants under consideration. This sensitivity further depends on the physiological status of the tissue (Pavar and Maheshvari 2004; Pirian *et al*., 2012). Similar results have been reported in *Withania somnifera* with leaf explants (Sivanandhan *et al*., 2014. Whereas, in *Centella asiatica*, maximum hairy root induction took place in nodal segment followed by leaves and petiole and no hairy roots were produced in roots (Gandi and Giri, 2012).

Table. 3.19. Effect of different explants on hairy root induction

Bacterial strain	Explants	Transformation frequency (%)	No. of roots/explant
MTCC 532	Petiole	54.7±2.0 $^{d e}$	3.3±0.3 ^{d}
	Leaf	**61.0±2.1** $^{\mathbf{c d}}$	**4.7±0.7** $^{\mathbf{d}}$
	Nodal segment	50.7±1.2 $^{e f}$	3.0±0.6 ^{d}
	Radical	47.0±1.7 ^{f}	2.7±0.3 ^{d}
A4	Petiole	71.7±1.9 ^{b}	12.7±0.9 ^{a}
	Leaf	**78.0±1.5** $^{\mathbf{a}}$	**18.0±1.5** $^{\mathbf{a}}$
	Nodal segment	70.7±1.2 ^{b}	11.7±1.2 $^{b c}$
	Radical	65. 3±1.7 ^{c}	9.0±0.7 $^{c d}$
LBA 9402	Petiole	60.7±1.6 $^{c d}$	9.3±0.9 $^{b c d}$
	Leaf	**71.0±2.3** $^{\mathbf{b}}$	**10.0±1.2** $^{\mathbf{b c d}}$
	Nodal segment	64.7±1.5 $^{b c}$	7.3±0.9 $^{d e}$
	Radical	57.0±1.2 ^{d}	7.3±0.8 $^{d e}$

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

Plate.10. Hairy root induction in leaf explant. (a) Establishment of leaf explant. (b) Hairy root initiation on MS basal medium. (c) Proliferation of hairy roots after 10 days of initiation on MS basal medium. (d) Further proliferation of hairy roots after 20 days on MS basal medium.

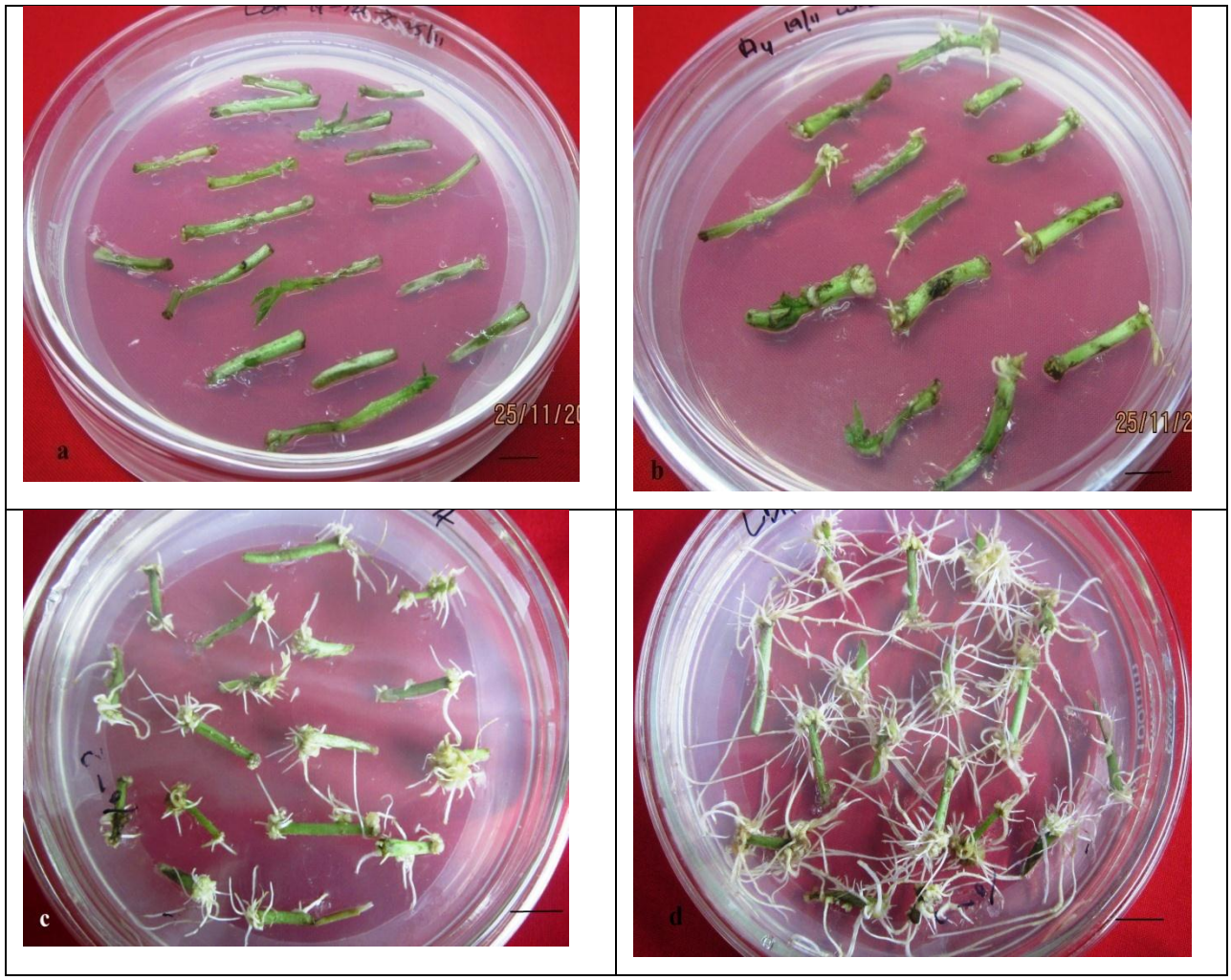


Plate.11. Hairy root induction in nodal segment. (a) Establishment of nodal segment. (b) Initiation of hairy roots on MS basal medium. (c) Proliferation of hairy roots after 20 days on MS basal medium. (d) Hairy root proliferation after 30 days.

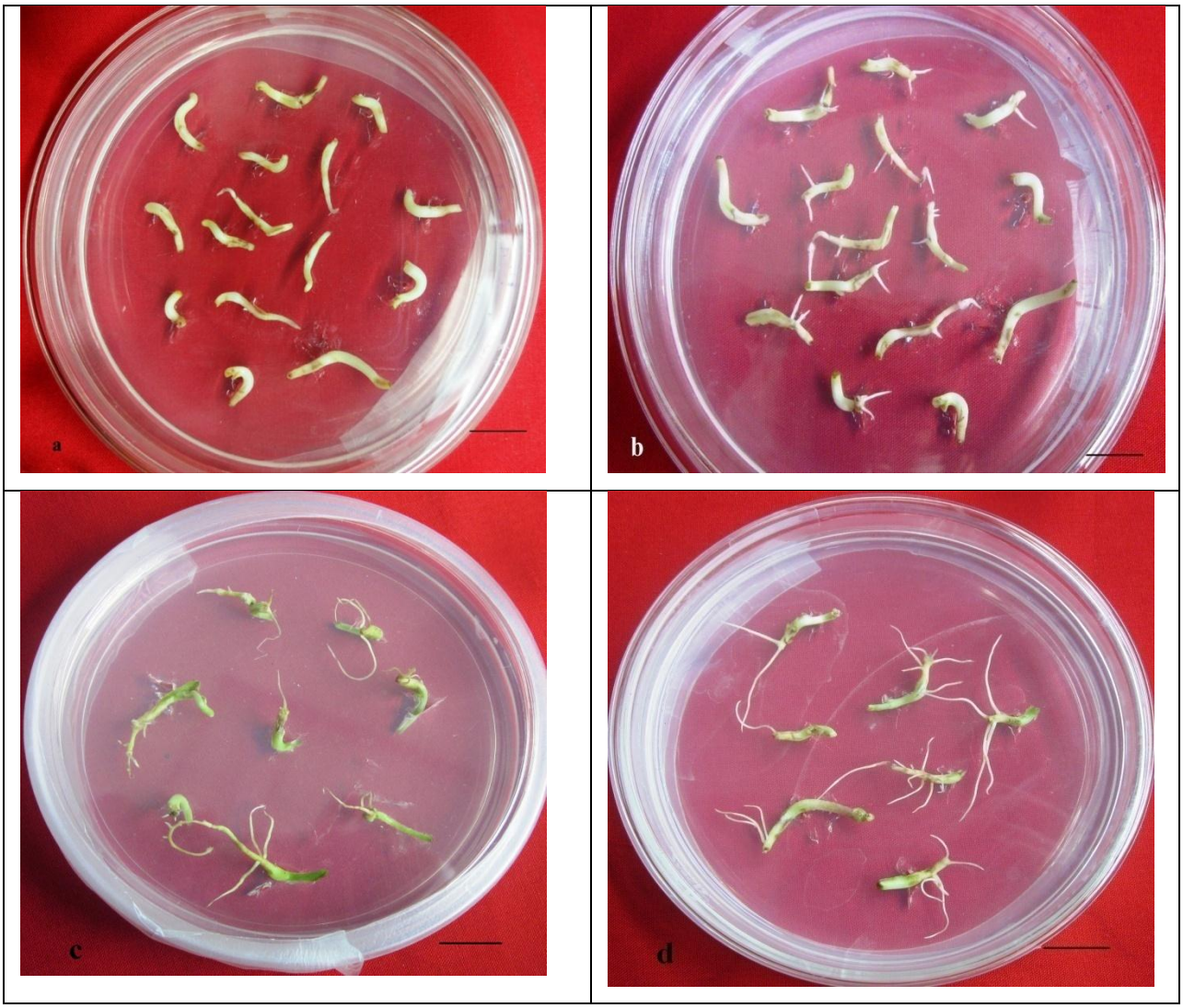


Plate. 12. Hairy root induction in radical explant. (a) Establisment of radicle explant. (b) Hairy root initiation on MS basal medium. (c) Proliferation of hairy roots after 10 days on MS basal medium. (d) Further proliferation of hairy roots after 20 days on MS basal medium.

3.5.2.6. Effect of wounding methods on transformation frequency

Wounding leads to natural secretion of some phenolic compounds from wound sites which are known to start the event of transformation (Swain *et al.,* 2012). Therefore method of making wound also affected the transformation frequency. Two methods of wounding were employed i.e. cutting the explants followed by dipping in bacterial suspension and pricking the explants with needle containing bacterial suspension. Of the two methods of making wound, cutting method was found to be superior, regardless of the strain. Pricking method gave significantly low transformation frequency in all the strains. However, in case of cutting method, degree of overgrowth of *A. rhizogenes* was much more than the pricking method.

In A4 strain cutting method gave 79% transformation frequency with average number of 9.7 roots per explant and pricking method gave 59.7% transformation frequency and average 7.3 roots per explant. In case of LBA 9402 culling the explant gave 70% transformation followed by pricking method with 52.3% transformation frequency. In MTCC 532 60% and 42.3% transformation frequency was obtained with cutting and pricking methods respectively (Table. 3.20).

In cutting method, explants are cut with blade, due to which *A. rhizogenes* might have got larger surface area to interact with the host plant and also larger amount of phenolic compounds are released to facilitate higher degree of transformation. Whereas, in pricking with needle, smaller surface area is being provided for bacterial interaction. But degree of overgrowth of *A. rhizogenes* was more in the former case.

Immersion of pie-pricked explants of *C. ternatae* in bacterial suspension was found to be most suitable for achieving transformation (Swain *et al.,* 2012). Similarly, immersion of wounded explants of *B. diffusa* (Sahu *et al*., 2013) and *Alpinia galangal* (Rao *et al*., 2012) was found to be effective.

Table. 3.20. Effect of wounding methods on transformation frequency

Bacterial strain	Wounding method	Transformation frequency (%)	No. of hairy roots	Degree of overgrowth of *A. rhizogenes*
A4	Cutting	79.0±1.15 [a]	9.7±0.9 [a]	++
	Pricking	59.7±2.40 [c]	7.3±1 [a b c]	+
LBA9402	Cutting	70.0±0.6 [b]	8.3±0.7 [a b]	++
	Pricking	52.3±2.2 [d]	5.3±1.2 [c d]	+
MTCC532	Cutting	60.0±1.0 [c]	6.7±1.2 [b c d]	++
	Pricking	42.3±1.5 [e]	4.3±0.8 [d e]	+

+ Less overgrowth, ++ Heavy overgrowth

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

Plate. 13. Effect of various methods of infections on transformation frequency. (a-b) Infection of nodal segment and leaf explant using cutting method. (c-d) Infection of nodal segment and leaf explant using pricking method.

3.5.2.7. Effect of antibiotic type and concentration on recurrence of A. rhizogenes after co-cultivation

In order to determine the effective antibiotic which are capable of suppressing the growth of *A. rhizogenes* after co-cultivation, two antibiotics were tested, which not only eliminated the *A. rhizogenes* but also supported better plant growth. After co-cultivation, the explants were subjected to two different antibiotic treatments with different concentration alone and in combinations.

Presence of *A. rhizogenes* was visible in all the explants immediately after the completion of co-cultivation period. After two weeks the control treatments were found to have an *A.rhizogenes* overgrowth. Addition of carbencillin at a concentration of 250 and 500 mg/L was not effective in suppressing the bacterial growth and 100 % contamination was reported. An increase in concentration to 750 mg/L eliminated the *A. rhizogenes* but transformation rate was hampered.

On the other hand, treatments with cefotaxime at a concentration of 250 mg/L did not eliminate the bacterium. Whereas, at a concentration of 500 mg/L reduced the bacterial overgrowth to 50 %, but when the concentration was further incremented to 750 mg/L, adverse effect on transformation frequency was observed.

Combination of cefotaxime and carbencillin was proved to be best for achieving maximum elimination of *A.rhizogenes.* At a concentration of 250+250 mg/L (cefotaxime + carbencillin) 100% contamination was recorded whereas, at a concentration of 500+500 mg/L zero percent contamination was observed. However, when concentration was increased to 750+750 mg/L, transformation frequency was lowered.

A. rhizogenes recurrence is frequently observed when explants are cultured in the absence of antibiotics (Wiebke *et al*., 2006). To determine the length of antibiotic treatment, hairy roots were cultured on a medium without antibiotic after 20, 30 and 40 days of treatment. *A. rhizogenes* recurrence was observed on hairy roots after 20 days, regardless of antibiotic type and concentration. However, the loss of hairy roots due to bacterium overgrowth was less in case of combination of the two antibiotics. On the other hand, after 30 and 40 days of treatment none of the hairy roots showed the *A. rhizogenes* recurrence at all antibiotic concentrations used.

Ideal antibiotic to be used in transformation process should be highly effective, inexpensive and a negative impact on plant growth (Cheng *et al*., 1998). Cefotaxime and carbencillin are extensively used in *Agrobacterium* mediated transformation. In *Agrobacterium* mediated transformation of soyabean, cefotaxime at a concentration of 350 and 500 mg/L was effective and carbencillin at 500 and 1000 mg/L was not effective (Wiebke *et al*., 2006).

In Pinus exposure to 350 mg/L cefotaxime upto 6 weeks did not eliminate the bacterium growth, while at a concentration of 500 mg/L, it was eradicated them from the explants. Further increase in the antibiotic concentration adversely affected the plant growth (Tang *et al*., 2000).

Table. 3.21. Effect of antibiotic type and concentration on recurrence of *A. rhizogenes* after co-cultivation

Antibiotic	**Concentration mg/L**	**Overgrowth of *A. rhizogenes* (%)**	**Transformation frequency (%)**	**Hairy roots with recurrence of *A.rhizogenes* (%)**		
Cefotaxime + Carbencillin				20 days	30 days	40 days
	250 + 250	100	65.00±1.53 [c]	40	0	0
	500 + 500	0	86.00±3.06 [a]	36	0	0
	750 + 750	0	72.33±1.86 [b]	30	0	0
Cefotaxime						
	250	100	54.33±2.40c	69	0	0
	500	50	73.00±1.52 [b]	60	0	0
	750	0	61.00±1.73 [c d]	58	0	0

Carbencilin						
	250	100	46.00±2.08	85	0	0
	500	100	55.33±2.03 ^{d}	80	0	0
	750	0	60.67±1.76 $^{c\ d}$	70	0	0

The mean value (±SE) of the three independent experiments is presented in the table showing the significance. The columns with same alphabet in superscript are non-significant based on SNK test at $p<0.05$ level.

Plate.14. Contamination reported during the transformation experiment. (a-b) Overgrowth of *Agrobacterium rhizogenes* due to extended co-cultivation period and longer infection time. (c-d) Recurrence of *Agrobacterium rhizogenes* after 20 days when antibiotics were used alone.

3.5.2.8. Growth of hairy roots in liquid medium

Fast growing hairy roots were selected and isolated from explant. Hairy roots were then cultured on hormone free MS liquid medium containing antibiotics for mass production. The roots grew rapidly and within 20-25 days the roots gradually changed from white to brownish white. After 30 days, hairy roots were harvested, dried and used for the extraction of camptothecin.

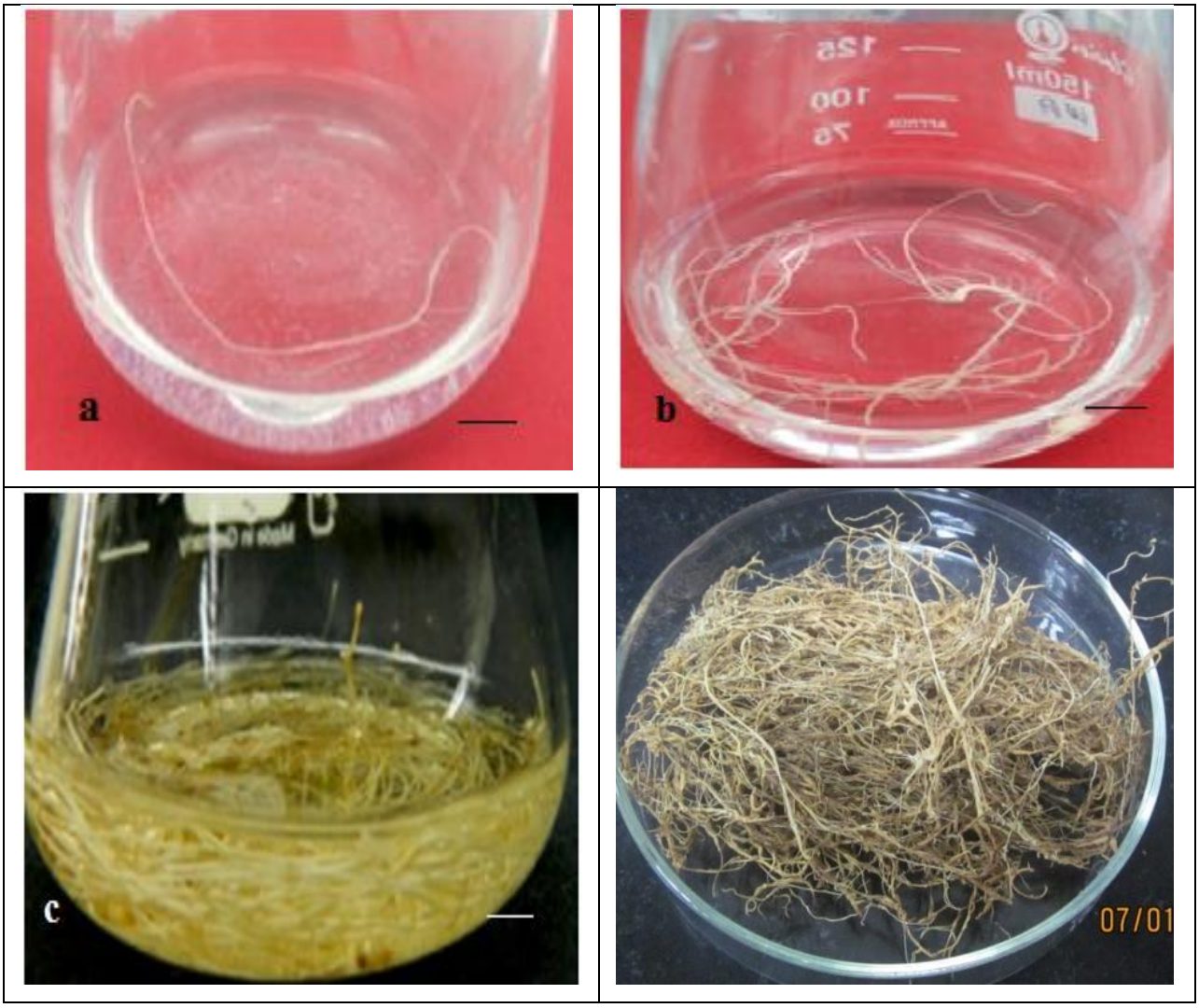


Plate.15. Growth of hairy roots in liquid medium. (a) Initiation of hairy root culture in liquid MS basal medium. (b) Proliferation of hairy roots in liquid MS medium after 15 days. (c) Proliferation of hairy roots in liquid MS medium after 25 days. (d) Harvesting of hairy roots after 30 days.

3.6. Confirmation of transformation by PCR analysis

Confirmation of transgenic status of the tissue was done by PCR amplification of the DNA isolated from the hairy roots, untransformed roots and *A. rhizogenes* using forward and reverse primers of *rol* B genes. A. rhizogenes was served as positive control and untransformed roots as negative control.

Transformed hairy root samples were found to be positive for *rol* B genes. The PCR products were of expected size (750 bp) and identical with those of positive control (*A. rhizogenes* LBA 9402). The untransformed roots (negative control) were negative for *rol* B genes. Presence of the amplified products of the expected size in positive control and hairy root samples, confirmed the identity of this amplification product and the transgenic nature of hairy roots. The results indicate that the *rol* B genes from the Ri plasmid of *A. rhizogenes* were integrated successfully into the genome of *N. foetida* hairy roots.

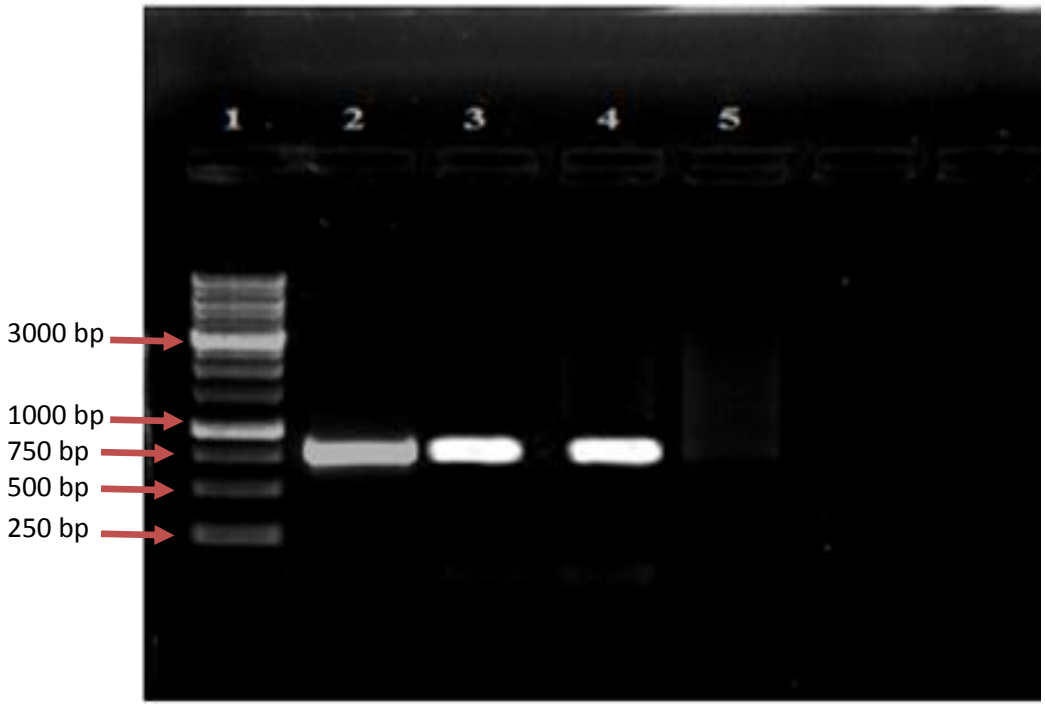


Plate. 16. **Confirmation of transformation by PCR analysis**. Lane 1: molecular weight marker (1 kb DNA ladder), lane 2: *Agrobacterium rhizogenes* DNA (positive control), lane 3 & 4: DNA from transformed hairy root, lane 5: DNA from untransformed root (negative control).

3.7. Camptothecin estimation and analysis

The amount of camptothecin in various parts of *N. foetida* was analyzed by HPLC. The authentic camptothecin standard gave retention time of 6.3 minutes (Figure). Extraction of camptothecin was carried out using three extraction techniques i.e., microwave extraction, stirring extraction and soxhlet extraction and the difference was compared. . Hairy roots induced with A4 and LBA9204 were used for the extraction of camptothecin. It was reported that there was a difference in camptothecin concentration among different parts of *N. foetida* as well as among the different extraction techniques.

Among the hairy roots, the one induced by A4 strain yield maximum camptothecin i.e., 0.2989%, 0.1630% and 0.1497% with microwave, soxhlet and stirring extraction respectively (Figure). In case of hairy roots induced by LBA 9204, the camptothecin content was found to be 0.197%, 0.1586% and 0.1414% with microwave, soxhlet and stirring extraction respectively (Figure). In *in vitro* roots it varied from 0.0996-0.0731% (Figure). Whereas in *in vitro* shoots it was further decreased from 0.0603-0.0687% (Figure). In *in vitro* leaves it varied from 0.0261-0.0412% (Figure).

Seed coat was also analyzed for the presence of camptothecin. In the seed coat with dried fruit, camptothecin was found to be more (0.273%) (Figure) than the seed coat without fruit (0.0048%) (Figure). In seeds unexpectedly higher yields of camptothecin was reported. It varied from 0.0982% with stirring extraction, 0.1047% with soxhlet extraction to 0.1165% with microwave extraction (Figure).

In case of *in vivo* leaves, two sources were selected for collection of leaves. The plant translocated from the Dapoli (Western Ghats) and the one propagated through seeds in Herbal Garden MDU. Both green and senescent leaves were collected from both the sources. In transplanted plants the green leaf yielded 0.022% camptothecin, whereas senescent leaves yielded 0.0068% camptothecin (Figure). In the seedling plants green leaves yielded 0.0125% camptothecin and in senescent leaves camptothecin yield was found to be 0.0059% camptothecin (Figure).

A study also reported difference in camptothecin yields in various parts of *N. foetida* i.e, maximum in roots, followed by fruits, stem and leaves (Namdeo *et al*., 2012). Moreover, *in vitro* grown plants yielded more camptothecin than the soil grown plants (Ravishankar *et al.,* 2013).

Table. 3.22. Camptothecin yields in hairy roots and various parts of *N. foetida* using different extraction techniques

	Camptothecin yield (%)		
Explants	**Microwave Extraction**	**Soxhlet extraction**	**Stirring extraction**
Hairy roots			
A4	0.299	0.163	0.150
LBA	0.197	0.159	0.141
***In vitro* root**	0.099	0.084	0.073
***In vitro* shoot**	0. 069	0.060	0. 067
***In vitro* leaf**	0.041	0.037	0.026
Seed coat			
With fruit	0.027	0.026	0.013
Without fruit	0.005	0.003	0.002
Seed	0.117	0.105	0.098
***In vivo* leaves**			
Translocated plant			
Green leaves	0.022	-	-
Senescent leaves	0.007	-	-
Seedling plant			
Green leaves	0.013	-	-
Senescent leaves	0.006	-	-

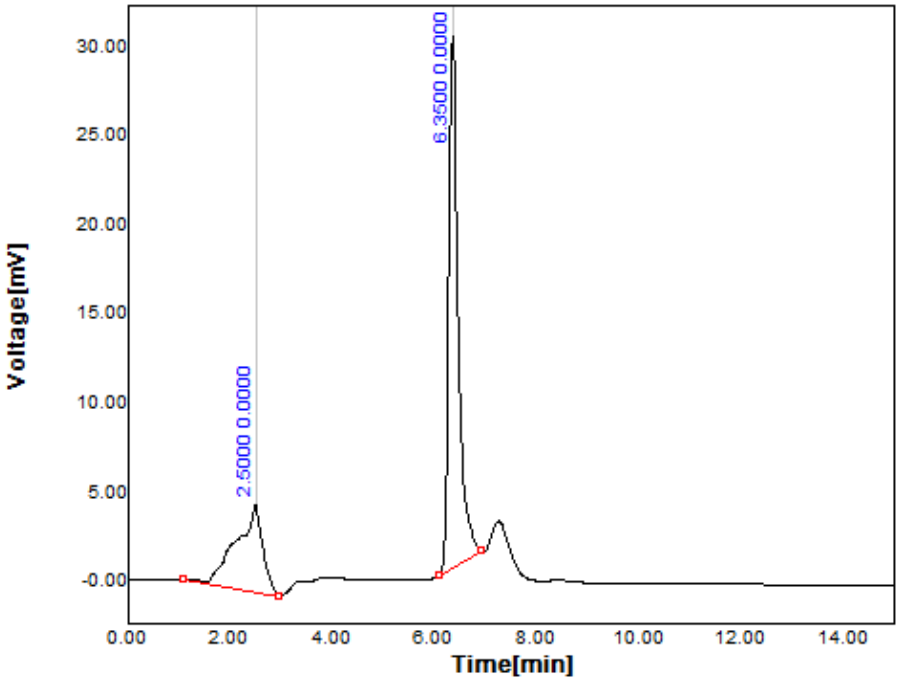


Figure. 6. HPLC chromatogram of authentic CPT (5ppm).

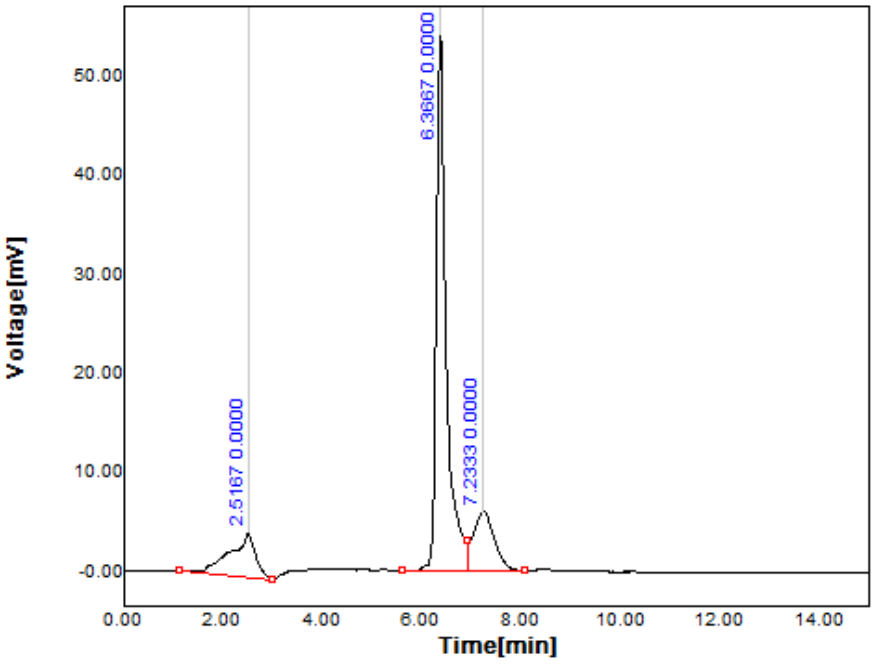


Figure.7. HPLC chromatogram of authentic CPT (10 ppm).

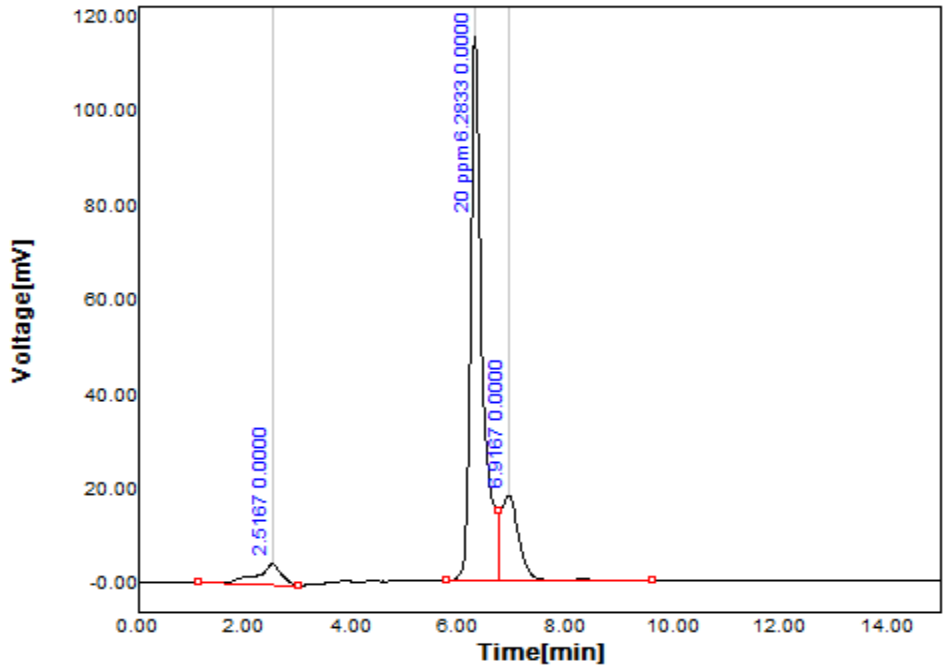


Figure. 8. HPLC chromatogram of authentic CPT (20 ppm).

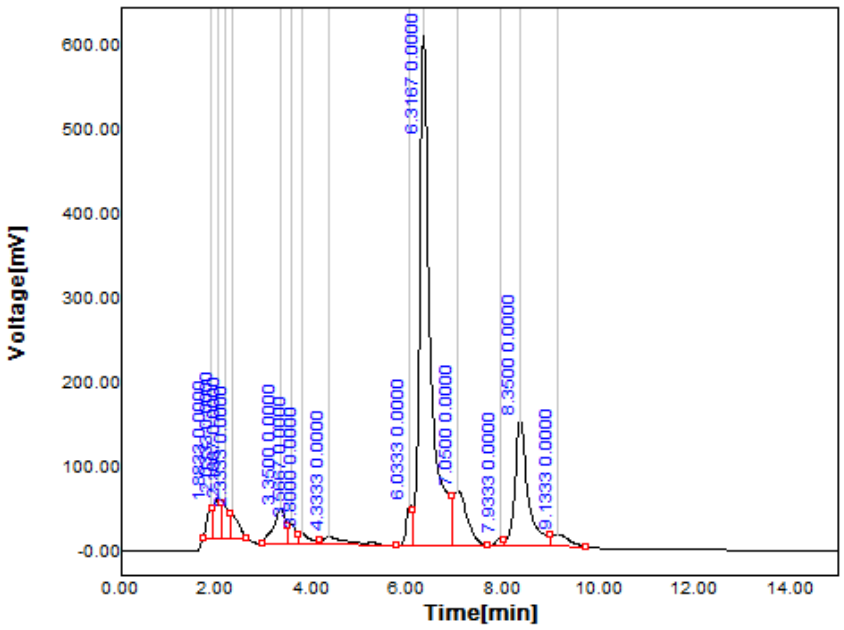


Figure.9. HPLC chromatogram of A4 strain induced hairy root sample

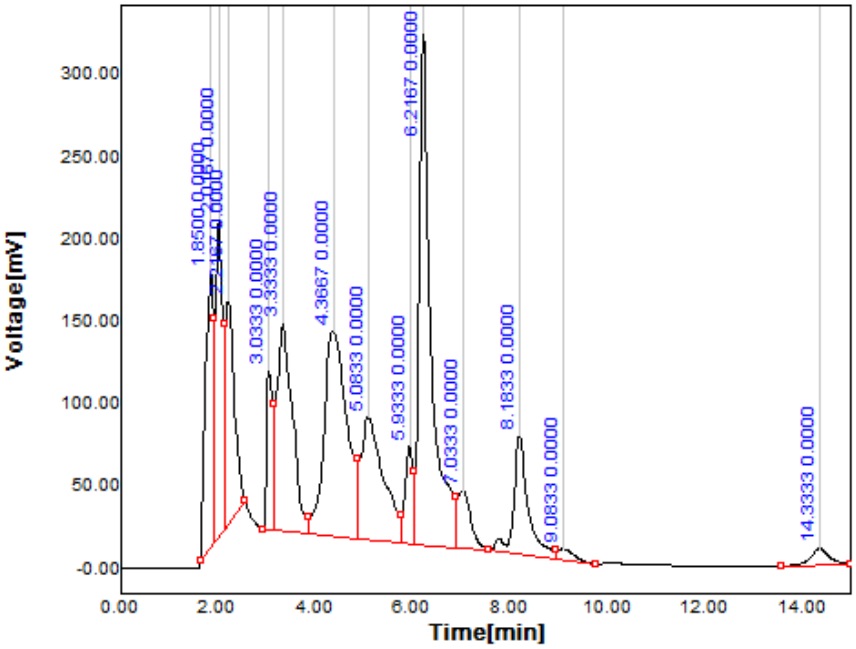


Figure.10. HPLC chromatogram of LBA 9204 strain induced hairy root sample

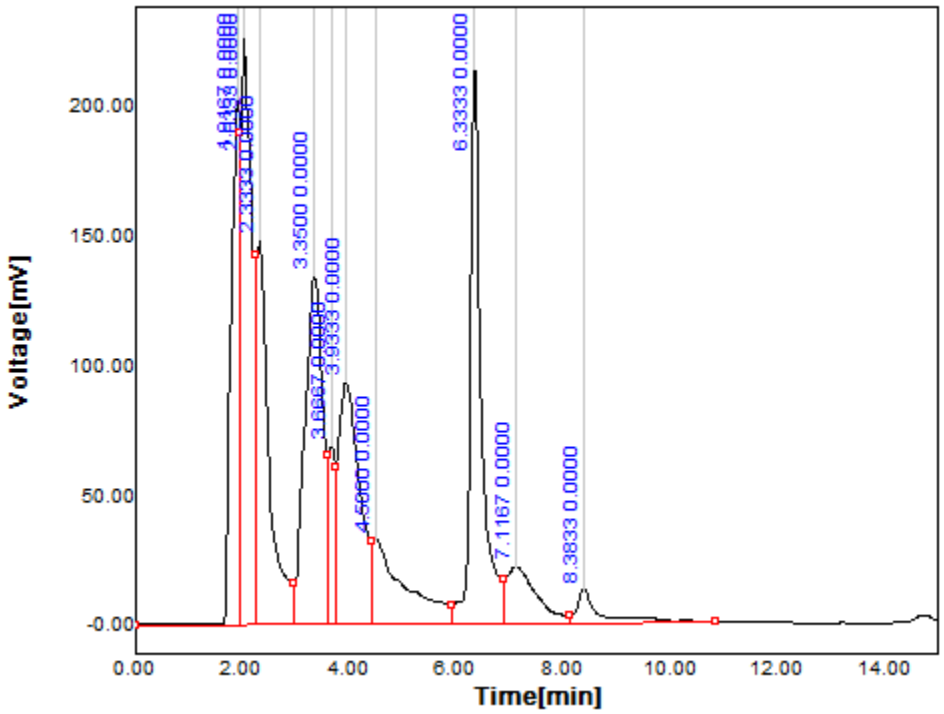


Figure.11. HPLC chromatogram of root sample

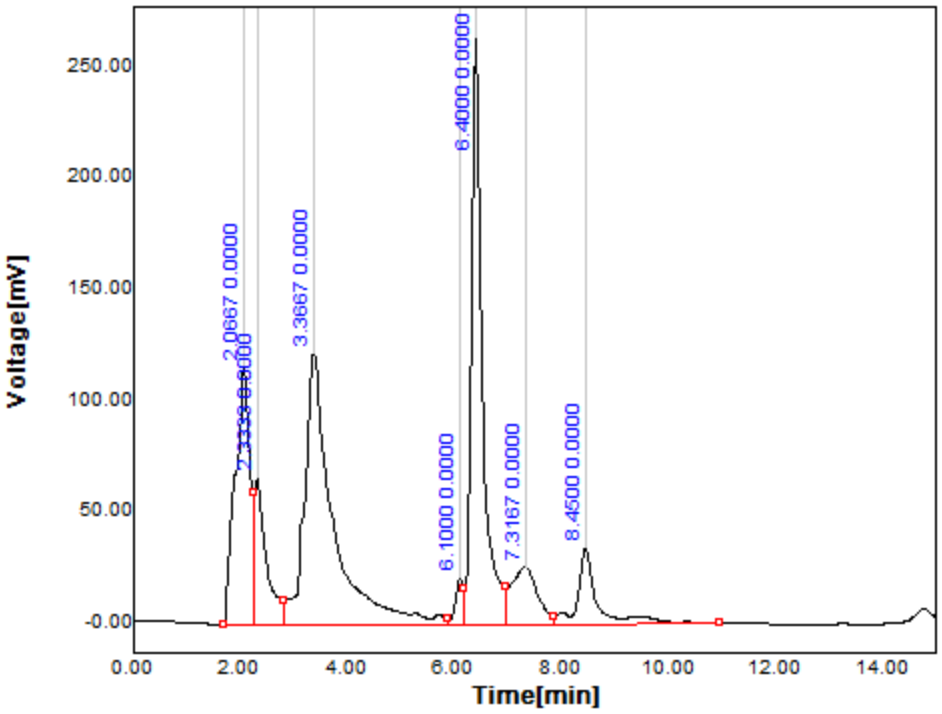


Figure. 12. HPLC chromatogram of seed sample

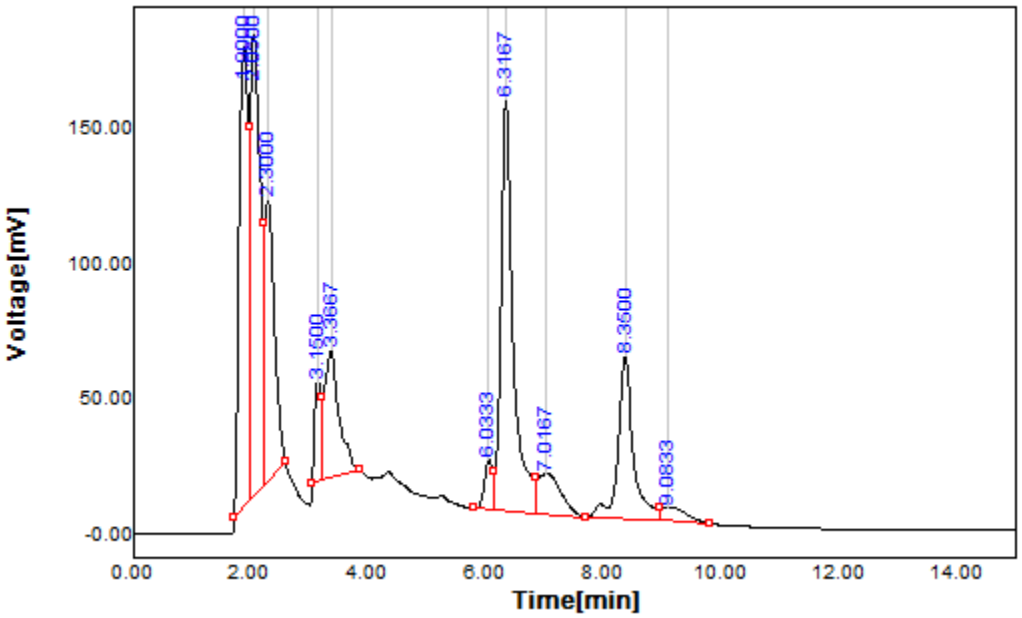


Figure.13. HPLC chromatogram of shoot sample

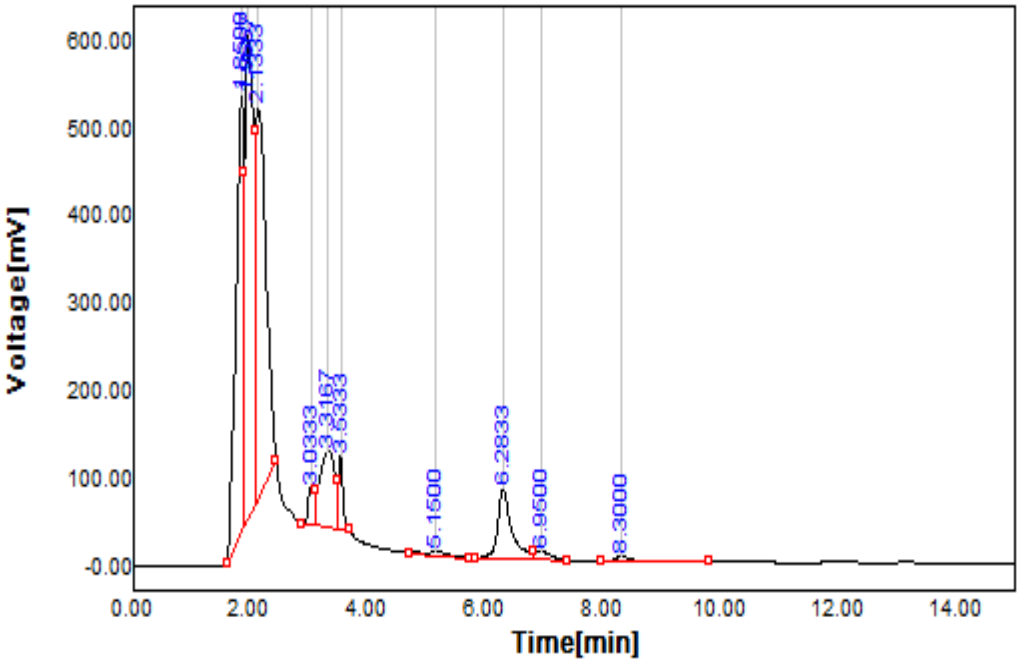


Figure.14. HPLC chromatogram of leaf sample

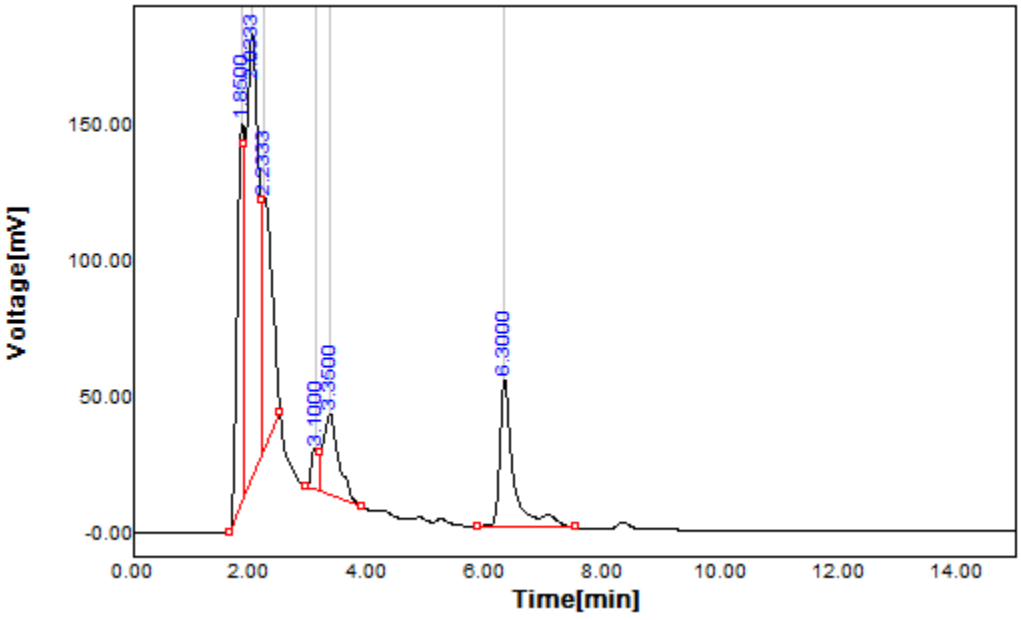


Figure.15. HPLC chromatogram of seed coat sample with fruit

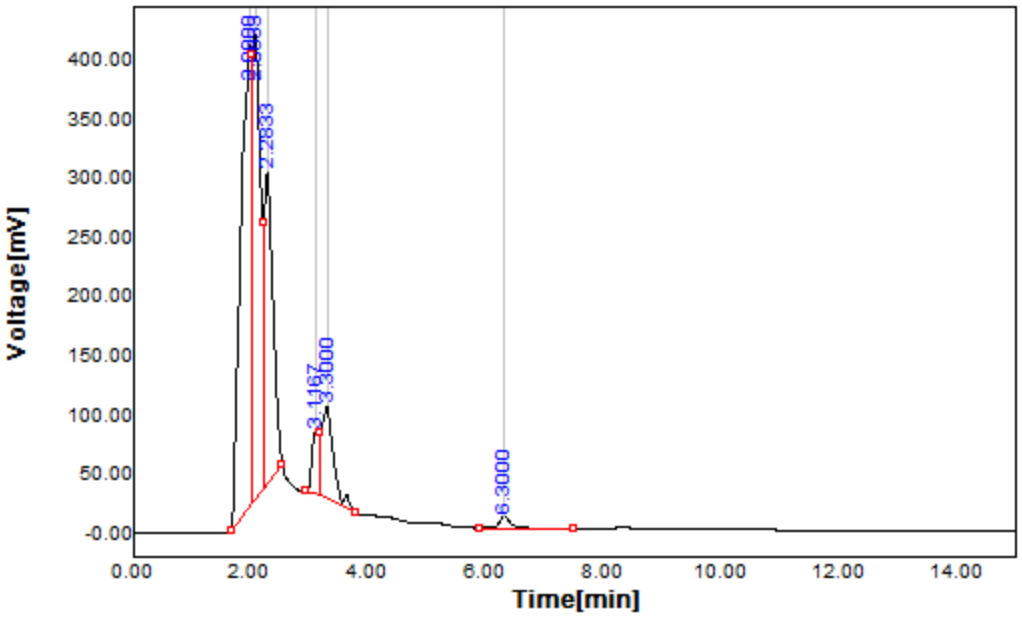


Figure.16. HPLC chromatogram of seed coat sample without fruit

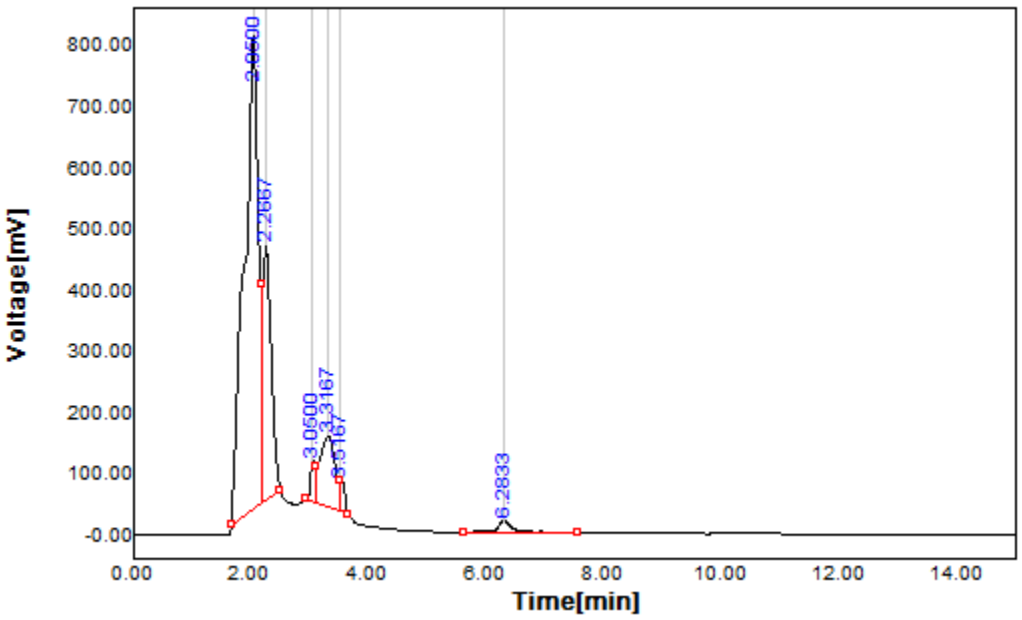


Figure.17. HPLC chromatogram of in vivo leaf of self propagated plants.

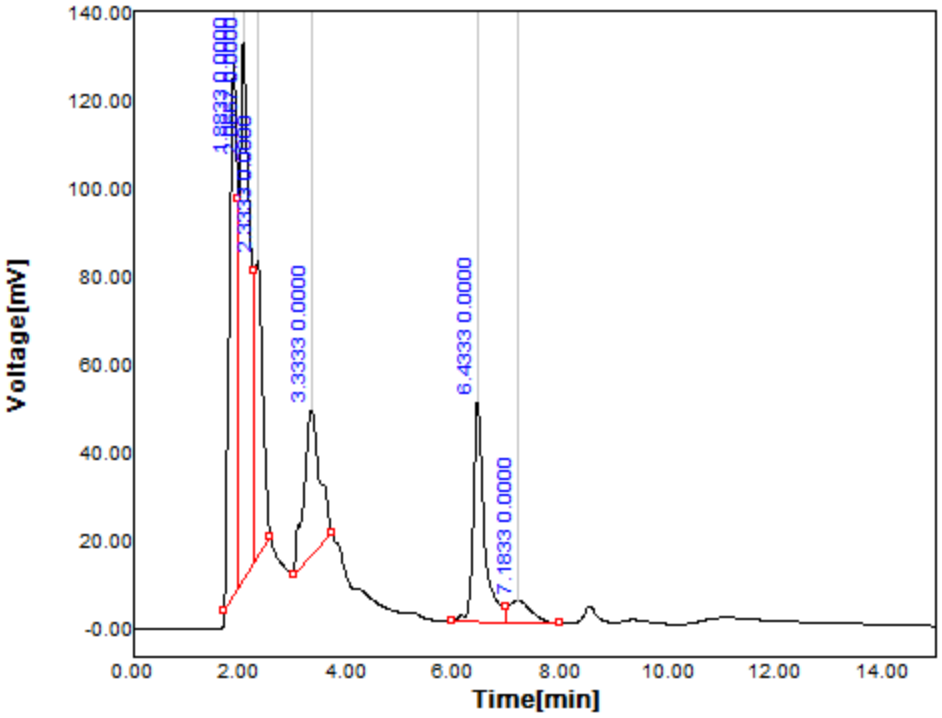


Figure.18. HPLC chromatogram of in vivo leaf of transplanted plants

Section IV

SUMMARY AND CONCLUSION

1. Work was carried out in the tissue culture laboratory and screen house of the department of environmental science, MDU, Rohtak

2. Plant material -Seeds and plants of *N. foetida* were collected from surrounding forests of Dapoli with collaboration from Forestry Department of Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. Maharashtra. Plants of Ophiorrhiza species were collected from the forests with collaboration from Tropical Botanical Garden and Research Institute (TBGRI), Kerela.
3. The best combination of sterilizing agents was found to be 70% ethanol for 1 minute followed by 2% NaOCl for 5 minutes, as this treatment gave minimum inhibition along with maximum seed germination with no contamination.
4. Out of various supporting medium tested, paper bridge method gave maximum (82.33%) seed germination in seeds without seed coat and 52.67% seed germination in seeds with seed coat. Germination initiation was less in seeds without seed coat as compared to seeds with seed coat i.e, 4 days in former and 12 days in latter
5. With the passage of time germination was found to decrease. Fresh seeds showed a percent germination of 84.66%, but only 8% seed germination was observed in seeds without seed coat after 8 months of storage at room temperature.. Whereas, the refrigerated seeds when germinated after 8 months gave a percent germination of 76.33%.. The germination potential can be increased with low temperature storage of the seeds of *N. foetida*.
6. Viability of fresh seeds of *N. foetida* was observed to be 93%. After one year of storage of seeds at room temperature the viability decreased to 45%, whereas, seeds stored at 4 ℃ gave a viability of 72%. After two years seeds stored at 4 ℃ showed 61% viability and seeds stored at room temperature showed no viability.
7. Among the various dormancy breaking treatments, maximum germination (68.33%) was obtained with 3% KNO_3 followed by GA_3 with 63.33% germination at 150 ppm concentration.

8. Maximum shoot multiplication of 71.66% was obtained on MS media supplemented with 0.5 mg/L TDZ with 15.33 number of shoots per explant. This was followed by MS medium supplemented with 1mg/L BAP + 1 mg/L TDZ with 70.33% shoot multiplication and 10.33 shoots /explant.
9. Shoot tip responded maximum with 75% shoot multiplication and 16.66 shoots per explant, followed by nodal explant, roots, leaves and least in cotyledons.
10. Maximum shoot elongation was reported in shoot tip(6.77 cm) explant in MS medium supplemented with 2mg/L BAP + 1.5mg/L GA_3. This concentration favored better elongation of shoots derived from all explants.
11. Test on effect of sucrose concentration, the percentage of shoot formation, number of shoot per explant and length of shoots increased gradually with increasing sucrose concentration in medium up to 3 %. 100% regeneration frequency was observed at 3% sucrose level with an average shoot length of 5.37 cm and number of shoots was 7.66.
12. Maximum number of explants responded to half strength MS medium supplemented with 2 mg/L BAP + 3mg/L IBA, followed with full strength MS medium and least in ¼ strength MS medium. Difference was also observed among the treatments with regard to duration of initiation of rooting. In full strength MS medium, rooting initiated after 2-2.5 weeks, followed by half strength MS medium (3 weeks) and fourth strength MS medium took 4- 4.5 weeks for root initiation.
13. Differential response was obtained among the shoots derived from different explants for root induction. Maximum percent response was observed in shoot tip derived explants i.e., 86 % in MS medium supplemented with 2 mg/L BAP + 3mg/L IBA. This was followed by shoots derived from nodal segment (80.33%) and least in leaf explant derived shoots (63%).
14. To avoid the problem of callusing and shoot tip necrosis during rooting, activated charcoal was added to the medium. Maximum rooting was observed in half strength MS medium supplemented with 2 mg/L IBA + 300 mg/L activated charcoal with 50 % shoot tip necrosis and least callusing.
15. The optimized transformation protocol for hairy roots production in *N. foetida* was as follows. After experimentation with five strains of *A. rhizogenes*, finally three strains of *A. rhizogenes* (A_4, LBA 9204 and MTCC 532) were selected.

16. All the three strains responded to *A. rhizogenes* infection for 25 minutes and 48 hours co- cultivation period. Maximum hairy roots were induced in A4 infected explants i.e. 80.67%, followed by LBA 9204(75.67%) and MTCC 532 (60%).
17. Out of the three strains of *A. rhizogenes*, A4 was found to be most virulent with respect to transformation percent and emergence time.
18. Supplementation of co-cultivation medium with 150 µM acetosyringone gave improved and maximum induction of hairy roots in all A. rhizogenes strains. A4 gave 82.66% transformation at 150 µM followed by LBA 9204 (72%) and least in MTCC 532 (61.67%).
19. Optical density of 1.0 of the bacterial suspension was found to be optimum in all the three strains. At OD=1.0 80.66% in A4, 72.33% in LBA 9204 and 60.66% in MTCC 532 transformation was obtained in leaf explant. Optical densities above and below OD=1.0 gave lower transformation percentages.
20. All the strains induced maximum hairy roots with leaf explant which was significantly higher from other explants (petiole, nodal segment and hypocotyls). A maximum of 78% in A4, 71% in LBA 9204 and 61% in MTCC 532 transformation was reported in leaf explant.
21. Among the methods of infection of explant with A. rhizogenes, cutting the explant followed by dipping in bacterial suspension was found to be better than other methods employed (pricking with needle containing bacterial suspension and unwounded explants). In A4 strain cutting method induced 79% hairy roots , followed by pricking method (59.67%) and least in unwounded explants (24.3%). Same trend was followed in other two strains.
22. For the elimination of *A. rhizogenes* after co- cultivation, a combination of cefotaxime and carbecillin (500+500 mg/L) was found to be best. This combination of antibiotics not only gave better elimination but also gave maximum transformation (86%).
23. *A. rhizogenes* recurrence was observed on hairy rots after 20 days , regardless of antibiotic type and concentration, but the loss of hairy roots was less in case of combination of two antibiotics. On the other hand , after 30 and 40 days none of the hairy roots showed the *A. rhizogenes* recurrence at all antibiotic concentrations used.

24. PCR amplification of *rol* B gene experiment, confirmed the presence of *rol* B gene in the hairy roots induced in N. foetida. While it was found to be absent in the untransformed roots.
25. Camptothecin yield was found to be: seeds (0.116%), in vitro roots (0.099%), in vitro shoots (0.067%), in vitro leaves (0.041%) and seed coat (0.0273%) and maximum in hairy roots (0.299%).

Achievements

Nothapodytes foetida and *Ophiorrhiza species* grows wild in the forests of Maharashtra, Karnataka,Tamil Nadu,Kerela,West Bengal and Assam . Since, the present study is carried out at M.D.University,Rohtak Haryana and both these plants collected from their natural habitat (forests of South India) are transplanted on campus under sub tropical conditions. The characterization of both these species under changed habitat (subtropical climatic conditions) has opened the gateway of dissemination of these species in North India also which shall be an excellent addition in the biodiversity resulting in conservation of these endangered species due to wider habitat range. The outcome of the finding of the study are useful in conservation of these endangered species , addition to biodiversity besides ensuring raw material supply to pharmaceutical industries for camptothecine production. The luxuriant growth of *Nothapodytes foetida under climate* of Rohtak has a potential as a cash crop for farmers of North India.

- In vivo and in vitro propagation of of *Nothapodites foetida* was succesfuly achieved through seed germination and tissue culture.
- *Agrobacterium rhizogene* mediated transformation was successfuly achieved with good hary root production.
- The production of camptothecine through in vitro produced tissues will ease pressure from the exploitation of destructive extraction of camptothecine from natural plant materials and contribute to conservation of this plant.
- It was observed that Nothapodytes foetida shows good vegetative growth in the herbal garden of the university, it also flowers but seed setting does not take

place. Camptothesin in the leaves of these plants is comparable to leaves from plant of Western Ghats.

- Nothapodytes foetida shows good vegetative growth in the climate of Rohtak. The possibility of introduction of this plant to North India as a cash crop for the extraction of camptothecine from vegetative parts needs to be studied. This will ease pressure on the exploitation of the plant in its native range.
- The camptothecine concentration in hairy roots is almost two times as compared to any other part , this protocol can be scalled up and used to produce camptothecine in the laboratory with out destruction the plants in the wild.
- This work will contribute to deminish the exploitation and ecosystem degrdation of this plant species for camptothecine extraction from its native ranges and ensure suply of camptothecine through hairy roots.

Publications

- Beniwal V.S., Sarina, Narkhade S.S., **Laura J.S. ,** (2012). In vitro studies on direct regeneration of *Nothapodytes Foetida* using shoot tip and nodal explants. Journal of Non-timber Forest Products. Vol. 19 No. 3, pp. 191-194.
- Sudha Sambyal Malik and **J.S. Laura**, 2014. Distribution of camptothecin through the plant kingdom. International Journal of Current Research. 6:6497:6507. ISSN: 0975-833X
- Sudha Sambyal Makil and **J.S. Laura**, 2015. Effect of Storage Temperature, Storage duration and Supporting Media on in Vitro Seed Germination of *Nothapodytes Foetida*: An Endangered Medicinal Plant of Western Ghat. Journal of Agroecology and Natural Resource Management. 2(2):130-133. ISSN No. 2394-0786
- Sudha Sambyal Malik and **J.S. Laura,** 2019. Agrobacterium rhizogenes mediated hairy root induction in endangered *Nothapodytes Foetida*. Journal of Pharmacognosy and Phytochemistry. 8(1): 2325-2328. ISSN: 2278-4136

RESEARCH ARTICLE | Published: 01 September 2012

In Vitro Studies on Direct Regeneration of *Nothapodytes Foetida* using Shoot Tip and Nodal Explants

V. S. Beniwal✉, Sarina, Satish S. Narkhede, J. S. Laura and Puneet Beniwal

Journal of Non-Timber Forest Products | Volume: 19 | Issue: 3 | Page No. 191-194 | 2012

DOI: https://doi.org/10.54207/bsmps2000-2012-349N16 | Cite this article

Abstract

The effect of different concentrations of auxins and cytokinins either alone or in combination with additives was assessed to be useful on the direct regeneration of shoot tip and nodal explants of *N.foetida*. BA and IBA proved comparatively better than others. The highest direct regeneration percent (95.0, 92.0 in shoot tip and nodal explants respectively) was observed on the MS medium supplemented with BA ($3.0mgL^{-1}$) + IBA ($1.0mgL^{-1}$) and additives (adenine sulphate ($50mgL^{-1}$) + glutamine ($100mgL^{-1}$) + L-arginine ($25mgL^{-1}$) + citric acid (0.0025%) + ascorbic acid (0.005%). Shoot tips as explants performed better by giving higher regeneration percentage and better growth of propagules in comparison to nodal explants in the establishment media.

Keywords

References

About this article

How to cite

Beniwal, V.S., Sarina, &., Narkhede, S.S., Laura, J.S. and Beniwal, P., 2012. *In Vitro* Studies on Direct Regeneration of *Nothapodytes Foetida* using Shoot Tip and Nodal Explants. *Journal of Non-Timber Forest Products*, 19(3), pp.191-194. https://doi.org/10.54207/bsmps2000-2012-349N16

RIS Download

Publication History

Manuscript Published on 01 September 2012

Share this article

Anyone you share the following link with will be able to read this content:

ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 6, Issue, 05, pp.6497-6507, May, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

REVIEW ARTICLE

DISTRIBUTION OF CAMPTOTHECIN THROUGH THE PLANT KINGDOM

Sudha Sambyal Malik and *Laura, J. S.

Department of Environmental Sciences, Maharshi Dayanand University, Rohtak, Haryana, India

ARTICLE INFO

Article History:
Received 15th February, 2014
Received in revised form
04th March, 2014
Accepted 19th April, 2014
Published online 20th May, 2014

Key words:

Camptothecin, Plant kingdom,
Endophytic fungi.

ABSTRACT

Many plant derived compounds have been used as drugs both in their original form and in semi-synthetic form. Camptothecin (CPT), a pyrole quinoline alkaloid, is one of the most promising anticancer drug of 21st century. It was first extracted from *Camptotheca acuminata*, since then it has been reported to exist in several plant species and also in plant endophytic fungi. Indiscriminate harvesting of these species for drug has led to a serious threat to these species. The distribution of CPT in the plant kingdom is being described here, so that the stress on the existing sources of CPT can be relieved by finding alternative sources.

INTRODUCTION

Nature has been recognized as a rich source of medicinal compounds for hundred of years. About 20% of the plant species contain alkaloids, which are a diverse group of low molecular weight, nitrogen-containing molecules. Many of these alkaloids are produced as a defense mechanism of plants against herbivores, microbes, viruses and competing plants (Wink, 2003). The potent biological activity of some alkaloids has traditionally been exploited by humans for the treatment of many diseases. The most serious proximate threats when extracting medicinal plants generally are habitat loss, habitat degradation, and over harvesting (Hamilton, 2003). An incredibly large number of people in the world rely on plants as a source of drugs (Raskin *et al.*, 2002; Fransworth *et al.*, 1988). It is estimated that over 50% of all drugs in clinical use are plant derived natural product (Fransworth *et al.*, 1976).

Plants are a common host to a number of microbes including endophytes, which may also influence the production of plant metabolites and may itself capable of producing metabolites as produced by the host plants. Plant endophytic fungi are also an important and novel resource of natural bioactive compounds with their potential applications in pharmaceutical industry. In past two decades, it has been discovered that endophytic fungi contain many valuable compounds with antimicrobial, insecticidal, cytotoxic and anticancer properties (Zhao *et al.*, 2010). There are several plant derived alkaloids, which are currently in clinical use. These include anticancer agent camptothecin (CPT), vincristine, taxol and vinblastine, the muscle relaxant tubocurarine, the analgesics codeine and morphine, the anti malarial quinine, the antiarrythmic ajmalicine, the antibiotic sanguinarine, the sedative scopolamine and the topical analgesic capsaicin (Raskin *et al.*, 2002). National Cancer Institute USA has screened over 435,000 plants for antineoplastic effects (Daniel *et al.* 2001). Considering the enormous significance of these compounds, an extensive research work is going on for the standard methodologies for their large scale production. It is estimatsd that plant derived compounds constitute more than 50% of anticancer agent (Newman *et al.*, 2003; Nikun *et al.*, 2011). More than 3000 plant species have been reported to be used in cancer treatment. Over 60% of currently used anticancer agents are derived from natural sources including plants, marine organisms and microorganisms (Cragg *et al.*, 2003).

Among the plant derived compounds, Camptothecin, a quinoline alkaloid, has emerged as successful antineoplastic agent. It was discovered in 1966 by M.E Wall and M.C Wani in systemic screening of natural products for anticancer drugs. It was isolated from the bark and stem of *Camptotheca acuminata* a tree native to China used as a cancer treatment. During the last half century, scientists have discovered its potential as a selective anticancer drug (Wu *et al.*, 1995). Camptothecin is an expensive chemical, costing about US$ 170/250 mg. The development and marketing of this drug have been approved for treatment of ovarian, breast, lung, and colon cancer (Giovanella *et al.*, 1989). It has been used for leukemia and diseases of liver, gall bladder, spleen, and stomach and to improve chemotherapy of patients with locally advanced or metastatic colon carcinoma (Suchita Kamble *et al.*, 2011).

**Corresponding author: Laura, J. S.*
Department of Environmental Sciences, Maharshi Dayanand University, Rohtak, Haryana, India.

Journal of Agroecology and Natural Resource Management
Print ISSN: 2394-0786, Online ISSN: 2394-0794, Volume 2, Number 2; April-June 2015 pp. 130-133

http://www.krishisanskriti.org/janrm.html

Effect of Storage Temperature, Storage duration and Supporting Media on in Vitro Seed Germination of Nothapodytes Foetida: An Endangered Medicinal Plant of Western Ghat

Sudha Sambyal Malik[1] and J.S. Laura[2*]

[1,2]Dept. of Environment Science, MDU, Rohtak, Haryana
E-mail: [1]sambyal.sudha@gmail.com, [2]jslmdu@gmail.com

Abstract—*Nothapodytes foetida Grah. (Mabb.) is a medicinal tree species from the Western Ghats which has gained a considerable importance in recent times for its alkaloid Camptothecin. Camptothecin (CPT) is a bio molecule, which has gained attention all over the world because of its role as anticancer drug. Due to the over exploitation of the plant for the extraction of camptothecin, this plant has became endangered. Moreover, seeds also show a viability of about 2-3 months, thus leading to problems in germination. The present study describes in vitro seed germination and storage behavior of Nothapodytes foetida. The results showed that germination percentage can be improved by the use of filter paper bridge as supporting media rather than agar. It is interesting to note that germination on media gelled with agar was only 57 % whereas seed with filter paper bridge as supporting media showed 82% germination. Seeds were tested for two storage conditions over time. Seeds stored at room temperature exhibit viability of less than a year (7-8 months), whereas low-temperature storage enhanced the viability to more than one year. High seed germination, low mean germination time and low rate of fall in seed germination percent of seeds stored at 4°C was reported. Above 50% seed germination even after 12 months storage at 4°C was reported which suggest this storage method as an appropriate technique. Cold storage of the seeds of Nothapodytes foetida is recommended for the conservation of germplasm in the seed banks.*

Keywords: *Camptothecin, Nothapodytes foetida, anticancer drug*

1. INTRODUCTION

Nothapodytes foetida is a medicinal plant and endemic to Western Ghats. This species is distributed in patches of the dry and moist deciduous as well as evergreen forests of the Western Ghats in India. It has immense value in treatment of many killer diseases especially cancer. The metabolite–camptothecin (CPT), is known as a potent drug that breaks single-strand DNA in the mammalian systems and tumours[1]. The stem, wood bark, seeds, leaves and various organs of the tree are rich sources of the potent antitumor agents like quinoline alkaloids, camptothecin (CPT) and 9-methoxy-camptothecin (9-OMeCPT).

The content of camptothecin in this tree is very high as compared to *Camptotheca acuminate* [2]. Due to huge global demand of camptothecin, *N. foetida* is being exploited. Perhaps this has led to the large scale exploitation and indiscriminate collection of this species from its wild habitat in the recent years [3]. The species has become endangered due to over-exploitation for medicinal use and habitat destruction in its distribution range area. Therefore, appropriate steps should be taken for its conservation. The present study was undertaken with an aim to test the effect of long-term storage of seeds under different storage conditions on seed germination.

In *N. foetida* seed germination in natural habitat is very less due to the lack of appropriate germination conditions and hard seed-coat. It has been reported that seeds lose their viability after 2-3 months, thus leading to problems in germination [4, 5]. Seed viability in terms of seed germination showed that seeds can be stored up to 60 days with about 30 per cent seed germination [6]. This is due to some sort of seed dormancy. Seed dormancy is caused by the conditions within the seed which prevent germination under ideal conditions. In *Nothapodytes foetida,* dormancy is because of seed coat, physiological conditions and presence of phenolic compounds in the seed coat leading to poor germination [7, 8]. This dormancy is overcome through the application of pre-treatment by many workers [7, 8, 9, 10]. These pre-treatments only help in breaking the seed dormancy and increased the germination, but failed to enhance the viability of *N. foetida* seeds.

Germination of seed is a function of duration of storage, storage temperature and moisture content at storage [11]. Therefore good management of storage temperature and duration may enhance germination. For the large scale production of camptothecin large number of plantation is required. But natural regeneration through seeds is low and vegetative propagation through cuttings is also not successful.

Journal of Pharmacognosy and Phytochemistry 2019; 8(1): 2325-2328

Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(1): 2325-2328
Received: 09-11-2018
Accepted: 13-12-2018

Sudha Sambyal Malik
Dept. of Environment Sciences
M. D. U. Rohtak, Haryana, India

Jitender Singh Laura
Dept. of Environment Sciences
M. D. U. Rohtak, Haryana, India

Correspondence
Jitender Singh Laura
Dept. of Environment Sciences
M. D. U. Rohtak, Haryana, India

Agrobacterium rhizogenes mediated hairy root induction in endangered *Nothapodytes foetida*

Sudha Sambyal Malik and Jitender Singh Laura

Abstract
Nothapodytes foetida is a member of family Icacinaceae and yields an anti-cancer drug: Camptothecin (CPT) which has a huge global demand. An alternative method for the production of camptothecin is highly desirable and can be attained by the application of hairy root culture. The present study was carried out to establish an efficient protocol for hairy roots in *N. foetida* using five different strains of *Agrobacterium rhizogenes* (A4, LBA 9204, MTCC 532, MTCC 2364 and NCIM 5140). *In vitro* grown seedlings were used for the transformation experiment. Young seedlings were used as explants for the induction of hairy roots. Explants were infected with different *A. rhizogenes* strains. Then the explants were co-cultivated with the respective strains in Murashige and Skoog media and subsequently transferred to MS media containing antibiotics. The strain A_4 was more effective than the other four strains in hairy root induction in *N. foetida.*

Keywords: Camptothecin, hairy roots, *Nothapodytes foetida*, *Agrobacterium rhizogenes*

1. Introduction
Secondary metabolite production from the plants can be achieved by the application of tissue culture combined with genetic transformation. Root derived phytochemicals can be obtained from hairy root culture (Giri and Narasu, 2000) [7]. *Agrobacterium rhizogenes* causes hairy roots at the site of infection. It has been reported that hairy roots yields higher amounts of secondary metabolites in comparison to intact plant roots and cell suspension cultures (Allan *et al.*, 2002; Hamill *et al*., 1995; Hashimoto and Yamada, 1983; Hartmann *et al.*, 1986) [2, 9, 11, 10]. *Nothapodytes foetida* (Grah.) Mabb. (Syn. *N. nimmoniana*, *Mappia foetida*), which yields camptothecin (CPT) used in anticancer drug formulation. This tree immediately needs conservation attention because it is the most convenient source for large-scale production of CPT. *N. foetida* has become endangered and is now confined only to the remnant of forest pockets. Due to loss of its habitat and over exploitation, the population of this species has declined by 50-80% (Singh *et al.*, 2010) [17]. RET (Rare, Endangered and Threatened) list of medicinal plants made in accordance to criteria given by International Union for Conservation of Nature and Nature Resources (IUCN), given by ENVIS also includes *N. foetida* in endangered categoery. As it has been reported that maximum concentration of CPT is found in the roots of *N. foetida*, therefore hairy root culture could be better option for the large scale production of CPT (Namdeo *et al.*, 2012) [13].
Importance of N. foetida in cancer treatment creates a huge necessity for the development of a protocol so that the production of camptothecin could be enhanced. Thus, the present study was undertaken to establish a proficient protocol to increase the camptothecin yield. This would lead to decline in burden on the plant species in its natural home range area, there upon halting the over harvesting of N. foetida in the wild.

2. Material and Method
2.1 Plant material
Seedlings grown in vitro were used as the explant (leaves, nodal segment, hypocotyl and radical) for the induction of hairy roots. The explants were excised and used for transformation studies.

2.3 Bacterial strains
Following *Agrobacterium rhizogenes* strains were used for transformation:

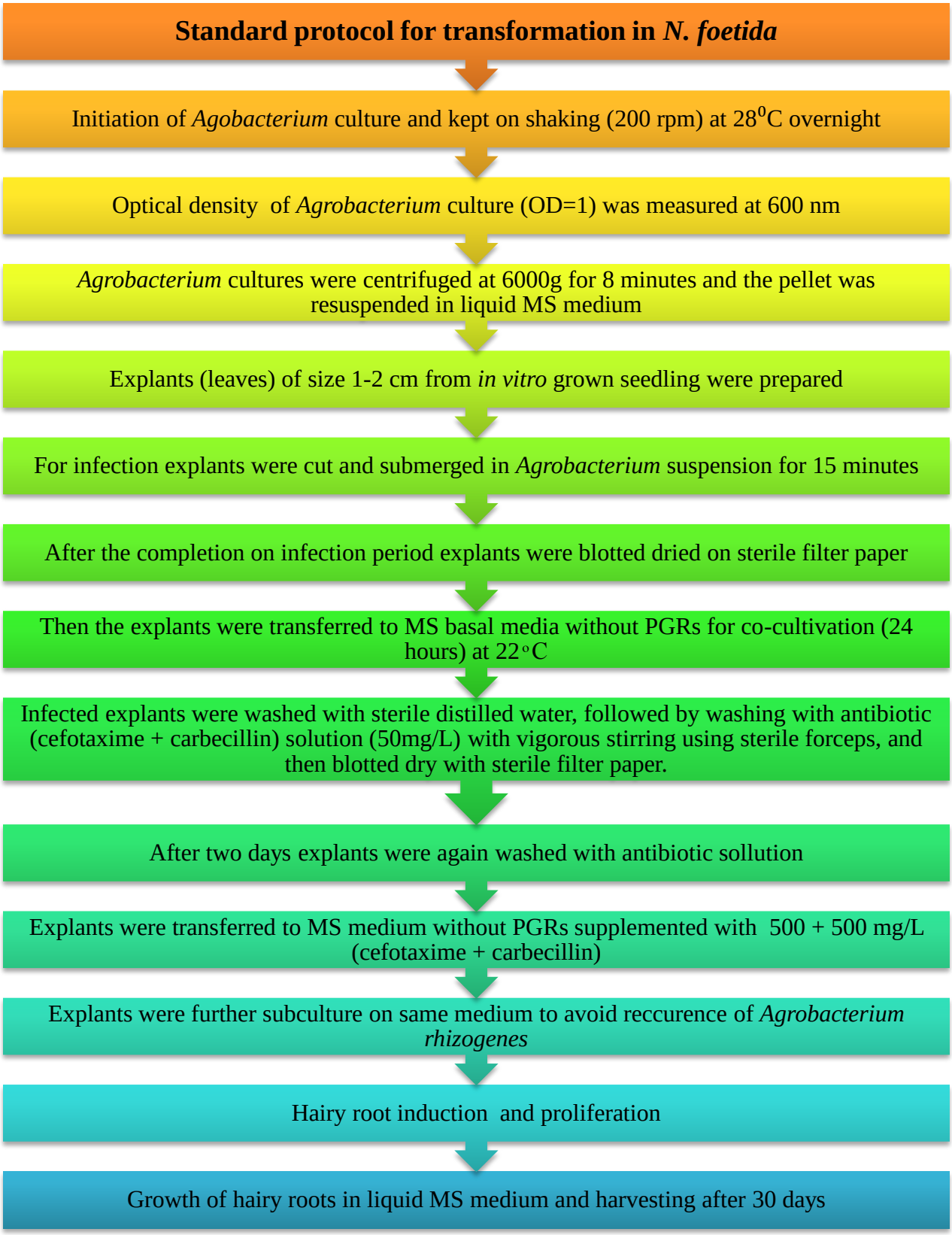


Figure. Flow chart of standard protocol for hairy root induction in *N. foetida*

LITERATURE CITED

- Aasim, M., Khawar, K. M. and Ozcan, S. 2013. Production of herbicide resistant cowpea (*Vigna unguiculata* L.) transformed with the bar gene. Turk. J. Biol. 37: 472–478.
- Agrawal, V., Prakash, S. and Gupta, S.C. 2002. Effective protocol for in vitro shoot production through nodal explants of *Simmondsia chinensis*. Biol Plant. 45: 449–53.
- Ahmed, M. R., and Anis, M. 2014. *In vitro* regeneration and the antioxidant enzymatic system on acclimatization of micropropagated *Vitex trifolia* L. Agroforest Syst. 88: 437-447.
- Ahmed, S., Sharma, A., Bhushan, B., Singh, A. K. and Wali, V. K. 2014. Effect of carbohydrate source, pH and supporting media on *in vitro* rooting of banaba (musa spp.) cv. Grand Naine. African Journal of Agriculture.9 (14):1135-1140.
- Alboresi. A., Gestin, C., Leydecker, M. T., Bedu, M., Meyer, C. and Truong, H.N. 2005. Nitrate, a signal relieving seed dormancy in *Arabidopsis*. Plant Cell. Environ. 28(4): 500-512.
- Allan, E. J., Eeswara, J. P., Jarvis, A. P. Mordue, A. J., Morgan, E. D., and Stuchbury, T. 2002. Induction of hairy root cultures of *Azadirachta indica* A. Juss. and their production of azadirachtin and other important insect bioactive metabolites. Plant Cell Rep. 21: 374-79.
- Amjad, M., Ziaf, K., Iqbal, Q., Ahmad, I. and Riaz, M. A. 2007. Effect of seed priming on seed vigor and salt tolerance in hot pepper. Pak J Agri Sci. 44(3):408-419.
- Arya, A., Kumar, S. and Kasana, M. S. 2014. Root initiation in cuttings and *in vitro* raised shoots of *Pinus roxburghii*. Journal of Phytology. 6: 7-13.
- Bansal, M., Kumar, A. and Reddy, S. 2014. Influence of *Agrobacterium rhizogenes* strains on hairy root induction and "bacoside A" production from *Bacopa monnieri* (L.) Wettst. Acta Physiol Plant. 36:2793–801.
- Basra, S. M. A., Farooq, M. and Tabassum, R. 2005. Physiological and biochemical aspects of seed vigor enhancement treatments in fine rice (*Oryza sativa* L.). Seed Sci. Technol. 33:623–628.

- Batra, J., Dutta, A., Singh, D., Kumar, S. and Sen, J. 2004. Growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root clones in relation to left- and right-terminilinked Ri T-DNA gene integration. Plant Cell Rep 23:148–154.
- Bewley, J.D. and Black, M. 1994. Seeds: Physiology of Development and Germination. 2nd Edn. Plenum Press, New York. 444.
- Blidar, C. F., Ardelean A. and Turcus, V. 2011. Efficient initiation of in vitro culture at wheat. Analele Universităţii din Oradea, Fascicula Biologie. 18(2): 176-181.
- Blidar, C. F., Ardelean A. and Turcus, V. 2012. Using the filter paper bridge technique for the initiation of vitrocultures of maize. Analele Universităţii din Oradea, Fascicula Biologie. 19(1): 17- 22.
- Blidar, C. F. and Ilea, C. 2014. Rapid initiation of in vitro cultures at Viola Wittrockiana Matrix F1 Red Blotch. Analele Universităţii din Oradea, Fascicula Biologie. 21(2): 74-78.
- CAMP. 2001. Conservation assessment and management plan for CAMP 2001. Conservation assessment and management plan for medicinal plants of Maharashtra state FRLHT.
- Chaudhuri, K. N., Ghosh, B., Tepfer, D. and Jha, S. 2005 Genetic transformation of *Tylophora indica* with *Agrobacterium rhizogenes* A4: growth and tylophorine productivity in different transformed root clones. Plant Cell Reports. 24:25-35.
- Cheng, Z. M., Schnurr, J. A. and Kapaun, J. A. 1998. Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. Plant Cell Rep. 17: 646-649.
- Cho, H. J., Widholsm, J. M., Tanaka, N., Nakanishi, Y. and Murooka, Y. 1998. *Agrobacterium rhizogenes* mediated transformation and regeneration of the legume *Astragalus sinicus* (Chinese milk vetch). Plant Science. 138: 53-65.
- Colgecen, H., Koca, U. and Toker, G. 2011. Influence of different sterilization methods on callus initiation and production of pigmented callus in *Arnebia densiflora* Ledeb. Turkish J. Biol. 35: 513–520.
- Cuenca, B. and Vieitez, A. M. 2000. Influence of carbon source on shoot multiplication and adventitious bud regeneration in *in vitro* beech cultures**.** Journal of Plant Growth Regulation. 32: 1–12.

- Danphitsanuparn, P., Boonsnongcheep, P., Boriboonkaset, T., Chintapakorn, Y. and Prathanturarug, S. 2012. Effects of *Agrobacterium rhizogenes* strains and other parameters on production of isoflavonoids in hairy roots of *Pueraria candollei* Grah. ex Benth. var. candollei. Plant Cell Tiss Org Cult. 111:315–322.
- Danso, K. E., Azu, E., Elegba, W. Asumeng, A., Amoatey, H. M. and Klu, G. Y. P. 2011. Effective decontamination and subsequent plantlet regeneration of sugarcane (*Sacchrum officinarum* L.) *in vitro*. Int. J. Integr. Biol. 11: 90–96.
- Das, A., Gantait, S. and Mandal, N. 2011. Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. International Journal of Agricultural Research. 6: 40-48.
- Dhabhai, K., Sharma, M. M. and Batra, A. 2010. *In vitro* clonal propagation of *Acacia nilotica* (L.) - a nitrogen fixing tree. Researcher. 2(3): 7-11.
- Dobránszky J, Teixeira da Silva JA (2010). Micropropagation of apple-a review. Biotechnol Adv 28:462-488.
- Eskandari, H. and Kazemi, K. 2011. Effect of seed priming on germination properties and seedling establishment of cowpea (*Vigna sinensis*). Not. Sci. Biol. 3(4): 113-116.
- Estrela, C., Sydney, G. B., Bammann, L. L. and Felippe, Jr. O. 1995. Mechanism of action of calcium and hydroxyl ions of calcium hydroxide on tissue and bacteria. Braz Dent J. 6:85-90.
- Evelyne, P. S. and Ravindhran, R. 2011. Micropropagation of *Lippia nodiflora* using shoot tip and nodal explants under in vitro conditions. International Journal of Current Research. 3(10): 043-047.
- Franca-Neto, J. B. 1999. Test de tetrazolio para determinacao do vigor de sementes. In: Krzyzanowski, F. C., Vieira, R. D., Franca-Neto, J. B. (eds.). vigor de sementes: conceitos testes. ABRATES, cap. 8: 1-7.
- Fullner, K. J. and Nester, E. W. 1996. Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens.* J Bacteriol. 178(6):1498-1504.
- Gairi, A. and Rashid, A. 2004. Direct differentiation of somatic embryos on different regions of intact seedlings of Azadirachta in response to thidiazuron. J. Plant Physiol .161:1073-1077.
- Gandi, S. and Giri, A. 2012. Genetic transformation of *Centella asiatica* by *Agrobacterium rhizogenes*. Journal of Pharmacognosy. 3 (2):82-84.

- Gelvin, S. B. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol Mol Biol Rev. 67: 16-37.
- Giri, A., Giri, C. C., Dhingra, V. and Narasu, M. L. 2001. Enhanced podophyllotoxin production from *Agrobacterium rhizogenes* transformed cultures of *Podophyllum hexandrum*. Natural Product Letters. 15: 229–235.
- Giri, A. and Narasu, M. L. 2000. Transgenic hairy roots: recent trends and applications. 3 Biotechnol. Adv. 18: 1-22.
- Girija, S., Kavitha, S. and Deepavathi, S. 2006. Direct multiple shoot regeneration from shoot tip and nodal explants of *Ocimum sanctum* L. (Tulsi): a medicinal herb. Plant cell Biotechnology and Molecular Biology. 7: 23-28
- Gordon, M.E. and Letham, D.S. 1975. Regulators of cell division in plant tissues. XXII. Physiological aspects of cytokinin-induced radish cotyledon growth. *Aust. J. Plant Physiol.* **2**: 129-154.
- Ghanti, K., Kaviraj, C.P., Venugopal, R.B., Jabeen, F.T.Z. & Rao, S. (2004). Rapid regeneration of Mentha piperita L. from shoot tip and nodal explants. Indian J Biotechnol. 3: 594-59.
- Gonbad RA, Sinniah UR, Aziz MA, Mohamad R (2014) Influence of cytokinins in combination with GA3 on shoot multiplication and elongation of tea clone Iran 100 (Camellia sinensis (L.) O. Kuntze). The Sci World Journal. 1-9.
- Hamill, J. D. and Rhodes, M. J. C. 1993. In Plant Biotechnology; Grierson, D., Ed.; Blackie: Glasgow. 3: 178-209.
- Zhaleh Soheilikhah1 , Naser Karimi*1 , Hamid Reza Ghasmpour2 , Ali Reza Zebarjadi. 2013. Effects of saline and mannitol induced stress on some biochemical and physiological parameters of Carthamus tinctorius L. varieties callus cultures . AJCS 7(12):1866-1874 .
- Sk Moquammel Haque, Biswajit Ghosh Micropropagation, in vitro flowering and cytological studies of Bacopa chamaedryoides, an ethno-medicinal plant. Environmental and Experimental Biology (2013) 11: 59–68.
- Huang, Y., Diner, A.M. and Karnosky, D.F. 1991. *Agrobacterium rhizogenes* mediated genetic transformation and regeneration of conifer: Larixdeciduas. *In vitro* Cell Developmental Biology. 27:201- 207.

- Jaiwal, P. K., Kumari, R., Ignacimuthu, S., Potrykus, I. and Sautter, C. 2001. *Agrobacterium tumefaciens*-mediated genetic transformation of mung bean (*Vigna radiata* L.) a recalcitrant grain legume. Plant Sci. 161:239-247.
- Jian, B., Hou, W., Wu, C., Liu, B., Liu, W., Song, S., Bi, Y. and Han, T. 2009. *Agrobacterium rhizogenes*-mediated transformation of super root-derived *Lotus corniculatus* plants: a valuable tool for functional genomics. BMC Plant Biol. 9: 1-14.
- M.Z. Karim, S. Yokota, M.M. Rahman, J. Eizawa, Y. Saito, M.A.K. Azad, F. Ishiguri, K. Iizuka and N. Yoshizawa, 2007. Effects of the Sucrose Concentration and pH Level on Shoot Regeneration from Callus in *Araria elata* Seem. *Asian Journal of Plant Sciences, 6: 715-717.*
- Kaushik, S., Georga, I., Koumoundouros, D. 2011. Growth and body composition of zebrafish *(Danio rerio*) larvae fed a compound feed from first feed onward: Toward implications on nutrient requirements. Zebrafish 8:87-95.
- Kavitha MS, Wesely EG, Mehalingam P (2012). Direct multiple shoot regeneration from shoot tip and nodal explants of Solanum Nigrum L. a medicinal herb. Journal of Ornamental and Horticultural Plants 2(2):65-72.
- Khawar, K. M., Sancak, C., Uranbey, S. and Zca, S. 2004. Effect of thidiazuron on shoot regeneration from different explants of lentil (*Lens culinaris*) via organogenesis. Turk J Bot. 28: 421–426.
- Kiani, B. H., Safdar, N., Mannan, A. and Mirza, B. 2012. Comparative Artemisinin analysis in *Artemisia dubia* transformed with two different *Agrobacteria* harbouring *rol ABC* genes. Plant Omics J. 5:386–391.
- Kolumbina, M., Blesing, M. and Mares, D. J. 2006. α-Amylase and programmed cell death in aleurone of ripening wheat grains. J. Exp. Bot. 57(4): 877-885.
- Kumar, R., Vishwanathan, H., Suresh, T. and Mohan, P. 2002. Antibacterial activity of *Mappia foetida* leaves and stems. Fitoterapia. 73(2):734-36.
- Kumar, S. G. B., Ganapathi, T.R., Srinivas, L. Revathi, C.J. and Bapat, V.A. 2006. Expression of hepatitis B surface antigen in potato hairy roots. Plant Sci. 170: 918–925.
- Kumar, V., Sharma, A., Prasad, B. C. N., Gururaj, H. B., Ravishankar, G. A. 2006. *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root

formation is enhanced by ultrasonication and acetosyringone treatment. Electronic Journal of Biotechnology. 9 (4): 349-357.

- Laura, J. S. and Beniwal, V. S. 2012. *In vitro* studies on direct shoot regeneration of *N. nimmoniana* (Graham) Mabberly through shoot tip and nodal explants. Int J Curr Res 4(9):23–26.
- Maina, S. M., Emongor, Q., Sharma, K. K., Gichuki, S. T., Gathaara, M. and Villiers, S. M. 2010. Surface sterilant effect on the regeneration efficiency from cotyledon explants of groundnut (*Arachis hypogea* L.) varieties adapted to eastern and Southern Africa. African J. Biotechnol. 9: 2866–2871.
- Marrero, P., Padilla, D. P., Valdes, F. and Nogales, M. 2007. Comparison of three chemical tests to assess seed viability: the seed dispersal system of the Macaronesian endemic plant *Rubia fruticosa* (Rubiaceae) as an example. Chemoecology 17, 47–50.
- Shakti Mehrotra* Arun Kumar Kukreja Suman Preet Singh Khanuja Bhartendu Nath Mishra. 2008. Genetic transformation studies and scale up of hairy root culture of Glycyrrhiza glabra in bioreactor. Electronic Journal of Biotechnology ISSN: 0717-3458 Vol.11 No.2,
- Miche, L. and Balandreau, J. 2001. Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. Appl. Environ. Microbiol. 67: 3046–3052.
- Almudena Montoliu, Aurelio Gómez-Cadenas and Rosa M. Pérez-Clemente. 2010. in Vitro Adventitious Rooting of Carrizo Citrange Microshoots. HortScience June 2010 vol. 45no. 6 988-990
- Morla, S., Rao, C. S. V. R. and Chakrapani, R. 2010. Factors affecting seed germination and seedling growth of tomato plants cultured *in vitro* conditions. J. Chem. Biol. Phys. Sci. 1: 328–334.
- Nakagawara, S., Goto, T., Nara, M., Ozawa, Y., Hotta, K. and Arata, Y. 1998. Spectroscopic characterization and the pH dependence of bactericidal activity of the aqueous chlorine solution. Anal. Sci. 14: 691–698.
- Namdeo, A. G., Sharma, A. 2012. HPLC analysis of the camptothecin content in various parts of *N. foetida* collected in different periods. Asian Pac J Trop Biomed. 389–393.

- Nanda, R. M., Rout, G.R. and Das, P. 2003. Studies on biochemical markers for embryogenic potential of *Acacia catechu*. Biologia Bratislava. 58: 69-76.
- Nourozi, E., Hosseini, B. and Hassani, A. 2014. A reliable and efficient protocol for induction of hairy roots in *Agastache foeniculum.* Biologia 69: 870-879.
- Nowak, B., Miczyński, K. and Hudy, L. 2004. Sugar uptake and utilization during adventitious bud differentiation on *in vitro* leaf explants of 'Węgierka Zwykła' plum (*Prunus domestica*). Plant Cell Tissue Organ Cult. 76:255–260.
- Padmanabha, B., Chandrashekar, M., Ramesha, B., Vasudeva, R., Ganeshaiah, K. and Shaanker, R. 2006. Patterns of accumulation of camptothecin, an anti-cancer alkaloid in *Nothapodytes nimmoniana* Graham in the Western Ghats, India: Implications for identifying high-yielding sources of the alkaloid. Curr Sci. 90(2):95–100.
- Pai, S. R., Pawar, N. V., Nimbalkar, M. S., Kshirsagar, P. R., Kolar, F. K. Dixit, G. B. 2013. Seasonal variation in content of camptothecin from the bark of *Nothapodytes nimmoniana* (Grah.) Mabb., using HPLC analysis. Pharmacognosy Res. 5(3): 219-223.
- Pal, A., Swain, S. S., Mukherjee, A. K. and Chand, P. K. 2013. *Agrobacterium* pRi TL-DNA *rol* B & TR-DNA opine genes transferred to the spiny amaranth (*Amaranthus spinosus* L.) –A nutraceutical crop. Food Technol Biotechnol. 51:26–35.
- Park, H.J., Lee, H.R., Pyee, J. and Cha, H.C. 2001. Regeneration of grape (*Vitis labruscana* cv. Kyoho) by shoot-tip culture. *J. Plant Biol.* **44**: 185-192.
- Pawar, P. K. and Maheshwari, V. L. 2004. *Agrobacterium rhizogenes* mediated hairy root induction in two medicinally important members of family Solanaceae, Indian Journal of Biotechnology. 3: 414–417.
- Pirian, K., Piri, K. H. and Ghiyasvand, T. 2012. Hairy roots induction from *Portulaca oleracea* using *Agrobacterium rhizogenes* to Noradrenalines production. International Research Journal of Applied and Basic Sciences. 3(3): 642-649.
- Porter, J. R. 1991. Host range and implications of plant infection by *Agrobacterium rhizogenes*. Crit Rev Plant Sci 10:387–421
- Pradhan, K. and Badola, H. K. 2012. Effect of Storage Conditions and Storage Periods on Seed Germination in Eleven Populations of *Swertia Chirayita*: A Critically Endangered Medicinal Herb in Himalaya. The Scientific World Journal. 1-9.

- Rai, V. R. 2002. Rapid clonal propagation of *N. foetida* (Wight) Sleumer—a threatened medicinal tree. *In Vitro* Cell Dev Biol Plant. 38(4):347–351.
- Rao, K., Chodisetti, B., Mangamoori, L. N. and Giri, A. 2012. *Agrobacterium*-mediated transformation in *Alpinia galanga* (Linn.) Wild for enhanced acetoxychavicol acetate production. Appl Biochem Biotechnol. 168:339–347.
- Rathore, M. S. and Shekhawat, N. S. 2013. *In vitro* regeneration in *Sarcostemma acidum* (Roxb.) - An important medicinal plant of semi-arid ecosystem of Rajasthan, India. Physiol Mol Biol Plant. 19:269–275.
- Sahu, L., Jenaab, S., Swainc, S. S., Sahoob, S. and Chand, P. K. 2013. *Agrobacterium rhizogenes*-mediated transformation of a multi-medicinal herb, *Boerhaavia diffusa* L.: optimization of the process and antimicrobial activity against bacterial pathogens causing urinary tract infections. Front Life Sci. 7:197–209.
- Samadi, A., Jafari, M., Nejhad, N.M. and Hossenian, F. 2014. Podophyllotoxin and 6-methoxy podophyllotoxin production in hairy root cultures of *Linum mucronatum* ssp. mucronatum. Pharmacogn. Mag. 10: 154–16.
- Sang, I. S., Moon, J. C., Cheol, S. J. and Kim, W. 2008. Effect of potassium nitrate priming on seed germination of seashore paspalum. Hortscience 43(7):2259–2262.
- Sathyagowri, S. and Seran, T. H. 2011. *In vitro* plant regeneration of ginger (*Zingiber officinale* Rosc.) with emphasis on initial culture establishment. Int. J. Med. Arom. Plants. 1(3): 195-202.
- Selvaraj M., Benjamin, J. H. F. and Rao, M. V. 2013. Influence of agar concentration and liquid medium on *in vitro* propagation of *Ceropegia thwaitesii* Hook- an endemic species. Journal of Agricultural Technology. 9(4): 1013-1022.
- Sharma, S. N., Puri, S. C. and Srivastava, T. N. 2000. Enhancement of seed germination in *N. foetida*. J Med Arom Plant Sci. 22:206–210.
- Shim SI, Cheol Moon J, Jang CS, Raymer P, Kim W. 2008. Effect of Potassium Nitrate Priming on Seed Germination of Seashore Paspalum . Hort Science 43, 2259 – 2262.
- Siddique I, Bukhari NA, Perveen K, Siddiqui I, Anis M. Pre-culturing of nodal explants in thidiazuron supplemented liquid medium improves *in vitro* shoot multiplication of *Cassia angustifolia*. Acta Biol Hung. 2013; 64: 377-384.

- Nayak, P., Behera, P.R., and Manikkannan, T. 2007. High frequency plantlet regeneration from cotyledonary node cultures of Aegle marmelos (L.) Corr. In vitro Cell Dev. Biol., 43: 231–236.
- Barik DP, Naik SK, Mudgal A, Chand PK. Rapid plant regeneration through in vitro axillary shoot proliferation of butterfly pea (*Clitoria ternatea* L.)—a twinning legume. In Vitro Cell Dev Biol Plant. 2007;43:144–148.
- R. K. Singh, R. K. Raipuria, V. S. Bhatia, A. Rani, Pushpendra, S. M. Husain, D. Chauhan, G. S. Chauhan, T. Mohopatra. SSR markers associated with seed longevity in soybean. Seed Science Technology 36 (2008) 162-167.
- Singh, J. and Tiwari, K. N. 2010. High-frequency in vitro multiplication system for commercial propagation of pharmaceutically important *Clitoria ternatea* L.- a valuable medicinal plant. Ind Crop Prod. 32:534–538.
- Sivanandhan, G., Selvaraj, N., Ganapathi, A. and Manickavasagam, M. 2014. An efficient hairy root culture system for *Withania somnifera*. African Journal of Biotechnology. 13(43): 4141-4147.
- Thiyagarajan M, Venkatachalam P. 2013. A reproducible and high frequency plant regeneration from mature axillary node explants of Gymnema sylvestre (Gurmur) – An important antidiabetic endangered medicinal plant. Ind Crop Prod 50: 517–524.
- Sujatha, G., Zdravkovic-Korac, S., Calic, D., Flamini, G. and Ranjitha Kumari, B. D. 2013. High-efficiency *Agrobacterium rhizogenes*-mediated genetic transformation in *Artemisia vulgaris*: hairy root production and essential oil analysis. Indust Crops Product. 44:643–652.
- Sut, D., Talukdar, P. and Singh, S. 2004. Micropropagation of ramie (*Boehmeria nivea* (L) Gaud) through shoot-tip and nodal segment culture. Indian J. Agric. Res. 38 (2): 131-134.
- Sutter, E. G. and Trigiano, R. N. 1996. Plant Tissue Culture Concepts and Laboratory Exercises, Gray (Eds), CRC Press, New York. Pp. 11–25.
- Swain, S. S., Sahu, L., Pal, A., Barik, D. P., Pradhan, C. and Chand, P. K. 2012. Hairy root cultures of butterfly pea (*Clitoria ternatea* L.): *Agrobacterium* × plant factors influencing transformation. World J Microbiol Biotechnol. 28:729–739.

- Talei, D., Saad, M. S., Yusop, M. K., Mihdzar, A. K. and Valdiani, A. 2011. Effect of different surface sterilizers on seed germination and contamination of King of Bitters (*Andrographis paniculata* Nees.) Am. Euras. J. Agric. Environ. Sci. 10: 639–643.
- Tang, H., Ren, Z. and Krezal, G. 2000. An evaluation of antibiotics for the elimination of *Agrobacterium tumefaciens* from walnut somatic embryos and for the effects on the proliferation of somatic embryos and regeneration of transgenic plants. Plant Cell Reports, Berlin/Heidelberg. 19: 881-887.
- Tehrim, S., Mirza, M.Y. and Sajid, G.M. 2013. Comparative study of different growth regulators for efficient plant regeneration in grapes. Pakistan J. Agric. Res. 26: 275-289.
- Tejavathi, D. H., Raveesha, H. R. and Shobha, K. 2011. Evaluation of different treatments to improve the seed germination among the populations of *N. foetida* (WT) Sleumer. Indian J Fund Appl Life Sci. 1(4):187–192.
- Thomas, T.D. 2008. The role of activated charcoal in plant tissue culture. Biotechnology Advances. 26: 618–631.
- Tiwari SK, Tiwari KP, Siril EA (2002) An improved micropropagation protocol for teak. Plant Cell Tiss Org Cult 71:1–6
- Turker, Arzu & Yucesan, Buhara & Ekrem, Gurel. (2010). Adventitious shoot regeneration from stem internode explants of Verbena officinalis L., a medicinal plant. Turk J Biol. 34. 297-304.
- Tyagi, Hemshikha, and Uttar Kumar Tomar. "Factors Affecting *in vitro* Shoot Proliferation and Rooting of Mature *Tecomella undulata* (Sm.) Seem Tree." *Research in Plant Sciences* 1.2 (2013): 38-44.
- Vejsadova, H. 2006. Factors affecting seed germination and seedling growth of terrestrial orchids cultured *in vitro*. Acta Biol. Cracoviensia Ser. Bot. 48: 109–113.
- Verma, P. C., Rahman, L., Singh, A., Dharm, N., Jain, C., Khanuja, S. P. S. and Banerjee, S. 2007. *Agrobacterium rhizogenes*-mediated transformation of *Picrorhiza kurroa* Royle ex Benth.: Establishment and selection of superior hairy root clone. Plant Biotechnol Rep. 1:169–174.

- Victor, J. M. R., Murthy, B. N. S., Murch, S. J., KrishnaRaj, S. and Saxena, P. K. 1999. Role of endogenous purine metabolism in thidiazuron-induced somatic embryogenesis of peanut (*Arachis hypogaea*). Plant Growth Regul. 28: 41-47.
- Wang, R., He, L. S., Xia, B., Tong, J.F., Li, N. and Peng, F. 2009. A micropropagation system for cloning of hemp (*Cannabis sativa* L.) by shoot tip culture. Pak. J. Bot., 41(2): 603-608.
- Wesely, E. G., Antonisamy, J. M. A., Subramanian, K. K. M. and Nallyan, S. 2011. Micropropagation of *Alternanthera sessilis* (L.) using shoot tip and nodal segments. Iranian Journal of Biotechnology. 9(3): 206.
- Wiebke, Beatriz, Ferreira, Fabricio, Pasquali, Giancarlo, Bodanese-Zanettini, Maria Helena, & Droste, Annette. (2006). Influence of antibiotics on embryogenic tissue and Agrobacterium tumefaciens suppression in soybean genetic transformation. *Bragantia*, *65*(4), 543-551.

Summary of the major research project

"*In vitro* and *in vivo* studies on micropropagation, transformation and camptothecin in *Nothapodytes foetida* and *Ophiorhiza species*"

UGC reference No. F.No.37-261/2009 (SR) dated 12/1/201

Funded by

University Grant Commission

Bahadurshah Zafar Marg

New Delhi-110002

Principal Investigator: Jitender Singh Laura

Department of Environmental Science,

Maharshi Dayanand University

Rohtak, Haryana

Summary

Camptothecin is in high demand worldwide with the pharmaceutical industries due to its anti cancer properties. *Nothapodytes foetida* (Grah.) Mabb. (Syn. *N. nimmoniana*, *Mappia foetida*), is a tree species found in Western Ghats along with various species of Ophiorrhiza are convenient source for large-scale production of camptothecin (CPT) used in anticancer drug formulation. Furthermore, the global market of camptothecin is growing day by day and it has no synthetic source. *Camptotheca acuminata* and *N. nimmoniana* are the plant sources most widely for large scale production of CPT. The supply of wood chips from bark and roots of *Nothapodytes foetida* there is a short fall lof 50% than the current demand. As CPT accumulates in stem and root bark of *N. foetida*, whole tree is cut to generate biomass for extraction. In Indian market, the current demand for its biomass is 500-700 metric tons. In Maharashtra, overexploitation and habitat destruction for raw material has led to population decline by 50-80 % in last decade (CAMP, 2001). Due to exploitation of these voluble bio resources, efficient methods for rapid propagation of these plant species is highly desirable for conservation and also meet the increasing demand for camptothecin.

Vegetative propagation of the tree through cuttings is also not successful. In addition to this, *in vivo* vegetative propagation techniques are also time consuming and season dependent (Kaushik *et al.*, 2011). Seeds of *Nothapodytes foetida* lose viability quickly and have poor germination capacity (Sharma *et al.*, 2000). *In vitro* production of elite plant and valuable secondary metabolites is considered to be a suitable alternative, in comparison to field production of plants. To ensure sustainable supply of camptothecin, it is important to domesticate and cultivate *Nothapodytes foetida*. Due to exploitation of this plant species, efficient methods for rapid propagation of *N. foetida* are needed. Genetic transformation of plants using *A. rhizogenes*, is an important alternative for the production of secondary metabolites (Giri and Narasu, 2000).

Hairy roots have been reported to yield higher amounts of secondary metabolites than cell suspension cultures and in some cases, intact plant roots (Allan *et al.*, 2002). Hairy roots have been researched for the synthesis of root derived phytochemicals (Hamill and Rhoder, 1993). As it has been reported that maximum concentration of CPT is found in the roots of *N. foetida*, therefore hairy root culture could be a better option for the large scale production of CPT (Namdeo *et al.*, 2012).

Keeping in view, the endangered status of the plant, the obstacles associated with its propagation, and huge global demand of camptothecin, the present study was undertaken with the following objectives:

1. To establish healthy and sterile cultures for future *in vitro* studies and large scale multiplication.
2. To standardized technique of *Agrobacterium rhizogenes* mediated transformation for inducing hairy roots to enhance the camptothecin content.
3. Assessment of differentiated and undifferentiated cultures for camptothecin (CPT) production and check for possible correlation between organization and secondary metabolites production.
4. To standardize the technique for raising liquid cultures from hairy roots obtained by using various explants directly or by infection with *agrobacterium rhizogenes* and analysis of these cultures for Camptothecin (CPT).
5. To estimate the content of Camptothecin (CPT) using different methods of extraction especially quantitatively in the *in vivo* & *in vitro* generated hairy roots.
6. To compare the camptothecin content in transformed and untransformed seedling roots and shoots.

SUMMARY AND CONCLUSION

Work was carried out in the tissue culture laboratory and screen house of the department of environmental science, MDU, Rohtak

1. Plant material -Seeds and plants of *N. foetida* were collected from surrounding forests of Dapoli with collaboration from Forestry Department of Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. Maharashtra. Plants of Ophiorrhiza species were collected from the forests with collaboration from Tropical Botanical Garden and Research Institute (TBGRI), Kerela.
2. The best combination of sterilizing agents was found to be 70% ethanol for 1 minute followed by 2% NaOCl for 5 minutes, as this treatment gave minimum inhibition along with maximum seed germination with no contamination.
3. Out of various supporting medium tested, paper bridge method gave maximum (82.33%) seed germination in seeds without seed coat and 52.67% seed germination in seeds with seed coat. Germination initiation was less in seeds without seed coat as compared to seeds with seed coat i.e, 4 days in former and 12 days in latter
4. With the passage of time germination was found to decrease. Fresh seeds showed a percent germination of 84.66%, but only 8% seed germination was observed in seeds without seed coat after 8 months of storage at room temperature.. Whereas, the refrigerated seeds when germinated after 8 months gave a percent germination of 76.33%.. The germination potential can be increased with low temperature storage of the seeds of *N. foetida*.
5. Viability of fresh seeds of *N. foetida* was observed to be 93%. After one year of storage of seeds at room temperature the viability decreased to 45%, whereas, seeds stored at 4 ºC gave a viability of 72%. After two years seeds stored at 4 ºC showed 61% viability and seeds stored at room temperature showed no viability.

6. Among the various dormancy breaking treatments, maximum germination (68.33%) was obtained with 3% KNO_3 followed by GA_3 with 63.33% germination at 150 ppm concentration.
7. Maximum shoot multiplication of 71.66% was obtained on MS media supplemented with 0.5 mg/L TDZ with 15.33 number of shoots per explant. This was followed by MS medium supplemented with 1mg/L BAP + 1 mg/L TDZ with 70.33% shoot multiplication and 10.33 shoots /explant.
8. Shoot tip responded maximum with 75% shoot multiplication and 16.66 shoots per explant, followed by nodal explant, roots, leaves and least in cotyledons.
9. Maximum shoot elongation was reported in shoot tip(6.77 cm) explant in MS medium supplemented with 2mg/L BAP + 1.5mg/L GA_3. This concentration favored better elongation of shoots derived from all explants.
10. Test on effect of sucrose concentration, the percentage of shoot formation, number of shoot per explant and length of shoots increased gradually with increasing sucrose concentration in medium up to 3 %. 100% regeneration frequency was observed at 3% sucrose level with an average shoot length of 5.37 cm and number of shoots was 7.66.
11. Maximum number of explants responded to half strength MS medium supplemented with 2 mg/L BAP + 3mg/L IBA, followed with full strength MS medium and least in ¼ strength MS medium. Difference was also observed among the treatments with regard to duration of initiation of rooting. In full strength MS medium, rooting initiated after 2-2.5 weeks, followed by half strength MS medium (3 weeks) and fourth strength MS medium took 4- 4.5 weeks for root initiation.
12. Differential response was obtained among the shoots derived from different explants for root induction. Maximum percent response was observed in shoot tip derived explants i.e., 86 % in MS medium supplemented with 2 mg/L BAP + 3mg/L IBA. This was followed by shoots derived from nodal segment (80.33%) and least in leaf explant derived shoots (63%).

13. To avoid the problem of callusing and shoot tip necrosis during rooting, activated charcoal was added to the medium. Maximum rooting was observed in half strength MS medium supplemented with 2 mg/L IBA + 300 mg/L activated charcoal with 50 % shoot tip necrosis and least callusing.
14. The optimized transformation protocol for hairy roots production in *N. foetida* was as follows. After experimentation with five strains of *A. rhizogenes*, finally three strains of *A. rhizogenes* (A_4, LBA 9204 and MTCC 532) were selected.
15. All the three strains responded to *A. rhizogenes* infection for 25 minutes and 48 hours co- cultivation period. Maximum hairy roots were induced in A4 infected explants i.e. 80.67%, followed by LBA 9204(75.67%) and MTCC 532 (60%).
16. Out of the three strains of *A. rhizogenes*, A4 was found to be most virulent with respect to transformation percent and emergence time.
17. Supplementation of co-cultivation medium with 150 µM acetosyringone gave improved and maximum induction of hairy roots in all A. rhizogenes strains. A4 gave 82.66% transformation at 150 µM followed by LBA 9204 (72%) and least in MTCC 532 (61.67%).
18. Optical density of 1.0 of the bacterial suspension was found to be optimum in all the three strains. At OD=1.0 80.66% in A4, 72.33% in LBA 9204 and 60.66% in MTCC 532 transformation was obtained in leaf explant. Optical densities above and below OD=1.0 gave lower transformation percentages.
19. All the strains induced maximum hairy roots with leaf explant which was significantly higher from other explants (petiole, nodal segment and hypocotyls). A maximum of 78% in A4, 71% in LBA 9204 and 61% in MTCC 532 transformation was reported in leaf explant.
20. Among the methods of infection of explant with A. rhizogenes, cutting the explant followed by dipping in bacterial suspension was found to be better than other methods employed (pricking with needle containing bacterial suspension and unwounded explants). In A4 strain cutting method induced 79% hairy roots ,

followed by pricking method (59.67%) and least in unwounded explants (24.3%). Same trend was followed in other two strains.

21. For the elimination of *A. rhizogenes* after co- cultivation, a combination of cefotaxime and carbecillin (500+500 mg/L) was found to be best. This combination of antibiotics not only gave better elimination but also gave maximum transformation (86%).
22. *A. rhizogenes* recurrence was observed on hairy rots after 20 days , regardless of antibiotic type and concentration, but the loss of hairy roots was less in case of combination of two antibiotics. On the other hand , after 30 and 40 days none of the hairy roots showed the *A. rhizogenes* recurrence at all antibiotic concentrations used.
23. PCR amplification of *rol* B gene experiment, confirmed the presence of *rol* B gene in the hairy roots induced in N. foetida. While it was found to be absent in the untransformed roots.
24. Camptothecin yield was found to be: seeds (0.116%), in vitro roots (0.099%), in vitro shoots (0.067%), in vitro leaves (0.041%) and seed coat (0.0273%) and maximum in hairy roots (0.299%).

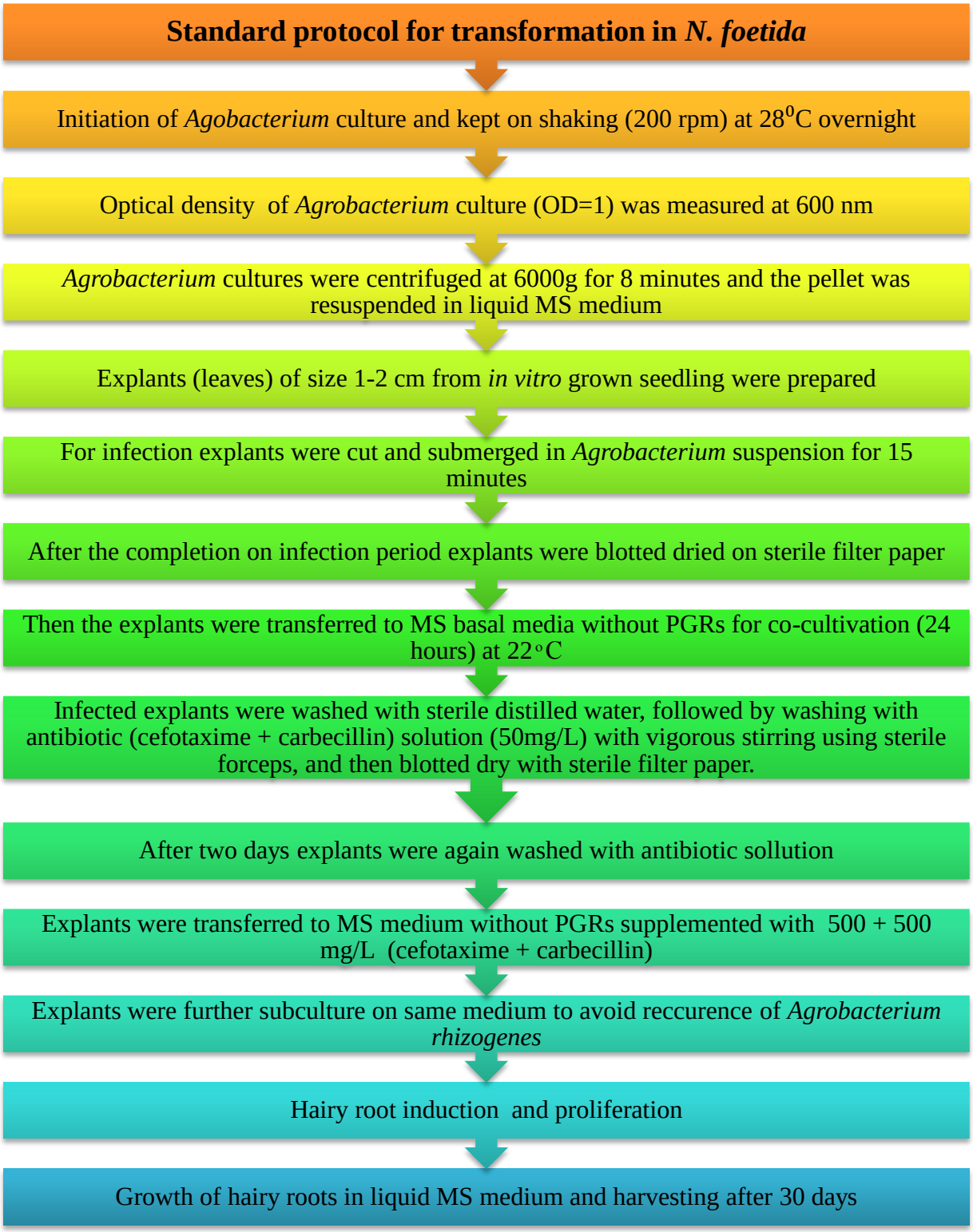
Achievements

Nothapodytes foetida and *Ophiorrhiza species* grows wild in the forests of Maharashtra, Karnataka,Tamil Nadu,Kerela,West Bengal and Assam . Since, the present study is carried out at M.D.University,Rohtak Haryana and both these plants collected from their natural habitat (forests of South India) are transplanted on campus under sub tropical conditions. The characterization of both these species under changed habitat (subtropical climatic conditions) is expected to open the gateway of dissemination of these species in North India also which shall be an excellent addition in the biodiversity resulting in conservation of these endangered species due to wider habitat range. The outcome of the finding of the study are useful in conservation of these endangered species , addition to biodiversity besides ensuring raw material

supply to pharmaceutical industries for camptothecine production. The luxuriant growth of *Nothapodytes foetida under climate* of Rohtak has a potential as a cash crop for farmers of North India.

- In vivo and in vitro propagation of of *Nothapodites foetida* was succesfuly achieved through seed germination and tissue culture.
- *Agrobacterium rhizogene* mediated transformation was successfuly achieved with good hary root production.
- The production of camptothecine through in vitro produced tissues will ease pressure from the exploitation of destructive extraction of camptothecine from natural plant materials and contribute to conservation of this plant.
- It was observed that Nothapodytes foetida shows good vegetative growth in the herbal garden of the university, it also flowers but seed setting does not take place. Camptothesin in the leaves of these plants is comparable to leaves from plant of Western Ghats.
- Nothapodytes foetida shows good vegetative growth in the climate of Rohtak. The possibility of introduction of this plant to North India as a cash crop for the extraction of camptothecine from vegetative parts needs to be studied. This will ease pressure on the exploitation of the plant in its native range.
- The camptothecine concentration in hairy roots is almost two times as compared to any other part , this protocol can be scalled up and used to produce camptothecine in the laboratory with out destruction the plants in the wild.
- This work will contribute to diminish the exploitation and ecosystem degrdation of this plant species for camptothecine extraction from its native ranges and ensure supply of camptothecine through hairy roots.

Flow chart of standard protocol for hairy root induction in *N. foetida*



LITERATURE CITED

- CAMP. 2001. Conservation assessment and management plan for CAMP 2001. Conservation assessment and management plan for medicinal plants of Maharashtra state FRLHT.
- Allan, E. J., Eeswara, J. P., Jarvis, A. P. Mordue, A. J., Morgan, E. D., and Stuchbury, T. 2002. Induction of hairy root cultures of *Azadirachta indica* A. Juss. and their production of azadirachtin and other important insect bioactive metabolites. Plant Cell Rep. 21: 374-79.
- Giri, A. and Narasu, M. L. 2000. Transgenic hairy roots: recent trends and applications. 3 Biotechnol. Adv. 18: 1-22.
- Hamill, J. D. and Rhodes, M. J. C. 1993. In Plant Biotechnology; Grierson, D., Ed.; Blackie: Glasgow. 3: 178-209.
- Kaushik, S., Georga, I., Koumoundouros, D. 2011. Growth and body composition of zebrafish *(Danio rerio*) larvae fed a compound feed from first feed onward: Toward implications on nutrient requirements. Zebrafish 8:87-95.
- Namdeo, A. G., Sharma, A. 2012. HPLC analysis of the camptothecin content in various parts of *N. foetida* collected in different periods. Asian Pac J Trop Biomed. 389–393.
- Sharma, S. N., Puri, S. C. and Srivastava, T. N. 2000. Enhancement of seed germination in *N. foetida*. J Med Arom Plant Sci. 22:206–210.