MATERIAL AND GENERAL METHODS

I) **Source of cultivars:**

Twenty nine cultivars of nine oil seeds were obtained from the Oil Seed Research Station (Marathwada Agricultural University, Latur, Maharashtra). The seeds were stored at \(22^\circ C\) in cloth bags and used whenever needed. The following cultivars were investigated for seed mycoflora.

1) **Arachis hypogea:** Linn
   
   cv k-4-11; cv No. 33; cv SB-IX;
   
   cv k-145-12; cv Kopargaon-1; cv Punjab-1.

2) **Helianthus annus:** Linn
   
   cv EC-68413; cv EC-68414; cv EC-68415;
   
   cv EC-69874; cv Latur local;
   
   cv Sunrise selection.

3) **Carthamus tinctorius:** Linn
   
   cv N-7; cv N-62.3

4) **Ricinus communis:** Linn
   
   cv Aruna NPH-1; cv S-248-2

5) **Glycine max:** Linn
   
   cv EC 2856; cv EC 9308; cv Clark 63; cv Bragg;
   
   cv Lee; cv Early pleacin; cv EC 16692;
   
   cv Kalitur EC 18309; cv Kalitur EC 574.

6) **Guizotia abyssinica:** Cass
   
   cv 12.3

7) **Sesamum indicum:** Linn
   
   cv N-85
8) *Linum usitatissimum*: Linn
   cv S-36

9) *Brassica juncea*: (L)Czern & Coss
   cv Local

II) **Assessment of seed mycoflora:**

   Three methods were used for isolation of externally and internally seed-borne fungi.

   i) **Plating seed washates:**

   Ten seeds of each variety were washed in 20 ml of sterile water and 1 ml of seed washings obtained thus, was plated on GNA. Fungi developed within 3 days were immediately transferred to PDA and/or GNA slants for further study.

   ii) **Standard blotter test:**

   Seeds were equidistantly spaced on moist sterile blotters without any pre-treatment of disinfectant in petriplate moist chambers. 10 petriplates of 9" diameter each containing 10 seeds were incubated at \(25 \pm 2^\circ\text{C}\) for four days. Observations were made for fungi appearing on seeds every 24 hours and growth, was carefully transferred to PDA slants for further studies. A minimum of 400 seeds were observed in each case.

   iii) **Agar plating:**

   Seeds were disinfected externally by treating with 10% sodium hypochlorite solution for 10 minutes. Such disinfected seeds were cut aseptically and pieces were plated on GNA plates. Colonies which developed during three days were picked up and maintained on PDA and/or GNA slants.
III) Media:

The following media were used in this study.

i) Potato dextrose agar (PDA).

Potato slices 200 g; Dextrose 20 g; Agar 20 g;
Tap water 1000 ml, pH 5.5.

ii) Glucose nitrate agar (GNA):

Glucose 10 g; KNO₃ 2.5 g; KH₂PO₄ 1 g; MgSO₄ 0.5 g;
Agar 20 g; Distilled water 1000 ml.

iii) Czapeck Dox's Agar (CZDA)

K₂HPO₄, 1g; NaNO₃, 2 g; MgSO₄·7H₂O, 0.001g; KCl, 0.50g;
FeSO₄·7H₂O, 0.001g; Sucrose, 30 g; Agar, 15 g &
Tap water 1000 ml.

iv) Seed paste suspension (SPS)

10 gms of seeds were crushed to a homogenous paste
in a little quantity of distilled water and then it was made
upto 1000 ml. This suspension was directly used as a medium.

v) Seed Paste Agar (SPA)

10 gms of seeds were crushed to a homogenous paste
and then made up to 1000 ml. 20 gms of agar agar was
added to it.

vi) Seed paste nutrient agar (SPNA)

Seed paste 10 g; KNO₃, 2.5g; KH₂PO₄, 1 g;
MgSO₄, 0.5g; Agar 20 g; Distilled water 1000 ml
vii) **Seed paste peptone agar (SPPA)**

Seed paste, 1; Peptone, 0.25; Agar agar 2.0
(all quantities are % w/v).

All the media were sterilized under 15 lbs pressure for 20 minutes. 5 ml of medium was used for preparing slants, 15 ml for pouring plates and 25 ml in experiments with liquid media.

IV) **Culture vessels:**

All the glassware was thoroughly cleaned with acid dichromate cleaning mixture first, then with hot, tap water and rinsed with distilled water and dried completely before use. Petridishes were sterilized in the oven at 160°C for two hours.

V) **Isolation, identification & preservation of fungal cultures:**

For identification of the fungi associated with seeds semipermanent slides were prepared at appropriate stage of growth of the fungus and the measurements of hyphae, conidiophore, conidia etc. were taken. These were then compared with those recorded earlier in various sources and identification was confirmed.

Cultures were preserved on PDA, GNA, & CZDA slants for further study. Assuming possibility of loss of lipase producing capacity of the fungi during continuous sub-culturing, fresh isolations were made directly from the stored seed periodically and used. Seed herbaria were maintained by preserving the seeds with the fungus on filter paper with the help of a transparent adhesive tape.
All the cultures were incubated at 22 ± 2°C and subcultures were made every 21 days.

VI) **Inoculation:**

2.5 ml of spore suspension from 5 day old culture was added to 25 ml medium in Erlenmayer flasks. In every case the spore suspension of all the isolates under experiment was standardized to contain 20 to 30 spores per microscopic field.

While inoculating agar media a loop full of standardized spore suspension was placed in the centre of the plates.

VII) **Spore germination studies:**

i) **Seed exudates:**

Seeds were surface sterilized and washed several times in sterile distilled water. 10 seeds were then spaced out on thin pads of moistened cotton in petriplates previously sterilized. After three days seeds were removed and exudates squeezed out of the pad. They were made up to 10 ml by addition of sterile water and used in germination studies.

ii) **Seed extracts:**

5 g of seeds were crushed to homogenous paste and then made up to 500 ml. The suspension was directly used in spore germination studies.
iii) **Assessment of germination:**

Spores from 5 day old PDA slant culture were used for studying spore germination. The spores from the culture were brushed off in 2 ml of distilled water or sterile test solution (seed leachets; seed extracts), hanging drop slides incubated at 25 ± 2°C in petriplate moist chambers were directly observed under the microscope and the percentage germination of spores was recorded. Spores were considered to have germinated when their germ tube lengths exceeded their size (Manners & Hossain, 1963). Five hundred spores from different microscopic fields were counted in each observation.

VIII) **Analysis of seed exudates:**

1) **Proteins & lipoproteins:**

Analysis of seed exudates for proteins and lipoproteins was carried out by Disk polyacrylamide gel Electrophorasis.

In this procedure electrophorasis is carried out in small glass tubes in the vertical dimension. Because of the sharpening effect achieved by a discontinuous buffer
system, the proteins and lipo-proteins are separated in narrow bands which appear as discs on staining. The method is adopted from Zweig & Whitaker (1967) with slight modification.

Electrophoresis was carried out in small glass tubes in vertical dimension. The components of the system were as follows:

The anode is at the bottom in the vertical arrangement.

(A) 7% running gel, stacks at pH 8.3, runs at pH 9.5.

Stock solutions:

(a) 48 ml. 1 N HCl, 36.6 gm Tris, 0.23 ml TEMED, and water to 100 ml, pH 8.9

(b) Approximately 48 ml 1 N HCl, 5.98 gm Tris, 0.46 ml TEMED, and water to 100 ml, pH 6.7. The pH is adjusted to exactly pH 6.7 with the 1 N HCl.

(c) 28.0 gm acrylamide, 0.735 gm bisacrylamide and water to 100 ml.

(d) 10.0 gm acrylamide, 2.5 gm bisacrylamide, and water to 100 ml.

(e) 4 mg riboflavin to 100 ml with water.

(f) 40 gm sucrose to 100 ml with water.

Working solutions prepared from stock solutions:

(a) Running gel solution a: - 1 part stock (a), 2 parts stock (c), and 1 part water, pH 8.8 to 9.0

(b) Running gel solution b: 0.14 gm ammonium persulfate per 100 ml with water (prepared fresh). To prepare the running gel combine equal parts working solutions
(a) and (b)

(c) Spacer and sample gel solution: 1 part stock (b), 2 parts stock (d), 1 part stock (e), and 4 parts stock (f), pH 6.6 to 6.8.

(d) 10 x buffer for electrode reservoirs: 6.0 gm Tris, 28.8 gm glycine, and water to 1 liter, pH 8.3 Dilute 10-fold before use. In some separations it is preferable

The apparatus was made of Acrylic plastic sheets of 6"x6"x6" size with 6 equal distant holes of appropriate diameter (to hold tubes) drilled in the bottom of the upper reservoir vessel. The gel containing tubes were fitted by means of rubber stoppers in the holes. Small platinum electrodes of No. 30 gauge were used. Gel containers - pyrex glass tubes - were cut to uniform length. The sample gel, running gel & spacer gel were then prepared. The 3 ml sample was applied on the top of the spacer gel in tubes held in perfectly vertical plane. Electrophoresis was carried out after the gel was set, at 1.5 mA per tube until the marker dye had nearly reached the bottom. This took 1 to 2 hrs. 12 ± 1°C temperature was maintained constant. The tubes were then removed, gel columns expelled under water with syringe and stained

For proteins gel was immersed in 0.001% bromophenol blue solution at least for an hour. It was then removed and rinsed with distilled water, bands of protein become evident on the disk.
For lipoproteins one gm of Amido black dye was dissolved in 100 ml of 7%, Acetic acid. The gel was immersed in this solution for 12 hrs. It was then removed and rinsed with 7% acetic acid.

Schematic drawing indicating approximate width & intensity of the bands of the gel was carried out in order to document the results.

ii) Lipid degradation products: For the detection of monoglyceride, diglyceride, triglyceride, free fatty acids, free & esterified cholesterol and phospholipids during the seed deterioration - a method adopted from Marzo et al (1971) was used.

Fifty gram silica gel was stirred vigorously with 100 ml of doubly distilled water and/sprayed in a layer 0.5 mm thick on to 5 x 20 cm plates. The plates were air dried, stored in a desiccator & were activated at 110°C for 30 minutes before use.

Seeds were crushed & directly used as sample. 0.5 ml sample was applied at equal distance on plate. The developing mixture was petroleum ether, diethyl ether, methanol & acetic acid in 90:7:2:0.5 v/v. The plates were then placed in a developing mixture & it was allowed to flow up to 20 cms from the place of sample application. Plates were removed and dried. The lipid containing bands made visible by exposing the plates to iodine vapours, traced on a paper and reproduced.
iii) **Amino acids:**

Amino acids in the exudate were detected chromatographically. About 0.005 ml of the sample of seed exudate along with known amino acids applied 2 cms apart to a Whatman filter paper No. 1. Butenol:Acetic acid:water mixture in the ratio 4:1:5 was used as a solvent for detection of amino acids. The lower layer of the solvent was placed at the bottom of the chromatographic cabinet and the filter paper was left in the cabinet for 6 hours to equilibrate. The upper layer of the solvent was then poured in to the trough and allowed to irrigate the paper for eight hours. The paper was then removed and dried in the air and sprayed with ninhydrin.

iv) **Reducing sugars:**

Using the same solvent system chromatograms were developed with Benzidine reagent.

v) **Fatty/organic acids:**

Butenol:formic acid: water in the proportion 10:2:5 was used as solvent. Chromatograms were developed with bromo cressol green & bromo thymol blue.

vi) **Phenols:**

Using the above solvent system chromatograms were developed with Folin Deni's reagent.
IX) **Seed germination studies:**

i) **Collection of fungal metabolites:** Fungi which were used in these studies were grown in SPS for five days. Culture filtrates were obtained by filtration through Whatman filter paper No. 42. The filtrates from six flasks were pulled together and made up to 150 ml with sterile distilled water.

ii) **Preparation of spore suspension:** In studies where spore suspensions were used the fungus was grown as lawn culture on SPA plates (9 cm diam) for five days. Sterile water was added to each plate and spore suspension from two plates was made up to 150 ml with sterile distilled water.

iii) **Seed germination test:** 100 seeds were added to 150 ml of either metabolite or spore suspension in 500 ml flasks. The flasks were shaken intermittently for 24 hrs and seeds were then spaced out on blotters in petriplate moist chambers and percentage germination was recorded over the required period.

X) **Effect of metabolites of seed borne fungi:**

i) **Radicle growth:** 10 germinated seeds were equidistantly spaced on moist blotter previously dipped in fungal metabolites. Sufficient metabolites, so as to keep the blotter wet was added to these blotters twice a day.

After every 24 hours increase in the length of radicle was measured in mm.
ii) **Alterations of respiration:** Alteration of respiration in seeds following treatments by fungal metabolites was measured by a method following Robertson (1938). The oxygen consumption was measured by Warburg Manometer using 7 manometers at a time at 29 ± 0.5°C temperature. Oxygen consumption is expressed in tables as ml.02 /gm wet wt/minute.

iii) **Leakage of Electrolytes:** Five grams of seeds were allowed to imbibe fungal metabolites (50 ml of metabolites) for 24 hours. Seeds were then removed, washed with distilled water and then with conductivity water. Seeds were then placed in 50 ml of conductivity water in 150 ml. Conical were flask/intermittently shaken. The liquid was removed after 2, 4, 6, 8 hrs and observed for loss of electrolytes with the systronics - 305 conductivity bridge (Cell constant 0.97).

iv) **Leakage of proteins:** Proteins were measured by Biuret method. 1.50 gms of CuSO₄·5 H₂O and 6 gms of Na.K.tartarate (NaKC₄·H₂O₆) were dissolved in 500 ml of water. With constant swirling 300 ml of 10% NaOH (prepared from stock, carbonate free 65 to 75% NaOH solution) was added. The solution was made upto 1 liter with water and stored in a paraffin lined bottles.

To 1.0 ml of solution 4.00 ml of biuret reagent was added by swirling and allowed to stand for 30 minutes at room temperature and optical density was read at 550 micro μ with systronics colorimeter, type 101. 4 ml of biuret reagent + 1.0 ml of water served as control.
The concentrations of protein in the sample was obtained by reference to a calibration curve established with a clear solution of Egg-albumin.

v) **Release of carbohydrates:** Five gram batches of seeds in cheese cloth bags were allowed to imbibe toxic metabolites (in 50 ml of c.f.) for 24 hours. Seeds were then washed with distilled water and later with conductivity water. Cheese cloth bags were then suspended in 50 ml of conductivity water in 150 ml conical flask and were shaken intermittently. The seeds were removed from the flask for 2, 4, 6 hrs and the liquid was used to determine total loss of carbohydrates from imbibed seeds. Total carbohydrates in the ambient solutions were then determined by Anthrone method (Oser, 1965).

To 5 ml of test solution 10 ml of (0.2% in 95% H$_2$SO$_4$) Anthrone reagent was added. The tubes covered with glass marble were heated in a boiling water bath for 10 minutes. The colour developed was then read at 620 micro m using systromics type 101, colorimeter and mg/ml carbohydrates were obtained from standard graph. 5 ml conductivity water plus 10 ml Anthrone reagent served as control.

vi) **Loss of fatty acids:** 2.5 gms of seeds were placed in 25 ml of metabolites. After 24 hours seeds were removed and washed twice by distilled water. Seeds were then placed in 25 ml of distilled water for 24 hours. After 24 hours
10 ml of liquid was pipetted out in 150 ml conical flask and titrated with 0.05 N NaOH using phenol-phthalin as an indicator. Amount of fatty acids released from seed is measured in terms of 0.05 N NaOH required to neutralise the released fatty acids. (1 ml of 0.05 N NaOH = 1 unit).

In other experiment equal volume of 1% seed paste and metabolites were allowed to incubate. Total fatty acids released were measured after filtration through Whatman No. 42 in terms of NaOH units. Seed paste/served as control.

XI) **Synthesis of lipase:**

   synthesizing
Lipase/activity of selected fungi was measured in terms of growth on fatty substrate, total fatty acids released, saponification value and iodine number.

   i) **Growth on solid media:** Growth was measured in terms of colony diameter in mm. Nature and type of growth as well as the day of sporulation was recorded.

   ii) **Growth on liquid media:** Mycelial mat was carefully removed after appropriate incubation period. It was washed several times with water and then was dried to constant weight on Whatman No. 42. Cultures were grown for 7 days on SPS and filtrates collected after every 24 hours were used for determination of total fatty acids released, saponification value and iodine number.
iii) Total fatty acids released:

To 10 ml of culture filtrate equal volume of account oil was added as the substrate to this solution 10 ml of phosphate buffer (7 pH) was added. The reaction mixture was allowed to incubate for 24 hours at 24 ± 1°C. 10 ml of distilled water in place of culture filtrate served as control.

The reaction mixture was then titrated with 0.05 N NaOH using phenolphthalein as indicator, units of NaOH required to neutralize the fatty acids released indicated the lipase activity.

iv) Saponification value:

The saponification value of an oil is defined as the amount of potassium hydroxide in milligrams required to saponify (hydrolyse) one gram of the oil. The method is as described below:

2 gms of the oil was weighed out in a conical flask and to this 5 ml of culture filtrate was added and incubated for 24 hours. To this solution 5 ml of 0.2 N alcoholic KOH was added. The flask was then heated with reflux air condensar on water bath for half an hour. The flask was intermittently shaken throughout the boiling and after half an hour cooled and diluted to 25 ml in a measuring flask. It was then titrated against 0.1 N HCl using phenolphthalein as indicator.
A blank was run by refluxing 5 ml of the alcoholic KOH solution, 2 gms of oil and 5 ml of distilled water. Since saponification value is the amount of KOH in milligram to saponify one gram of a fat. It was calculated as

$$\text{Saponification value} = \frac{R - R_1 \times 0.9845 \times 5610}{1000 \times W}$$

Where:

- $W =$ weight of the oil
- $R =$ reading of 0.1 N HCl for blank
- $R_1 =$ Reading of 0.1 N HCl for the actual experiment.
- $R - R_1 =$ ml of 0.1 N HCl actually required \(=\) $R - R_1 \times 0.9845$ of 0.1 N KOH = Amount of alkali used up in terms of 0.1 N HCl.

v) Iodine number:

For determining iodine number Wijs method was used (Oser, 1965). Two grams of coconut oil was weighed out in a conical flask to this 5 ml of culture filtrate was added and allowed to incubate for 24 hours. To this solution 5 ml of Wijs solution was added. In a similar conical flask two grams of water was taken as a control. Stopper of/conical flask was replaced after moistening with potassium iodide and flasks were kept in the dark place for half an hour. 5 ml of 15% potassium iodide was then added to both the flasks. The mixture was then diluted with
25 ml of distilled water and the iodine liberated was determined by titration with 0.1 N sodium thiosulphate solution using starch as indicator

\[
\text{Iodine value} = \frac{(a - b) \times F \times 100}{W}
\]

Where

\[
\begin{align*}
a &= \text{reading for the blank experiment} \\
b &= \text{reading for the actual experiment} \\
f &= 0.01269 = \text{wt of iodine in gram which is equivalent to 1 ml of 0.1 N sodium thiosulphate} \\
W &= \text{weight of the oil used}
\end{align*}
\]