Chapter II

MATERIALS AND METHODS
A. Purification of the test pathogens:

All the test pathogens were obtained from Mycology Laboratory, Department of Botany, University of Sagar, Sagar. These pathogens were isolated in the laboratory from their respective hosts by various workers. The cultures were purified by single spore isolation and/or hyphal tip methods. Potato-Dextrose-Agar slants containing purified cultures were stored in refrigerator until used.

B. Procedure for respirometric studies:

The rate of endogenous mycelial respiration under different experimental conditions was measured by noting the rate of oxygen consumption by mycelial suspensions prepared from starved mycelial mats of all the four test organisms. Standard manometric techniques as described by Umbreit et al. (1964) were followed for these studies using Warburg's respirometer.

(i) Preparation of mycelial suspension:

The cultures of different test pathogens were freshly grown in sterilized Petriplates containing Potato-Dextrose-Agar medium. Mycelial discs from such freshly grown cultures were cut with
the help of sterilized cork borer of 4 mm. diameter, and these discs were transferred to sterilized 100 ml. Erlenmeyer flasks containing 25 ml. of the basal medium which contained dextrose, 50 g; malt extract (Difco), 50 g; K₂HPO₄, 0.012 M; KH₂PO₄, 0.02 M; NH₄NO₃, 0.0375 M; MgSO₄7H₂O, 0.009 M; and distilled water to make one liter (Darby and Goddard, 1950). The basal medium was sterilized by autoclaving (20 minutes at 120°C) separately in two sets, (1) the phosphates and (2) the nitrate and sulfate. This was done to avoid the formation of insoluble magnesium phosphates. The glucose and malt extract were sterilized by autoclaving with the nitrate and sulfate.

The inoculated flasks were then incubated at 25°C (± 1) as this temperature was found to be the most suitable one and after 5 days of incubation period in case of G. papayae, R. solani and F. oxysporum while after 4 days of incubation period in case of C. capsici, the thick mycelial mats were removed and washed thoroughly in 0.02 M phosphate buffer pH 7.0. These washed mycelial mats were then placed separately in beakers containing 40 ml. fresh buffer for 30-40 minutes at room temperature for the starvation of fungi by allowing them to use their endogenous reserves so as to get a better respiratory responses to the exogenous substrates. About 2 g. of starved mycelial mat of each fungus was fragmented in 50 ml. buffer in Waring blender for 30 sec. at full speed. These fragments were then washed several times on the refrigerated centrifuge. The supernatant was rejected and the mycelial suspension of required
dilution from the residue were made. This mycelial suspension was used for respirometric studies.

(ii) Preparation of respiratory flasks:

Warburg's flasks for experimental use were prepared by pipetting out 0.2 ml. of 20% KOH solution in the central well and 2.7 ml. of starved mycelial suspension in the main compartment using clean and thoroughly rinsed pipettes. A piece of filter paper made into wick was then inserted in the central well containing KOH to provide a large surface for the absorption of carbon dioxide. 0.3 ml. of the treatment solution prepared freshly for noting the effect of the substances was pipetted out into the side arm of the flask. In the control flask 0.3 ml. of buffer was placed in the side-arm in place of any treatment solution. Thermobarometer contained only 3 ml. of buffer (no treatment solution in the side-arm) in the main compartment. Required aliquots of mycelial suspension (2.7 ml.) were also taken out and dried in an electric oven adjusted at 80°C for 24 hours and then removed to a desiccator, cooled, and determined the constant dry weight of the mycelium so as to present the results on dry weight basis.

(iii) Determinations of oxygen uptake:

Thoroughly cleaned and dried manometers were filled with standard manometer fluid (Brodie's fluid). These manometers
were adjusted to 250 mm. levels on both the arms with stopcock open. Now, the respiratory flasks prepared as detailed above, were attached with their respective manometers with the help of rubber bands. The manometers with their flasks were then tightened on the Warburg's apparatus filled with water adjusted at 28°C. Care was taken to keep the flasks completely dipped inside the water. The flasks were now allowed to equilibrate for 10-15 minutes with constant shaking and stirring. The treatment solutions of the side arms were tapped into main compartment of the flasks and manometer levels were again adjusted, if required, to 250 mm. with stopcock open. The stopcock was now closed and the whole system was made air tight. Readings were taken at 15 minutes intervals for 120 minutes by noting the level of left arm of the manometer while level in the right arm was kept constant at 250 mm. Appropriate corrections were made for the changes in level of the manometric fluid if any, caused by changes in atmospheric pressure and temperature with the help of thermobarometer readings. The values so obtained in each case were then multiplied by 'flask constant' so as to convert the readings in ml of oxygen consumed per unit time. The 'flask constant' was determined for each pair of flask and manometer before starting the experiments by standard methods using mercury, following the instructions described by Umbreit et al. (1964).
C. Procedure for growth studies:

In second set of experiment the effects of different substances (which have been used as treatment solutions during respiratory determinations) were also determined on fungal growth for the purpose of correlation if any, between respiration and fungal growth.

For these studies each fungus was incubated in 100 ml. Erlenmeyer flasks containing 25 ml. of the basal medium with a required amount of the substance for the period of 15 days at 28°C. After harvesting, the mycelial mat was dried and weighed; the dry wt. of mycelium was determined after 15 days, since the organisms had attained more or less optimum dry weight by this time.