Chapter - 3

MATERIAL AND METHODS

A) Isolation of Fungal pathogen *Fusarium oxysporum*

The present investigation was carried out at Chemind Diagnosis & Biosolutions, Jaipur.

**Cleaning and sterilization of glassware**

The Petri dishes, pipettes, conical flasks, test tubes, beakers, etc. used in the experiment were thoroughly washed and dried. The Petri dishes and pipettes were wrapped in a silver foil and sterilized in hot air oven at 1600°C for 2 hrs. All the media and glassware required for isolation were sterilized in autoclave 121°C at 15 lbs pressure/inch.

**Preparation of media for pathogen**

**Collection of fungal disease materials**

Roots and stem parts were collected from infected cumin plant showing characteristic symptoms of wilt. The disease plants were taken in Paper bags, brought to the laboratory for isolation of *F.oxysporum f.sp. cumini*.

**Collection of Soil samples**

The soil samples with microflora & plant material were collected from rhizosphere of spice plant cumin along with their open areas from four Cumin cultivated areas of Rajasthan viz. Ahore, Jalore, Jodhpur, Jobner.
Table 3.1: Localities and their attributes selected for collection and study of Cumin

<table>
<thead>
<tr>
<th>Location</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Average Temperature (Degree C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahore</td>
<td>25° 21' N</td>
<td>72° 51' E</td>
<td>30-36</td>
</tr>
<tr>
<td>Jalore</td>
<td>25° 22' N</td>
<td>73° 00' E</td>
<td>30-35</td>
</tr>
<tr>
<td>Jodhpur</td>
<td>26° 18' N</td>
<td>73° 04' E</td>
<td>27-35</td>
</tr>
<tr>
<td>Jobner</td>
<td>26° 58' N</td>
<td>75° 23' E</td>
<td>28-32</td>
</tr>
</tbody>
</table>

Isolation and Identification of fungal pathogen *Fusarium oxysporum*

The mycelium of genus *Fusarium* is delicate, white or peach but usually with a purple tinge, sparse to abundant then floccose, becoming felted and sometimes wrinkled in older cultures. Micro conidia borne on simple phialides arising laterally on the hyphae or from short sparsely branched conidiphores. Micro conidia generally abundant, variable, oval ellipsoid, cylindrical, straight to curved, 5-2 x 2.2-3.5 Macro conidia, sparse in some strains are borne on more elaborately branched conidiphores or on the surface of sporodochia. They are thin-walled, generally 3-5 septate, fusoid and pointed at both ends, occasionally fusoid-falcate. Macro-conidia are found with a somewhat hooked apex and a pedicellate base. Chlamydospores, both smooth and rough walled, are generally abundant and are terminal and intercalary, generally solitary but occasionally found in pairs or in chains. (Plate 3.2)
The experimental material for the present investigation consists of soil samples. Collection of soil sample was done from rhizosphere of Cumin plant. Fungal isolates from cumin plants that showed symptoms of *F. oxysporum* were collected. Soil samples (1g) was taken representing different places and dispersed in 10 ml 0.85% saline water. Top root and side root samples were trimmed, washed in running tap water to remove soil, blotted dry and cut into 10-mm segments. Root segments of Cumin were sterilized with 0.5% NaOCl and each longitudinal section was transferred to Petri dishes containing potato dextrose agar (PDA; Hi media). Plates were incubated on the laboratory bench for 5 days at 25 ± 2°C. Pure colonies which developed from the bits were transferred to PDA slants and incubated at 27 ± 10°C for sporulation for 15 days.

**Single spore isolation**

After that serial dilutions was done to get single colonies. 2% of filtered clear water agar was taken and 10 ml was poured into sterile petriplates. After solidification diluted 100μl of spore suspension was spread uniformly on agar plates after brief vortexing. Then such plates were examined under a microscope. Then it was incubated for 24 hours at 37°C and colony formation was observed on agar plates. 20-25 colonies per plate was taken and purified through streaking. Fungi were transferred to freshly prepared PDA to eliminate contamination. Single spore isolation was done from each colony (Plate 3.3.1, 3.3.2). Isolates were identified morphologically to species based on characteristics of macroconidia, phialides, microconidia, chlamydomospheres and colony growth traits (*Leslie and Summerell 2006*). Total ten isolates of *F. oxysporum* were recovered from root rotted parts of cumin plants Single spore pure isolates were prepared from cultures of Fusarium species (*Nelson et al., 1983; Leslie et al., 2006*). All the soil samples was preserved at -80 °C. The collected soil samples was homogenously mixed for each sample separately and was used for further bacterial isolation, DNA isolation and other studies.
Preparation of *F. oxysporum f.sp. cumini* inoculum in Broth

*F. oxysporum f.sp. cumini* mycelium were transferred to the agar slants and then incubated at 28°C for three days. Then the fungal culture obtained from the slants were inoculated in five 250 ml conical flasks that contained 100 ml of sterilized PD broth. Then the mycelia mat was obtained by filtration of fungal suspension on Whatman filter paper after incubation of conical flask for 10 days. It was then blended with required sterilized water and blended for 30 seconds so as to obtain mycelial suspension.
Plate 3.1: Isolation of *Fusarium oxysporum* from infected Cumin plants
Plate 3.2: Morphological characteristics of *Fusarium oxysporum* isolated from Cumin on PDA (A,B) and Macroconidia (C,D)
Plate 3.3.1: Purification of *Fusarium oxysporum*
Plate 3.3.2: Purification of *Fusarium oxysporum*
VCG Diversity of Fusarium sps:

PDA was mixed with 3% and 5% (w/v) potassium chlorate to produce one hundred and twelve nit mutants from 10 isolates. (Klittich and Leslie 1988, Kistler et al. 1998). Those isolates that grow fast on Puhalla’s Minimal Medium (MM) were considered to be nit mutants (Puhalla 1985). These mutants do not utilize nitrogen that helps as visual indicator of heterokaryon formation. These nit mutants were divided into three phenotypic classes viz. nit 1 where there is a mutation of nitrate reductase structural locus, nit 3 which has a mutation of nitrate-assimilation pathway specific locus and Nit M in which mutations affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity. Classification of these nit mutants was done phenotypically by growing them on basal medium mixed with any nitrogen source (MM without NaNO₃). Then pairing of Nit1 and Nit M mutants of all isolates of Fusarium oxysporum was done in all possible combinations on MM. It was then incubated at 25°C in dark. The isolates that were vegetatively compatible were observed by their extensive growth at the interface of two colonies after 9 days. (Klittich and Leslie 1988). This was done on the basis of their growth on media containing different nitrogen sources like sodium nitrate, sodium nitrite, hypoxanthin, ammonium tartrate. The most frequent phenotype was nit1 (50%), followed by nit3 (20%) and nitM (30%) among total 112 mutants. Nit mutants were used to force heterokaryon and to determine distribution of VCGs and their relation to pathogenicity and geographic origin. Six VCGs were recovered for F. oxysporum isolates. Based on pairing complementary nit mutants of all isolates, the 10 isolates were grouped into six VCGs. (Smith White et al., 2001).

Isolation of Fungal DNA

Extraction of total genomic DNA of fungus was done using the method of Doyle and Doyle (1990) from five days old fungal mycelium grown on PDA. Then the testing of quality of extracted DNA was done on 0.8% agarose gel after RNase treatment. Finally the DNA was quantified using spectrophotometer (Optigen
The DNA samples were diluted to 25 ng µl-1 for PCR amplification (Arif et al. 2011). Steps of isolation of fungal DNA is as follows.

1. Mixing of tissue was done in liquid nitrogen till it was converted to a powder. It was then transferred into sterilized eppendorf tube.

2. CTAB buffer with BME (1.0 ul of BME per 1.0 ml of CTAB) was then added. CTAB was used at 2.0 mL per gram of tissue. For 0.1 grams of tissue, 500 ul CTAB and 0.5 ul of BME was used.

3. Extracts were incubated for one hour at 65°C and the samples were shaken every 10 minutes so that proper mixing of material could take place.

4. Then it was centrifuged at 13,000 rpm for 1 minute and the supernatant was taken into a new tube.

1. Multiple extractions of the same pellet was done and supernatants were combined to get a better extraction.

2. The combined supernatants were then extracted with one volume of chloroform.

3. Tubes were spun at 1000 X G (RCF) for 5 minutes (4°C).

4. The aqueous layer was pulled off and transferred to a new tube.

5. To get more DNA out we used “back extract” the chloroform with one volume ddH2O. The “back extracted” tubes are spun at 1000 X G (RCF) for 5 minutes (4°C) and the aqueous phase (top layer) is transferred to the same tube as in step 8.

6. Next the DNA was precipitated with 2/3 volume of isopropanol. The solution was allowed to incubate at −20°C for at least an hour and up to overnight.

7. Tubes were centrifuged at max speed for 5 minutes and the supernatant is discarded.

8. Pellet was washed with 500 ul of 80% ethanol and then centrifuged at max speed for 5 minutes.
9. Supernatent was discarded and a small (0.1-10 µL) pipette tip was used to pull out as much liquid as possible.

10. Pellets were dried by either leaving the tube open in a safe location or by using the speed-vac.

11. Once pellets were dried they were resuspended with 50-100ul of 1xTE Buffer.

12. It was Run on agarose gel and Spec was used to quantify DNA (use 5ul DNA/750ul 1x TE for OD [260/280]. Store aliquots at either –80° C or -20°

To find amount of DNA OD$_{260}$ = 1/50 ug/ul for d.s. DNA

**Quantification of DNA**

The quantitation of DNA was done by observing it at 260 nm and 280 nm wavelengths by using a UV- VIS spectrophotometer (UNICAM) as follows:

- 1500 µl T.E. buffer was taken in a cuvette and spectrophotometer was calibrated at 260 nm as well as at 280 nm wavelengths.

- 5 –15 µl of DNA was added, mixed properly and absorbance (A) was recorded at both 260 and 280 nm.

DNA concentration was estimated by employing the following formula:

$$\text{Amount of DNA (µg / µl) = } \frac{A_{260} \times 50 \times \text{dilution factor}}{1000}$$

Quality of DNA was judged from the ratio of A values recorded at 260 and 280 nm. (Table 3.2)

**Isolation of Fungal DNA**

Total genomic DNA was extracted using the method of **Doyle and Doyle (1990)** from five days old fungal mycelium grown on PDA. The quality of extracted DNA after RNase treatment was assessed on 0.8% agarose gel and finally the DNA
was quantified using spectrophotometer (Optigen 2020plus). The DNA samples were diluted to 25 ng µl-1 for PCR amplification (Arif et al. 2011).

13. Tissue was grinded under liquid nitrogen until it became a powder. Then it was transferred into sterilized eppendorf tube.

14. CTAB buffer with BME (1.0 ul of BME per 1.0 ml of CTAB) was then added. CTAB used at 2.0 mL per gram of tissue. For 0.1 grams of tissue, 500 ul CTAB and 0.5 ul of BME was used.

15. Extracts were incubated for one hour at 65°C and the samples were shaken every 10 minutes to ensure that things are well mixed.

16. Then it was spun at 13,000 rpm for 1 minute and the supernatant was pulled off into a new tube.

17. Multiple extractions of the same pellet was done and supernatants were combined to get a better extraction. If we want to extract again, we have to repeat from step 2.

18. The combined supernatants were then extracted with one volume of chloroform.

19. Tubes were spun at 1000 X G (RCF) for 5 minutes (4°C)

20. The aqueous layer was pulled off and transferred to a new tube.

21. To get more DNA out we used “back extract” the chloroform with one volume ddH2O. The “back extracted” tubes are spun at 1000 X G (RCF) for 5 minutes (4°C) and the aqueous phase (top layer) is transferred to the same tube as in step 8.

22. Next the DNA was precipitated with 2/3 volume of isopropanol. The solution was allowed to incubate at –20°C for at least an hour and up to overnight.

23. Tubes were spun at max speed for 5 minutes and the supernatant is discarded.
24. Pellet was washed with 500ul of 80% ethanol the spun at max speed for 5 minutes.

25. Supernatent was discarded and a small (0.1-10 µL) pipette tip was used to pull out as much liquid as possible.

26. Pellets were dried by either leaving the tube open in a safe location or by using the speed-vac.

27. Once pellets were dried they were resuspended with 50-100ul of 1xTE Buffer.

28. It was Run on agarose gel and Spec was used to quantify DNA (use 5ul DNA/750ul 1x TE for OD [260/280]. Store aliquots at either –80° C or -20°

   To find amount of DNA OD_{260} = 1/50 ug/ul for d.s. DNA

**Quantification of DNA**

The quantitation of DNA was done by observing it at 260 nm and 280 nm wavelengths by using a UV- VIS spectrophotometer (UNICAM) as follows:

- 1500 µl T.E. buffer was taken in a cuvette and spectrophotometer was calibrated at 260 nm as well as at 280 nm wavelengths.
- 5 –15 µl of DNA was added, mixed properly and absorbance (A) was recorded at both 260 and 280 nm.

DNA concentration was estimated by employing the following formula:

\[
\text{Amount of DNA (µg / µl)} = \frac{A_{260} \times 50 \times \text{dilution factor}}{1000}
\]

Quality of DNA was judged from the ratio of A values recorded at 260 and 280 nm. *(Table 3.2)*
Table 3.2: Quantification of Fungal DNA

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Strains</th>
<th>Ratio of A260 /A280</th>
<th>Conc. of DNA (ng/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A1</td>
<td>1.97</td>
<td>599.46</td>
</tr>
<tr>
<td>2</td>
<td>A2</td>
<td>1.89</td>
<td>632.67</td>
</tr>
<tr>
<td>3</td>
<td>J1</td>
<td>1.8</td>
<td>743.08</td>
</tr>
<tr>
<td>4</td>
<td>J2</td>
<td>1.83</td>
<td>675.80</td>
</tr>
<tr>
<td>5</td>
<td>J3</td>
<td>1.87</td>
<td>686.21</td>
</tr>
<tr>
<td>6</td>
<td>Jod1</td>
<td>1.90</td>
<td>598.75</td>
</tr>
<tr>
<td>7</td>
<td>Jod 2</td>
<td>1.77</td>
<td>768.32</td>
</tr>
<tr>
<td>8</td>
<td>Jod 3</td>
<td>1.84</td>
<td>697.45</td>
</tr>
<tr>
<td>9</td>
<td>Job1</td>
<td>1.82</td>
<td>589.95</td>
</tr>
<tr>
<td>10</td>
<td>Job2</td>
<td>1.79</td>
<td>725.10</td>
</tr>
</tbody>
</table>
Material and Methods (cont...)

B) Characterization of Antagonistic Rhizobacteria

Isolation of bacteria from rhizospheric soils

Root samples of cumin with adhered soil were collected from same four cumin cultivated areas of Rajasthan (Ahore, Jalore, Jodhpur, Jobner). Cumin plants from the moistened soil and the soil adhering to the root region was collected by shaking the root gently along with terminal root portion were cut off and brought to the laboratory in Paper bags for isolation of rhizobacteria. Loosely adhering soil with roots was collected and 1 g of the soil was suspended in the test tube 10 ml. of sterilized water and was shaken thoroughly to mix soil particles to get uniformly dispersed. Serial dilution 10⁻², 10⁻³, 10⁻⁶ are made by pipettes measured volumes (1ml in 9ml of sterilized H₂O). Finally 1 ml aliquot of 10⁻⁶ are added to sterilized, petriplates, triplicates to which are added 15 ml. (approx.) of the sterilized cool molten (45°C) medium. About 1g root sample with adhered soil was dispensed in sterilised distilled water and was serially diluted using sterilised distilled water in 10-fold series up to 10⁻³ and 100-mL aliquots of all dilutions was spread plated in triplicates using nutrient agar and King’s medium B (King, Ward and Raney 1954) media separately. King’s B medium was used for the isolation of Pseudomonas spp. and Nutrient agar (NA) was used for Bacillus spp. After solidification, the plates were incubated in an inverted position for 3 days at 28±0°C. They were examined under UV light and colonies with yellow green and white colors pigmentation were marked and recorded. The number of colonies appearing on dilution plates were counted, averaged and multiplied by the dilution factor to find the number of cells/spores per gram of the sample.

\[
\text{Number of cells/g.} = \frac{\text{Colonies} \times \text{Dilution factor}}{\text{Dry Wt. of Soil}}
\]
All the isolates were purified by re-streaking, their cultural and microscopic characters were recorded, and the cultures were maintained as glycerol stocks. The selected strain was characterized based on morphological and biochemical tests by referring to the Bergey’s Manual of Determinative Bacteriology. The chosen strain was examined for its colony morphology, pigmentation, cell shape and gram reaction as per the standard procedures given by Anonymous (1957) and Bartholomew and Mittewar (1950).

Pure culture of bio-agents was streaked on King's B agar medium and nutrient agar medium visual observation was made after 48 hours of incubation at 28± 0°C for identification.

- Morphological characterization
- Biochemical characterization
- Functional characterization

The young cultures were Gram stained by smearing loopful culture on grease free clean slides stained accordingly and examined microscopically for shape, arrangement and Gram reaction.

**Gram staining**

Actively growing Pseudomonas spp. and Bacillus spp. culture was taken for preparing a thin smear on clear slide. The smear was then air dried on flame gently. Now the smear was passed through crystal violet stain solution for 30-40 second. Slide was rinsed with running tap water to remove excess of stain adhered on slide. Then slide was passed through iodine solution for 1-2 min. Slide was again rinsed with using tap water. Further the slide was dipped in alcohol for 2 seconds and then placed to the safranin solution for 3 minutes. The slide was again rinsed with water and air dried. The slide was examined under oil-immersion objective using one drop of cedor oil. Further strain was identified as Pseudomonas fluorescens and Bacillus
subtilis according to Bergey's Manual of Systematic Bacteriology (Kreig and Halt, 1984).

Preparation of Bacterial Cell Culture

Bacterial culture was grown overnight in 25ml nutrient broth in thermo shaker. The bacterial culture was then centrifuged at 5000 rpm for 3 minutes to obtain pellets and supernatant will be discarded.

- 10 ml LB culture was grown until saturation and the cells were platted centrifugation for 10min. at 5000rpm.
- Pallet was resuspended in gently 9.5ml TE Buffer {10mM Tris, 1mM Na2 EDTA, and pH-8}, 1.5ml of 10% SDS and 50μl of a 20mg/ml protinase K solution.
- Mixed gently and incubated 1hour at 37’c.
- After incubate add 1.8ml of 5M NaCl, mixed then add 1.5ml of 10% CTAB in 0.7m NaCl and Mix for 10min and incubate 20min. at 65’c.
- Added equal volume of TE saturated phenol/chloroform {1:1} solution pH-8, mixed for 30min and transfer in fresh tube then centrifuge at 10000 g for 30min. at 4’c.
- Supernatant was transfer in fresh tube, then mix gently add equal volume of chloroform / isoamylalcohol {24 : 1} and centrifuge at 10000 g for 30 min. at 4’c.
- Upper phase was transfer new tube then added 0.6 volume isopropanol until a white DNA pallet precipitated, centrifuge at 10000 g for 10 min.
- 1ml of 70% alcohol was added and centrifuged at 5000g for 10 min at 4 ‘C.
- DNA was washed and held in room temperature over night.

Finally washed DNA was dissolved in 1ml T10E1buffer. {PH-8}.
Quantification of DNA

The quantitation of DNA was done by observing it at 260 nm and 280 nm wavelengths by using a UV-VIS spectrophotometer (UNICAM) as follows:

- 1500 µl T.E. buffer was taken in a cuvette and spectrophotometer was calibrated at 260 nm as well as at 280 nm wavelengths.
- 5-15 l of DNA was added, mixed properly and absorbance (A) was recorded at both 260 and 280 nm.

DNA concentration was estimated by employing the following formula:

\[
\text{Amount of DNA (µg / µl)} = \frac{A_{260} \times 50 \times \text{dilution factor}}{1000}
\]

Quality of DNA was judged from the ratio of A values recorded at 260 and 280 nm. (Fig 3.3)
Table 3.3: Quantification of DNA from Antagonistic Rhizobacterial Strains

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Rhizobacterial Strains</th>
<th>Ratio of A260/A280</th>
<th>Conc. of DNA (ng/ l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CrJ10</td>
<td>1.92</td>
<td>532.67</td>
</tr>
<tr>
<td>2</td>
<td>CrJ19</td>
<td>1.78</td>
<td>743.08</td>
</tr>
<tr>
<td>3</td>
<td>CrJ21</td>
<td>1.86</td>
<td>622.70</td>
</tr>
<tr>
<td>4</td>
<td>CrJ25</td>
<td>1.83</td>
<td>578.35</td>
</tr>
<tr>
<td>5</td>
<td>CrJ28</td>
<td>1.89</td>
<td>592.86</td>
</tr>
<tr>
<td>6</td>
<td>CrJ30</td>
<td>1.77</td>
<td>643.45</td>
</tr>
<tr>
<td>7</td>
<td>CrA1</td>
<td>1.80</td>
<td>711.56</td>
</tr>
<tr>
<td>8</td>
<td>CrA5</td>
<td>1.89</td>
<td>731.25</td>
</tr>
<tr>
<td>9</td>
<td>CrA8</td>
<td>1.78</td>
<td>695.50</td>
</tr>
<tr>
<td>10</td>
<td>CrA9</td>
<td>1.81</td>
<td>587.62</td>
</tr>
</tbody>
</table>

Antagonistic activity of Rhizobacteria against *Fusarium oxysporum*

Antagonistic activity of the isolates was evaluated by measuring the growth inhibition of soil borne plant pathogenic fungi *Fusarium oxysporum* of cumin on PDA. Well diffusion method where pathogenic fungal was spreaded and bacterial cultures were put in corresponding wells (5 mm diameter) other as well as fungus on PDA plates, was followed. Two plates of each treatment were incubated at 28oC for 4 days to examine zone of inhibition (*Berg, Fritze, Roskot and Smalla 2001*) and rating of inhibition zone was visually done.

Antagonistic activity On PDA

Antagonistic activity of the isolates was evaluated by measuring the growth inhibition of soil borne plant pathogenic fungi *Fusarium oxysporum* of Cumin on PDA. Well diffusion method where pathogenic fungus was spreaded and bacterial cultures were put in corresponding wells (5 mm diameter). Plates were incubated at 280C for about a week; however, selection and re-streaking of different types of colonies was started after 36 h of incubation and new colonies were picked and
streaked as and when they appeared in the plate. Two plates of each treatment were incubated at 28oC for 4 days to examine zone of inhibition (Berg, Fritze, Roskot and Smalla 2001) and rating of inhibition zone was visually done.

\[
\% \text{ inhibition} = \left[ 1 - \frac{\text{Fungal growth}}{\text{Control growth}} \right] \times 100.
\]

**Antagonistic activity in broth:**

Potato dextrose broth was dispensed into conical flasks, autoclaved and four treatments namely no inoculation (control), inoculation with bacteria, inoculation with pathogenic fungus (250 M) alone and inoculation with bacteria plus fungi both, were imposed. All the treatments were done in duplicates. The bacterial inoculums were prepared by growing cells in King’s medium B for 48 h. Cells were centrifuged and resuspended (0.575 OD at 650 nm) in sterilised water. About 500 mL of bacterial cell suspension and three discs (5 mm diameter) of 7-day-old fungal culture raised on Potato dextrose agar plates, were inoculated in to flasks as per treatments. All the flasks were incubated for 4 days in a shaker incubator at 280C and 110 rpm. The contents of the flasks were centrifuged, washed with sterile water and dried at 65oC till constant weight.

Percentage reduction in fungal growth was calculated as follows: \([R - (R2 - R1)/R]*100\). Where R is dry weight of fungus alone, R1 is dry weight of bacterial cells and R2 is dry weight of fungal mycelium_bacterial cells. Replicates were averaged and expressed as per cent reduction in fungal dry weight.

**Identification of efficient antagonistic rhizobacteria**

The best-selected rhizobacterial isolates were tentatively identified as to the genus, according to the method described in Bergey's Manual of Determinative Bacteriology (Sneath 1986; Holt et al. 1994).

Effect of liquid biofertilizer on plant growth

1. Soil collected from a field is to be sterilized using autoclave.
2. Equal amount of soil is added on each pot along with the control.
3. Control represents soil with no bacterial suspension.
4. 30 seeds were firstly dipped in liquid suspension for 1 hr.
5. Seeds were then inoculated into the pot.
6. As the germination occurs seeds were given first boosting dose of liquid suspension (100µl) of a specific bacteria.

**Plant Growth Promotory Activity of Rhizobacteria (PGPR)**

Plant growth promotory activities if different rhizobacterial strains were determined using different qualitativie assays.

**Production of Indole Acetic Acid (IAA)**

Production of Indole acetic acid (IAA) was determined by the method described by **Bric et al, 1991**. Bacterial isolates were initially inoculated in nutrient agar added with L Tryptophan. Then it was incubated at 37°C for 48hrs and fully grown cultures were centrifuged at 3000rpm for 30 minutes. After that two drops of orthophosphoric acid and 4ml of Salkowski Reagent (50 ml 35% of perchloric acid, 1ml of 0.5mFeCl3 solution), was added to the 2ml of supernatant. Development of pink colour indicated production of IAA. The absorbance at 530 nm was measured.

**Phosphate Solubilisation:**

Bacterial strains were evaluated for their ability to solublize inorganic phosphate. Agar medium containing calcium phosphate as the inorganic form of phosphate was utilized in this assay. A loopfull of each culture was placed on the plates; five per plate, and the plates were incubated at 27°C for 3 days. A zone of clearing around the colonies after 3 days was scored as positive for phosphate solubilization. The experiment was performed twice with five replicates for each bacterial strain. Phosphate solubilising ability of different antifungal rhizobacterial isolates was examined using Pikovskaya agar (**Pikovskaya, 1948**) containing tricalcium phosphate. The zone of clearance around the spotted culture in the plate was measured. Appropriate dilution was spread on Pikovskaya agar plate containing insoluble ticalcium phosphate (**Johnson et al., 1972**) Plates were incubated at 30±0.1 0C for 24-48 h. Colonies showing zone of clearance were considered as P-solubilizer and were purified by repeated streaking.
Production of protease enzyme

Protease of the isolates was assayed following Nielsen and Sorensen (1997); however, instead of skimmed milk, casein was used in the casein minimal agar medium. Four bacterial cultures were spot inoculated on a plate in triplicate. Zone of clearance was measured after 4 days incubation at 30°C to calculate protease ratio (R2/R1), where R1 is the diameter of bacterial colony and R2 is diameter of zone of clearance plus bacterial colony.

Nitrogen Fixing Capacity

Different antifungal rhizobacterial isolates were grown on nitrogen free malate medium to study N2 fixing capacity (Okon, Albrecht and Burris 1977) qualitatively. Growth with change of the colour of the medium from greenish to blue was recorded positive (+) for nitrogen fixation and otherwise was recorded negative (-).

Molecular Analysis

Identification and characterization of beneficial rhizobacteria was done on the basis of morphological, physiological and molecular characteristics assessed by fatty acid analysis, mol (%), G + C contents, DNA–DNA hybridization, and 16S rRNA sequencing. Taxonomy of PGPR can be defined with the help of these characteristics.

The rhizobacterial isolate that gave good results in the preliminary test was grown in Luria broth for 24 hrs at 370°C for extraction of genomic DNA through enzymatic lysis. The total genomic DNA extracted was dissolved in water (protease, nuclease free) and stored at 4°C. PCR amplification was done by using 16S universal primer resolved in 1.2% agarose gel and visualized on gel documentation system (BIORAD, USA). The purified 16S rRNA gene was performed using as a template in cycle sequencing reaction with fluorescent dye- labeled terminators (Big dye, Applied Biosystems) of isolate each cluster with same primer and run in 3130 XL ABI prism automated DNA sequence. The sequence was compared with 16S rRNA gene sequence available in the NCBI GeneBank database using BLASTn program.
Purification of DNA

Microbial DNA isolated by above method was not pure so purification of DNA is essential for PCR analysis. Impure DNA was purified by two methods of purification

Purification using RNase treatment:

RNA was removed by treating the sample with DNase free RNase. Protein including RNase was removed by treating with chloroform: Isoamylalcohol (24:1).

The purification was carried out in following steps-

- 2.5 l of RNase was added to 0.5 ml of crude DNA preparation (2.5 l of RNase = 25 g of RNase, so treatment was 50 g/ml of DNA preparation).
- Gently it was mixed thoroughly and was incubated at 37 C for 1 hour.
- After 1 hour, a mixture of 0.3 – 0.4 ml of chloroform: Isoamyl alcohol (24:1) was added and mixed thoroughly for 15 minutes till an emulsion was formed.
- Centrifuged for 15 minutes at 15000 RPM.
- Supernatant was taken avoiding the whitish layer at interface.
- The DNA was re-precipitated by adding double quantity of absolute alcohol.
- To pellet the DNA the tube was centrifuged for 5 minutes at 5000-10000 RPM for 10 minutes.
- The transparent and viscous pellet was washed with 70 percent alcohol and dried over night.
- The DNA was re-dissolved in 200 l of T10E1 buffer).

Gel Analysis

The integrity of DNA was judged through gel analysis in following steps:

- 150 ml Agorose gel (0.8%) was casted in 0.5X TBE (Tris Borate EDTA) buffer containing 0.5 µg / ml of Ethidium Bromide.
2 µl of DNA per sample was loaded in each well.

Known amount of uncut Lambda phage DNA was also loaded as control.

Electrophoresis was conducted at 50 V for 1 hr.

Gel was visualized under UV light using transilluminator.

Presence of single compact band at the corresponding position to phage DNA indicated high molecular weight of isolated DNA.

Dilution of DNA for PCR:

The quantitated DNA was diluted to final concentration of 25 ng/µl in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) for RAPD amplification.

RAPD analysis

Requirements for PCR

(1) Random primers: 40 primers from set # 1 and 10 primers from set # 2 obtained from the University of British Columbia, Vancouver, Canada.

(2) dNTPs: The four individual dNTPs such as dATP, dGTP, dCTP and dTTP were obtained from m/s Bangalore Genei, Pvt. Ltd. Bangalore.

(3) Taq DNA polymerase: Taq DNA polymerase and 10x Taq assay buffer A were obtained from M/S Bangalore Genei, Pvt. Ltd., Bangalore.

(4) Thermo cycler: (Model-CGI-96, Corbett Research, Australia)

For RAPD analysis were screened. Out of 50 primers only 9 primers were amplified and eight of these primers were removed because of their monomorphic nature and poor reproducibility. The PCR reactions were performed in a 25 µl reaction mixture containing 1x assay buffer, 0.5 units of Taq DNA polymerase, 200 µM of each dNTPs (Bangalore Genei), 0.2 µM primers and 50 ng of template DNA. The PCR reactions were carried out in DNA thermal cycler using a single primer in each reaction. The PCR reactions were repeated thrice for each primer to ensure the reproducibility of RAPD results. Only highly reproducible and polymorphic primers were included in the study.
Table 3. 4 Sequence details of random primers used in RAPD analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCC 106</td>
<td>CGTCTGCCCCG</td>
</tr>
<tr>
<td>GCC 116</td>
<td>TACGATGACG</td>
</tr>
<tr>
<td>GCC 126</td>
<td>CTTTCGTGCT</td>
</tr>
<tr>
<td>GCC 132</td>
<td>AGGGATCTCC</td>
</tr>
<tr>
<td>GCC 137</td>
<td>GGTCTCTCCCC</td>
</tr>
<tr>
<td>GCC 184</td>
<td>CAAACGGCAC</td>
</tr>
<tr>
<td>GCC 189</td>
<td>TGCTAGGCCTC</td>
</tr>
<tr>
<td>GCC 193</td>
<td>TGCTGGCTTT</td>
</tr>
<tr>
<td>GCC 196</td>
<td>CTCCTCCCCCC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Profile</th>
<th>Step</th>
<th>Temperature</th>
<th>Duration (mix)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>94</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>37</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>Elongation</td>
<td>72</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Extension</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

The PCR amplification conditions for RAPD consisted of initial extended step of denaturation at 94°C for 4 min followed by 44 cycles of denaturation at 94°C for 1 min, primer annealing at 37°C for 1 min and elongation at 72°C for 2 min followed by a final step of extension at 72°C for 4 min.

DNA from different rhizobacteria strains was used for RAPD analysis following the method recommended by Bhat and Jarret (1995) with required modification. Amplification reaction mixture was prepared in 0.2 ml thin walled PCR tubes containing following components. The total volume of each reaction mixture was 20µl. The cocktail for the PCR amplification was found to be optimum.
were

1. Template DNA (25 ng/μl) – 1.0 μl
2. 10x assay buffer with 15mM MgCl2 – 4.00 μl
3. dNTP’s mix (2.5 mM each) – 2.00 μl
4. Primer (5pM/μl) – 2.00 μl
5. Taq DNA polymerase (3 units/μl) – 0.2 μl
6. Sterile distilled water – 12.8 μl

Except template DNA the master mix was distributed to PCR tubes (19μl/tube) and later 1 μl of template DNA from the respective PGPR strains was added making the final volume of 20 μl.

Separation of amplified products by agarose gel electrophoresis

**Requirements**

1. Electrophoretic unit: Gel casting trough, gel combs, power-pack and UV Transilluminator
2. Agarose (1.2%)
3. Bromophenol blue
4. Ethidium bromide (0.5 μg/ml)
5. **50 x TAE (stock):** Tris-free base - 60.5 g
   Glacial acetic acid - 14.25 ml
   0.5 M EDTA - 25 ml
   Make up the volume to 250 ml pH 8.0
6. **Working solution (1 x TAE):** 20 ml of 50 x TAE was made upto 1000 ml by using distilled water.

**Procedure**

- One gram of agarose was weighed and added to a 250 ml conical flask containing 100 ml of 1 x TAE buffer.
- The agarose was melted by heating the solution on a microoven and the solution was stirred to ensure even mixing and complete dissolution of
agarose.

- The solution was then cooled to about 50°C.
- Two to three drops of ethidium bromide (0.5 μg ml-1) was added.
- The solution was mixed and poured into the gel casting plat form after inserting the comb in the trough.
- While pouring sufficient care was taken for not allowing the air bubbles to trap in the gel.
- The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus containing sufficient buffer (1 x TAE) so as to cover the wells completely.
- The amplified products (20 μl) to be analysed were carefully loaded into the sample wells, after adding bromophenol blue with the help of micropipette.
- Electrophoresis was carried out at 60 volts until the tracking dye migrated to the end of the gel.
- Ethidium bromide stained DNA bands were viewed under UV – transilluminator and photographed for documentation.

The PCR reaction products were mixed with 4μl of 6x DNA loading buffer and fractionated on 1.2% agarose for RAPD containing 0.5 μg ml-1 ethidium bromide. Gels were electrophoresed until the indicator dye reached 10 cm from the well at 55 mA for 4 hrs. After separation gels were documented using Avigene Gel Doc syastem (Koria).

50 RAPD markers generated with single primers of arbitrary nucleotide was used for the identification of isolates. (Arici et al. 2010). Out of 50 primers only 9 primers were amplified and eight of these primers were removed because of their monomorphic nature and poor reproducibility. Genetic similarity estimates based on RAPD banding patterns were calculated using method of Jaccard’s coefficient analysis. The similarity coefficient matrix generated was subjected to algorithm “Unweighted Pair Group Method for Arithmetic Average (UPGMA)” to generate clusters using NTSYS 2.02 pc program (Rohlf, 1998,2000).
Scoring the amplified fragments

Only clear and reproducible bands were scored for the data analysis, but a major band corresponding to a faint band in repetition was also included in the study. RAPD data were scored for the presence (1) or absence (0) and each band was regarded as a locus. The amplified profiles for all the primers were compared with each other and the bands of DNA fragment were scored as ‘1’ for presence and ‘0’ for the absence of a band generating the ‘0’ and ‘1’ matrix. λ DNA EcoRI/HindIII double digest marker was used as a standard for the estimation of molecular weight of the RAPD products. Bands with same molecular weight and mobility were considered as a single locus. The total number of alleles, polymorphic alleles, average number of alleles per primer and polymorphism percentage were calculated by using the formula:

\[
\text{Per cent polymorphism} = \frac{\text{No. of polymorphic bands}}{\text{Total number of bands}} \times 100
\]

Analysis of RAPD-PCR amplified fragments

Pair wise genetic similarities between different strains were estimated and clustering was done using the symmetric matrix of similarity coefficient and cluster obtained based on unweighted pair group arithmetic mean (UPGMA) of NTSYS-PC program version 2.0 (Exeter software, New York, USA) described by Rohlf (1998).

Sequencing of 16S rRNA

The isolates were grown on nutrient agar medium at 280C for 24 h. Genomic DNA was isolated from cells that were treated with lysozyme (50 mg mL⁻¹), SDS (10%, w/v), proteinase K (20 mg mL⁻¹), DTT (1M), and EDTA (0.5M) and then subjected to serial extractions with phenol_chloroform (1:1, v/v) and precipitation with NaCl and ethanol. The pellet was re-suspended in TE buffer (10 mM Tris and 1 mM EDTA) and stored at 200C till use. PCR was performed in 25 mL reaction volume with the following reaction components: 2.5 mL of 10XPCR buffer, 1.5 mM MgCl₂, 200 mM of each dNTP, 1U Taq DNA polymerase, 5 pmol of each primer
and 25 ng of DNA. (Edwards, Rogall, Blocker, Emde and Bottger 1989). The PCR amplification of 16S rRNA gene was carried out in a palm cycler (Corbett Research, USA) using the following universal primers:

forward  27F (5' AGAGTTTGATCCTGGCTCAG 3' )
Reverse  519R (5' GWATTACCGCGGCKGCTG-3')

DNA ladder of 1 kb (Bangalore Genei) was used as a size marker. PCR product, approximately 500 bp, was sequenced from Chromous Biotech Pvt. Ltd. (Bangalore) using the same forward primer.

16S rDNA gene amplification was described by following method:

The following sequences for two primer sets were used:

- Set 1 Primer-1 5' AGAGTTTGATCCTGGCTCAG 3’
- Primer-2 5’ TACCTTGTTACGACTT 3’
- Set 2 Primer-1 5’ CCGGCTTTCCCATTCGG 3’
- Primer-2 5’ TGCGGCTGGATCTCCTT 3’

PCR Reactions were performed in final volume of 25 µl containing 2.5µl 10X Assay Buffer with MgCl2 (Bangalore Genei), 0.19 µl of Taq DNA polymerase (3U/ µl) (Bangalore Genei), 2.0 µl dNTPs (10 mM) (Bangalore Genei), 1µl primer-1 (10 pM/µl), 1µl primer-2 (10 pM/µl) (OPERON TECHNOLOGIES), 16.3µl Deionised water and 2.5µl template DNA (25 ng/µl). The PCR was performed in ‘Corbett pamlcycler’ using the following cycling parameters:

**Cycle 1:**

- Denaturation (94°C) 5 Minutes
- Primer annealing (45°C) 1 Minutes
- Primer Extension (72°C) 2 Minutes
Cycle 2 - 30:
- Denaturation (94°C) 1 Minute
- Primer annealing (45°C) 1 Minute
- Primer Extension (72°C) 2 Minutes

Cycle 32:
- Denaturation (94°C) 1 Minute
- Primer annealing (45°C) 1 Minute
- Primer Extension (72°C) 7 Minute

Following the amplification, the PCR products, after addition of 2μl of trekking dye were loaded on 1.2% Agarose gel (Himedia, molecular grade), which was prepared in 1X TBE buffer containing 0.5 μg/ml of the Ethidium Bromide. The amplified products were electrophoresed for 3 - 3.5 hrs at 100 V with cooling. After separation the gel was viewed under UV trans-illuminator and photographed by digital camera.

Amplified 16S Ribosomal DNA Restriction Analysis

Restriction endonuclease digestion was performed with HaeIII and HpaII endonucleases in separate reaction mixtures containing 1 ml of endonuclease (10 U/ml), 1.5 ml of matched incubation buffer, and 12.5 ml of PCR product; the mixture was incubated for 1.5 h at 37°C.

Scoring of PCR Products

In order to score and preserve banding pattern a photograph of gel was taken by digital photographic system, under UV Trans illuminator. PCR bands were designated on the basis of their molecular sizes (length of polynucleotide amplified). 100 bp ladder was loaded with each primer products to estimate the molecular size. The distance run by amplified fragments from the well was translated to molecular sizes with reference to molecular weight marker. The presence of each band was scored as ‘1’ and its absence as ‘0’.
The scores (0 or 1) for each band obtained from photograph were entered in the form of a rectangular data matrix (qualitative data matrix). The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard’s similarity coefficient. The equation for calculating Jaccard’s similarity coefficients ‘f’ between two samples A and B is:

\[ f = \frac{n_{xy}}{n_1 - n_z} \]

Where:
- \( n_{xy} \) = Total Number of bands common to sample A and sample B.
- \( n_1 \) = Total number of bands present in all samples.
- \( n_z \) = Number of bands not present in sample A or B but found in other samples.

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tool was used for Cluster analysis in order to determine genetic distances. These genetic were for constructing dendrogram that depicted the relationships of the similar and dissimilar strains using computer program NTSYS pc version 2.02 (Rohlf, 1998).

The statistical calculations were done using Ntysis pc 2.02e. Similarity matrix for RAPD primers was constructed using the Jaccard’s similarity coefficient values to find out genotypic relationship. The average distance of a single variety from rest of the genotypes was also calculated. The 0/1 matrix data obtained from RAPD were arranged to get separate similarity matrices which were subjected to UPGMA (unweighted pair-group method with arithmetic averages) analysis to generate dendrogram and compared using the Mantel matrix correspondence test (MxComp module of NTSYSpc).

Sequence alignment and Phylogenetic analysis

Phylogenetic analysis as a part of taxonomic approach was done by comparing partial sequences of approx. 420 bp of the isolates with sequences registered in the GenBank non-redundant nucleotide database. This was done by using BLAST version 2.0 (Altschul, Jenkins, Kristiansen and Froholm 1990) NCBI Basic or RDP. Then alignment of these partial sequences was done using
Clustal W software (Thompson, Gibson, Plewniak, Jeanmougin and Higgins 1997) which was then compared with those of the known bacteria. The calculation of genetic distances was done according to 144 S. Kimura’s two-parameter method (Kimura 1980). Neighbour-joining method was used in preparation of phylogenetic tree (Saitou and Nei 1987). Bootstrap analysis was based on 500 re-samplings and The MEGA 3.1 package (Kumar, Tamura and Nei 2004) was used for all analyses. *Escherichia coli* was taken as out group during construction of tree and the isolates having similarity less than 95% were included in the phylogenetic analysis. The partial sequences of 16S rRNA genes of the antagonistic rhizobacterial isolates that showed variation from from 420 to 500 bp were analysed using BLAST.