MATERIALS
AND
METHODS
MATERIALS AND METHODS

I. SELECTION, COLLECTION AND ISOLATION
   i) Isolation of mycoflora 40
      a) Blotter method 40
      b) Agar plate method 41
   ii) Disease incidence and degree of infection 42
   iii) Pathogenicity 42

II. SEED QUALITY TEST
   i) Germination efficiency test 43
   ii) Mineral composition of seeds 43
   iii) Oil analysis 44
      a) Determination of oil percentage 44
      b) Qualitative analysis of lipids 49
      c) Thin layer chromatography of oil 49

III. CULTURAL CHARACTERISTICS
   i) Basal media selection 50
   ii) Effect of Ph 51
   iii) Effect of temperature 52

IV. BIOCHEMICAL CHARACTERISTICS OF PATHOGENS
   i) Protein test 52
   ii) Amino acid 53
   iii) Enzymes (Protease, Amylase and Lipase) 54

V. DISEASE PROTECTION
   i) Antibiotics (Griseofulvin, Tetracycline, Norfloxacine) 56
   ii) Fungicides (Bavistin, Jatayu and Kitazin) 57
   iii) Angiospermic sources (Spices viz. Clove, Turmeric, Asafoetida, Nutmeg and Blackcumin) 57
MATERIALS AND METHODS

1. SELECTION, COLLECTION AND ISOLATION

Five types of commonly used oil producing seeds *viz*- *Helianthus annuus* (L.) (sunflower), *Carthamus tinctorius* (L.) (safflower), *Glycine max* (Merr.) (soybean), *Arachis hypogaea* (L.) (groundnut) and *Zea mays* (L.) (maize) were selected for isolation of surface mycoflora as well as internally seed-born fungi. Seed sample were collected from market and Agriculture College Raipur (C.G.) and stored at room temperature. For each crop 100 seeds of both healthy and diseased categories were differentiated on morphological basis of discoloration, size and smoothness of seed surface. The seed mycoflora was isolated from selected seeds by standard techniques i.e. Blotter method and Agar plate method.

i. Isolation of mycoflora:

a) Blotter method

For the isolation of surface mycoflora blotter method was used. Discs of sterile blotting paper were placed in sterilized petri plates (90mm dia.) and moistened with sterilized distilled water. The non disinfected and infected seeds were placed on 3 layered water soaked blotter in controlled temperature at $28^0$ C ($\pm 2^0$ C). The fungi growing on the seeds were examined after 7 days of inoculation and the data was recorded.
b) **Agar plate method**

For the isolation of internal seed-borne mycoflora agar plate method was used. Selected seeds were surface sterilized with 0.01% aqueous mercuric chloride solution, washed with sterile distilled water and then transferred to boiling test tubes one in each containing 15ml water agar medium under aseptic condition (Neergaard 1997).

**Water agar medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>200.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0ml</td>
</tr>
<tr>
<td>pH</td>
<td>4.5</td>
</tr>
</tbody>
</table>

The internally seed borne fungi emerging in between the seed coat and cotyledons were transferred to the plates containing potato dextrose agar (PDA) medium and isolated. The isolated organisms were purified, identified and maintained on PDA slants.

**Potato dextrose agar medium (PDA)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>200.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0ml</td>
</tr>
<tr>
<td>pH</td>
<td>4.5</td>
</tr>
</tbody>
</table>
Altogether 26 different fungal isolates were detected, 15 from blotter method (surface mycoflora), Table-2(ii) and 11 from agar tube method (internally seed borne mycoflora)Table-2(iii). These internally seed-borne isolates viz- *Curvularia lunata* (Boedijn) was isolated from *Glycine max*, *Trichoderma viride* (Pers.ex.S.F.Gray), *Alternaria alternata* (Fr.) Keissler and *Fusarium oxysporum* (Schlecht. ex. Fr.), from *Helianthus annuus*. *Aspergillus versicolor* (Vuill.) Tiraboschi, *Aspergillus terreus* (Thom. and Raper.) and *Aspergillus niger* (Van Tiegh) from *Arachis hypogaeae*. *Paecilomyces lilacinus* (Bain). and *Chaetomium globosum* (Kunze ex. Fr.) from *Carthamus tinctorius*. The mycoflora of *zea mays* was detected as *Aspergillus flavus* (Link ex. Fr.) and *Aspergillus fumigatus* (Fres.). These internally seed-borne isolates were selected for further studies.

**ii. Disease intensity**

The percent disease intensity was recorded after 7 days of germination of selected varieties of oil seed crops using following formula.

\[
\text{% Disease intensity} = \frac{\text{Total no. of infected seeds}}{\text{Total no. seeds taken}} \times 100
\]

**iii. Pathogenicity test**

The healthy seeds of sunflower, safflower, soybeans, maize and groundnut were surface sterilized with 0.01% mercuric chloride and dipped in the spore suspension of its original culture for 5 min. The treated seeds were then transferred
to the culture tube containing 25ml of sterilized agar medium. Size of the conidia of original and pathogenicity character were measured by micrometry after 7 days of incubation. The reisolation of the causal organism proved pathogenicity.

2. SEED QUALITY TEST

i. Germination efficiency test

The germination efficiency of seeds of both categories (healthy and diseased) of all the selected varieties of oil seeds were recovered after 7 days of incubation by using following formula,

\[
\text{% Germination efficiency} = \frac{\text{Totals no. germinated seeds}}{\text{Total no. of seeds taken}} \times 100.
\]

ii. Determination of mineral compositions of seeds

For the determination of mineral composition, five selected seed varieties of oil seeds were taken and morphologically categorized into healthy and diseased seeds. Detection of mineral composition was done by ash method of Brooks (1972).

Ash method

Pre weighed silica crucibles were filled with 5gms of selected seeds each for healthy and diseased category. All the crucibles containing seeds were then transferred in electric muffle furnace at a temperature of 300°C to get the ash.
When the seeds were charred and completely turned into grey colored ash, the crucibles were removed and placed in desiccators for cooling. Next day crucibles were finally weighed to calculate the net weight of ash. The ash was then transferred in pre sterilized tubes, plugged tightly to prevent from moisture. 0.1gm. ash of each seed category was taken in presterilized 12mm X 150mm pyrex tubes and numbered serially for identification. 10 ml of 2M HCl was added in each tube and placed in water for 15 minutes. The residue was filtered unless it settled readily. The solubilization of trace element is usually hundred percent complete. The clear solution was than tested for sodium, potassium, manganese, zinc, copper and lead by absorption spectrophotometer as the detection of Na and K requires further addition of 10 ml of lithium carbonate in the solution.

iii. Lipid analysis of oils

Lipids are one of the major constituents of foods, and they are a major source of energy and provide essential lipid nutrients. Oils and fats are relatively interchangable names for a variety of chemical compounds. Each type of oil has a different profile of lipids present which determines the precise nature of its nutritional and physiochemical properties. The studies for lipid analysis were carried out in both seeds as well as commercially available oil samples.

a) Determination of oil percentage

The selected seeds of both diseased and healthy were powdered using mixer grinder and their respective oil percentage values were estimated by soxhlet
method (Lalitha Kumari et al., 1970) using petroleum ether as solvent and taking five grams of seed powder for thimble making.

b) **Qualitative analysis of lipids**- Acid value (AV), saponification value (SV), Iodine Value (IV) and Peroxide value (PV).

Qualitative analysis of lipids of both healthy and rancid oils was done in terms of Acid value (AV), saponification value (SV), Iodine Value (IV) and Peroxide value (PV). (Plummer, 1988, Narwar 1996 and Horwitz 2002), to analyse the overall nature of lipids. Branded refined oil of these five seeds were purchased from market and these were considered as healthy (ready to eat) edible oils. Rancidity was created on exposing oil in air for six weeks.

- **Acid Value (AV)**

  The acidity of fat and oils is expressed as its acid value or number which is defined as milligram KOH required to neutralize the free fatty acid present in 1 gram of fat or oil. The acid value of oil gives an indication about the age and extent of deterioration. Acid value was calculated as per following procedure.

  5 ml of oil was added to 25 ml of fat solvent (95% ethanol : ether, 1:1) was taken in a conical flask and after shaking few drops of phenolphthalein indicator was added and it was mixed thoroughly. It was than titrated with 0.1 N KOH until faint pink colour persists for 20- 30 sec. and volume of KOH was noted. The above procedure was repeated with blank (sample not containing any oil) and
calculated by using the formula. Titrated value is the difference between the test and blank readings.

\[
\text{Acid value (mg KOH/g fat)} = \frac{\text{Titrated value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of sample (g)}}
\]

1 ml of 1 N KOH contains 56.1 mg of KOH. Hence a factor of 56.1 is incorporated in the numerator in the above equation to obtain weight of KOH from the volume of 0.1 N KOH solution used during this titration.

• **Saponification value (SV)**

The saponification value is defined as milligram of KOH required to saponify 1g of the given fat. Saponification value was calculated as per following procedure.

After weighing accurately 1 g of oil in a conical flask it is dissolved in 3 ml of fat solvent (95% ethanol : ether, 1:1) Then 25 ml of 0.5N alcoholic KOH was added and it was refluxed on boiling water bath for 30 min. The content was cooled to room temp., then few drops of phenolphthalein was added to it and it was titrated with 0.5N HCL till the pink colour disappears. Above stated steps were repeated with blank. The difference between the blank and test reading gives titre value.
Materials and Methods

\[
\text{Saponification value} = \frac{28.05 \times \text{titre value}}{\text{Weight of sample (g)}}
\]

The multiplication factor of 28.05 in the above equation is included since 1 ml of 0.5 N KOH contains 28.05 mg of KOH.

- **Iodine Value (IV)**

  It is defined as gram of iodine absorbed by 100 g of fat. 10 ml of fat solution was taken in stoppered bottle and 20 ml of Wij’s solution was added to it. After shaking thoroughly it was allowed to stand in dark for 1 hr. A blank was prepared in which fat solution was replaced by chloroform.

  After the reaction time in of 1 hr in dark the neck of the bottle was rinsed with 50 ml of water and 10 ml of 10% potassium iodide solution. The liberated iodine was titrated with standard 0.1 N sodium thiosulphate solution till the content of the flask become pale yellow in colour. (0.1 N sodium thiosulphate solution was prepared by dissolving 24.82 g of Na\(_2\)S\(_2\)O\(_3\)·5H\(_2\)O in 1 L water). After adding few drops of 1% starch solution it was again titrated with sodium thiosulphate solution till the blue colour disappears.

  The difference between the blank and test readings gives the amount of 0.1 N sodium thiosulphate required to reach with an equivalent volume of iodine. One
L of 0.1N iodine solution contains 12.7 g of iodine. The iodine number was calculated as follows:

\[
\text{Iodine number} = \frac{(x - y) \times 12.7}{100} \times \frac{100}{\text{wt. of sample (g)}}
\]

\[
X = \text{Volume of 0.1 N sodium thiosulphate used for blank}
\]

\[
Y = \text{Volume of 0.1 N sodium thiosulphate used for sample}
\]

- **Peroxide value (PV)**

It is widely used as a measurement of the extent to which rancidity reaction have occurred during storage (for creating rancidity oils were kept in open in air for six weeks and stored at room temperature). Peroxides are intermediates in the autoxidation reaction. It was calculated by measuring iodine released from potassium iodide and titrating it with sodium thiosulphate. It is useful for assessing the extent to which spoilage has advanced, when the peroxide value is between 20 to 40 meq/kg rancidity is noticeable.

5g of test oil sample was taken in a 250 mL stoppered conical flask and 30 mL of solvent mixture (three parts glacial acetic acid and one part chloroform) was added to it and thoroughly mixed. After that 0.5 mL saturated potassium iodide solution was added and mixed. The contents of conical flask were allowed to stand in dark for 1 min with occasional shaking then 30 ml of distilled water was added to it. The liberated iodine was titrated with sodium thiosulphate (0.01 N). using 0.5
mL of starch indicator solution (soluble starch 1% w/v) until the blue colour just disappears.

Peroxide value is expressed as milliequivalent of peroxide oxygen per kg of oil sample (mEq kg\(^{-1}\)).

\[
\text{Titer value} \times N \times 1000 \\
m\text{Eq kg}^{-1} = \frac{\text{--------------------------}}{\text{Weight of the oil sample (g)}}
\]

Where, \(N\)=Normality of the sodium thiosulphate solution used.

c) Thin Layer Chromatography of oils

Thin layer chromatography of both healthy and rancid oils were performed using glass sheets coated with silica gel. Oil samples were treated with isopropanol, warmed up, filtered and crude lipid extract were obtained. The neutral lipid fractions were developed with n-hexane, diethylether, acetic acid (80:20:1) v/v/v. The polar fractions were developed with chloroform, methanol and water (65:25:4) v/v/v. Iodine vapour was used as coloring agent and spots were eluted with different organic solvents for further detection. The Rf value of different spots were calculated using the formula given below

\[
\text{Rf} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}
\]
Different concentration of ethyl ether (1%, 4%, 8%, 20% and 100%) and methanol (20%, 30%, 50%, and 70%) were used for elution of lipids from silica gel plates and different type of lipids were detected.

3. CULTURAL CHARACTERISTICS OF THE ISOLATED PATHOGENS

i. Basal media selection

The isolated pathogens were grown on different basal media viz. - Richard’s broth (RB), Potato dextrose broth (PDB), Asthana Hawker’s broth (AHB), Sabouroud’s broth(SB), Czapek- Dox broth (CDB), for optimum growth. 25 ml of each medium was poured in 100ml conical flasks, and growth in respect of biomass was recorded after 7 days of incubation in triplicates.

**Richard’s broth (RB)**

<table>
<thead>
<tr>
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<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>10.0g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>5.0g</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>2.5g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>50.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>5</td>
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</tbody>
</table>

**Potato dextrose broth (PDB)**

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Potato peeling</td>
<td>200.0g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Ph</td>
<td>5</td>
</tr>
</tbody>
</table>
Materials and Methods

**Asthana Hawker’s broth (AHB)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.00g</td>
</tr>
<tr>
<td>KNO₃</td>
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<tr>
<td>KH₂PO₄</td>
<td>1.75g</td>
</tr>
<tr>
<td>MgSO₄7H₂O</td>
<td>0.75g</td>
</tr>
<tr>
<td>Distilled water</td>
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</tr>
<tr>
<td>pH</td>
<td>5</td>
</tr>
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**Saubaroud’s broth (SB)**

<table>
<thead>
<tr>
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</tr>
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<tbody>
<tr>
<td>Peptone</td>
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<tr>
<td>Dextrose</td>
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<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
</tr>
</tbody>
</table>

**Czapek- Dox broth (CDB)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>2.0g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.0g</td>
</tr>
<tr>
<td>MgSO₄7H₂O</td>
<td>0.5g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5g</td>
</tr>
<tr>
<td>FeSO₄ 7H₂O</td>
<td>10.0g</td>
</tr>
<tr>
<td>Sucrose</td>
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<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
</tr>
</tbody>
</table>

**ii. Effect of pH**

The isolates were inoculated on Richard’s broth medium whose pH was adjusted to 3, 4, 5, 6, 7 and 8, in three replicates. 100 ml flasks containing 25ml media was inoculated with 5 mm agar medium plug containing active mycelium.
The flasks were incubated at $27 \pm 1^0C$ for seven days. The mycelia produced were harvested by filtration through pre-weighed filter paper. Mycelial dry weight and pH of culture filtrate were determined. The pH of the media was adjusted with 1N NaOH and 1N HCl.

iii. Effect of temperature

All eleven isolates were inoculated in Richard’s broth medium (pH 5) at temperature range of $20^0C$ to $50^0C$ with a difference of five degree in each case. The effect of temperature was recorded in terms of biomass after 7 days of growth.

4. BIOCHEMICAL CHARACTERISTICS OF ISOLATES

i. Protein test– Protein was estimated by method of Lowry et al., (1951)

a) Preparation of sample for protein measurement

The test organisms were cultured in Richard’s broth medium for 7 days. Protein quantity was estimated from mycelial extract. 1 ml of aliquot was taken in centrifuge tube to which 1 ml of 10% trichloroacetic acid (TCA) was added to precipitate the protein. The mixture was allowed to stand on ice bath for 15 min. and then centrifuged. The supernatant was discarded. This procedure was repeated twice. The pellet was washed with ethanol–ether mixture and dissolved in 10ml of 1 N NaOH. This sample was used for protein estimation.
b) Reagent required:

(i) Preparation of alkaline CuSO₄

Solution A: Alkaline sodium carbonate solution (2% Na₂CO₃ in 0.1 N NaOH).

Solution B: Copper sulphate- sodium potassium tartrate(0.5% CuSO₄ in 1% Na, K tartrate), 50 ml of solution A was mixed with 1 ml of solution B.

(ii) Folin-Ciocalteu’s Phenol reagent.

c) Procedure:

1ml of aliquot was taken in a test tube to which 5 ml of freshly prepared alkaline CuSO₄ solution was added. After 5 minutes, 0.5ml of Folin Ciocalteu’s Phenol reagent was added and the solution was immediately shaken. After 15 minutes optical density (OD) of the developed colour was read on systronics digital spectrophotometer at 750 nm against a blank of 1ml N NaOH. Standard graph was prepared by using different concentrations of bovine serum albumin and total protein content of the test sample was calculated with the help of standard graph.

ii. Chromatographic Analysis of amino acids

The qualitative analysis of the amino acids was done in all the isolates by using ascending paper chromatography. For this purpose Whatman Paper no. 1 was used as an absorbent.
The isolates were cultured in Asthana Hawker’s medium and incubated at 28±1\(^0\)C for 7 days. On 8\(^{th}\) day the culture filtrates were collected and concentrated to about its half volume at 60\(^0\)C. The concentrated samples were used as aliquots for chromatographic analysis of amino acids. Aliquots of the test samples were plotted with 20\(\mu\)l micropipette and dried at room temperature.

The chromatography was developed in n– butanol: acetic acid : water (4:1:1.6 v/v) solvent system and ninhydrin (0.2% in acetone) was used as the detecting reagent, and different amino acids present in all the isolates were detected and compared with standards Rf values.

iii. Enzymes

*In vitro* production of extracellular enzymes viz- protease, amylase and lipase were determined in all the isolates.

a) Protease:

This test was performed on solid Nutrient Agar medium, pH7.2 containing 2% gelatin following the method of Hankin’s and Anagnostakis(1975).

**Nutrient Agar medium (NAM)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>3.0g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0g</td>
</tr>
<tr>
<td>Agar-agar</td>
<td>15.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>
Observations were taken after 7 days of incubation for the zone formed in the plate after acid mercuric chloride (12 gm Hgcl$_2$ +16 ml conc. HCL+84ml Distilled water) reaction of the culture medium. The radial size of enzyme diffusion zone represented the relative amount of the enzyme and the capacity for proteolysis.

b) Amylase:

Enzyme activity was measured on solid medium (Nutrient agar medium containing 0.2 % starch) after 7 days of growth by using Lugol’s iodine solution (1 gm iodine +3gm Potassium iodine + 125 ml Distilled water) A yellow zone, around the colony in an otherwise blue medium indicated amylolytic activity of the organism. The diameter (mm) of the zone of the activity represented the amount of the enzyme produced by the organism.

c) Lipase:

Study of lipase was done with Tween-20 (TT) as a standard substrate which is known to support lipase production. Peptone media with 1% Tween-20 was used for detection of lipase activity. Two days old isolates from peptone agar slants were used as inoculum. pH of media was maintained at 7.5 and temperature maintained at 30 ±2°C.
Materials and Methods

**Lipase assay medium (LAM)**

- Peptone - 8.0g
- CaCl₂ - 100.0m
- Tween-20 - 10ml
- Agar-agar - 20.0g
- Distilled water - 990ml

The radial size of enzyme diffusion zone (white crystalline halo around developing colony) represented the amount of enzyme produced by the organism.

**5. CONTROL EXPERIMENTS**

The different control measures viz- antibiotics, fungicides and angiospermic sources(spices), were used for *in vitro* control of test organisms. The poisoned food technique was followed both in liquid and semisolid media (Nene and Thapliyal, 1966). Potato dextrose agar medium was used for plate method and Richard’s broth medium was used to analyze the morphological variation in growth as well as biomass in treated and control samples at, $28 \pm 1^\circ$C for 5 days incubation period. The efficacy of control measures were expressed as percent of radial growth and biomass over control, which was calculated using formula suggested by Vincent (1949). The pH of the media was maintained at 5.

\[
\frac{\text{Growth in control} - \text{growth in treatment}}{\text{Growth in control}} \times 100
\]

\(\%\text{Growth inhibition} = \frac{\text{Growth in control} - \text{growth in treatment}}{\text{Growth in control}} \times 100\)
i. Antibiotics

Three antibiotics viz- griseofulvin, tetracycline and norfloxacin with 0.01%, 0.02% and 0.03 % aqueous concentrations were selected to test out their effectivity against all the isolates.

ii. Fungicides

Bavistin, Jatayu and kitazin, with aqueous concentrations (0.01%, 0.02% and 0.03 %) were selected to control the growth of all the test isolates.

iii. Angiospermic Sources (Spices)

Five commonly used Indian spices (powder) with three different concentrations. of 0.02%, 0.04%, 0.06% were selected viz- Syzygium aromaticum (Clove), Curcuma longa (Turmeric), Myristica fragrance (Nutmeg), Ferula asafoetida (Asafoetida) and Nigella sativa (Blackcumin). The antifungal mechanism of spices was studied under two categories i.e., by evaluating their biomass in Richard’s broth and by measuring their radial growth on PDA.

a) Growth on broth media for spices:

Richard’s broth was prepared with 0.02 %, 0.04 %, 0.06 % of spice concentrations. The medium was thoroughly shaken for uniform mixing of the extract. 25 ml of this medium was poured into conical flask and each flask was inoculated with mycelium of 5mm size disk cut out from periphery of actively growing culture control were also maintained by growing pathogen on broth media.
without spices. These flasks were incubated at 28 ± 1°C for five days and biomass of pathogens was taken.

b) Growth on semisolid media for spices:

To study the antifungal mechanism of spices on PDA media the poison food technique was used (Nene and Thapliyal 1982). For this powdered spices were added to PDA media to get 0.02%, 0.04%, 0.06% concentration. The medium was thoroughly shaken for uniform mixing of the extract. 20 ml of poisoned medium was poured into each of sterile petriplates. Each plate was inoculated with mycelium of 5mm size disc cut out from actively growing culture. Control were also maintained by growing the pathogen on PDA plates without spices. The plates were inoculated at 28± 1°C for 5 days and radial growth was measured, when maximum growth occurred in plates.