PART I

CHAPTER I

GENERAL INTRODUCTION
Organisms derive useful energy and intermediates for synthesis from oxidation of compounds in a process known as cellular respiration. The most frequently occurring form of cellular respiration is aerobic oxidation, which represents two broad phases: (1) glycolysis, or the conversion of substrate to pyruvic acid, and (2) the tricarboxylic acid cycle (TCA cycle), which further oxidizes pyruvic acid to carbon dioxide and water, utilizing oxygen as the final hydrogen acceptor. The most common glycolytic pathway is the Embden Meyerhof (EM) pathway, although the hexose monophosphate (HMP) pathway (known under a wide variety of names such as the hexose monophosphate oxidative shunt, the Warburg-Lipmann-Dickens pathway or the pentose phosphate cycle), Entner-Doudoroff (ED) pathway and Glucuronate-Xylulose pathway may provide alternative mechanisms. The respiratory metabolism in fungi, has been comprehensively reviewed by Blumenthal (1965) and Niederpruem (1965). The EM and HMP pathways have been reported very commonly in the mechanism of respiration of fungi whereas the ED pathway was recognised conclusively only in the hyphae of Caldariomyces fumago (Ramchandran and Gottlieb, 1963) and in the spores of Tilletia caries (Newburg and Cheldelin, 1958). The presence of Glucuronate-Xylulose pathway in fungi has only been indirectly evidenced by Sastry and Sarma (1957).
The occurrence of an active tricarboxylic acid cycle among fungi such as *Aspergillus* and *Penicillium* was long suspected; however, the presence was not confirmed. The evidence for existence of TCA cycle in fungi came from the nutritional studies of Barnett and Kornberg (1960). A further evidence of an operative TCA cycle in fungi came from studies involving the mode of action of iodoacetic acid on *Neurospora crassa*, in which the respiration and growth were inhibited appreciably by physiological levels of iodoacetate (Ryan et al., 1944). Subsequently, certain mutants of *N. crassa* were found to utilize various growth substrates associated with the TCA cycle (Lewis, 1948).

After considerable efforts involving refinement of existing techniques a functional TCA cycle has been found to be widespread among the fungi. The respiration of whole cells and extracts, is usually measurable in terms of gas exchange-oxygen uptake or carbon dioxide formation. The basic instrument in most laboratories is the Warburg constant volume manorespirometer, described in the manual of Umbreit et al. (1964) and the manometric procedure for measuring oxygen utilization has been successfully applied in the studies on a number of fungal forms.

The determination of whether a substrate is oxidized or not, at a rate higher than of the endogenous rate, is very useful to identify possible metabolic pathways of respiration.
To determine various aspects of oxidative metabolism in fungi, physiological experiments of a great variety have been conducted by a large number of workers (Darby and Goddard, 1950; Cochrane, 1956, 1958, 1963; Cochrane et al., 1963; Sechtol and Throneberry, 1966; Harhash, 1966; Mcfeters and Ulrich, 1972; Vyas, 1971; Chourasia, 1976). These experiments included the study of utilization of various substrates carbon and nitrogen sources - and respiratory responses to various metabolic poisons, fungicides, antibiotics, respiratory intermediates, plant growth regulators, age of the culture and pH of the system.

Determinations of respiratory metabolism in pathogenic fungi is obviously very important for understanding the physiological and biochemical aspects of pathogenesis. It is now fairly well admitted that the causation of disease is a complex series of inter-connected events impinging upon many vital processes of the host. Evidently, the studies of various factors on the metabolic processes are bound to affect the interactions between host and the parasite which is often evidenced through the effect on respiration in various ways. Moreover, if a pathogen is found to have respiratory pattern other than the conventional and universally present ones. Then this difference may be exploited for the design of some specific chemotherapeutic agents. Similarly a number of antibiotics, fungicides and certain amino acids have been
reported to act by blocking or disrupting the respiratory metabolism in fungi and have proved to be useful for devising effective control measures against certain plant diseases.

With this in view, a plant pathogen: *Botryodiplodia theobromae* Pat., was selected for the present investigation. Due to its wide host range and aggressive pathogenic abilities, *Botryodiplodia* may be regarded as one of the most destructive fungal pathogens, particularly in the tropical and subtropical regions. A voluminous work has been done on this pathogen by a number of workers (Srivastava and Tondon, 1969; Goose et al., 1961; Saksena and Kumar, 1961; Tondon and Bhargava, 1962; Willamson, 1964; Prasad, 1963; Sukla and Sarkar, 1972; Shreemali, 1973; Umezurike, 1969; 1970; Nagaraja Rao et al., 1971; Ali, 1977; Mishra, 1978). The literature reveals that majority of the approaches concerned with host-parasite relationship, nutritional aspects, production of pectic and cellulolytic enzymes and cell-wall-degrading enzymes. However, oxidative metabolism of this pathogen appears to remain unexplored, therefore, the present investigation on endogenous mycelial respiration of the six different isolates of *B. theobromae* was undertaken. Besides this, for the purpose of correlation, all the factors, which were employed in respirometric investigations, were also studied in relation to their effect on fungal growth.
The format of the thesis has been planned as follows:

Part I

This part deals with the isolation, purification and pathogenicity tests of the test isolates, and general procedures for respirometric and growth studies.

Part II

In this part, morphological studies of different isolates have been included.

Part III

This part included respirometric and growth studies of the pathogenic isolates, viz., effects of the age of culture, starvation period, pH, carbon sources, inorganic and organic nitrogen sources, metabolic inhibitors, antibiotics and fungicides on endogenous mycelial respiration and mycelial growth.

Part IV

This part includes general summary, conclusions and bibliography.
PART I

CHAPTER II

MATERIALS AND METHODS
A. Isolation and Purification of the Test Pathogen:

Diseased fruits of Apple (*Pyrus malus* L.), Lemon (*Citrus medica* L.), Guava (*Psidium guajava* L.), Mango (*Mangifera indica* L.), Orange (*Citrus aurantium* L.) and Musambi (*Citrus sinensis* L.) were collected from local market and brought to the laboratory. The fruits were washed and surface sterilized for 2 minutes with 0.1 per cent solution of mercuric chloride. After giving several washings with sterilized distilled water, pieces were cut from infected tissue and were transferred in petriplates containing Potato-Dextrose-Agar (PDA) medium. The inoculated plates were incubated at 28°C for 6 days. The fungal hyphae growing out from the diseased tissue, were picked up and transferred into petri plates. The isolates were then purified by hyphal tip method and maintained on PDA slants.

B. Pathogenicity Tests:

Pathogenicity tests were made to confirm the pathogenic ability of the isolated pathogens on their respective hosts. Small cavities of about 4 to 6 mm in diameter were made upon the surface sterilized host fruits with a sterilized cork borer. The inoculum consisting of an agar disc containing mycelium cut from freshly grown colony was placed into the cavity, and the inoculated healthy fruits were placed in moist
chambers and incubated at 28°C. Appearance of typical rotting symptoms were taken as positive indication of pathogenicity. Repeated isolations and inoculations were performed to confirm the pathogenicity.

C. Procedure for Respirometric Studies:

The rate of endogenous mycelial respiration under different experimental conditions was measured by observing the rate of oxygen consumption by mycelial suspensions prepared from the mycelial mats of all the six test isolates of *B. theobromae* Pat. Standard manometric techniques as described by Umbreit et al. (1964) were followed for these studies using Warburg's constant-volume-respirometer.

(i) Preparation of mycelial suspension:

The cultures of different test isolates were freshly grown in sterilized Petriplates containing Potato-Dextrose-Agar medium. Mycelial discs of 10 mm diameter from such freshly grown cultures were cut with sterilized cork borer, 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; K$_2$HPO$_4$, 0.012 M; KH$_2$PO$_4$, 0.02 M; NH$_4$NO$_3$, 0.0375 M; MgSO$_4$·7H$_2$O, 0.009 M and distilled water to make it one litre (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 28°C (± 1).

After incubation of 3½ days in case of isolate A, G, M and O, and 3 and 4 days in Mu and C isolates, respectively, the thick mycelial mats were removed and washed thoroughly in 0.02 M phosphate buffer (pH 7.0). One gram of starved mycelial mat of each fungus was fragmented in 50 ml buffer in waring e blender for 30 sec. at 500 r.p.m. This mycelial suspension was used for respirometric studies.

(ii) Preparation of Respiratory Flasks

For experimental use, Warburg's flasks were prepared by pipetting out 2.7 ml of mycelial suspension in the main compartment. A piece of filter paper, made into a wick, was inserted in the central wall of the flask to provide large surface for absorption of carbon dioxide; to this 0.2 ml of 20 per cent KOH was added. To the side arm of the flask, 0.3 ml of treatment solution was pipetted out for observing the effect of various substances on respiration. In case of control 0.3 ml of buffer was used in the side arm. Thermobarometer contained 3 ml buffer in the main compartment and 0.2 ml of 20 per cent KOH in the central wall. The mycelial suspension (2.7 ml) was taken out and dried in an oven 80°C for 24 hours, it was used to determine the dry weight of the mycelium. The results of
respiration are presented on the dry weight basis.

(iii) **Determination of oxygen uptake**:

**Warburg's Direct - Total Uptake Method**:

The dried manometers were filled with standard manometric fluid (Brodie's fluid). These manometers were adjusted at 200 mm levels on both the arms with stop cock open. The respiratory flasks, prepared as above were then attached to the respective manometers. The manometers with flasks were then fixed on to the Warburg's apparatus. The trough of apparatus was filled with water and adjusted to 20^\circ C. The flasks were completely dipped in the water and allowed to equilibrate for 15 minutes with constant shaking and stirring. The treatment solution of the side arm was tipped into main compartment of the flasks and manometer levels were open again adjusted to 200 mm with stop cock. The stop cock was then closed and the whole system was made airtight. Readings were taken usually at 10 minutes intervals for 60 minutes by noting the level of left arm of the manometer, keeping the level of right arm at 200 mm. By the help of thermobarometer reading, appropriate corrections were made for changes in the level of the manometric fluid caused by deviation in atmospheric pressure and temperature. The values so obtained in each case were then multiplied by flask constant so as to convert the readings in $\mu$l of oxygen consumed per unit time.
(iv) **Determination of flask constant**: 

The flask constant for each pair of flask and manometer was determined before starting the experiments by using mercury as described by Umbreit *et al.* (1964). In Warburg's apparatus, oxygen consumption was noted by the difference in pressure in mm. This value was multiplied by flask constant which gave directly the volume of oxygen consumed in microlitre (µl) at N.T.P. Flask constant was calculated by the following formula:

\[
K = \frac{\frac{273}{T} + V_f \chi}{P_0}
\]

Where:

- \( K \) = Flask constant.
- \( V_g \) = Volume of gas phase in flask including connecting tubes down to the reference point (200 mm on closed arm of manometer).
- \( V_f \) = Volume of fluid in vessel.
- \( P_0 \) = Normal pressure, which is 760 mm Hg or 10,000 mm Brodie's fluid.
- \( T \) = Temperature of bath in absolute degrees \((273 + \text{temp. in } ^\circ\text{C})\).
- \( \chi \) = Solubility in reaction liquid of gas involved (values of \( \chi \) taken from the book *Manometric Techniques* by Umbreit *et al.*, 1964).
Once $K$ is known, then the amount of gas exchange is:

$$X = h K$$

$$\frac{V_g 273}{V_f T} + V_f \alpha$$

$$= h \frac{273}{P_0}$$

Where:

$h$ = the reading of the manometer in mm. The value of rate of oxygen uptake has been expressed as $Q_{O_2}$ (μl of $O_2$ consumed per hour per mg dry weight of mycelium at 28°C).

D. Procedure for Growth Studies:

To study the effect of different chemicals on radial growth of the six isolates of *B. theobromae*, Czapek’s (Dox) Agar medium, contained sucrose, 10 g; NaNO$_3$, 2 g; K$_2$HPO$_4$, 1 g; MgSO$_4$·7H$_2$O, 0.5 g; KCl, 0.5 g; FeSO$_4$·7H$_2$O, 0.01 g; Agar-Agar, 15 g; and distilled water to make it one litre. The required quantity of the chemicals (except antibiotics) were added in the medium and autoclaved at 15 lb for 20 minutes, before pouring into the sterilized petriplates. For inoculum, mycelial discs of 10 mm diameter were cut from the freshly grown culture of the concerning isolates. The inoculated petriplates were incubated at 28°C for 3 days. Three replicates of each variable were run and colony diameter was recorded at every
12 hours interval up to 72 hours. Arithmetic mean of these replicates with SE (Standard Error) is given in the tables, along with the percent change over control. For control the six isolates were grown on pure Czapek's (Dox) Agar medium.