CHAPTER-III

MATERIALS AND METHODS

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3.1 Collection of plant materials

Healthy plant tissues of leaves, rhizomes and roots of *Curcuma longa* were collected aseptically in sterile plastic bags from different places of Guwahati. Plant materials were sealed immediately after collection and preserved at 4^0 C until they were processed.

3.2 Isolation of endophytes

Endophytes were isolated from surface sterilized plant parts. Surface sterilization was performed using the modified method of Guo et al. (2008). Plant materials were washed in running tap water and then sterile distilled water. This was followed by consecutive immersion in 75% ethyl alcohol for 1 min, 3.25% sodium hypochlorite for 5 min and again 75% ethyl alcohol for 30 seconds and washed with sterile distilled water, then dried with sterile tissue paper. The excess water was dried under laminar air flow chamber. Outer tissues of the collected samples were removed and inner tissues were cut into small pieces with sterile scalpel and sets of four segments were evenly placed in each petridish containing potato dextrose agar (PDA) and nutrient agar (NA). The plates were incubated in incubator at 27 ± 1^0 C (for fungi and bacteria) and 37 ± 1^0 C (for bacteria) until microbial growth appeared on the plates. After several days of incubation the endophytic microorganisms that appeared on the plates were transferred with a sterile needle to freshly prepared petriplates containing potato dextrose agar medium or nutrient agar and incubated for 1-3 weeks and were checked for its purity. The purified strains were preserved at 4^0 C.

3.2.1 Efficiency of surface sterilization

The efficiency of surface sterilization procedure was necessarily checked by imprint method (Schulz et al.,1993), also sample washed distilled water was inoculated in to broth media as a control to check growth in liquid medium. Most endophytes generally sporulates after few weeks either in darkness or in daylight. Endophytes are slow growing

microbes (*In vitro*), so need patience while working with them. As some isolates require months, or even a year or more, in culture before they sporulate. Endophytic fungi stop sporulating after they have been transferred several times (Strobel and Daisy, 2003).

3.2.2 Precautions to avoid contamination

Healthy and without negative symptoms medicinal plants were selected for isolation of endophytes. Long time preservation of plant material was avoided and precaution taken to avoid further contamination after collection of plant material. The imprint of isolation plates were examined for the sterility. If any growth observed on the imprint plate then the whole batch was discarded. In case of surface growth, plate containing the samples was discarded. During isolation petridishes were sealed with parafilm and incubation period for each microbe was recorded. The day of earliest visual growth was observed from the plating date and considered as an incubation period for growth.

3.2.3. Preservation of cultures

Endophytes in the pure culture were preserved on the slant at 4^0C and each tube was labelled with code number of the host plant and isolate code with date of isolation. Replicate were made for each isolates and appropriate media was used according to the need of the organisms.

3.3 Identification of Fungal Endophytes

The isolates were identified on the basis of their morphological and spore characteristics with the help of standard fungal identification manuals of Gillman (1998) and Nagamani (2006). Fungi that unable to sporulate were categorized as sterile mycelia (SM).

3.4 Antimicrobial screening of the isolated fungal endophytes

For antimicrobial screening, fresh culture of the isolated endophytic strains was inoculated into 250 ml Erlenmeyers flasks each containing 150 ml potato dextrose broth (PDB). The flasks were incubated for 4-5 weeks at 27 ± 1° C with periodic shaking at 150 rpm. After

incubation, the fungal cultures were filtered through sterile cheesecloth to remove the mycelia mats. The filtrates were collected for antimicrobial screening. For this, nutrient plates were inoculated with 0.2 ml of overnight grown culture of each test bacterial suspension. Like this, potato dextrose agar plates were also inoculated with 0.2 ml of the test fungi. The plates were evenly spread out with the help of a sterile cotton swab. Agar cups were prepared by scooping out the media with cork borer (6 mm in diameter). Four cups were prepared in each plate. The agar cups were then loaded with the filtrate of each fungal endophytic strain. The plates were inoculated at 37 ± 1^0 C for 24h. Zone of inhibition were measured. The endophytes with broad spectrum activity were considered as potent strains.

3.4.1 Extraction of crude metabolites and evaluation of antimicrobial activity of the fungal potent strains

Fungal potent strains were inoculated into 30 numbers of 250 ml Erlenmeyers flasks each containing 150 ml potato dextrose broth (PDB). The flasks were incubated for 4-5 weeks at 27 ± 1^0 C with periodic shaking at 150 rpm. After incubation, the fungal cultures were filtered through sterile cheesecloth to remove the mycelia mats. The liquid broth was collected and extracted with equal volume of ethyl acetate in a separating funnel by vigorous shaking for 10 minutes. The mix suspensions were then allowed to stand for 5 min to separate the organic solvent (ethyl acetate) extract and cell mass. The lower portion containing cell mass was discarded and upper solvent portion so obtained was collected. Ethyl acetate was evaporated with the help of rotary evaporator. The resultant component was dried with $MgSO_4$ (Santos and Filho, 2003) to yield the crude extract. The crude extract was dissolved in dimethyl sulphoxide (DMSO) for antimicrobial activity against the test organisms. The antimicrobial assay was performed by agar cup diffusion method

as suggested by Grammer (1976). The diameter of zone (mm) was recorded after 24 h of incubation at 37^0 C. Three replicates were maintained in each case.

3.5 Data Analysis

Colonization frequency (CF %) of endophytic fungi was calculated using the following formula:

$CF = (N_{COL}/N_t) \times 100$

Where, N_{COL} = number of tissue segments colonized by specific fungus

N_t = total number of tissue segments plated

Frequency of dominant endophytes was calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes X 100 [Kumaresan and Suryanarayan, 2002].