CHAPTER II

MATERIALS AND METHODS
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SCREENING OF FUNGI FOR THEIR ABILITY TO PRODUCE PLANT GROWTH REGULATORY METABOLITES IN THE CULTURE FILTRATE

About 80 fungi were tested for their ability to produce plant growth regulatory metabolites in their culture filtrates. Some of these test fungi were isolated from various soil samples and others were collected from microbiological research laboratory, Department of Botany, University of Saugar, Sagar (M.P.).

Each of the test fungus was purified by single spore/hyphal tip isolation method and stored in the slants.

All the fungi were maintained in Czapek's dox medium and monthly subcultures were regularly made for the maintenance of cultures in good active condition.

For the secretion of plant growth regulatory fungal metabolites, each fungus was grown in the Czapek's broth containing following ingredients.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>30 gm</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>3 gm</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>1 gm</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>500 mg</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>500 mg</td>
</tr>
<tr>
<td>Ferrous sulphate ((7\text{H}_2\text{O}))</td>
<td>Trace</td>
</tr>
</tbody>
</table>
Tryptophan : 200 mg
Distilled water : 1000 ml
pH : 6.5

Medium was sterilized in an autoclave at 15 lb/sq inch pressure for 15 - 20 minutes.

The test organism was inoculated with 8 mm disc of the fungal culture from petridishes in 250 ml Erlenmeyer flask containing 50 ml of broth medium. Inoculated broths were kept for 10 days at 28°C in the incubator.

After harvesting, the cell free culture filtrates were obtained by filtering the contents with bacteriological filters in sterilized conditions. The clear solution thus obtained was tested for the plant growth regulatory responses on avena sections.

BIO ASSAYS:

Avena Coleoptile straight growth test

Avena coleoptile straight growth test (Nitsch and Nitsch, 1956) was used for the present screening. Seeds of avena variety 'Victory' were used for this purpose. Surface sterilization of these seeds was carried out by treating them with 0.2% mercuric chloride solution for 5 minutes. Then the seeds were washed for ten to fifteen times with sterilized distilled water. These seeds were grown in wide-mouth
test tubes at 28°C in dark till the coleoptiles had attained the length of 25 - 30 mm. The coleoptiles were then harvested and from each of these the apical portion of 2 - 4 mm was removed. The section of 4 mm length were then cut out from each coleoptile cylinder. These 4 mm coleoptile sections were then transferred to sterilized distilled water containing traces of magnesium sulphate and kept for two to three hours.

Ten uniform avena sections were transferred to the sterilized petriplates containing 20 ml of culture filtrate of the test fungi. Uninoculated medium was used as a control. All operations were carried out in dark under weak red light. After 36 hours of incubation at 28°C in dark, the sections were observed. Final length of each section was measured and mean of each set determined. Results have been expressed as percentage change over control. Following formula was applied for the calculation of percent change in the growth of avena sections.

\[
\frac{\text{Length of treated avena section} - \text{Length of avena section in control}}{\text{Length of avena section in control}} \times 100
\]

= Percent change in growth over control.
Pea seedling's growth test

Out of the total 80 fungi already screened by using avena straight growth test, 32 fungal species were selected for investigating the effects of their culture filtrates on the growth and morphology of pea seedlings. The selection was mainly based on their relative performance in the screening test and all the fungi found capable of causing strong stimulatory or strong inhibitory effects on the growth of avena sections were selected for secondary screening.

The culture filtrates were prepared as already described earlier. The cell free filtrates were stored in refrigerator until used.

For bioassay purpose pea seeds of variety T 165 were obtained from J.N.K.V.V. Jabalpur (M.P.). The seeds were first surface sterilized with 0.2% HgCl₂ solution for 5 minutes. These seeds were then washed repeatedly 8-10 times with sterilized distilled water. The surface sterilized seeds were sown aseptically in 500 ml, capacity Erlenmeyer flasks. Each flask contained 100 ml of 2% plain agar. In each 5 ml of cell free culture filtrate of test fungus was added after solidification of sterilized agar medium. Experiments were done in duplicates. The control sets contained 5 ml of respective uninoculated broth in place of fungal filtrate.
These flasks were then incubated at 28°C for 7 days under florescent light.

Various parameters including mainly the length of shoots, length of roots, number of nodes, number of secondary roots, diameter of the shoot below the first internode, length of first, second, third and fourth internodes, number of leaf premordia and other growth abnormalities if any were taken into consideration for ascertaining the effect of fungal metabolites on the growth and morphology of pea seedlings. The recorded data have been presented in Tables 10 - 15 and Fig.10 - 15.

**EXTRACTION, PURIFICATION AND IDENTIFICATION OF ACTIVE GROWTH FACTORS**

On the basis of good performance with regards to the growth stimulatory activity of culture filtrates, about 12 test fungi were selected for detailed biochemical analysis. For this study, culture filtrates were obtained as described earlier. From the culture filtrates extra cellular auxin and gibberellins like compounds were extracted, following the procedure of Phelps and Sequeira (1967) and Katznelson and Cole (1962, 1965) and Cross et al. (1963).

**Extraction of Indoles**

Indole compounds were separated into two parts:-
(i) Ether soluble acidic fraction

(ii) Ether soluble basic fraction.

For extraction, the pH of the culture filtrates (10 ml) was made up to 8.0 with saturated sodium bicarbonate solution and the basic fractions were removed by extracting with peroxide free ether. The peroxide free ether was prepared according to the procedure of Reimers (1943).

The pH of the aqueous layer was adjusted to 3.0 by adding 1 N HCl and acidic derivatives were removed by extraction with the peroxide free solvent ether (diethyl ether).

The two fractions were then evaporated till dryness, and residue of each was finally dissolved in 0.5 ml of methanol.

**Extraction of Gibberellins**

For the extraction of gibberellins, the method described by Cross et al. (1963) was used. Free gibberellins were extracted from the culture filtrates of fungi. The culture filtrate (15 ml) was acidified to pH 2.5 and extracted with ethylacetate. The aqueous phase was re-extracted with 1% sodium hydrogen carbonate solution and again re-extracted with ethylacetate.
The extract was evaporated at room temperature to dryness. The crystals obtained were used for further studies.

For the identification of extracted and crystalised materials, thin layer chromatography was used. Thin layer plates of silica gel G were made with the help of T.L.C. preparator. From each of the concentrated fractions, 50 µl samples were spotted on the TLC plates.

Indole-auxins like compounds were separated with following solvent system:

Isopropanol: ammonia: water (80:5:15)

Gibberellin like compounds were separated with ethyl-acetate: chloroform: acetic acid (15:5:1).

The plates were kept in the solvent system and the solvent front was allowed to run up to 2/3 of length of the TLC plate. The plates were then taken out and dried at room temperature until all the solvent had evaporated. For each sample four plates were spotted, and out of these four, one was developed separately with Salkowski's reagent and the second plate with concentrated sulphuric acid with 5% ethanol, for the location of Indole and gibberellins respectively (Salkowski reagent was prepared in the proposition of 50 part of 35% HClO₄ (perchloric acid) and 1 ml of 0.5 M Ferric chloride). The sprayed plates were then dried for 15 minutes
in chromatographic oven at 60°C in case of indole-auxins, and at 110°C in case of gibberellins. Gibberellins were detected in ultraviolet light (254 nm). All the gibberellins give fluorescence properties in UV light.

Indole compounds after heating the TLC plates gave coloured spots with the Salkowski's reagent.

Rf values and colours were recorded for indole and gibberellin like compound (Tables 16 - 18). In each case data were compared with available authentic samples prepared simultaneously by the same procedure.

**Estimation of Indoles**

The corresponding spot's of unsprayed TLC plates were then eluted and used for the purpose of quantitative and biological analysis of the indole compounds.

Elution were made with 3 rinses of 2 ml portion of methanol allowing 5 minutes for each rinse.

The methanol elution was taken to dry ness and the residue was dissolved in 1 ml of diethyl-ether in the test tube. To this was added 2 ml of Salkowski's reagent, and reaction mixture was incubated for 30 minutes at room temperature. The coloured samples were then read at 530 nm, using Zeiss Jena Spekol, against a reagent blank. The amount of indole
compounds in each sample was then calculated from the standard curve prepared for IAA under similar conditions.

From similar sets of TLC plates, the unsprayed regions of corresponding spots were used for the measurement of their biological activity. For this purpose, the corresponding regions of unsprayed spots were dissolved in 10 ml of 2% buffered (pH 5.1) sucrose solution and directly used for avena bioassay test.

**Estimation of Gibberelline**

The amount of gibberellic acid produced was estimated spectrophotometrically by the method described by Holbrook et al. (1961). It is based on the conversion of gibberellic acid into gibberellic acid, followed by the measurement of the absorption of the latter compound at 254 nm. This method has been shown to be specific for gibberellic acids.

Following method was adopted for gibberellic acid estimation in culture broth.

**Reagents**

(i) Dilute HCl 30% : 300 ml of conc. HCl (specific gravity 1.18) was diluted up to 1 litre with distilled water in a volumetric flask and cooled to 20°C.

(ii) Dilute HCl 5% : 100 ml of conc. HCl (specific gravity 1.18) was diluted up to 2 litres with distilled water.
(iii) Standard gibberellic acid solution.

(iv) Zinc acetate solution: 21.9 gm of crystalline zinc acetate (Zn(CH₃COO)₂·2H₂O) was dissolved in about 80 ml of distilled water, 1.0 ml of glacial acetic acid was added and the volume was made up to 100 ml with water.

(v) Potassium ferrocyanide solution: 10.6 gm of analytical reagent grade potassium ferrocyanide was dissolved in 100 ml of water.

Calibration graph was prepared according to the procedure described by Holbrook et al., 1961.

15 ml of culture filtrate was transferred to the test tube and 2 ml of zinc acetate solution was poured in. After two minutes, 2 ml of potassium ferrocyanide solution was added. Precipitation was separated with the help of centrifuge. From each sample 5 ml of clear solution was taken in two tubes. One tube containing 5 ml culture filtrate was treated with 30% HCl and second tube was preserved for blank reading with 5% HCl. 30% HCl containing volumetric flask was kept soon after treatment in BOD chamber at 20°C for 75 minutes. Blank reading was taken immediately after treatment with 5% HCl at UV range (254 nm) in spectrophotometer UV-2 Carlzeiss model.

Actual amount of gibberellic acid was calculated as follows:
O.D. of samples - O.D. of blank = Amount of GA present in culture filtrate.

Bioassay of Gibberellins

Bioassay of total gibberellins in the extract was carried out by barley endosperm test according the procedure described by Palag (1965) and Combe et al. (1967).

Surface sterilisation of barley seeds was done with the help of 0.2% HgCl₂ solution for 2-5 minutes. This was to prevent the contamination by microorganisms which may produce enzyme affecting amylase activity of barley. All these operations were carried out in aseptic conditions. Seeds were suspended in the sterilized distilled water for over night. The seeds were cut into two half and embryonic portion was separated from the endosperm. Culture extract was sterilized with the help of seitz filter. Two ml of this sterilized extract was poured in small sterilized petri plate, and 10 halves of barley endosperms were added in this solution. The plates were then kept for incubation at 30°C. After 48 hours of incubation, the clear solution was removed for sugar estimation.

Colorimetric estimation of reducing sugars

Two ml of test solution was mixed with 2 ml of Nelson's reagent. The reaction mixture was then boiled for exactly
20 minutes on a hot water bath. Tubes after removing from hot water bath were immediately transferred to an ice cold water bath to stop the reaction. Then 2 ml of ars enomolybdate reagent was added and tubes were shaken vigorously, until no further deep blue colour developed; then the coloured solution was transferred to spectrophotometric tubes and read at 540 nm. Sugars were determined in term of the optical density of the solution. The quantity of reducing sugars was calculated by referring to a previously prepared standard curve for glucose.

**Solutions and reagent used**

(i) Nelson's reagent

(a) Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃</td>
<td>12.5 gm</td>
</tr>
<tr>
<td>Pot. sod. tartarate</td>
<td>12.5 gm</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>100.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500 ml</td>
</tr>
</tbody>
</table>

(b) Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO₄·7H₂O</td>
<td>7.5 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 ml</td>
</tr>
<tr>
<td>H₂SO₄ (Conc.)</td>
<td>One drop</td>
</tr>
</tbody>
</table>

Solution A and B are stored separately and 25 ml of solution (A) should be mixed with 1 ml of solution (B) just before use.
(ii) Arsenomolybdate solution

(a) Solution A

\[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}\] 25 gm
Distilled water 450 ml
\[\text{H}_2\text{SO}_4\] 21 ml

(b) Solution B

\[\text{Na}_2\text{HAsO}_4\cdot 7\text{H}_2\text{O}\] 3 gm
Distilled water 25 ml

Solution A and B are mixed and stored in a brown bottle.

Data were recorded in Table 19 and the results were then compared with standard graph of reducing sugars released by known amount of gibberellic acid.

EFFECT OF VARIOUS CULTURAL AND NUTRITIONAL CONDITIONS ON THE PRODUCTION OF AUXINS AND GIBBERELLINS

On the basis of previous results seven fungi were selected for further detailed study for the production of indole auxins and gibberellin like compounds in their culture filtrates. For the production of auxin Fusarium moniliforme strain I, Botryodiplodia theobromae strain K52M, B. theobromae strain KB, Curvularia lunata strain S and Penicillium notatum were employed. While Fusarium moniliforme strain II, Botryodiplodia theobromae strain K52M, Curvularia lunata strain N and Penicillium notatum were used for the production of gibberellins.
These fungi were grown in Czapek's broth medium as already described earlier. Various factors including incubation period, influence of different media, carbon sources, nitrogen sources were investigated. Different substances were added in different compositions where ever required to the basal medium (Czapek's broth before autoclaving).

Carbohydrates:

Sucrose of the basal medium was replaced by equal amount of following carbohydrates:

Lactose
Maltose
Glucose
Arabinose
Fructose
Starch

Nitrogen sources:

Sodium nitrate of the basal medium was replaced by equal amount of following nitrogenous substances.

Peptone
Ammonium di-hydrogen phosphate
Ammonium nitrate
Ammonium sulphate

The determination of indole and gibberellin like substances were carried out by the same spectrophotometric
method as described earlier. In each case dry mycelial weight of 100 ml culture broth was also recorded.

For investigating the effect of culture media the test fungi were grown in following media. Each medium was supplemented with 0.02% tryptophan.

1. Czapek's broth

\[
\begin{align*}
\text{NaNO}_3 & \quad 3.0 \text{ gm} \\
\text{K}_2\text{HPO}_4 & \quad 1.0 \text{ gm} \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.5 \text{ gm} \\
\text{KCl} & \quad 0.5 \text{ gm} \\
\text{FeSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.01 \text{ gm} \\
\text{Sucrose} & \quad 30.0 \text{ gm} \\
\text{Distilled water-1000 ml, pH 6 to 6.5}
\end{align*}
\]

2. Richard's broth

\[
\begin{align*}
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.25 \text{ gm} \\
\text{KH}_2\text{PO}_4 & \quad 5.0 \text{ gm} \\
\text{KNO}_3 & \quad 10.0 \text{ gm} \\
\text{Sucrose} & \quad 10.0 \text{ gm} \\
\text{FeCl}_3 & \quad \text{Trace} \\
\text{Distilled water} & \quad 1000 \text{ ml} \\
\text{pH} & \quad 5.5
\end{align*}
\]

3. Glucose asparagine broth

\[
\begin{align*}
\text{Asparagine} & \quad 1.0 \text{ gm} \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 1.0 \text{ gm}
\end{align*}
\]
KH₂PO₄  5.0 gm
FeCl₃  0.01 gm
Glucose  10.0 gm
Distilled water  1000 ml
pH  5.5

Soma et al. broth medium

Glucose  40 gm
Sucrose  40 gm
Glycerol  20 gm
KH₂PO₄  0.5 gm
MgSO₄·7H₂O  0.5 gm
K₂SO₄  0.2 gm
NH₄NO₃  0.5 gm

Trace element solution  2 ml
Distilled water  1000 ml
pH  4.5

Trace element solution contained FeSO₄·7H₂O 0.1 gm, CuSO₄·5H₂O 0.015 gm, ZnSO₄·7H₂O 0.16 gm, MnSO₄·7H₂O 0.01 gm, (NH₄)₆Mo₇O₂₄·4H₂O 0.01 gm dissolved in 100 ml of distilled water).

Emerson's Yps medium

Beef extract  4 gm
Peptone  4 gm
Sodium chloride  2.5 gm
Yeast extract  1 gm
Glucose  10 gm
Distilled water  1000 ml
Gibberellic acid fermentation

Large scale trials were carried out in 10 litre capacity jar fermentors of M/S Emenze (Model F-3). The fermentors had the facilities for adjusting the agitation and aeration as desired (Plate 5-7).

Two fungi were selected for this study, i.e. Botryodiplodia theobromae and Gibberella fujikuroi.

Nine litre of Czapek's broth with following composition was prepared for each fermentor vessel.

Sucrose \(300 \text{ gm}/10 \text{ litre}\)
Sodium nitrate \(30 \text{ gm}/10 \text{ litre}\)
Potassium dihydrogen phosphate \(10 \text{ gm}/10 \text{ litre}\)
Magnesium sulphate \(5 \text{ gm}/10 \text{ litre}\)
Ferrous sulphate Trace
Distilled water 10 Litres

To investigate the effect of glucose the second run of the fermentors was also done with glucose containing medium for both the fungi. The composition of medium was same excepting glucose was added in same amount in place of sucrose.

In all the three fermentor vessels 9 litre of medium was added and sterilized for 60 minutes at 15 lb pressure in steam autoclave. In separate flasks 10% silicon antifoam was also sterilized. After coaling, the fermentor vessels
were kept in suitable conditions. Agitation of 300 rpm and 0.25 v/v air supply were maintained. Temperature was adjusted to 30°C. 100 ml of silicon antifoam was added in each fermentor so as to suppress the foaming during fermentation.

Inoculum was prepared separately in Czapek's broth and one litre of inoculum was added in each fermentor vessel.

Fermentors were kept running for 10 days continuously. In all four runs of the fermentors were done for two fungi and two carbon sources. During fermentation the pH changes were also recorded. Samples were analysed after every 24 hours for gibberellic acid contents using Holbrook's method as described earlier. Data were recorded in Tables 28 to 31.

EFFECT OF PHYSICAL AND CHEMICAL MUTAGENEO ON THE PRODUCTION OF GIBBERELLIC ACID BY TEST FUNGI

Gibberella fujikuroi strain Gf, and Botryodiplodia theobromae strain K52M were taken for this study. Methods were followed as described by Imshenotsky and Ulyanova (1962a,b) and Clowes and Keys (1968).

For mutation work mycelial suspension in case of G. fujikuroi and spore suspension in case of B. theobromae
filtered through a sterile plankton net and diluted with sterile distilled water was made up to $10^{-3}$ dilution for ultraviolet treatments. 10 ml of suspended material was taken in 12 sterilized petriplates in duplicates. The bacteriocidal ultraviolet lamp (range 2600 Å) was used for irradiation.

The time of exposure was varied from 5 to 30 minutes. After exposing, the petriplates were covered with black thick paper and kept for over night in the low temperature (0°C).

0.1 ml of treated fungal suspension was plated in petriplates containing PDA. Inoculated petriplates were kept in incubator at 28°C. The colonies of untreated and treated samples were counted and recorded in the Table 32 and 36. Each colony was transferred to PDA slants.

However for experimental use all of the 8 colonies of G. fujikuroi and 3 of B. theobromae survived colonies of 15 minutes exposure were taken for testing of gibberellic acid production because this period of exposure was found to be more effective in causing mutagenic effects. Gibberellic acid production was estimated by the same Holbrook's (1961) method as already described earlier.

For chemical mutagenesis the suspension of fungal mycelia and spores suspension were taken as described in case
of previous experiment. Different concentrations of N-methyl-
N' -nitro-N-Nitrosoquianidine (NTG) were prepared in sterilized
distilled water. One ml of fungal suspension and 9 ml of
NTG solution of different concentrations were shaken for
10 minutes. Suspensions were then centrifuged several times
after repeated washings with distilled water. The washed
mycelia were suspended in 10 ml of distilled water containing
tween 80. 0.1 ml of this fungal suspension was plated in
petri plates containing PDA and incubated at 28°C. Colonies
were counted and recorded in the Table 34 and 37. For
fermentation work however three colonies of 10,000 ppm were
taken for both test fungi.