3. Materials & Methods ............

Development ............... hairy roots in *Alpinia galanga* .................. bioactive compounds
The chapter gives an overview of the materials the methods used in the present study. These include – micropropagation, genetic transformations, extraction of secondary metabolites and demonstration of antimicrobial activity by different *Alpinia* extracts.

**Material procurement for the proposed study**

*Alpinia galanga* plant material (A.G Biotek, Hyderabad); acetoxychavicol acetate (LKT laboratories, USA); plant media (Murashige and Skoog, Gamborg’s, Linsmaier and Skoog) and bacterial media (Mueller-Hinton broth and agar, Nutrient broth, Nutrient agar)(Himedia); pH capsules (Himedia); HCl and NaOH (Himedia); plant growth regulators (Duchefa, Netherlands); syringe driven 0.22 µm filters (Millipore); mercuric chloride (Qualigens); C-18 HPLC column (Phenomenex); HPLC grade solvents (Merck); saffranine (Himedia); antibiotics – cefotaxime and amikacin (Himedia); *Agrobacterium* strains – LBA 9402, A4 (ATCC, USA), 532, 2364 (MTCC, India) and PRTGus (Oksman caldentey, Finland); DNA extraction kit (Nucleospin); PCR reagents (Bioron international); 1Kb ladder (Qiagen); ethidium bromide (Merck); Microorganisms – *S. aureus*, *S. epidermis*, *E. faecalis*, *S. typhimurium*, *K. pneumonia*, *E. aerogene* and *E. cloacae* (clinical isolates, Global Hospitals); *B. subtilis* MTCC 2391, *E. coli* MTCC 1563 and *P. aeruginosa* MTCC 6642 (MTCC, India); analytical grade solvents (Himedia); glassware (Merck).
Callus showing root like structures before the emergence of shoots

3.1 Micropropagation

The maintenance of aseptic (free from all microorganisms) or sterile conditions is essential for successful tissue culture procedures. The in vitro culture technique requires all culture vessels, media, instruments, as well as plant tissue (explants) to be sterilized. Culture vessels and instruments were sterilized in a hot-air-oven.
3.1.1 Culture media

MS (Murashige and Skoog, 1962), B₅ (Gamborg, 1968) and LS (Linsmaier and Skoog, 1965) media were used for the in vitro culture of *A. galanga*.

3.1.2 Media sterilization

The stable components were sterilised by autoclave, whereas the thermolabile solutions were filter sterilized using (0.22 µm) syringe driven filtration units. The plant growth regulators were sterilised along with media as they are thermostable. But zeatin – a thermolabile natural phytohormone, it was filter sterilised before usage.

3.1.3 Phytohormones

In addition to the nutrients, plants require certain growth regulators such as auxins and cytokinins. The stock solutions were prepared at a concentration of 1 mg/ml. Generally, auxins are dissolved in ethanol/dilute NaOH and cytokinins in dilute HCl. In case of thidiazuron, a cytokinin, DMSO is used as a solvent.
3.1.4 **Plant material**

*Alpinia galanga* (L.) Willd is a monocot belonging to the family *Zingiberaceae*, distributed in India and various parts of Southeast Asia. Different parts of *A. galanga* were excised and used for initiation of aseptic cultures and to carry out further experiments.

3.1.5 **Surface sterilization**

Surface sterilization of different explants of *A. galanga* was done by treating the explants with some chemical agents called surface sterilants (ethyl alcohol, mercuric chloride).

Explants were washed thoroughly under running tap water for 15 minutes, later with a mild detergent solution followed by four to five rinses with distilled water. In some cases, where fungal contamination is prominent (the present case, where the explant is loaded with microbes), an additional alcohol treatment is preferred to relieve the explants of the spores. After washing thoroughly, the explants were surface sterilized with 0.1% mercuric chloride for 5 – 8 minutes, followed by 8 – 10 rinses with sterile distilled water, to remove traces of mercuric chloride.

The sterile leaf, leaf sheath, rhizome and root explants were dissected aseptically to remove the superficial layers and transferred to MS media supplemented with various plant growth regulators. Care was taken not to damage the tender rhizome buds. The young shoots that arise from these explants were used for further experiments.
**Stepwise sterilization process**

<table>
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<tr>
<th>Process</th>
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<tr>
<td>Explant dissection</td>
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<tr>
<td>Wash under running tap H₂O (15 min)</td>
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<tr>
<td>Detergent wash (10 min)</td>
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<td>Mercuric chloride treatment (5-8 min)</td>
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<td>Sterile H₂O wash (7-8 rinses)</td>
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<td>Transfer to media</td>
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3.1.6 Culture conditions

The cultures were incubated under standard culture conditions at 25 ± 2°C under a 16/8 h (light/dark) regime with 40 – 50 mol m⁻²s⁻¹ light provided by the cool fluorescent tube light.

3.1.7 In vitro propagation

The explants were cultured on semi solid MS media supplemented with different concentrations of cytokinins viz. kinetin (Kn), zeatin (Zn), thidiazuran (TDZ), benzyl aminopurine (BAP) for multiple shoot induction and auxins viz. α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), 3-indolebutyric acid (IBA) for root induction.

3.1.8 Callus Induction

Various explants like leaf, rhizome and root were excised as circular discs and placed on MS media fortified with different combinations of bioactive compounds.
auxins and cytokinins. The explants were incubated under standard culture conditions.

3.1.9 Indirect Organogenesis

The callus obtained from the explants was transferred onto MS basal media and sub-cultured every fortnight. The compact mass of cells thus formed was transferred to MS media supplemented with different cytokinin concentrations for shoot induction and later to auxin containing media for root initiation respectively. Among the different growth regulators, the combination that gave the best result was used for further micropropagation experiments.

3.1.10 Histological studies

Histological studies were carried out for samples showing organogenesis. These samples were fixed in a solution (formaldehyde : glacial acetic acid : ethyl alcohol; 5:5:90; v/v). Following the fixation, tissues were dehydrated in an ethanolic graded series and then embedded in paraffin as recommended by Johansen (1940). Serial sections of 6 – 10 µm in thickness were cut using a Leitz Rotary Microtome (1512). The sections were obtained from freshly prepared wax blocks with tissue specimen by adjusting the microtome and stained with saffronine.

3.1.11 Extraction of ACA

The sample preparation was done by extracting it in methanol. The sample was macerated and filtered first using a Whatman filter paper and later by 0.22 µm filter before injecting into HPLC.
3.1.12 **Phytochemical analysis in callus and in vitro cultures by HPLC**

The callus cultures along the line of morphogenetic response were analysed for phytoconstituents and compared to that of the normal plants. The analysis was carried out using the Shimadzu – LC-10AT VP series HPLC system equipped with a Supelco column (250 x 4.6 mm, C18, ODS with particle size of 5 µm). ACA was detected at 210 nm with a UV-VIS detector (Shimadzu UV-Visible SPD-LC 10A VP series). The mobile phase consisted of 60% methanol and 40% water at a flow rate of 1 ml/min (Xiaogen et al., 2009), with an injection volume of 20 µl. Standard ACA was prepared in HPLC grade methanol at a concentration of 1 mg/ml.

3.1.13 **Acclimatization**

The regenerated plantlets were transferred to the plastic pots for hardening in a mixture containing autoclaved vermiculite and soil (1:1) under controlled conditions. The potted plants were irrigated with MS basal salt solution (1/8 strength) every 4 days continuously for 3 weeks (Bakrudeen and Arun, 2009). Plants were sprayed with 0.1% Bavistin – an antifungal agent, once a week. The poly pots were then covered with a rigid plastic cover and water was sprayed to maintain 70 – 80% relative humidity and maintained for 4 weeks. Once hardened in the greenhouse, the plants were transferred to the fields.
3.2 Genetic Transformations

3.2.1 Assessment of different explants for transformation frequency

In vitro grown A. galanga plants were dissected into different parts like leaf, leaf sheath, rhizome and root; and evaluated separately for transformation efficiency. The explants were wounded and incubated in bacterial suspension and later assessed for the response, i.e. emergence of hairy roots. The transformation frequency (TF) was determined as follows:

\[
\text{TF} = \frac{\text{No. of explants giving rise to hairy roots}}{\text{Total no. of explants infected with } A. \text{ rhizogenes}} \times 100
\]
3.2.2 Genetic transformations in *A. galanga*, using different strains of *A. rhizogenes*

The bacterial strains, known to cause the hairy root disease, were cultured on nutrient agar (Hi media) and stored at 4°C and sub-cultured every fortnight. The pH of nutrient media was maintained neutral at 7.0. The bacterial suspensions were initiated in nutrient broth (Hi media) for use in genetic transformation experiments at a cell density of 10⁹ cells/ml (Soham et al., 2009).

3.2.3 Study of infection technique and duration of bacterial treatment on genetic transformations (Bacterial growth)

Different infection methods (techniques) such as wounding the explants – pricking with needle/cutting with scalpel were followed by dipping in bacterial suspension. All the explants were incubated with bacterial suspension at different time durations ranging from 5 min to 20 min, for assessing the degree of bacterial infection after 48 h.

3.2.4 Effect of co-cultivation duration on growth and transformation of explants

The influence of co-cultivation duration on the degree of bacterial infection was studied. Co-cultivation allows integration of bacterial T-DNA into the plant genome. Different explants, after infection with *A. rhizogenes* strains were allowed to grow on MS basal media, for varying duration’s from 12 to 72 h.
3.2.5 **Role of temperature in hairy root induction**

The explants showing *Agrobacterium rhizogenes* infection, were sub-cultured on MS basal media and incubated at varying temperatures. The incubation temperatures ranged from 18°C, 20°C, 22°C, ……… to 28°C. The response of the explants at different temperatures was analyzed after six weeks of culture.

3.2.6 **Effect of media pH on growth and proliferation of Hairy roots**

Evaluation of media pH was carried out to study the influence of pH on the growth and proliferation of hairy roots. The pH of MS media was set over a range of 4.5 – 7.0. The study was conducted for a period of 4 weeks.

3.2.7 **Influence of different concentrations of cefotaxime (antibiotic)**

In order to eliminate residual bacterial growth, explants were cultured with MS media containing antibiotic (cefoxaxime 300 μg/ml). As the antibiotic is heat labile, it is filter sterilized before addition to the media. Different concentrations of antibiotic (cefoxaxime) were evaluated for effective elimination of bacteria after co-cultivation.

3.2.8 **Growth of transformants**

Growth kinetics of transformed plants obtained from the rhizome explants of *Alpinia galanga* using different strains of *Agrobacterium rhizogenes* were evaluated for a period of 6 – 7 weeks. The plants were
inoculated into fresh MS semi-solid media and left in the incubator under a 16/8 h (light/dark) regime at 25 ± 2 °C. The increase in fresh weight of each transformed root line was observed every 5 days. The transformed plants were cultured on different types of media i.e. MS, B5 and LS media. The concentration of sucrose was maintained at 3%. Control/untransformed plants were also maintained on the same media alongside the transformants for comparison.

3.2.9 Growth of Hairy roots in liquid media

The hairy roots obtained at the site of infection were sub-cultured every 3 weeks and subsequently transferred to MS basal liquid media for evaluation of growth. The flasks were placed on a gyratory shaker at 120 rpm under standard culture conditions. The growth pattern was studied every 4 days for a period of 4 weeks.

3.2.10 Quantification of ACA in transformants by HPLC

Two grams (wet weight) of control and transformed roots were extracted in HPLC grade methanol. The fine paste obtained was passed through a syringe driven membrane. 20 µl of this concentrated extract was used for HPLC analysis. ACA in the transformed and untransformed root cultures was quantified and compared.

3.2.11 Molecular confirmation of transformants

The bacteria free roots of normal plants (control) and transformed plants were excised for DNA extraction to carry out the molecular analysis. Polymerase chain reaction was employed for the detection of Ri T-DNA integration in hairy roots of A. galanga. The roots were ground in the
presence of liquid N$_2$ and the DNA extracted using a Nucleospin extraction kit. A set of rol A specific primer pair was used for the amplification of 308 bp rol A gene fragment. The sequence of primers used for amplification were forward - 5'-AGAATGGAATTAGCCG GACTA3' and reverse - 5'-GTATTAATCCCGTAGGTTT TTT3'. One Kb ladder was used as a standard reference (Qiagen).

The template DNA from normal and transformed roots was taken at a concentration of 50 ng acts as, 1 X PCR buffer, 25 pmoles of each primer, 2.5 mM of dNTPs and 1 unit of Taq polymerase were present in the PCR mixture (25 µl). PCR for rol A was carried out by amplifying with initial denaturation at 94°C for 5 min followed by 35 cycles of 1 min denaturation at 94°C, 1 minute annealing at 55°C and 1 minute extension at 72°C with a final extension of 72°C for 10 minutes using a thermal cycler (Eppendorf, Germany). The PCR products obtained were analysed using 1% agarose gel, stained with ethidium bromide. The DNA bands thus obtained were observed and documented using a transilluminator equipped with a gel documentation system (Bio-Rad, USA) (Vinod kumar et al., 2006).

3.2.12 Statistical Analysis

Results calculated from triplicate data were expressed as means ± standard deviations. The data were compared by least significant difference test using Statistical Analysis System (SAS, ver. 9.1)
Depictation of the events during Genetic transformations

- Explant selection
- Agrobacterium infection (20 min)
- Co-cultivation (48 hrs)
- Transfer to antibiotic containing media
- Induction of Hairy roots
- Study of Growth Kinetics
- ACA quantification
- Molecular confirmation of Transformants

Potted plants used for extraction of the active principles in different solvents

Development ............. hairy roots in Alpinia galanga .................... bioactive compounds
3.3 Antimicrobial Action

3.3.1 Plant Extracts

The plants of *Alpinia galanga* (L) Willd collected from AG biotek, were thoroughly washed and separated into three different parts i.e. rhizome, root and leaves. They were oven dried at 60°C for 24 h to remove moisture and finely ground into a powder using an electric blender. Eighteen extracts were obtained with methanol, acetone and diethyl ether as solvent systems using a soxhlet apparatus. The phytoconstituents were extracted in acidic (5.5) and neutral pH range. The pH was adjusted using 0.1N HCl. The extracts were concentrated under reduced pressure using Rotavapour (Heidolph- Rotacool, Germany). The sequence of extraction is shown below in the scheme:
3.3.2 Bacterial Cultures

A combination of gram +ve and gram –ve pathogenic microorganisms were used for the present study. All the cultures were tested for purity by standard microbiological methods. The bacterial cultures were maintained on Mueller Hinton agar (Himedia, India) slants at 4°C with a subculture period of 15 days. Each bacterial strain was reactivated from the stored slants to Mueller Hinton broth (Himedia, India) and cultured overnight at 37°C before the antimicrobial assay.

3.3.3 Determination of Antibacterial activity

The Antimicrobial activity of Plant extracts was investigated by the Agar well diffusion method (Perez et al., 1990). The Mueller-Hinton agar plates were poured onto the petri-plates with an inoculum size of 10⁶ colony forming units/ml of bacteria. The wells were made in the MHA plates with the help of a borer, with a diameter of 8 mm. Each well was dispensed with different plant extracts at a concentration of 500 µg in...
the respective solvents with amikacin as the positive control. Amikacin is a broad spectrum antibiotic that acts by inhibiting protein synthesis. The concentration of amikacin used was 50 µg. The zone of inhibition around the wells was measured after 24 h of incubation at 37°C. The sensitivity of microbial species to the plant extracts was determined by measuring the diameter of the inhibitory zones around the wells (including the diameter of the well). All the experiments were performed in triplicate and the results embodied.

3.3.4 Determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration)

The minimum inhibitory concentration was determined by broth dilution method (Chattopadhyay et al., 1998a). Two-fold serial dilutions of the crude extracts as well as the positive antibiotic control (amikacin) were prepared in Mueller-Hinton broth (Chattopadhyay et al., 1998b). A direct suspension of microorganisms was prepared in 5 ml sterile distilled water from a 24 h old suspension in Mueller-Hinton broth. Turbidity of the suspension was adjusted to match a 0.5 Mc Farland standard (McFarland, 1907), which corresponds to $1.5 \times 10^8$ cfu/ml. For broth dilution tests, 50 µl of standardized suspension of bacteria was added to each tube containing crude extracts at concentrations ranging from 0.005 – 5.120 mg/ml and incubated at 37°C. The lowest concentration that did not show any visible growth after macroscopic evaluation was considered as MIC.
After the determination of MIC, the tubes which did not show any visible growth were diluted 100-fold with drug free Mueller Hinton broth and incubated at 37°C for 48 h. The lowest concentration of the tube that did not show any visible growth was considered as the Minimum Bactericidal Concentration (MBC). The assays were performed in triplicate.

3.3.5 Analysis by GC-MS (Gas Chromatography - Mass Spectroscopy)

For GC-MS Analysis, the samples were injected into a HP-5MS capillary column (30 m length X 0.25 m i.d X 0.25 µm film thickness), Agilent Technologies, USA GC-MS model, consisting of 6890N Gas Chromatograph coupled with 5973 insert MSD [Mass Selective Detector]. The injector was set at 250°C and the detector at 280°C. The stepped temperature program was as follows: held at 50°C for 2 minutes, then, from 50°C to 280°C at the rate of 10°C/min, held for 5 minutes. The total run time was for 30 minutes. The GC-MS interface temperature was 280°C and the injection volume, 1 µl. The solvent delay was 2 minutes and injected in a split ratio of 1:10. The MS scan range was from 35-6000 Da. Compound identification was done by comparing the retention times with those of the authentic samples and the spectral data obtained from library data of corresponding compounds.