MATERIALS AND METHODS

The present investigation entitled “In vitro multiplication and Agrobacterium rhizogenes mediated genetic transformation of Nothapodytes foetida” 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.

3.1. Materials

3.1.1. Plant Material:
For the present investigation, fresh seeds of *N. foetida* were procured from Forestry Department of Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. Seeds were picked in the month of January 2014 and received in February 2014. The seeds packed magenta boxes with proper labeling were stored at room temperature which was subjected to average temperature of 10-20 °C during winter and 30-40 °C during summer. The second lot of the seeds was stored at 4 °C in the refrigerator in magenta box sealed with paraffin tape. The seeds of *N. foetida* were sown in the Experimental Medicinal Nursery of MDU, Rohtak for raising the seedlings. The seeds were also cultured *in vitro* for raising aseptic seedlings. Plant material of *Nothapodytes foetida* translocated from Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth (Agricultural University) Dapoli, Ratnagiri Maharashtra and grown in Herbal garden M.D.University Rohtak was used.

3.1.2. 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 (Intitute of Microbial Technology) Chandigarh in the form of freeze dried cultures and one strain: NCIM 5240 was procured from NCIM (National Collection of Industrial Microorganisms) Pune and two other strains i.e. A4 & LBA 9402 were obtained from PBSM (Plant Biotechnolology and Secondary Metabolites) Lab, BARC(Bhaba Atomic Research Centre), Mumbai.
3.1.3. 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.

3.1.4. 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.

3.2. Method
3.2.1. 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 composition 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. Na₂ EDTA and FeSO₄ 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-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.

3.2.2. 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.

3.2.3. 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 polypropylene 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.

3.2.4. 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.

3.2.5. 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 exposure 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.

3.2.6. Calculation
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:

\[
\text{Percentage of response} = \left( \frac{\text{No of explants responded}}{\text{Total no. of explants inoculated}} \right) \times 100
\]
3.2.7. 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 3.1. Composition of MS medium (Murashige and Skoog, 1962)

<table>
<thead>
<tr>
<th>Essential element</th>
<th>MS concentration mg/L</th>
<th>Volume of stock solution taken for one liter</th>
<th>Concentration used for stock solution preparation (gm/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macro elements</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>100 ml</td>
<td>16.5</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
<td></td>
<td>19.0</td>
</tr>
<tr>
<td>CaCl₂2H₂O</td>
<td>440</td>
<td></td>
<td>4.4</td>
</tr>
<tr>
<td>MgSO₄7H₂O</td>
<td>370</td>
<td></td>
<td>3.7</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td><strong>Micro elements</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>10 ml</td>
<td>0.83</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td></td>
<td>6.2</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>22.3</td>
<td></td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>8.6</td>
<td></td>
<td>8.6</td>
</tr>
<tr>
<td>Na₂MoO₄2H₂O</td>
<td>0.25</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO₄5H₂O</td>
<td>0.025</td>
<td></td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Iron source</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄7H₂O</td>
<td>27.8</td>
<td>10 ml</td>
<td>0.278</td>
</tr>
<tr>
<td>Na₂EDTA-2H₂O</td>
<td>37.3</td>
<td></td>
<td>0.373</td>
</tr>
<tr>
<td><strong>Organic supplement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mynoinositol</td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>0.5</td>
<td>2.5 ml</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>0.1</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>30000</td>
<td></td>
<td>30 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>8000</td>
<td></td>
<td>8 gm</td>
</tr>
</tbody>
</table>
Table 3.2. Various *A. rhizogenes* medium with composition

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Components</th>
<th>Concentration (gm/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTCC 2364</td>
<td>Xanthomonas medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CaCO₃</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>20</td>
</tr>
<tr>
<td>MTCC 532</td>
<td>Nutrient agar medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Beef extract</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Peptone</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15</td>
</tr>
<tr>
<td>NCIM 5240</td>
<td>Yeast extract Medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Beef extract</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Peptone</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>2</td>
</tr>
<tr>
<td>A4 and LBA 9402</td>
<td>Yeast Mannitol Medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K2HPO₄</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>MgSO₄.7H₂O</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Sodium chloride</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

3.3. Seed germination

3.3.1. 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 the completion of 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.

3.3.2. 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.

3.3.3. 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.

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

3.3.3.1. 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.

3.3.3.2. 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. KNO₃ (0.2, 0.3 and 0.4%) - 24hr
2. Water - 24 hr
3. GA₃ (100, 150 and 200 ppm)

3.4. *In vitro* Shooting

3.4.1. 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.

3.4.2. 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.

3.4.3. 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.

3.4.4. 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 fortnightly intervals for sixty days total duration.

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

3.5.1. 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.

3.5.2. 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.

3.5.3. 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.

3.5.4. 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.

3.6. 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 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 ratio in green house of herbal garden at Maharshi Dayanand University Rohtak.

3.7. *Agrobacterium* mediated genetic transformation

3.7.1. Plant material and explants preparation

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

3.7.2. Bacterial strains

Following *Agrobacterium rhizogenes* strains were used for transformation:

Table.3.3. Bacterial strains used for infection

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Bacterial strains</th>
<th>Growth medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Agrobacterium rhizogenes</em> NCIM 5140</td>
<td>YEB</td>
</tr>
<tr>
<td>2</td>
<td><em>Agrobacterium rhizogenes</em> A4</td>
<td>YMB</td>
</tr>
<tr>
<td>3</td>
<td><em>Agrobacterium rhizogenes</em> MTCC 532</td>
<td>YEB</td>
</tr>
<tr>
<td>4</td>
<td><em>Agrobacterium rhizogenes</em> MTCC 2364</td>
<td>Xanthomonas</td>
</tr>
<tr>
<td>5</td>
<td><em>Agrobacterium rhizogenes</em> LBA 9204</td>
<td>YMB</td>
</tr>
</tbody>
</table>

3.7.3. Culture conditions and media

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

\[
\text{Transformation frequency} = \frac{\text{Number of explants forming hairy roots}}{\text{Total number of explants taken}} \times 100
\]

**Co-cultivation medium**: MS basal medium without PGRs

**Growth medium**: MS basal medium with antibiotics

**Proliferation medium**: MS basal liquid medium without PGRs
3.7.3. Transformation

3.7.4.1. 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 \textit{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, and 1.6). Different concentrations of \textit{Agrobacterium rhizogenes} were identified for the different strains of \textit{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 \textit{in vitro} grown seedlings (leaves, petiole, nodal segment and radicle) 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 \textit{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.

### 3.7.4.2. 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.

#### 3.7.4.2.1. 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.

#### 3.7.4.2.2. Effect of co-cultivation temperature on hairy root induction

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

#### 3.7.4.2.3. 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.
3.7.4.2.4. 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.

3.7.4.2.5. Effect of explants on hairy root induction
Various types of explants (leaf, radicle, nodal segment and petiole) were used for transformation by three different strains of *A. rhizogenes* (A4, LBA9204 and MTCC532).

3.7.4.2.6. 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.

3.7.4.2.7. 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).

3.7.4.3. 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).
3.8. Polymerase chain reaction (PCR)
DNA isolation from plant and plasmid DNA for PCR analysis was carried out using following procedures:

3.8.1. 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 Na₂EDTA
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) β-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 Na₂EDTA 2.0 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 (2/3rd 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) 2/3rd 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.

3.8.2. 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 and 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 x g 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 centrifuged for 1 minute at 11000 x g.
3.8.3. PCR analysis

3.8.3.1. 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’

3.8.3.2. 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 template DNA, 1µl of 5U dNTPs, 2 µl of 25 mM MgCl₂, 5 µL of 5X PCR Buffer, 1µl each 10 µM forward and reverse primers, 0.2 µl 5U of Taq DNA polymerase. DNA amplifications were performed in a thermal cycler (Agilent SureCycler 8800, Agilen Technologies) using the programme: initial template 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. 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⁺).

3.9. CPT estimation and analysis

3.9.1. 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).
3.9.2. 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.

3.9.2.1. 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.

3.9.2.2. 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.

3.9.2.3. 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.

3.9.3. 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.

3.9.4. 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