Pathological and Biochemical Investigations on Stem end rot disease in Pineapple

(*Ananas comosus* (L.) Merr.)

A Synopsis Submitted to the

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For the award of the degree of

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By

Mr. Swaroop Kumar H.M., M. Sc.

Department of Fruit and Vegetable Technology

Central Food Technological Research Institute, Mysore-570 020

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Synopsis

The research undertaken towards understanding of intricate relationship between host and pathogenic factors of SER disease in pineapple var. 'queen' caused by *Ceratocystis paradoxa,* which is essential to devise a nontoxic, zero residue control measures, is briefed below.

Chapter 1

As a first step, to be familiar with decaying agent, the fungus has been isolated from naturally infected fruit, identified and established its pathogenicity, nature of invasion and colonization. The contribution of pathogen to severity of the disease in fresh fruit is dependent on the level of inoculum, spore germination and rate of vegetative growth. Minimum threshold concentration of spores, enhanced spore germination and rate of growth of mycelia of *C. paradoxa* in the presence pineapple fruit juice was demonstrated. A consistent symptom of SER disease of pineapple by *C. paradoxa* is the production of soften peduncle that typically involve the death and maceration of infected and surrounding tissue, which makes the host tissue predispose for fungal invasion and colonization. Most revealing account exhibited in diseased tissue was rapid spread and colonization of pathogen with advance of ripening. Whether such progress relates to the difference in the chemical compositions of fruit in context with maturity and ripening were thoroughly investigated.

Chemical factors that govern two stages of maturity viz. 1) physiological and 2) commercial maturity stages were defined. The susceptible stage of pineapple fruit to *C. paradoxa* infection has been recognized. The green and 25% ripe fruit exhibited resistant to SER pathogen. The chemical factors like pH above 4, less acidity and more sugars associate with 50% ripe fruits were found be the major chemical factors that favour the *Ceratocystis paradoxa* for infection.

"Stem end rot disease manifestation is the ultimate expression of its final display of all its complex interrelationships with its pathogen. Histological account of SER disease has been worked out to elucidate cellular and structural changes in the host (pineapple fruit) tissues with invasion of pathogen (*C. paradoxa*). Scanning Electron Microscopic observations of infection process of *C. paradoxa* revealed a range of colonization status. For the first time, direct penetration of fruit tissue by *C. paradoxa*

infected hyphae and their hemi-biotrophic colonization were demonstrated. Extensive disorganization and disintegration of diseased tissues of cell wall were found to be responsible for collapse of fruit tissue. The most striking features of host reaction were viz., plasmolysis of cells. Blackening of tissue was due to excessive production black mycelium and single or chain of chlymydospores of *C. paradoxa.*

A detail investigation on constitutional and inductive chemical factors of pineapple and pathogenic factors of Ceratocystis paradoxa was carried out and presented in second chapter.

CHAPTER 2

There is little information to correlate SER infection with the cell protective system of pineapple. Given its significance in other species, it would be of value to examine the relationship of the antioxidant enzymes to blackheart development in pineapple fruit. In this chapter, the role of PPO, POD and PAL were investigated along with total phenol, and cell wall constitutive chemicals in pineapple fruit. These changes were examined at physiological, commercial maturity stage, at different stages of ripening and also in SER disease tissue of pineapple.

The present investigation demonstrated the possible role of antioxidant enzymes like PPO, POD and PAL in pre symptomatic biochemical changes like softening and translucency of tissue surrounding infection loci. Excessive or de-novo synthesis of these enzymes in disease mainly involve in predisposition of tissue by discoloration subsequently browning of tissue. The other constitutional changes in pineapple tissue, as a consequence of *C. paradoxa* infection were also emphasized.

It appears that there are two major influencing factors that determine the resistance of fruit against *C. paradoxa* infection. Among the parameters more suitable to grade pineapples into classes of maturity and ripe stage were (in the case of the intact fruit), skin colour (shell colour) and (in the case of the flesh), flesh colour. The selected parameters to predict resistance in pineapples were: components of cell wall, polyphenols, total proteins, total soluble solids (TSS), titrable acidity, TSS/acid (also known as the Brix/acid ratio), pH, colour and translucency. Shell colour, Brix/acid ratio, pH, ferulic acid content of cell wall showed a definite role as fruit resistance index. The changes of these chemicals as a function of ripening provided the suitable host-index parameters for disease resistance against *C. paradoxa* infection.

The cut end of peduncle provides an avenue for entry of pathogen. A state-of-the-art relation between host and pathogenic factors regarding physical, biochemical and pathological status, revealed that pineapple fruit start ripening immediately after attainment of physiological maturity. Inordinate delay in disease expression in intact, green fruit may well coincides with the firmness, high ferulic acid content in cell wall, high pH, low Brix/acid ratio. Ripening changes like softening of tissue, rapid decrease in moisture, TSS, Ascorbic acid, pH, concomitant increase in total sugars, and cellulase enzymes were found to be the key factors for susceptibility of the fruit for infection. A delay in these changes in pineapple may be responsible for extended period of latency of *C. paradoxa.*

Though pineapple produces cell wall degrading cellulase enzyme during ripening the concentration may be insufficient or qualitatively different. The cell wall derivatives may induce production of cellulase enzyme in *C. paradoxa* In the present investigation 10 to 15 time increase in cellulase enzyme in disease tissue may indicate that *C. paradoxa* not only contributes its enzyme to the host, but also induced increase production of this enzyme. Cellulase proved as a virulent factor of *C. paradoxa* against defense system of the pineapple fruit. It is also responsible for massive maceration of host tissue and decompartmentation of cell organelles. For the first time role cellulase enzyme in pathogenesis of SER disease in pineapple was distinctly demonstrated. The failure to produce cellulase enzymes may be responsible for avirulence of the pathogen.

CHAPTER 3

Cellulase being key virulent pathogenic factor in *C. paradoxa* infection, the pathogen isolate was tested for production of cellulase and pectinase enzymes in pure culture. To overcome the difficulty to obtain single batch, broth culture extracellulase in sufficient quantity a bioreactor was designed and successfully commissioned. This is essential to ward of both quantitative and qualitative variation in production of extracellulase cell wall degrading enzymes due to nutritional and cultural conditions. The configuration of bioreactor was illustrated. The important conditions optimized for bioreactor function for continuous production of cellulase were viz., broth volume, yield and duration of culture growth. Maximum yield of 8 gm per liter of broth was achieved for a period of 15 days. The culture filtrate of bioreactor was tested for extracellulase (Endoglucanase) activity. *C.paradoxa* cellulase enzyme found to be highly potent even at

high temperature (50°C) and both in acidic and alkaline pH range (5-9). This pathogenic adaptability may be the underlying reason to cause heavy post harvest SER disease in pineapple at tropical, arid conditions like India.

In the present investigation the crude enzyme concentrate was subjected to SDS-PAGE., followed by protein staining was used to identify the protein pattern of extracellulase enzyme of *C. paradoxa*. It exhibited presence of four distinct bands. Each band was tested for endoglucanse activity by CMC stain. Of the four protein bands the 37 k.Da. only showed the endoglucanse activity. It appears cellulase produced by *C. paradoxa* is a mixture of four proteins/enzymes. The other components might be responsible for cellulase's biological effects. This can also form as a taxonomic criterion to study the extent of biochemical and pathogenic variation among and with in the species of *C. paradoxa* from different host, which is worth to be explored.

CHAPTER 4

Non toxic or zero residues, effective post harvest control of SER disease in pineapple fruit was tried by dual methods viz. 1) Use of physical method like UV, irradiation and hot water treatment and 2) use of constitutive or inductive chemicals of host along with FDA recommended preservatives.

Hot water treatment (55^{o}C for 5-10 minutes) to cut stem end of pineapple were effective in inhibiting spore germination and also the incidence of SER disease in pineapple fruit. Among an array of eighteen chemicals tried Benzoic acid, ferulic acid and chlorogenic acid at the concentration of 500 ppm. were found to be effective for significant inhibition of spore germination and also induced significant reduction in infection and incidence of SER in pineapple fruit. The combination of these two treatments can be a new strategy to provide non-toxic, zero residue control measure to SER disease in pineapple.

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General Introduction

Pineapple fruit is widely distributed in tropical regions and in recent years has become one of the most demanded exotic fruits. It is important economically, nutritionally and also highly valued for its nutraceutical properties. Bromelain is a complex mixture of substances that can be extracted from the stem, core and fruit of the pineapple, with a wide variety of health benefits. Bromelain is known for its clinical and therapeutic applications, particularly for modulation of tumor growth, third degree burns, and improvement of antibiotic action, for the oral systemic treatment of inflammatory, blood-coagulation-related and malignant diseases. It also used in food processing for meat tenderization and as a dietary supplement.

Pineapple *Ananas Comosus* (L.) Merr., Is a member of the Bromeliaceae family (Monocotyledons) and comprises about 2000 species, with an annual world wide production of over 14 million tons. It is the eighth most abundantly produced fruit in the world, while the third largest grown fruit in India. Pineapple is a perennial monocotyledonous plant having a terminal inflorescence and a terminal multiple fruit. The fruit is a multiple fruit or syncarp or sorosis, formed by the fusion of spirally arranged fruitlets produced by flowers of the inflorescence (Coppens d'Eeckenbrugge and Leal, 2003). Each fruitlet can be identified by an "eye," the rough spiny marking on the

pineapple's surface. Each fruitlet fuses with neighbour ones, which is distinctly marked by slight depression and the region being called as leachets. The edible part of the fruit consists mainly of the ovaries, of the bases of sepals and bracts of the cortex of the axis. The fruit shell is mainly composed of sepal and bract tissues and the apices of the ovaries (Okimoto, 1948). Pineapples have a wide cylindrical shape, green leaves and fibrous yellow flesh. The principle varieties are 'Queen' 'kew', and 'Moratius. Var., 'Queen' contributes to more than 50% of total production in India. All the varieties grown in India are highly susceptible for stem end rot disease during transportation, ripening, storage and marketing.

Stem end rot (SER) is also called Black rot or butt rot or *Thielaviopsis* rot caused by the facultative parasitic fungus *Chalara paradoxa* (De Seyn.) Sacc. = *Thielaviopsis paradoxa* (De Seyn.) Hohn., teleomorph: *Ceratocystis paradoxa* (Rohrbach and Apt, 1986) is a major worldwide pineapple post harvest disease. The disease starts in the field when the causal pathogen enters the fruit through the broken peduncle, other wounds (e.g. bruises), or natural openings in the fruit shell. The high temperature and humidity prevailing in tropical countries like India further aggravate post-harvest losses due to fungi. Post harvest losses of about 70% in pineapple are reported in India. It is a wide spread major fungal disease of economic importance that occurs frequently in India. This is a highly specialized pathogen that causes SER disease in pineapple fruit only. The other commercial crop being sugar cane, however they were also isolated from other forest trees and organic debris of various industrial effluents.

Post-harvest losses are costly in terms of money and manpower and it can be catastrophic for developing countries like India. At present, Black rot of pineapple is controlled by the application of fungicides. Despite high cost, the problem of pesticide residue is becoming acute, as they were reported to have carcinogenic, teretogenic,

oncogenic, neurotoxic and genotoxic health hazards. However, increasing consumer resistance and the restrictions imposed on the use of these chemicals, have created an urgent need for the development of safe and effective alternatives. In case of *C. paradoxa* the problem to device control strategies is multifarious due to its facultative pathogenic nature of infection.

Stem end rot disease establishment and manifestation in pineapple is the ultimate expression of complex interrelationships with its pathogen, the details of which are not well documented. Hence, the present investigation on host-pathogen interaction of SER disease on pineapple var. Queen caused by *C. paradoxa* was undertaken to enumerate the etiology of disease, host biochemical and pathogenic factors, along with histo-pathological account during infection. Further, development of less toxic, zero residue and host friendly control measures for SER disease on Pineapple have also been emphasized.

Review of Literature

The present literature review mainly concern with host and pathogenic constitutive and/or induced factors that are responsible for causing SER disease in pineapple by *Ceratocystis paradoxa* and other fungal diseases only.

Pineapple Fruit

Pineapple is a non-climacteric tropical fruit. The cultivated pineapple (*Ananas comosus* (L.) Merrill, belongs to the family *Bromeliaceae*. Any reference to pineapple in this document refers to *Ananas comosus* var. Queen. The pineapple is the leading edible member of *Bromeliaceae* which consists about 2,000 species, mostly epiphytic and many strikingly ornamental. The pineapple shares the distinction accorded to all major food plants of the world of having been selected, developed, and domesticated by peoples of prehistoric times and passed on to us through earlier civilizations.

Pineapple is the 3rd largest fruit produced India next to Mango and banana. The total production of pineapple is around 14 million tons. In India, pineapple is produced in the state of Bihar, Kerala, West Bengal, Andhra Pradesh, Karnataka, Goa, Tamil Nadu, Orissa and North Eastern States. North Eastern states produces major share of pineapples

in India. Even though India is one of the major producers of fresh pineapples its position in world export is significantly low. Less than 2% of total production is used for processing. Among the various reasons, lack of proper post harvest handling, Infrastructure, and storage facilities crowned with heavy post harvest spoilage mainly stem end rot (SER), which affect long distance export of pineapple fruits in India. In developing countries like India, pineapple losses can be as high as 70% (Salunkhe *et al.,* 1991).

Systemic classification:

Kingdom : Plantae

Division : Magnoliophyta

Class : Liliopsida

Order : Poales

Family : Bromeliaceae

Subfamily : Bromeflioideae

Genus : Ananas

Species : *Ananas comosus* var. Queen *(L.)* Merrill.

Pineapple is a perennial monocotyledonous plant having a terminal inflorescence and a terminal multiple fruit. It continues to grow after fruiting by means of one or more axillary buds growing into vegetative branches with a new apical meristem The main morphological structures of the plant are the stem, the leaves, the peduncle (stem which bears fruit), the multiple fruit or syncarp or sorosis (fusion of many fleshy fruitlets), the crown, the shoots and the adventitious roots (Coppens d'Eeckenbrugge and Leal, 2003). The inflorescence consists of 50-200 individual flowers, capped by a crown composed of

numerous short leaves (up to 150) on a short stem. *A. comosus* var. *comosus* flowers are normally self-sterile and fruit development is parthenocarpic (does not require fertilisation) (Py *et al*, 1987). The edible part of the fruit consists mainly of the ovaries, of the bases of sepals and bracts and of the cortex of the axis. The fruit shell is mainly composed of sepal and bract tissues and the apices of the ovaries (Okimoto, 1948).

Maturity index and harvest

Maturity

The stage of maturity, method of harvest, handling and packing are largely determined by the ultimate destination of the fruit. It was revealed during the survey that adequate attention is not paid by the growers to harvest the fruit at the proper stage of maturity and both ripe and unripe fruits are indiscriminately gathered during harvest.

Pineapple undergo changes during the development, where the immature eyes are gray or light green and the small brackets or bracts which cover half of each eye are gray giving the fruit grayish appearance. As the fruit matures, the space between the eyes fills out and the colour gradually changes from light to dark green that indicate complete maturation of fruit (physiological maturity- referred as 80% maturity). As the fruit ripens, the eyes changes from pointed to flat with slight hollowness at the centre, the fruit becomes enlarged, less firm and more aromatic. The fruit is picked for domestic use when the fruit attained the yellowing (Harvest maturity-referred as 100% maturity). At this stage of maturity, the fruit has higher T.S.S. and low acidity. When the growing areas are far away from the market, the fruit is manually harvested at 80% matuity stage. Thus it takes 2-3 weeks before fruits are fully ripe. The growths of the ridge on the margin of the eyes are added indices for its harvesting if shipped for long distances For fruits intended for processing located near growing areas may be delayed till more colour develops on

the fruit. Change in surface colour that begins at peduncle end and progress towards crown region of the fruit, Based on which maturity/ripe stages are categorized into Green, 25%, 50%, 75% and 100% ripe (Collins 1968)

Storage of pineapple

Pineapple is a non climacteric, chilling sensitive fruit. Fruits tend to develop chilling injury symptoms if stored below 10°C. The susceptibleness is depending upon maturity stage at harvest, cultivar and storage condition. Matured Pineapple var. queen is successfully stored at 12±1°C and 90±5% RH for 16 days with optimum ripening and eating qualities. But these conditions fail to stop the *C. paradoxa* infection among field infected fruits. The SER infection slows down in lower temperature but continues to grow internally. SER disease of pineapple forms the major limiting factor during storage.

Pathology of Pineapple fruit

The present review focused on fungal diseases of pineapple fruit diseases exhibited following harvest. Stem end rot disease caused by *C. paradoxa* is of commercial importance. Many fruit disease symptoms have been described on pineapple. In addition or a few, specific pathogens followed by massive infection by broader spectrum secondary invaders are also reported (Collins, 1968; Rohrbach and Apt, 1986). The bacterial diseases and physiological disorders were not presented in this review, since it is out of scope of the present investigation.

Stem end rot (SER)

Stem end rot (SER), also called Black rot, *Thielaviopsis* fruit rot, water blister, soft rot or water rot, is caused by the fungus *Ceratocystis paradoxa* (De Seynes) Sacc. (syn. *Thielaviopsis paradoxa* (De Seyn.) Hohn (*telemorph Chalara paradoxa* (Dade) C. Moreau). Phylogenetic and taxonomic evaluation of the pathogen *Chalara paradoxa* (De Seyen.) Sacc. (syn.) *Thielaviopsis paradoxa* (De Seyen.) Höhn., teleomorph: *Ceratocystis paradoxa* (*Dade*) *C. Moreau* has been conducted by Paulin-Mahady et al. (2002). The disease is a universal fresh-fruit problem. The severity of the problem is dependent on the degree of bruising or wounding during harvesting and packing, the level of inoculum on the fruit and the storage temperature during transportation and marketing. Black rot does not occur in the field unless fruit is overripe or injured. The pathogen gains entry into host tissue through wounds and causes black rot disease in pineapple (Rohrbach and Phillips, 1990). Black rot of the pineapple fruit is characterized by a soft watery rot, which usually starts at the point of detachment of the fruit. Diseased tissue turns dark in the later stages of the disease because of the dark coloured mycelium and chlamydospores.

Infection of the pineapple fruit occurs through wounds resulting from harvesting and postharvest handling. Susceptibility Under conditions of high humidity, conidia may readily be produced on pineapple residue and be disseminated by wind to the unharvested fruit. Inoculum levels on fruit at harvest vary according to the environmental conditions prior to harvest (Rohrbach and Schmitt, 1994). The high correlation between moisture (rainfall duration) prior to harvest and disease following harvest has resulted in the name ‘water rot’. Infection occurs within 8–12 h following wounding. Refrigeration at 9°C during transportation will slow development of the

disease, but, when fruit are returned to ambient temperatures, disease development will resume (Rohrbach and Phillips, 1990). Fruitlet core rots (black spot) FCR (Oxenham, 1962; Rohrbach and Apt, 1986) or black spot (Keetch, 1977) (also called fruitlet brown rot and eye rot (Snowdon, 1990)) is a descriptive term for a brown to black colour of the central part of an individual fruitlet.

Fruitlet core rots (black spot)

Each major pineapple production area appears to have characteristic pathogens associated with the FCR symptom, probably as a result of the environmental conditions of the area (Rohrbach, 1980). For example, in Hawaii, Penicillium and Fusarium species are most commonly associated with FCR (Rohrbach and Apt, 1986). In South Africa, Penicillium species are most commonly found (Keetch, 1977), while, in Brazil, Fusarium species are most commonly associated with the FCR symptom (Bolkan *et al.,* 1979).

FCR (Oxenham, 1962; Rohrbach and Apt, 1986) or black spot (Keetch, 1977) (also called fruitlet brown rot and eye rot (Snowdon, 1990)) is a descriptive term for a brown to black colour of the central part of an individual fruitlet. FCR is caused by an infection by a pathogen or, more commonly, a group of pathogens. Botanically the central area of the fruitlet core is the septa (inverted Y) between the three seed cavities or locules. Because individual or mixtures of pathogens may be associated with the FCR symptom, there is considerable confusion in the literature. The Penicillium and Fusarium fungi (Rohrbach and Apt, 1986), the round yeasts and bacteria.

The degree to which these symptoms develop appears to depend on the time of infection, the pathogen or mixture of pathogens present, the cultivar and environmental conditions. The IFC symptom can also be caused by boron deficiency in which case the symptoms are indistinguishable.

It is theorized that the very low levels are the result of botanical malformations of individual fruitlets caused by disruptions in the normal phyllotaxis of the fruit (Kerns *et al.,* 1936). Malformation of the fruitlet allows infection of the stylar canals and nectary ducts by a range of pathogens. In contrast, true epidemics result from the coincidence of optimum environmental conditions resulting in predisposed flowers, production of inoculum of the pathogen(s) and transport of the inoculum to potential infection sites. The disease could become more important if some of the more susceptible, low-acid 'Smooth Cayenne' cultivars and hybrid cultivars are grown commercially for fresh-fruit markets.The FCR symptom is generally characterized by browning of the inverted 'Y' tissues.

As mentioned previously, the FCR complex involves the fungi P. funiculosum and F. subglutinans, and the round yeast *Candida guilliermondi*. The pineapple tarsonemid mite, *S. ananas*, and the pineapple red mite, *D. floridanus*, are also associated with the FCR complex. Considerable information is known and published on the Penicillium- and Fusarium-induced fruit diseases and the role of the pineapple tarsonemid mite (Rohrbach and Pfeiffer, 1976b; Rohrbach and Taniguchi, 1984). FCR symptoms produced by Penicillium species are dark to medium brown in colour, usually with a grey, water-soaked centre FCR symptoms from yeast infections are usually light brown.

Fusariosis

Fusariosis is caused by the fungus *F. subglutinans* which is the conidial stage of *G. fujikuroi* Edwards. Whether or not *F. subglutinans* in Brazil is the same as the Fusarium species causing FCR is not definitive in the literature. Laville (1980) considers *F. subglutinans* a distinctly different species from the Fusarium causing FCR. Other authors have attributed FCR to *F. moniliforme* (Oxenham, 1962; Guerout, 1974;

Rohrbach, 1983). The disease, first described in Argentina in 1954, was first reported in Brazil in 1964 and within 10 years had spread over the entire country (Laville, 1980; Rohrbach, 1983). The fruit symptoms at low severity levels are similar to those of FCR, which vary from light through medium to dark brown, extending partially to completely down the fruitlet core. FCR from Fusarium sp. is usually a 'dry' type of rot. In Brazil, the symptom is not limited to a single infected fruitlet, as in typical FCR reported in other pineapple production areas. Fruit symptoms involve multiple fruitlets, with the infected area of the fruit surface appearing off-colour initially and later becoming sunken, with profuse pink sporulation and exudation of gum. Gum exudation can be confused with the exudation from Thecla wounds (Laville, 1980).

Optimum temperatures for growth are 25°C, with a range of 5°C to 35°C (Camargo and Camargo, 1974). Control of fusariosis is most effective by planting disease-free seed material and by controlling insects, particularly the bud moth (Laville, 1980). Fungicides, such as captafol at 700, starting at differentiation through harvest at 20-day intervals, have given good control of the fruit-rot phase in Brazil (Bolkan *et al.,* 1978).

Miscellaneous fruit rots

Fruit rots caused by *Aspergillus flavus*, *Botryodiplodia theobromae* and *Rhizopus oryzae*. Internal browning is a physiological disorder caused by chilling injury. Pests, Diseases and Weeds 241 or Rhizopus stolonifer have been reported as postharvest diseases (Snowdon, 1990). A fruit rot caused by *Hendersonula toruloidea* (Natt.) has been reported by Lim (1985) on the Mauritus cultivar. Green fruit rot caused by *Phytophthora* species occasionally causes large losses of lodged first-ratoon fruit in

Australia under very wet conditions. These pathogens generally require some form of wounding for infection. Commercially, these diseases are of very minor importance.

Biochemical changes in Pineapple fruit during ripening and pathogenesis

Polyphenol oxidase (PPO)

However, the browning after mechanical or physiological injury, suffered during either harvest or cold storage, and during *C. paradoxa* infection affects consumer acceptability and palatability. Most browning in fruits is caused by enzymatic oxidation of natural phenolic compounds. Polyphenol oxidase (EC 1.10.3.1; PPO) is the major enzyme that catalyzes the oxidation of phenolic compounds to quinones, which further polymerize to brown pigments (Lee, 1991). The pineapple PPO was found in three isoforms (Das, Boht, & Eowda, 1997), with the major isoform being a tetramer of identical subunits of 25 kDa and having optimum activity between pH 6 and 7. The PPO is stable to heat when extracted, but loses over 50% of its activity following 20 min exposure to 60 °C in vivo (Teisson, 1977). PPO characteristics have been thoroughly investigated in apple (Janovitz-Klapp *et al.,* 1989), grape (Sánchez-Ferrer *et al.,* 1988), potato, and mushroom (Chen *et al.,* 1992). Although a number of studies on the morphological characteristics and physical and biochemical changes during pineapple fruit development are available (Gortner and Singleton, 1965; Kermasha *et al.,* 1987; Bartolome *et al.,* 1995), there is little or no information on PPO. In order to understand the role of PPO in the browning of pineapple fruit during cold storage, purification of PPO from pineapple was attempted.

Polyphenol oxidase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is an oxidative enzyme widely distributed in the plant kingdom and has been detected in most fruits and vegetables. PPO has been partially

purified from many fruits, including grape (Sanchez-Ferrer *et al.,* 1989), apple (Oktay *et al.,* 1995), guava (Augustin *et al.,* 1985), peach (Laveda *et al.,* 2000), banana (Sojo *et al.,* 1998), pear (Siddiq *et al.,* 1994), kiwi (Park & Luch, 1985), strawberry (Ebeling & Montgomery, 1990), plum (Siddiq *et al.,* 1992), cherry (Pifferi & Cultera, 1974), and pineapple (Das *et al.,* 1996). The localization of the enzyme in the plant cell depends upon the species, age, and in fruits and vegetables – on maturity. In potato tubers nearly all-sub cellular fractions were found to contain PPO. In freshly harvested apples, the enzyme is localized almost exclusively in chloroplasts and mitochondria (Vamos-Vigyazo, 1981). Where as its substrates (phenols) are localized in the vacuoles.

PPO is a copper-containing enzyme, which catalyses two entirely different reactions (a) the hydroxylation of monophenols to the corresponding o-dihydroxy compounds; (b) The oxidation of o-dihydroxy phenols to o-quinones. The reactions require molecular oxygen. The quinones formed from above reactions are very unstable and rapidly react with amino acids or proteins, generating brown pigments by polymerization (Garciýa-Carmona *et al.,* 1988). These reactions are more important in fruits with high phenol contents such as eggplant, apple, potato (Sakamura & Obata, 1963; Bajaj *et al.,* 1979). In the healthy cellular system PPO is separated from its substrates due to membrane compartmentation. Upon the loss of membrane integrity due to ripening/senescence/physical injury in the cells of fruit and vegetables, the contact of the enzyme and its substrates initiates browning reactions (Moskowiz and Hradzina, 1981; Mayer, 1987).

Techniques have been developed to prevent browning and PPO activity, each requiring a different approach depending on the characteristics of the plant tissue and the PPO (Martínez and Whitaker, 1995; Walker and Ferrar, 1995). Post harvest treatments such as heat, waxing, atmosphere control and application of 1-methylcyclopropene

(Rohrbach and Paull, 1982, Selvarajah and Herath, 1997 and Selvarajah *et al.,* 2001) have been tested as alternatives to prevent internal browning without success. In general, exposure of PPO to temperatures of 70–90 °C destroys their catalytic activity (Vamos-Vigyazo, 1981). Thermal inactivation profiles of PPO in fruit and vegetable processing follow first-order reaction kinetics with the time required varying with the product. Of the studies on heat inactivation of PPO only a few have included the calculations of Arrhenius and the kinetic parameters of heat inactivation of PPO from various foods. These include apple (Strübi, Escher, & Neukom, 1975), Sultana grapes (Aquilera, Oppermann, & Sanchez, 1987), apricot (Heil, McCarthy, & Merson, 1988), rice (Ansah, 1989) and mango (Askar, El-Ashwah, Omran, & Labib, 1994). No information is available for pineapple browning of tissue due to *C. paradoxa* infection, on the quantitative effects of temperature and time on the inactivation of PPO.

Peroxidase (POD)

Peroxidase (donor: hydrogen peroxide oxidoreductase, E.C.1.11.1.7) are, similar to PPO, member of the group of oxidoreductases and both enzymes catalyse more than one reaction and acts on a great number of substrates; Both are involved in enzymatic browning of fruit and vegetables (Williams *et al.,* 1985; Nicolas *et al.,* 1994). In plant cells POD is located in cytoplasm as a soluble form, and partly cell wall bound, which is in insoluble form, (Vamos-Vigyazo, 1981). Plant peroxidase is an iron-containing enzyme, which catalyses four types of reactions: (1) peroxidatic, (2) oxidatic, (3) catalatic, and (4) hydroxylation. It degrades hydrogen peroxide in the presence of a hydrogen donor. POD is highly specific to peroxide substrate, where as it has very low specificity for the hydrogen donor substrate. It uses wide variety of hydrogen donor substrates to decompose hydrogen peroxide. It can oxidize phenols to quinones, and then

condense tannins to brown polymers in the presence of H_2O_2, which may then contribute to enzymatic browning (Robinson, 1991). Increased POD activity has been observed in pineapple upon exposure to ozone, pollution, nutritional disorders, wounding, and chilling injury (Campa, 1991). The browning of pineapple and litchi fruit has been attributed to the POD activity (Zhang *et al.,* 2005).

Phenyl alanine lyase (PAL)

Being a key enzyme in phenolic biosynthesis, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) has also been considered to be associated with browning and the accumulation of chlorogenic acid and lignin-like materials (Hahlbrock & Scheel, 1989). PAL has been reported to play an important role in the browning process of many fruits and vegetables (Ke & Saltveit, 1989). During infection process of pathogens, the PAL activity was increased in order to render resistance to invading pathogen by excessive synthesis of polyphenols. Further oxidation reaction of these polyphenols leads to browning and cell death (Saltveit, 2000). Thus browning mechanisms in fruit tissues may involve any one of the above phenomenons or many interlinked phenomena. Different fruits have showed different mechanism of browning.

Blackheart development in pineapple fruit (*Ananas comosus*, Smooth Cayenne) has been attributed to activity of PPO, peroxidase and phenylalanine ammonia-lyase (Zhou *et al.,* 2003). For example phenolics, PPO, PAL, and iron play important role in blackening reactions of stored artichoke heads (Lattanzio, *et al* 1994), whereas PPO, peroxidase and anthocyanase enzymes together play important role in browning of litchi fruit (Zhang *et al.,* 2005).

Bromelain

Bromelain is a crude, aqueous extract from the stems and immature fruits of pineapples (Ananas comosus Merr., mainly var. Cayenne from the family of bromeliaceae), constituting an unusually complex mixture of different thiol-endopeptidases and other not yet completely characterized components such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates, among others [Cooreman (1978); Rowan and Buttle (1994)]. In addition, bromelain contains several proteinase inhibitors [Lenarcic, *et al.,*(1992), Hatano, *et al.,* (1996)]. Stem-bromelain (EC. 3.4.22.32) is distinguished from fruit-bromelain (EC. 3.4.22.33), previously called bromelin [Rowan and Buttle (1994)]. Today bromelain is prepared from cooled pineapple juice by centrifugation, ultrafiltration and lyophilization. The process yields a yellowish powder, the enzyme activity of which is determined with different substrates such as casein (FIP units), gelatine (gelatine digestion units) or chromogenic tripeptides [Filipova, *et al.,* (1984) Harrach, *et al.,* 1995)]. Bromelain contains several distinct cysteine proteinases that have similar but distinct amino acid sequences, as well as differences in proteolytic specificity and sensitivity to inactivation. Stem bromelain (EC 3.4.22.32, formerly EC 3.4.22.4) is the most abundant proteinase within bromelain preparations derived from pineapple stem. Other proteinases that are present at lesser amounts include fruit bromelain (the major proteinase present in pineapple fruit; EC3.4.22.33, formerly EC 3.4.22.4 and 3.4.22.5) and ananain (EC 3.4.22.31). Stem bromelain preferentially cleaves the Z-Arg-Arg model substrate, whereas fruit bromelain and ananain show minimal activity against this substrate. In contrast, fruit bromelain and ananain but not stem bromelain efficiently cleave the Bz-Phe-Val-Arg substrate (Laura, *et al.,* 2005).

. By high-resolution fast protein liquid chromatography (FPLC) and other biochemical methods, basic (stem bromelain, ananain, comosain) and acidic thiol-proteinases have been isolated from crude bromelain, partially or fully sequenced and characterized in more detail [Harrach, *et al.,*(1995); Harrach, *et al.,* (1998); Napper, et al (1994)]. They mainly comprise glycosylated multiple enzyme species of the papain superfamily with different proteolytic activities, molecular masses between 20 and 31 kDa, and isoelectric points between > 10 and 4.8. Two major basic proteinases, F4 and F5, were further characterized and showed molecular masses of 24,397 and 24,472 Da, respectively [Harrach, *et al.,*1995] These enzymes also differ in their susceptibility to inactivation.

Enzyme Activities

The activities comprise a wide spectrum with pH optima between 5.5 and 8.0 [Yoshioka, *et al.,* 1991]. The substrate spectrum is similarly broad, extending from synthetic low molecular mass amides and dipeptides up to high molecular substrates such as fibrin, albumin, casein, angiotensin II, bradykinin. Bromelain preferentially cleaves glycyl, alanyl and leucyl bonds. Commercial bromelain preparations are evaluated according to their proteolytic activity. The platelet aggregation inhibitory and anti-inflammatory action seems to be related to the protease activity. However, other effects such as inhibition of tumor cell growth and metastasis as well as debridement of burns are associated with other nonproteinolytic components contained in bromelain. Thus, the determination of the proteolytic activity alone may not be sufficient to completely characterize the pharmacological properties of bromelain [Taussig and Batkin (1988)]. Ananain is reported to be rapidly inactivated by the chicken egg white proteinase inhibitor cystatin and by the suicide substrate, E-64 (*trans*-epoxysuccinyl-l-leucylamido(4-guanidino)butane), but these inhibitors either very slowly or only

minimally inactivate stem and fruit bromelain (Laura, *et al.,* 2005). In aqueous solution, bromelain rapidly deteriorates through self-digestion. The addition of serum containing a2-macroglobulin will prevent self-digestion.

Organic acids

Fruit acidity and sweetness are two of the major factors that determine pineapple fruit eating quality. Other measures of fruit quality include shell color, fruit size and shape, aroma, crown size, crown to fruit ratio and the absence of disease and blemishes (Paull and Chen, 2003). Variation of pineapple fruit acidity and sweetness are associated with the pineapple clone used, fruit maturation and growing conditions (Singleton and Gortner, 1965, Py *et al.,* 1987 and Bartolome *et al.,* 1995).

Acidity

Fruit acidity increases during pineapple fruit growth and as the fruit approaches maturity and starts to ripen, the acidity declines (Singleton and Gortner, 1965 and Smith, 1988). Citric acid shows the greatest changes during fruit growth, increasing and then reaching a peak prior to ripening, whereas malic acid shows little change during development (Singleton and Gortner, 1965 and Chan *et al.,* 1973). Fruit sweetness gradually increases during the later stages of fruit growth (Bartholomew and Paull, 1986) with the sugar to acid ratio being recommended as a harvest index (Paull and Chen, 2003). However, citric acid alters sucrose perception (Schifferstein and Fritjers, 1990) and pineapple clones may have sufficient sugars but high citric acid may mask some of the sweetness perception. This masking of sucrose means that higher acid fruit may be perceived as being sour.

Low acid pineapple clones have been available for a number of years though high acid clones are preferred for canning. The availability of cultivars or clones of other economically important fruits differing in acid content, has facilitated comparative studies of organic acid metabolism in apple (Beruter, 2004), peach (Genard *et al.,* 1999 and Moing *et al.,* 1998a), citrus (Sadka *et al.,* 2001) and grape (Diakou *et al.,* 2000 and Terrier *et al.,* 2001). The final organic acid content of fruit is determined by the net balance of acid synthesis, degradation, utilization and compartmentation (Laval-Martin *et al.,* 1977, Ruffner *et al.,* 1984, Muller *et al.,* 1996 and Yamaki, 1984). The enzymes potentially involved in fruit acid metabolism are citrate synthase (CS, EC 4.1.3.7), aconitase (ACO, EC 4.2.1.3) (Sadka *et al.,* 2000a, Sadka *et al.,* 2001 and Luo *et al.,* 2003), phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), malate dehydrogenase (MDH, EC 1.1.1.37) and malic enzyme (ME, EC 1.1.1.40) (Hirai and Ueno, 1977, Moing *et al.,* 2000 and Diakou *et al.,* 2000). MDH catalyzes the interconversion of malate and oxaloacetate in the cytoplasm. CS catalyzes the acetylation of oxaloacetate using acetyl-CoA to yield citrate that is isomerized by ACO to isocitrate. PEPC condenses phosphoenolpyruvate with bicarbonate to yield oxaloacetate and ME reduces and decarboxylates malate to pyruvate. Although studies had been reported on the compositional changes in some pineapple fruit clones (Kermasha *et al.,* 1987, Bartolome *et al.,* 1995 and Brat *et al.,* 2004), information on fruit acid metabolism is limited. Potassium ion is thought to be involved in organic acid charge balancing (Lang, 1983) and potassium fertilization does increase pineapple titratable acidity (Py *et al.,* 1987 and Spironello *et al.,* 2004), though potassium accumulation in pineapple fruit has not been reported in relation to fruit acidity.

Ferulic acid

The hydroxycinnamic acid (HCA) ferulic acid (FA) occurs ester-linked to the primary cell walls (PCWs) of all families of the commelinoid group of monocotyledons. This group contains approximately half of all the monocotyledon families and comprises the following orders recognized by the Angiosperm Phylogeny Group (1998): Arecales, Commelinales, Poales, Zingiberales and some unplaced taxa (Harris; Rudall; Harris; Smith and Harris). However, most of the research done on FA ester-linked to monocotyledon cell walls (CWs) has been done on economically important species of the family Poaceae (Poales) which comprise the grasses and cereals. In contrast to the monocotyledons, FA occurs ester-linked to the PCWs of dicotyledons only in the order Caryophyllales that contains the family Chenopodiaceae that has a number of economically important species, including spinach (*Spinacia oleracea*) and sugar beet (*Beta vulgaris*). FA is ester-linked to polysaccharides in PCWs, but it is linked to different polysaccharides in the PCWs of the Poaceae and Chenopodiaceae. The polysaccharides to which the FA is ester-linked and the exact location of the link were determined by treating isolated PCWs with commercial fungal 'cellulases' such as 'Driselase' which is from the fungus *Irpex lacteus* and contains a mixture of endo- and exo-glycanases, but lacks hydroxycinnamoyl esterase activity.

Feruloyl oligosaccharides (FOs) were isolated from such enzymic digests and their structures determined. FA is ester-linked to glucuronoarabinoxylans (GAXs), typically the most abundant non-cellulosic polysaccharides in these PCWs (Kato; Ishii; Ishii and Wende). The FA is esterified via its carboxyl group to the C(O)5 hydroxyl of single *α*-L-Ara*f* residues located on the C(O)3 of Xyl*p* residues in the polysaccharide backbone. In contrast, the two most abundant FOs in enzymic digests of PCWs of the Chenopodiaceae were usually *O*-[2-*O*-*E*-feruloyl-*α*-L-Ara*f*]-(1→5)-L-Ara*f* and *O*-[6-*O*-*E*-

feruloyl-α-D-Gal*p*]-(1→4)-D-Gal*p* from the PCWs of spinach and sugar beet (Fry; Fry; Ishii; Colquhoun; Ishii and Ralet). The structures of these indicated that the FA is ester-linked to pectic polysaccharides, the most abundant non-cellulosic polysaccharides in these PCWs (Renard and Thibault, 1993). More detailed studies of the polysaccharides in the PCWs of species in families of the order Poales (Smith and Harris, 1999) and of the polysaccharides in the PCWs of pineapple (*Ananas comosus*, Bromeliaceae) fruit (Smith and Harris, 1995) confirmed the presence of GAXs, similar in structure to those in the PCWs of the Poaceae.

Pathogenesis

Cell wall degrading enzymes produced by *Ceratocystis paradoxa*

Extracellularly expressed exudates from pineapple fruit infected by *Ceratocystis paradoxa* were partially purified and the exudate-filtrate preparations were assayed for the presence of three hydrolytic enzymes. The preparations were found to contain cellulolytic and proteolytic enzymes when inoculated on carboxymethylcellulose and casein as substrates, respectively, while pectin-methylesterases and polygalacturonases were detected in the exudate-filtrate when apple pectin and sodium pectate were used as substrates, respectively. The activities and stabilities of these groups of enzymes were found to be optimal at pH 7.0 and at 30 °C. The exudates also exhibited macerating action when potato discs showed complete loss of coherence and maceration after a 24 hr incubation. More total reducing sugars were detected in exudates of infected fruit than those obtained from healthy fruit.

Cellulase

Plant cell walls are a major reservoir of fixed carbon in nature. In recent years there has been considerable interest in the utilization of plant material as a renewable source of fermentable sugars that could be subsequently converted into useful products such as liquid fuels, solvents, chemicals, food, or feed (Clarke, A. 1997; Bothast, and Saha, 1999). Such bioconversion processes are particularly attractive for the elimination of residues and wastes produced by agriculture and forestry. As a result of this interest, a wealth of knowledge on cellulolytic enzymes has accumulated (Béguin and Aubert, 1994; Tomme, *et al.,*1995; Ohmiya, *et al.,*1997). The past fifty years have witnessed remarkable progress in (a) isolation of microorganisms producing cellulases (Reese, and Maguire,1971) (b) improving the yield of cellulases by mutation, and protoplast fusion (Brown, *et al.,*1986), (c) purifying and characterizing the cellulase components (Bhat, K.M. *et al.,* 1989,1990; Christakopoulos, *et al.,* 1994; Wood, and McCrae,1977, Wood, and McCrae, 1977, 1982; 1986) (d) understanding the mechanism of cellulose degradation (Wood, *et al.,*1988) (e) cloning and expression of cellulase genes (Beguin, and Anbert, 1993; Begnin, *et al.,*1983; Begnin, *et al.,* 1983, 1988) (f) determining the 3-D structures of cellulase components (Davies,*et al.,* 1993; Davies, and Henrissat,1995, Ducros, *et al.,* 1995), Spezio, *et al.,*1993) (g) understanding structure-function relationships in cellulases (Claeyssens, and Tomme,1989;Davies, and Henrissat, 1995) and (h) demonstrating the industrial potential of cellulases (Beguin, and Anbert, 1993; Coughlan,1985; Mandels, 1985) Further background information can be found in other recent reviews (Esterbauer, 1991; Kubicek,1992; Ljungdahl, and Eriksson,1985; Ljungdahl, L.G. and Eriksson, K.E. (1985). This review summarizes the cellulase activity of *C. paradoxa* and other important pathogenic fungi on fruits and vegetables

Cellulase in *C. paradoxa* and other pathogenic fungi

The first report on endoglucanase production by Chalara (syn. Thielaviopsis) paradoxa isolated from olive mill waster water disposal pond was carried out recently Lucas etal 2001. However, its role as a SER disease pathogen has not been established yet. Production of cellulolytic enzymes has been reported in other plant pathogenic fungi, such as Macrophomina phaseolina (Wang, and Jones, 1995,), Phytophthora infestans (Sachslehner, *et al.,* 1998), or Sclerotium rolfsii (39 Bodenmann, *et al.,* 1985). Cellulolytic enzymes should play a role in the penetration of plant cell walls (Bateman, D. F., 1976). The cellulolytic system of *C. paradoxa* CH32 consists of at least one endoglucanase and one â-glucosidase, which has been characterized recently (Lucas, *et al.,* 2000). Production of endoglucanase activity in liquid cultures takes place during the late trophophase, coincidently with glucose exhaustion. This behavior seems to be in agreement with the general observation that cellulase systems are repressed in the presence of more easily metabolizable carbon sources, for example, glucose (Bisaria, and Mishra, 1989). Under these conditions, endoglucanase formation in various fungi starts only when the repressing carbohydrate glucose is completely metabolized (Ronne,. 1995). In this respect, it resembles endoglucanases produced by other fungi such as *Aspergillus niger* (Okada, 1985) or *Aspergillus niveous* (Taj-Aldeen, and Alkenany, 1996).

Cellulolytic enzymes are produced by a relatively few fungi and bacteria produce high levels of extracellular cellulase capable of solubilizing crystalline cellulose extensively (Johnson, E.A., *et al.,* 1982; Wood, 1985; Wood, 1991). So far, most of the studies have been on the cellulase system of aerobic fungi *Trichoderma viride, Trichoderma reesei, Penicillium pinophilum, Sporotrichum pulverulenlum, Fusarium solani, Talaromyces emersonii* and *Trichoderma koningii* (Eriksson, and Wood,1985; Gong, *et al.,* 1979; Eriksson, and Wood, 1985; Gong, *et al.,* 1979;). Only recently, it has

been recognized that the other microorganisms such as thermophilic aerobic fungi (*Sporotrichum thermophile*, *Thermoascus aurantiacus*, *Chaetomium thermophile, Humicola insolens*), mesophilic anaerobic fungi (*Neocallimastix frontalis, Piromonas communis, Sphaeromonas communis*) mesophilic and thermophilic aerobic bacteria (*Cellulomonas* sp., *Cellvibrio* sp., *Microbispora bispora*, and *Thermomonospora* sp.), mesophilic and thermophilic anaerobic bacteria (*Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens, Bacteroides succinogenes, Ruminococcus albus, Ruminococcus flavefaciens, and Clostridium thermocellum*) as well as actinomycetes (*Thermomonospora fusca*) also produce active cellulases [Ait, *et al.,*1979; Bartley, *et al.,*1984; Beguin, and Anbert, 1993; Begnin,*et al.,* 1983; Bhat,*et al.,* 1993; Bhat, and Maheshwari, 1987). Among the above noted microorganisms, the cellulolytic thermophilic microorganisms are of particular interest, because of their ability to produce thermostable cellulases which are generally stable under a variety of severe conditions including highly acidic and alkaline pH as well as temperatures up to 90°C.

Endoglucanases of *C. paradoxa* have been reported to act preferentially on amorphous substrates (Wood, 1991). However, degradation of crystalline cellulose by the low molecular mass endoglucanase S from Streptomyces sp. LX has also been reported (Li, *et al.,* 1998). Strains of *C. paradoxa* also produce other extracellular enzymes. Amylase production by *C. paradoxa* isolated from the pith of the sago palm was reported (Kainoma *et al.,* 1985). More recently, several strains of *C. paradoxa* isolated from olive mill wastewater disposal ponds were found to produce laccase activity (Robles. *et al.,* 2000). Among them, the strain *C. paradoxa* CH32 also produced â-glucosidase activity (Lucas, *et al.,* 2000). The endoglucanase enzyme from *C. paradoxa* CH32 showed optimum activity under acidic pH values and under moderate temperatures of incubation. In this respect it resembles other endoglucanases produced by mesophilic fungi, although

endoglucanases differ markedly in their pH and temperature optima for activity (Schulein, 1998).

Mechanism of cellulase activity

Cellulase is an inducible enzyme system (Kubicek, 1992, Ryu, and Mandels, 1980). All microorganisms studied so far have produced the highest level of cellulase when grown on cellulose (Wood, 1985). Cellobiose, lactose and sophorose are also known to facilitate the production of either complete or incomplete cellulase system by few microorganisms (Mandels, and Reese, 1960, Nisizawa, 1971, Ryu, and Mandels, 1980). Synthetic compounds such as palmitate and acetate esters of disaccharides and thiocellobiose have also been shown to function as inducers of cellulases (Ryu, and Mandels, 1980). However, cellulose was found to be the best carbon source for the production of high levels of cellulase by many microorganisms (Ryu, and Mandels, 1980, Wood, 1985).

The most generally accepted view of induction process is that the low levels of cellulase constitutively produced by the microorganism, first hydrolyses cellulose to soluble sugars (Beguin, and Anbert, 1993, Kubicek, 1992). These sugars are presumably converted into true inducers, which enter the cell and either directly or indirectly influence DNA binding protein and promote cellulase gene expression. It has also been suggested that in case of Trichoderma, the conidial bound cellobiohydrolase hydrolyses the cellulose and releases cellobiose and CBL (cellobiono-tS-1,5-1actone). The cellobiose and CBL are taken up by the mycelia and promote cellulase synthesis (Kubicek, *et al.,* 1988). The extracellular cellulase components of most fungi are generally found to exist as individual entities (Coughlan, 1985). Although many fungi secrete separate cellulase components into

culture medium, it is not yet clearly known how these components interact on the surface of crystalline cellulose and affect the extensive hydrolysis of cellulose.

Pectinase

The pectic enzymes were found to cause the maceration and cell killing that is the characteristic of the disease. Pectic enzymes and their products have also been implicated in elicitation of different host defense reactions such as elicitation of phytoalexin (Bruce and West, 1982; Davis *et al.,* 1986). These enzymes were also secreted by post-harvest pathogens viz., *Botrytis cinerea, Sclerotinia fructigena, Fusarium oxysporum*, *Pyrenochaeta lycopersici, C. lindemuthianum, Colletotrichum gloeosporoides and Alternaria alternata* as part of their strategy for penetrating the plant host cell walls (Scott and Fielding, 1985). They were primarily involved in the degradation of macro-molecules of host cell wall to facilitate their entry (Fogarty and Kelly, 1979). A convincing role of these enzymes in pathogenesis has been established by several workers (Lakshmesha, 2006; Brown and Adikaram, 1982).

Pathogenicity of *C. paradoxa* was found to depend upon the ability to produce cell wall degrading enzymes viz., cellulase rather than pectinase. A detail review of literature is not presented. In addition it also depends upon the stage of ripening of fruits and vegetables during storage. Wherein, there was an increase in the production of endogeneous cellulase and pectinase with an advance in ripening or during storage period in pineapple and other fruits and vegetables (Kertesz, 1951; Padmini Nagaraj, 1987).

Control Measures

Physical control measures

Various physical methods like UV illumination and Radiation was tried on *C. paradoxa* in vitro. The result indicated prolonged time of UV illumination and high and unpermissible level dosage (>8 kGy) to get desired result of inactivation of *C. paradoxa* reproductive propugules and young mycelia. Further practical difficulties to obtain uniform illumination of UV or radiation for pineapple fruit warrant restricting the work for in vitro only. The results presented were of academic interest. Important literature review of these treatments is presented in this review. However available literature regarding hot water both in vitro and in vivo is presented.

Ultraviolet illumination

Ultraviolet (UV) illumination is known to damage plant DNA and to affect several phusiologicl processes (Stapleton, 1992). However, a special interest has recently een drawn to the ability of low doses of UV-C light (wavelength of 190-200 nm) to induce disease resistance in a wide range of fruits and vegetables due to induction of phytoalexins (Rodov *et al.,* 1995; Rodov *et al.,* 1992; Droby *et al.,* 1993; Lu *et al.,* 1991) Pathogenesis related (PR) proteins (Portal *et al.,* 1999; El Ghaouth, 1994; Mercier *et al.,* 1993). The temporary effect of UV-C treatments was demonstrated in citrus frurits inoculated with *P. digitatum* (Droby *et al.,* 1993a).

Radiation

Radiation is used for food preservation, the inhibition of sprouting in potatoes and onions, control post harvest diseases (Thomas 1985; Barkai-Golan 1992), delay ripening (Maxie and Abdel-Dader, 1966; Mukherjii *et al.,* 1995) and control of insect infestation for quarantine purpose. It may however, adversely affect quality (Barkai-Golan 1992) . Radiation sources that have been used include gamma rays (cobalt 60 or caesium 137) and fast electrons (linear accelerators) each have its merits and limitations. Radiation systems are costly but can be easily integrated with other storage and handling methods and are now used mostly in the food packaging industries.

Radiation approval

List of commodities approved for radiation by health authorities in various countries has been considerable lengthened. Gamma irradiation up to a dosage of 1 kGy was approved by the Food and Drug Administration of the United States (Barkai-Golan 1992).

Hot water treatment

Stem end rot or Black rot of pineapple is controlled by the application of specific fungicides (Liu and Marcano, 1973, Sridhar, 1975 and Cho *et al.,* 1977). However, increasing consumer resistance and the restrictions imposed on the use of these chemicals have created an urgent need for the development of safe and effective alternatives.

Heat treatment technology is a safe and environmentally friendly procedure with increasing acceptability in commercial operations. It is used successfully, to control the incidence of postharvest disease in several commodities (Fallik, 2004). Pre-storage heat treatments to control decay are applied for short periods of time (min), as target pathogens are present in the outer-most layers of host tissue. Water is the preferred medium of application as it is a more efficient medium of heat transfer than air (Lurie, 1998). Pre-storage hot water treatments, methods of hot water immersion and reatment duration have been reviewed by Fallik (2004). The thermal death point of *C. paradoxa* is recorded as 52.5-53 °C (Ames, 1915). Hot water dip treatment at 53 °C followed by TMTD (Thiram) resulted in a limited control of the pathogen in sugarcane propagules (Buergo *et al.,* 1989). However, a hot water dip treatment of 1 min at 52 °C slowed the rate of rot development in litchi (Olesen *et al.,* 2004). Thus, the use of hot water dip treatments as a means of controlling incidence of black rot in Mauritius variety ineapples was investigated.

Chemical control measures

A detail account of chemical measure employed to control SER disease of pineapple has been presented. However in the present investigation extensive in vitro and in vivo work was carried out by using GRAS, preservatives and antioxidants which are either constitutive in fruits or inductive due to infection. These chemicals are used for the first time on *C. paradoxa*. Hence review of literature in this regard is not available. However appropriate literatures were cited to interpret out results.

Control measures for SER disease of pineapple

Black rot is commercially managed by minimizing bruising of fruit during harvest and handling, by refrigeration and with chemicals. Fruit must be dipped in an appropriate Fungicide within 6–12 h following harvest prior to packing and shipping (Rohrbach Phillips, 1990). Internal-browning symptom development can be reduced by waxing with paraffin polyethylene waxes at wax-to-water ratios of 1: 4–9 (Rohrbach and Apt, 1986). Waxing has been shown to increase internal CO_2 concentrations, thereby lowering O_2 concentrations, which results in reduced polyphenol oxidase (Paull and Rohrbach, 1985). The Penicillium-induced FCR, LP and IFC fruit diseases have been reduced by applications of endosulphan (3.35 kg a.i. /ha in 2338 l water) at forcing and 3 weeks following forcing. Reductions have been significant but only under low to moderate disease levels (Le Grice and Marr, 1970; Rohrbach *et al.,* 1981; Rohrbach and Apt, 1986). Fungicides, such as benomyl, have not been effective unless applied directly into the open heart as the inflorescence emerges (K.G. Rohrbach, unpublished results). Control of typical FCR induced by F. subglutinans has not been demonstrated. Control of fusariosis is most effective by planting disease-free seed material and by controlling insects, particularly the bud moth (Laville, \1980). Hot-water treatment of seed material at 54°C for 90 min with benomyl at 50 g /100 L is effective for disinfestation but will retard growth and kill up to 50% of the plants (Maffia, 1980). Fungicides, such as captafol, at 700 g a.i./ ha, starting at differentiation through harvest at 20-day intervals, have given good control of the fruit-rot phase in Brazil (Bolkan *et al.,* 1978). Resistance to fusariosis occurs in Ananas and Pseudoananas (Laville, 1980). Scale can be controlled relatively easily by preharvest applications of an appropriate registered insecticide, taking into consideration last application to harvest residue restrictions.

Both pineapple butt and black rot are caused by the fungus *C. paradoxa*. The severity of the problem in fresh fruit is dependent on the degree of bruising or wounding during harvesting and packing, the level of inoculum on the fruit and the storage temperature during transportation and marketing. Currently, these diseases are controlled by dipping the crown or fruit in a fungicide prior to planting or shipping of the fruit (Cho *et al.,* 1977). Treatment must be done in 12 h or less from the time the crown or fruit is removed from the plant (Rohrbach and Phillips, 1990). Inoculum levels on fruit at harvest vary according to the environmental conditions prior to harvest. The high correlation between moisture (rainfall duration) prior to harvest and disease following harvest has resulted in the name water rot. Storing seed material on the mother plants during dry weather, where there is good air circulation and minimal exposure to inoculum-infested 244 K.G.

Stem end rot is commercially controlled in fresh fruit by minimizing bruising of fruit during harvest and handling and with chemicals. Fruit must be dipped in a fungicide within 6–12 h following harvest prior to packing and shipping. Currently fruit can be dipped in triadimefon. The 'Queen' cultivar is generally more susceptible to *C. paradoxa*.

In accordance with the objectives and work carried out in this investigation, review of literature was emphasized on the etiology of disease, biochemical changes in pineapple in relation to ripening and during pathogenesis of *Ceratocystis paradoxa*. Cell wall degrading enzymes a major pathogenic factor of *C. paradoxa* was presented accordingly.

Scope of the Present Investigation

Pineapple is the third major commercial, high valued fruit crop grown all over India. Pineapple is an important fruit both nutritionally and nutraceutically. It is rich in Bromelain (1.5 %). Bromelain is currently utilized for therapeutic purposes like- antioxidant, anti-inflammatory, platelet aggregation inhibitory, inhibition of tumor cell growth and metastasis as well as debridement of burns.

Pineapple fruits usually have a very short post harvest life. Decay due to Stem end rot (SER) caused by *C. paradoxa* is an important factor, which limits the storage life of pineapple, and results in appreciable losses at wholesale, retail, and consumer levels. The tropical climate that prevails in India further aggravates the spoilage in pineapple. Post-harvest losses of around 70 % have been reported in India. Economic losses caused by post-harvest pathogens are costly in terms of money and man power, can be catastrophic for developing country like India. Hence there is an urgent need to minimize post-harvest loss due to Stem end rot spoilage in pineapple.

The conventional approach to control fungi has been the use of synthetic anti-fungal compounds. However, recently doubts have increasingly been expressed about the safety of many fungicides. It has emerged that, a significant number of commonly used fungicides pose threat to human health and environment. This necessitates developing an alternative, effective, nontoxic or zero residues, host friendly and pathogen specific

treatments to control post-harvest pathogens. This demands a clear understanding of intricate relationship that exists between host and pathogenic factors. Hence a detail investigation was undertaken with the following objectives.

1. Etiology of stem end rot (SER) disease in pineapple.
2. Host-pathogen factors in SER disease of pineapple fruit
3. Cellulase production and its activity in *Ceratocystis paradoxa*.
4. Physical and chemical measures to control SER disease

Chapter 1

Etiology of stem end rot (SER) disease in pineapple

Introduction

Pineapple fruit loss due to spoilage after harvest was estimated to be around 70% in India (Salunkhe 1992). Stem end rot (SER) makes fruits unavailable both for fresh consumption and for processing, thus forming a major limitation for the pineapple Industry in India. Post harvest losses are costly both in terms of money and manpower. Further it also engrosses production cost. In India pineapple cultivar 'queen' is extensively grown (>50%) and preferred fruit for fresh consumption. It was found to be more susceptible for SER infection. Tropical condition endowed with high temperature and humidity aggravates the rate of SER spoilage. The epidemiology of disease depends upon the variety, strain of pathogen, handling practices and storage conditions. Conservation of fresh fruits from SER disease is of utmost importance due to increasing demand for fresh consumption and also due to its nutraceutical properties. Precise pathogenic invasion, its colonization, maturity/ripe stage and underlying chemical factors at which pineapple fruit becomes susceptible for *C. paradoxa* infection has not been clearly documented. This situation warrants identification of susceptible stage and associated factors of pineapple fruit for SER disease. It is equally important to investigate pathogenesis of *C. paradoxa*, which is involved in SER disease of pineapple. The details of the work carried out in this regard are presented in this chapter.

Materials and Methods

Pathogenic isolates (*Ceratocystis paradoxa*)

C. paradoxa was isolated from the stem end of a naturally infected pineapple showing characteristic symptoms of black rot disease. Infected fruit is split longitudinally by a sterile knife. Growing margin of the lesion or water soaked region or leading edge region is carefully cut into small pieces of 3-4 mm blocks using sterile scalpel and surface sterilized it by dipping in 70% ethanol for 1 min. The blocks were washed with sterile water and plated on PDA media. The plates were incubated at 27±1^{o}C for 4-5 days. The major fungal colony developed was re-isolated from the growing margin of the colony and pure cultured on fresh PDA plates. Pure cultures of the isolate were maintained on potato dextrose agar (PDA) at 4^{o}C. Pathogenicity of the culture was maintained by inoculation and re-isolation of the pathogen at regular intervals on the pineapple (El-Neshawy and Wilson, 1997;).

Pathogenesity test of the isolates

Isolated pure cultures were subjected to Koch's postulate studies to establish stem end rot host-pathogen relation in the current scenario. Spores are harvested from 7 day old pure fungal culture obtained from infected pineapple fruit showing characteristic stem end rot symptom. Using sterile water containing 0.1% Tween -20, spore suspension of 10^5 spores/ml was prepared and spread on the freshly cut peduncle of matured pineapple. Ten matured fruits are used for the Pathogenicity test (Piano *et al.,* 1997). The fruits are incubated at room temperature (27°C) for 6 days. The fruits are split open to investigate the incidence and severity of the infection. In all the cases the fruit was found to be infected and showing characteristic symptom of stem end rot.

Culture maintenance and deposition

The culture was tested periodically by inoculation on the Pineapple and reisolation of the culture. The culture was maintained on PDA and stored at low temperature (4^{o}C). The culture was deposited in microbilogical collection center of the Institute (Acc. No. cftri/fvt 3695).

Preparation of spore suspension

A spore suspension of 0 to 10^6 spores /ml were prepared from 5 day old cultures grown on PDA at 27±1°C. Cultures were flooded with sterile distilled water containing 200-400 µl/ L Tween-20 and the surface of the culture was carefully scraped with a sterile, disposable loop without disturbing the agar. The resulting suspension was vortexed for ca. 30 s to break up any chains of spores. Spore concentration was determined with a heamocytometer after filtration through layered sterile muslin and suspensions were used within 2–3 h.

Minimum threshold concentration of spores (MTCS)

Minimum threshold concentration of inoculum for in vivo studies was determined by preparing spore concentrations in sterile distilled water with 0.1% tween-20, ranging from 10 to 10^6 spores/ml of *C. paradoxa*. Peduncles of ten fruit were inoculated for each spore concentration. All inoculations were done by atomizing 1ml of prepared spore suspensions on the cut peduncle end of the pineapple fruit. For each concentration 10 fruits are used. The fruits were incubated for 6 days at room temperature 28 ±2°C, the incidence and severity was stem end rot was analysed as described in Collins (1968). A set of ten non-inoculated fruit served as controls. Peduncles were trimmed to a length of 2 cm before inoculation and washed with sterile distilled water. The experiment was

repeated thrice. 10^5 spores/ml concentrations have given 100% incidence with highest severity in disease consistently. Hence 10^5 spores /ml were used in all artificial infection procedures in this investigation. Separate set of three sterile screw cap test tubes, each containing 10 ml of the spore suspension was used.

In-vitro Spore germination

Spore suspension (1×10^5 spores ml^{-1}) was prepared using sterile pineapple juice filtrate. The pineapple juice filtrate was prepared by extracting the juice from field-ripened fruit using a juice press. The juice was filtered through six layers of cheesecloth and an equal amount of distilled water was added. The juice filtrate was filtered through sterile Whatman No. 1 filter paper then with a 0.22 micron syringe filter (Micron Separations, Inc., Westboro, MA. The juice was diluted with sterilized distilled water in the ration of 1:1 vol. The solution was inoculated with spores in sterilized cavity slides and incubated at 27°C. The germination of spores were observed periodically at 12, 24, 48 hrs. (Reyes *et al.,* (2004)

Radial measurement of fungal growth

Two 4 mm-discs of *C. paradoxa* grown on PDA for 7 days or 200µl *C. paradoxa* spore suspension (1 $x10^5$ spores /ml) was added to the plate. Plates were incubated at 27° C for 7 days. Radial growth of *C. paradoxa*, was observed according the method (Reyes *et al.,* (2004) using vernier caliperse.

In-vivo experiments

For in vivo experiments, the inoculum of *C. paradoxa* spores was prepared as described previously in the in vitro studies. Concentration of inoculum for in vivo studies

was determined by preparing spore concentrations in sterile distilled water, ranging from 10 to 10^6 spores/ml of *C. paradoxa*. Peduncles of ten fruit were inoculated for each spore concentration. A set of ten non-inoculated fruit served as controls. Peduncles were trimmed to a length of 3–4 cm before inoculation. The cut portion was washed with steril distilled water. Fruit were incubated at 25 ±1° C and 85-90 % RH for 6 days, and examined for incidence and severity of disease. The experiment was repeated twice. Results confirmed that the 10^5 spores/ml were optimum thresh hold limits to cause 100% incidence of diseases. Thereafter, 0.1 ml of the spore suspension (10^5 spores/ml) was used to inoculate the peduncles soon after trimming and washing with sterile distilled water. With non-inoculated treatments, 0.1 ml of sterile distilled water was placed on the cut surface of the peduncle. All fruit were incubated at room temperature 28+2^{o}C.

Procurement of Pineapple

Healthy fresh and matured Pineapple fruit (*Ananas comosus* var. Queen) used for infection studies, were obtained from Fruit wholesale market at Mysore where harvested fruits reach the market within 6-8 hours from the production centre. Fruits are selected as to contain 4–6 cm of peduncle on the fruit, to prevent possible contamination of the fruit with field inoculum.

Infection Vs Maturity of pineapple fruits

Maturity indices of pineapple fruits are determined by de-greening of shell color of the ‘eye’ or lechet at the stem end region. Based on Chemical constitutents like total soluble solids, acidity and sugars the pineapple fruits are categorized into two different maturity Physiological maturity (80%) and Commercial maturity (100%) as described by Collins (1968). The Physiological maturity (80%) fruits showed no difference in its

ripening behaviour and sensory profile but showed 7 days increase in storage period. Hence both the maturities were used in this study.

Infection Vs Ripening

Ripening was characterized by degreening or change of shell colour after harvest of pineapple. Harvested pineapple were stored in room temperature 28+2^{o}C and the fruits were inoculated with 10^5 spore/ml concentration on pineapple at different stages of ripening. Ripening stages were characterized by 0 or green, 25% yellow, 50% yellow, 75% yellow and 100% yellow fruits as described (Brat et.al 2004). Ten fruits were kept in separate plastic crates at room temperature for each observation. The experiments were repeated thrice.

Tissue preparation for scanning electron microscopy

Blocks of diseased tissue (3-4 mm) from *C. paradoxa* infected and healthy pineapple fruit, were fixed in formalin – propionic - proponaol (10:10:80) (FPP) for two hour. Sections were dehydrated in concentration graded (from 50 % to 90 %) isopropyl series and then infiltrated stem tissues were embedded in paraplast (Johansen, 1940). The specimens were softened in a solution of 1 % sodium lauryl sulfate for 48 hours. Tissue was dried, mounted on aluminum stubs with double - stick tape. They were sputter-coated with 20 nm gold palladium and observed under scanning electron microscope (Model No. LEO 435 VP, Sl. No. 435-08-02, Leo Electron Microscopy Ltd., Chiton Road, Cambridge, CBI 3QH, England).

Results and Discussion

Etiology of SER Disease

Ceratocystis paradoxa infection starts from cut stem/peduncle or from surface wounds of the fruit after harvest. The pathogen gains entry to the fruit and

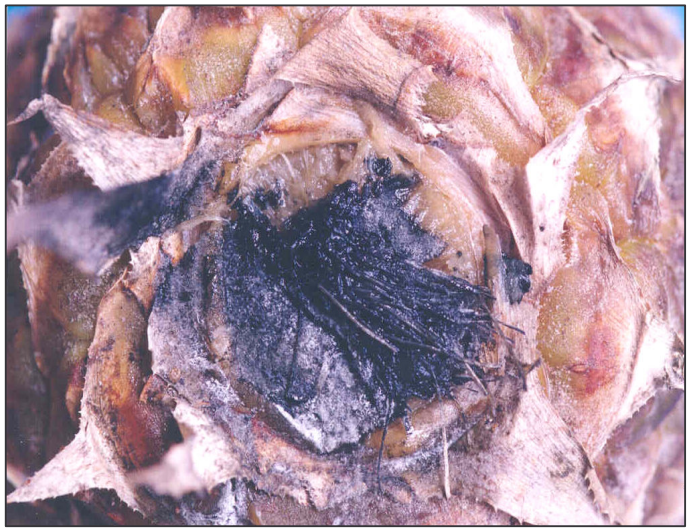


Fig.1.1:
Symptoms of SER disease on pineapple

rapidly establishes in the core region of the fruit. Once established physiological contact with the fruit the fungus grow quickly along the stem region and from this stage on the visible symptoms are distinct as water soaked patches upon dissection of the stem of the fruit referred as 'leakers' . These are the fruits where the broken peduncle remains wet, soft and associated with tissue translucency. The disease progress rapidly under ambient conditions of tropics, typify by high temperature and humid conditions. With advance of Disease the external symptoms manifestation being softening of peduncle or commonly referred as stem end of pineapple. Subsequently productions of black spores make the stem end soft and leaky (Fig 1.1). Hence the fruit rot that initiate at the cut peduncle and progress rapidly referred as Stem End Rot (SER). Hence forth the disease is referred in abbreviated for as SER. Under advance stage of disease soft tissues around the conducting strands in the stem disintegrates resulting fibrous appearance (Fig 1.1).

The fungi colonized regions become soft, water soaked, translucent tissue. It appears initially in the core region then slowly they spread to pulp region of the fruit. Internal 'Leaky' appearance of both core and pulp tissue heralds the onset of SER disease

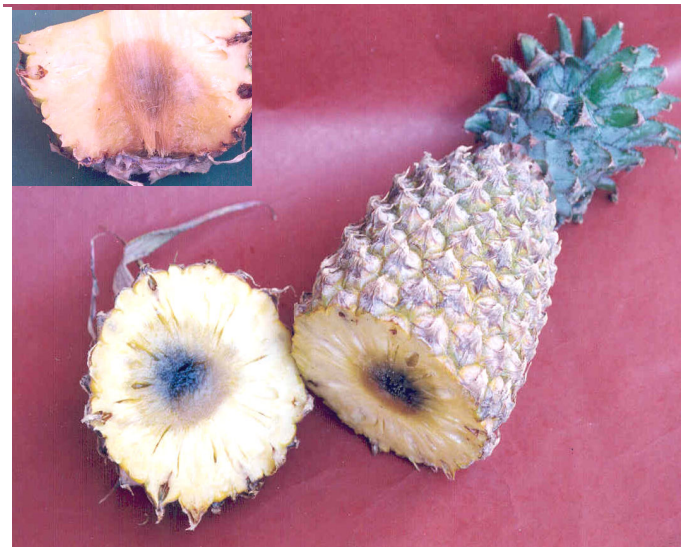


Fig.1.2:

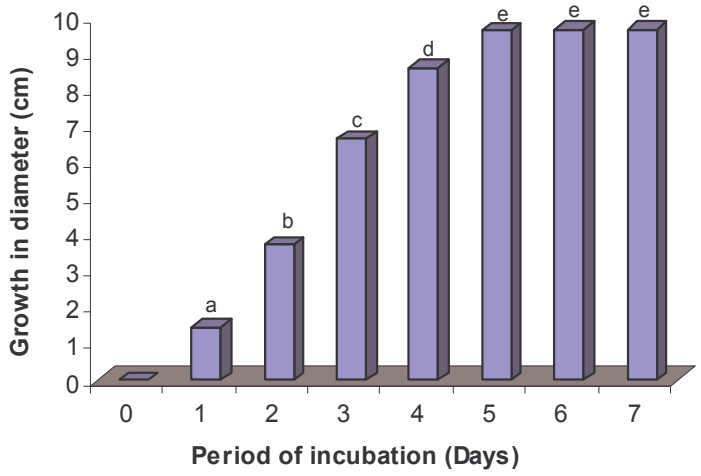
Cross sections of pineapple infected with *C. paradoxa*

in pineapple. The infected fruits are referred as 'Leakers', these are the fruit where both core and surrounding pulp at the stem end region appears wet and is associated with tissue translucency (Fig 1.2). Thus it appears *C. paradoxa* predispose the fruit tissue before its invasion and establishment of disease. The predisposed tissue was of interest study the pathogenesis, the work carried out is presented in chapter 2.

The disease appears to spread rapidly through the core region of the pineapple. When ¼ of the fruits are infected i.e., 3-5 days after infection, the fungus enters the sporulating phase; the sporulation also starts in the core region. Initially the predisposed sort tissue region that appears translucent turns black in colour due to overproduced mass of *C. paradoxa* spores (Fig 1.2). This symptom and its ultimate manifestation give the fitting name as black rot disease. The SER or black rot of pineapple was first reported from southeren part of India by Sridhar (1975) and subsequently by Aradhya (1982).

Morphological characters of *C. paradoxa*

The fungus exhibited luxuriously growth in Potato Dextrose Agar (PDA) media incubated at 27° C. The rate of growth was found to be a function of time. Two fold average increase



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Fig.1.3:

Radial growth of C. paradoxa on Potato Dextrose Agar media

in growth was observed for every 24hrs of incubation (Fig 1.4). The culture on PDA media appears black in colour with white margin after 4days of inoculation (Fig 1.4). This is due to production of black conidiospore as confirmed by microscopic observation. The culture was tested periodically by inoculation on the Pineapple and reisolation of the culture. The culture was maintained on PDA and stored at low temperature (4°C). The culture was deposited in microbilogical collection center of the Institute.

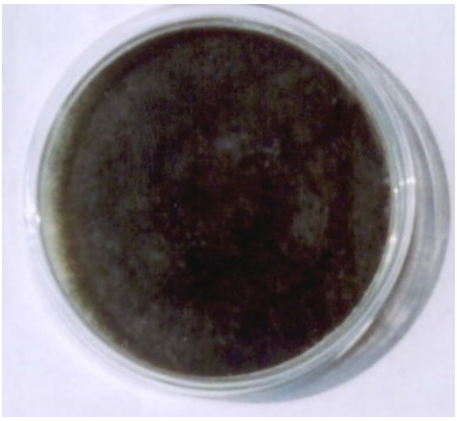


Fig.1.4:

Seven day old pure culture of *C. paradoxa* on Potato Dextrose Agar media

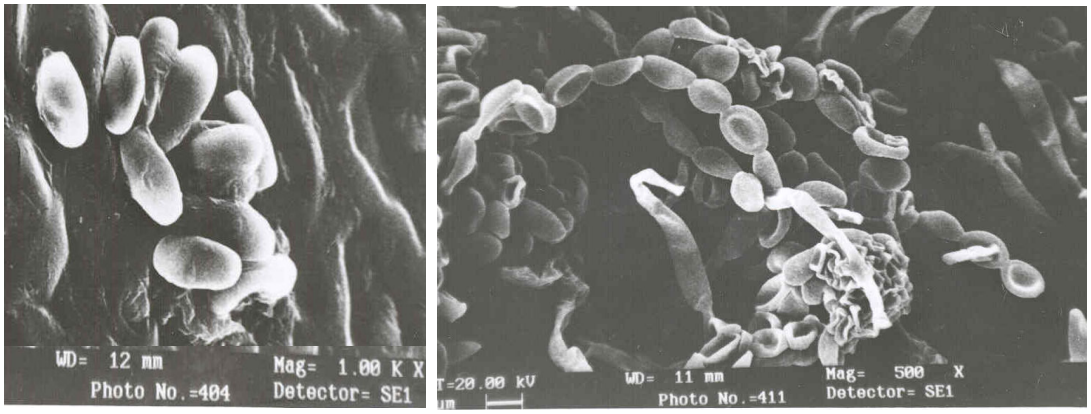
Scanning Electron Microscopy (SEM) of SER Disease

"Stem end rot disease manifestation is the ultimate expression of its final display of all its complex interrelationships with its pathogen". Scanning Electron Microscopy (SEM) is especially valuable in the study of the morphology and ontogeny of infection structures formed by *C. paradoxa* in the fruit and stem tissues. So far we have not found studies on the ultra structure of the *C. paradoxa* on pineapple fruit. In the present study SEM has been employed to elucidate the establishment and physical ultra structural relationships of pathogen within the host tissues. Hence, studies on host-parasite relationships to investigate the spatial arrangement of intracellular fungal structures by scanning electron microscopic studies were carried out. The objective of this study is to elucidate the histopathological tissue patterns under delimitation of pathogen colonization in pineapple fruit tissues. Understanding the nature, intensity and structural relationship of pathogen with host may helps in evolving effective methods to develop strategies for fruit protection. In the present investigation histopathological changes both healthy and *C. paradoxa* infected pineapple tissue were carried out by SEM.

The SER disease causing fungi was isolated on PDA media from naturally

Fig.1.5:

Single and chain of conidiospores of *C. paradoxa* (SEM)



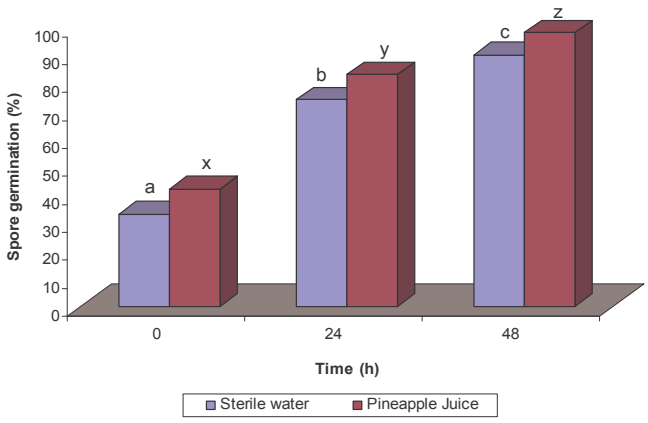
infected pineapple fruit. The pathogenic nature of the fungi was confirmed by cosch

postulation method. The taxonomic identification of the pathogenic fungi culture was carried out by its colony characteristic, morphological and reproductive structures. Based on the above features pathogen has been identified as *C. pardoxa* (Dade) Syn. *Thielaviopsis paradoxa* or *Chalara paradoxa* by using manual (Paulin-mahady *et al*., 2002). The chlamydospores are black in colour, oval in shape. Single or they are produced in chains (Fig 1.5).

In vivo experiments were carried out to test the spore germination, thresh hold concentration of spores required to cause infection and also to test the influence of pineapple juice on germination of spores and incidence of disease. The results are discussed below.

Percentage germination of *C. paradoxa* Spores in water and pine apple juice

It appears germination *C. paradoxa* conidiophores is a function of time and relative humidity. An increase in percentage of condiospres with increase in time of inoculation with water was observed. A significant increase in percentage of germination was observed with supplementation of water with pineapple juice by 1:1 volume (Fig 1.6) at all the duration of incubation. Presence of sugars and nutrional factors are the major reasons attributed to increased



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

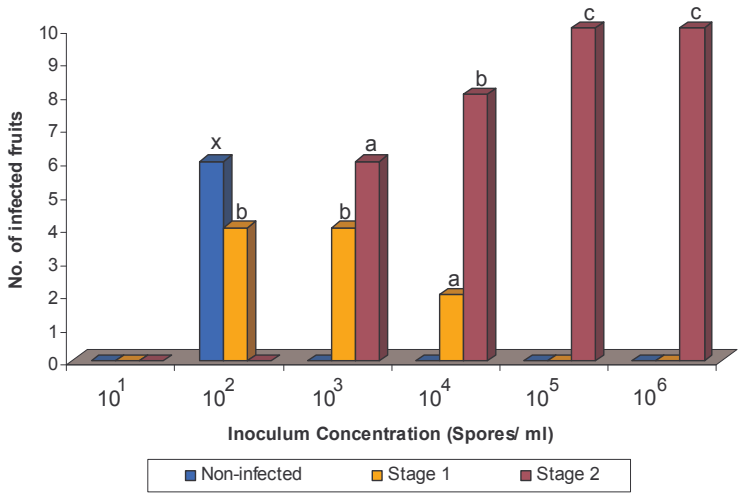
Fig.1.6:

Percentage germination of C. paradoxa spores

germination of conidiospores in *C. paradoxa*. apart from this certain compounds present in the host tissues might , on certain occasions, affect the host susceptibility to infection

Fig.I.7:

Incidence of SER disease at different concentration of *C. paradoxa* spores



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

in many fungi (Eckert and Ratnayake 1994; Arimoto *et al.,* 1995 and Fourie and Holz 1998) many fruits are more susceptible to the pathogen when their tissue are ina turgic state through being under high RH. The increased decay rate should be attributed to moisture held within the wounds (Eckert 1978). This finding demonstrated the practical importance of removal of pineapple fruit exudates at the cut stem end that is stimulatory to germination of pathogenic condisospores of *C. paradoxa*.

The results from the invivo experient to determine the effective concentration of spores for development of SER in pineapple are shown in (Fig 1.7). Infection was failed to occur in 10^1 and 10^2 spores/ml concentration. Incidence of infection and severity of infection was less than 50% at the concentration 10^3 spores/ml concentrations. Further logerthimic increase in concentration of spores increase in disease incidence and severity. All the fruits showed typical SER infection at the end of 6 days after inoculation of the fruit at 10^5 and 10^6 spores/ml concentrations. Thus the threshold concentration of spores required for effective infection was fixed as10^5 spores/ml. This concentration appears to be higher when compared to Mauritius and other varieties as reported by (Sridhara 1975).

This may be due difference in inherent quality of pineapple varieties and difference in strains of *C. paradoxa*

Treatment	Inoculation period after SD water wash	Incidence (%)	Severity (%)
Sterile distilled H_2O	0	0	0
	2	100 [b]	57 [f]
	4	100 [b]	53 [e]
	8	100 [b]	48 [d]
	16	100 [b]	39 [c]
	24	100 [b]	32 [b]
	48	97 [a]	30 [a]

Data were analyzed using Waller–Duncan K-ratio t-test.

Means within a column followed by the same letter were not significantlydifferent (n = 20).

Table 1.1:
Effect of surface moisture on incidence and severity of SER in pineapple fruit inoculated with *C. paradoxa*

isolated. Successful infection was found to be dependent of the level of the inoculum available (Gauman 1946, Eden *et al.,* 1996). This finding demonstrated the practical importance of reducing the inoculum level at the cut stem end of pineapple in order minimize infection due to *C. paradoxa*. Since *C. paradoxa* epend on a wound to enable them to penetrate into the host, it has generally been accepted that disease development is related to both the pathogen spore load and the availability of surface moisture on the cut stem end of pineapple. Hence study was undertaken to find the effect of surface moisture for incidence of SER disease in pineapple.

Surface water availiability or Relative humidity on the fruit surface plays an important role in fungal infection, mainly through germination of conidospores. True to this germination of chlamydospores in *C. paradoxa* is greatly influenced by the surface

moisture. Delay caused between the water wash and inoculation period resulted in significant reduction in severity of the disease (Table 1.1). However incidence of infection was not affected till 48hr delay in inoculation. The reduction in severity of SER disease is mainly attributed to relative humidity that influence the percentage of spore germination. It has been reported that *C. paradoxa* require 100% relative humidity for condial germination (Oruade Dimaro & Ecundaya 1992). Another possible explanation is that the epiphytic antagonist Yeast and saprophytic fungi which are reported on the pineapple fruit may colonize rapidly and offer resistance for *C. paradoxa* to infect (Oruade Dimaro & Ecundaya 1992). An alternative explanation would be the reduction of growth inhibitor, though not reported for these organisms.

Infection Vs Maturity of pineapple fruits

Pineapple fruits of different maturity viz. 1) Physiological maturity (80%) and 2) Commercial harvest maturity (100%) were selected based on their physico chemical properties. The results revealed that 100% matured fruits showed early infection, when

Table 1.2:

Major chemical characteristics of pineapples at different maturities

Chemical characteristics	Green (% maturity)		Ripe fruits (% ripening)			
	80 Physiological	100 Commercial	25	50	75	100
T.S.S (° Brix)	10.1± 1.2	11.0 ± 1.5	12.3± 1.4	13.0± 1.8	13.4± 1.0	14.2± 0.7
Acidity (%)	1.0 ± 0.9	0.87 ± 1.0	0.5 ± 0.2	0.48 ± 0.5	0.42 ± 1.2	0.4 ± 1.1
pH	3.1 ± 1.3	3. 37 ± 0.4	3.5 ± 0.9	4.1 ± 1.2	4. 8 ± 1.0	5.0 ± 0.8
Total sugar (mg/100gm)	6.0 ± 1.5	6.8 ± 2.2	7.9 ± 1.9	8.2 ± 2.6	8.5 ± 2.2	9.4 ± 1.6
Vitamin C (mg/100gm)	40.0 ± 1.8	39.4 ± 1.3	37.7 ±0.9	35.5 ± 2.2	31.5 ± 2.9	28.0 ± 1.4

compared to fruits at 80% maturity. High TSS and Vitamin C along with low sugar, acidity and pH in pineapple fruits at 80%, maturity were attributed to delayed expression of SER symptoms. (Table 1.2). Green fruit harvested at physiological maturity (80%)

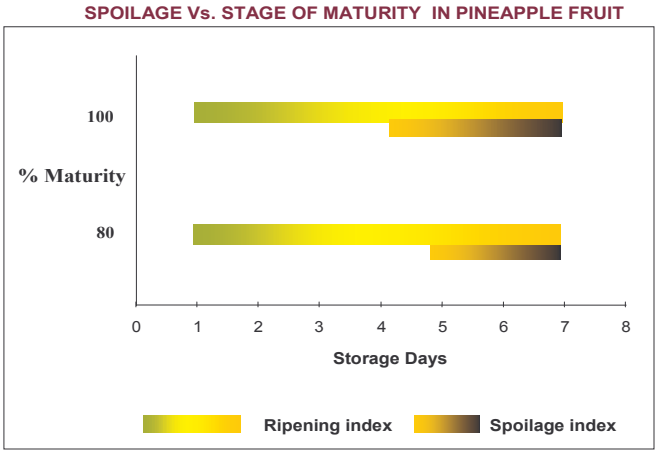


Fig.1.8:

Incidence of SER disease at different maturity and storage of pineapple fruits

takes 8-10 days to turn to complete characteristic orange-yellow colour while, 10-12 days for attaining 100% ripe. Interestingly the fruits were found to become susceptible for infection when it attained 50% ripe (Fig. 1.8). It appears that pH<4 and acidity <5% along with increase in sugar may be a key factors in increase in incidence and severity of disease. Increase in maturity and ripening stages of fruits with increased susceptibleness to disease is well documents by earlier workers in different fruits (Eckert 1987).

Infection Vs Ripening

Ripening was characterized by degreening or change of shell colour after harvest of pineapple. Harvested pineapples were stored in ambient conditions. Ripening of pineapple fruits by change in shell colour from green to orange yellow was observed daily. For matured Green fruit it takes 6-8 days for complete change of colour. Thus ripening stages in pineapple fruits were categorized into 5 stages viz. 100% green, 25% yellow, 50% yellow, 75% yellow and 100% yellow fruits. Matured green fruit took 5-6 days, while 100% ripe or complete yellow fruit took 2-3 days for expression of external SER symptoms (Fig 1.9). This study clearly indicated that incidence of disease

and expression SER symptoms by *C. paradoxa* are depends on the stage of ripeness in pineapple fruit. The early incidence and expression of symptoms with increase level of ripens in the pineapple fruits. Otherwise ripe pineapple fruits are more susceptible to *C. paradoxa* infection. Immature, green and 25% ripe fruits were found to be less or no SER incidence (Fig 1.9). The mechanism of maturity effect on SER incidence appears to be pre-exist factors like low acidity, pH, and less sugars in green fruit. The low pH (< 4) of the pineapple fruit is probably an important factor in their general resistance to *C. paradoxa* infection similar to other fruits (Lund 1983; Bartz and Eckert 1987). Fungal growth was found to increase with advance in ripening this may due to increase in sugars or sucrose in many fruits (Fourie and Holz, 1998)

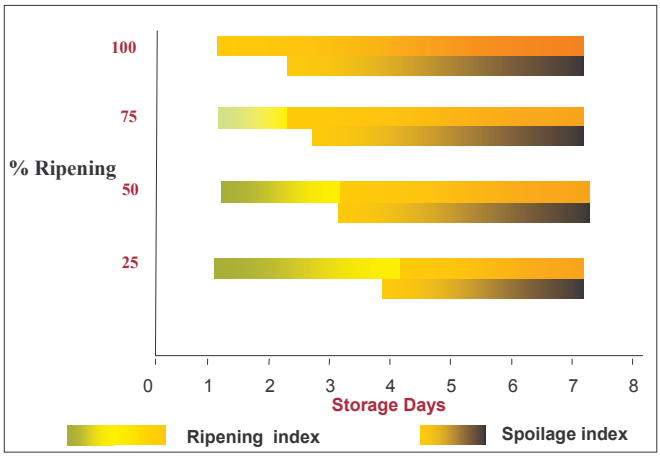


Fig.1.9:

Incidence of SER disease at different ripening stage and storage of pineapple fruits

The susceptibility of harvest pineapple fruit to *C. paradoxa* appear to depends mainly on their ripening stage. The result indicated that 50% ripe stage of fruit is more susceptible. Increase in pH, sugars and reduction in acidity at this stage may be optimum factors (Table1.2) associated with infection of *C. paradoxa*. Other factors affecting the impact of the ripening stage of pineapple fruit on SER disease susceptibility involve the enhanced virulence of the pathogen. The cellular changes underlying susceptibility during ripening and pathogen infection was carried out by scanning electron microscopy.

Healthy fruit tissue

The transverse section and longitudinal sections of core and pulp region of healthy pineapple fruit showed many layered large celled parenchymatous cells that constitute

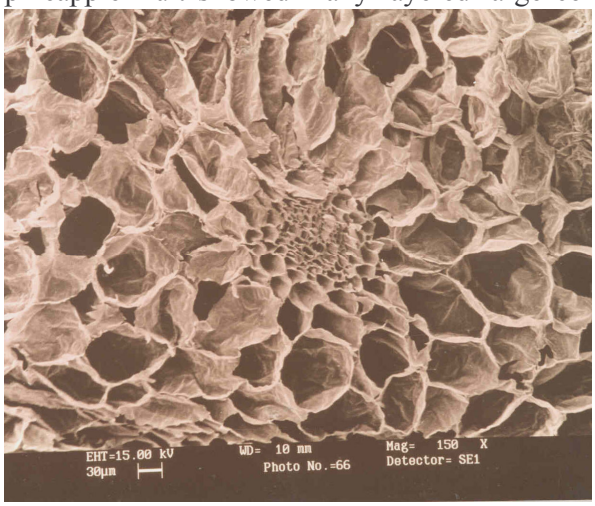


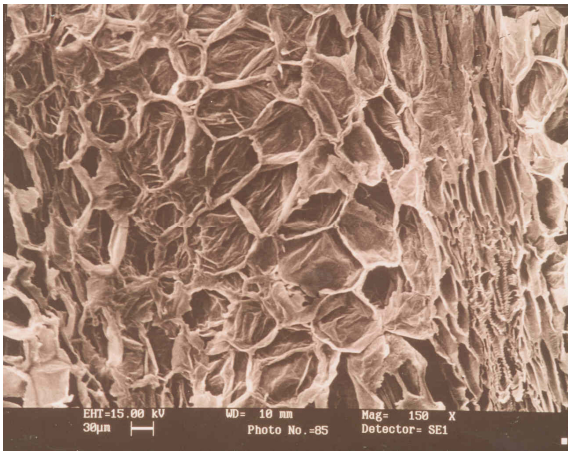
Fig.1.10:

SEM of transeverse section of healthy raw pineapple fruits

pulp region. The central narrow thick walled regions represent the core region of pineapple fruit. The narrow cylindrical cells arranged linearly represent the conducting tissue of the fruit which is an extension of peduncle. These conducting tissues constitute xylem and phloem. The adjacent conducting strands are connected by thin walled

Fig.1.11:

SEM of longitudinal section of healthy raw pineapple fruits



parenchymatious tissues (Fig 1.10). Infection begins with cut peduncle and spreads reapidly through central core regions. It appears that the thin wall paraenchymatous connecting tissue is preferred site of invasion for *C. paradoxa*, since the conducting tissue are thick and lignin in nature. Thus the infected core region gives fibrous appearance (Fig 1.11).

Ripening changes in healthy Pineapple fruits

The major and edible pulp portion of pineapple fruit tissue is constituted by thin walled compactly arranged parenchymatous cells (Fig 1.12). During normal process of

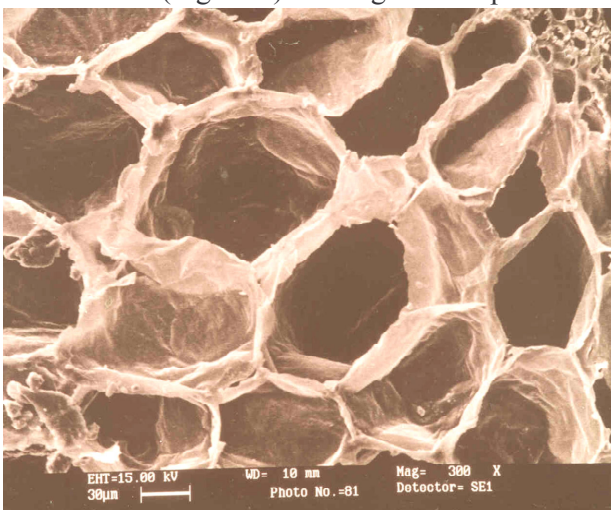


Fig.1.12:

SEM of transverse section of healthy ripe pineapple fruits

ripening and senescence these parenchymatous cells separate and become loosely arranges tissue. The separation and disintegration of cell wall resulting disfiguring of cell (Fig 1.13). This is a common phenomenon during ripening fruits (Barkai-Golan 1992). Cell wall disintegration along with accumulation of sugars during ripening of fruits is

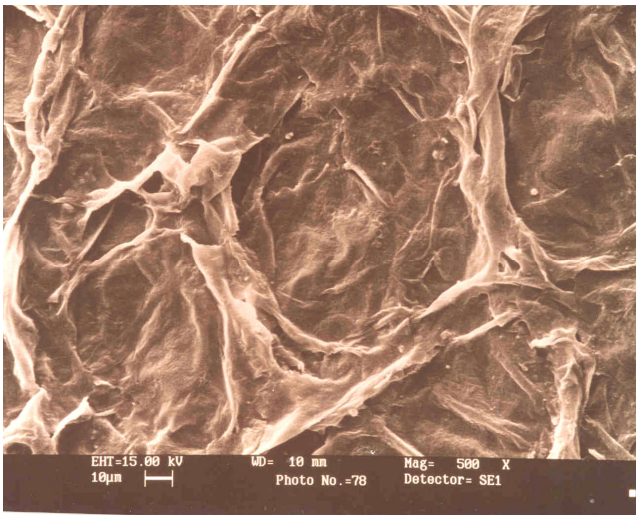


Fig.1.13:

SEM of transverse section of healthy ripe pineapple fruits

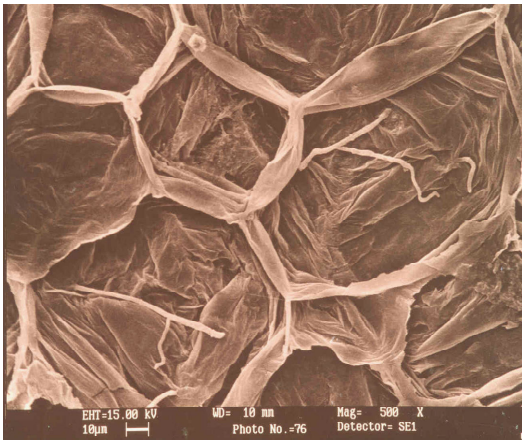
major factors responsible for susceptiblness of the fruit for infection (Eckert 1978).

Diseased fruit tissue

The infected tissue in pineapple tissue showed *C. paradoxa* hypha. It appears fungal hypha can traverse through the cell membrane (Fig 1.14), whether this process is

Fig.1.14:

SEM of transverse section of SER diseased pineapple fruit tissue



through plasmodesmata or altered membrane system due to infection process remains to be confirmed. At the initial stage of infection core and pulp tissue was ramified by hyphae without pronounced loss of structural integrity. Hyphae are swollen where their cell wall encountered, narrow as these pass through and enlarged again in the distal side of penetrated cell walls (Fig1.15). At the advance stage of infection there were apparent

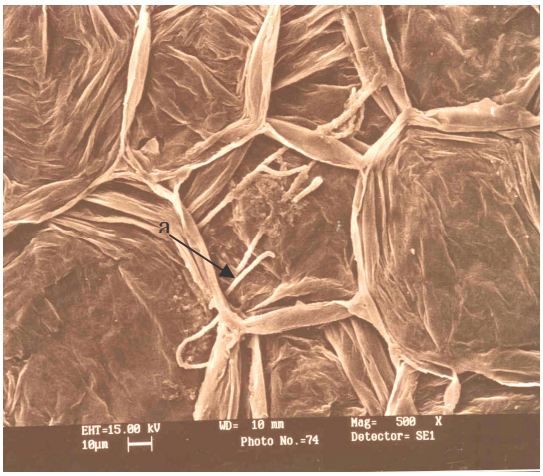


Fig.1.15:

SEM of transverse section of SER diseased pineapple fruit tissue

a*- Intracellular invasion and colonization of C. paradoxa mycelium*

changes like severe plasmolysis exhibited in cortical cells of fruit tissues. Baily *et al.* (1992) described two major invasion strategies *i.e.,* intracellular invasion and hemibiotrophic colonization (1.15). In pineapple infection intiates at the cut portion of

peduncle hence, subcuticular invasion is absent. However progress of infection was observed through intracellular hemibiotrophy.

Summary

Stages of infection processes of *C. paradoxa* in pineapple were defined. It showed range of colonization strategies viz., 1) Germination of conidspores 2) Penetration of host tissue through cut stem/peduncle end 3) Predisposal of host tissue 4) Intracellular invasion and 5) hemibiotrophic colonization. A minimum threshold concentration for infection of *C. paradoxa* spores account to be 10^5 spores/ml. Incidence and severity of SER disease in pineapple was found to be a function of degree of ripeness. Our results showed that the fruit maturity significantly affected SER incidence. Immature, green and 25% ripe fruits were found to be resistant for SER incidence. It appears that pre-exist factors like low acidity, pH, and less sugars in green fruit are responsible for reseistance of infection. The result indicated that 50% ripe stage of fruit is susceptible for *C. paradoxa* infection. Increase in pH, sugars and reduction in acidity with advance of ripening appears to be the key factors associated with infection of *C. paradoxa*. For the first time, SEM illustration of intercellular invasion and intracellular hemibiotrophic colonization of *C. paradoxa* were established in SER disease of pineapple.

Further characterization of the constitutive or induced pathogenic substrates involved in pineapple and *C. paradoxa* virulence factors associated with SER disease would help to better understand intrinsic factors and relationship between the host and pathogen. The work pursued in this regard is presented in the following chapter.

Chapter 2

Host - pathogen factors in SER disease of pineapple fruit

Introduction

Pineapple fruits usually have a very short post harvest life. Decay due to Stem end rot caused by *C. paradoxa* is an important disease, which limits the storage life of pineapple; results in appreciable losses at wholesale, retail, and consumer levels. The disease develops into a soft rot under warm humid conditions. Our earlier observations indicate black rot on fresh fruit occurs throughout the year especially when fruit referred to as "leakers". "Leakers" are those fruit where the broken peduncle remains wet and is associated with fruit translucency. Such fruit tissue translucency and subsequent browning of tissue surrounding infected or necrotic tissue of *C. paradoxa* was recognized as predisposition of host tissue for infection and colonization. Stem end rot disease establishment and manifestation in pineapple is the ultimate expression of complex interrelationships with its pathogen, the details of which is not well documented. *Ceraticystis paradoxa* is a facultative parasite, gains entry into pineapple through wounds and cut peduncle end. Despite the required threshold concentration of spores and favorable physical conditions, there was an inordinate delay in infection and incidence of stem end rot in green and 25% ripe fruits. In contrast 50% ripe fruits readily expressed infection and spreads rapidly with advance of ripening. The biochemical and pathological factors that govern pineapple fruit resistance in context with ripening and *C. paradoxa* infection are still unknown, which prompted us to undertake this study. The work carried out in this regard is presented below.

Materials and Methods

Pineapple fruits

Fresh matured pineapple fruits were obtained from local wholesale market in Mysore city and transported to the lab in plastic crates. The fruits were sorted to remove diseased or damaged and immature fruits. Based on size and colour development on the shell the fruits are further grouped into ten and kept in separated plastic crates. The plastic crates are arranged inside the cold room facility of dept maintained at 25 ± 1°C and 85-90 % Relative humidity. The measurement of physical, physiological, physicochemical, chemical and biochemical determination was carried out on daily basis, taking the pineapple fruits from storage and recording the storage day and ripening stage. The measurements are the mean of at three replicates of the experimental results.

Firmness

Texture evaluation was carried out according to the Kramer Shear test in an Instron 4301 texture analyzer. Shear force was measured using a 500 kg load cell and a stroke speed of 200 mm / min. The firmness was expressed as Newton / gm pineapple fruit (Priya Sethu *et al.,* 1996).

Colour

The pineapple fruit surface colour reflectance was measured (AOAC 1990a) using a Hunter lab color measuring system (Model *Lab Scan XE*, Hunter Associates Laboratory Inc, Virginia, USA) and recorded as *L a b*. The *L a b* color system consists of a luminance or lightness component '*L*' and two chromatic components: the component 'a'

for green (-*a*) to red (+*a*) and the '*b*' for blue (-*b*) to yellow (+*b*) colors. The colorimeter was calibrated using a standard white plate. Values of the white standard were L = 99.899, a = +0.236, b = +0.042.

PLW (Physiological loss of weight)

Loss of weight due to respiration and transpiration is a common feature in fresh commodity. The weight of the pineapple fruits stored at ambient conditions was recorded on daily basis for the 15 days and the decrease in fruit weight is expressed as % PLW over time. (Ranagnna, 2001)

Respiration

Respiration was measured as described by (Douglas *et al.,* 2003; Marrero, and Kader, 2006) Weighed fruits of each stage were enclosed in 5000 ml hermetic containers for one hour.Gas samples were taken from the outlet tubes of the containers. O2 and CO2 concentrations were measured using using Dansensor, Oxygen and Carbondioxide gas analyzer; model Checkmate 9900 O_2/Co_2, Denmark. The CO_2 evolution was calculated in ml/ Kg-h by using the formula:

$$CO_2 = \frac{\text{Density of } CO_2 \textbf{ x } CO_2 \text{ released (\%) } \textbf{x} \text{ Container Volume } \textbf{x} \text{ 60}}{\text{Weight of the Sample (Kg) x Enclosure time (min) x 100}}$$

Sample preparation

About 100 g of pineapple fruit was homogenized, and squeezed in two-layered muslin cloth, to extract the complete juice. The juice was centrifuged at 8000 rpm for 20 min at 4°C and used to determine pH, titrable acidity, total soluble solids (TSS), sugar content, protein content, and phenolic content.

pH

The pH of Pineapple juice was measured using a *Control Dynamics* pH meter, calibrated using standard buffers of pH 4 and 7 (HiMedia). The pH of the juice was measured by placing about 20 ml of juice in a 25 ml beaker and immersing the pH electrode in to the sample. Refrigerated samples were allowed to room temperature prior to pH measurement. Each measurement was replicated (Ranganna, 2001)

Total soluble solids

The °Brix, a measure of soluble a solid was determined by placing 3-4 drops of pineapple juice over a prism of the digital refractometer (ATAGO RX-5000), calibrated with triple distilled water. The °Brix value was recorded at 25°C, directly from the digital readout of the refractometer as % sucrose content. Each measurement was replicated (Ranganna, 2001).

Titrable acidity

Titrable acidity was determined by AOAC method [1990]. About 2-3 g of freshly extracted pineapple juice was titrated against 0.1N NaOH with phenolphthalein as indicator until pink color persisted. Titrable acidity was calculated using the following formula and expressed as *% citric acid* content. Or mg/100 gm FW of pineapple.

$$\text{Titrable acidity (\% citric acid)} = \left(\frac{64 \text{ x } N_{(NaOH)} \text{ x Titre value}}{\text{Wt. of sample x 1000}} \right) \text{x 100}$$

Ascorbic acid content

Ascorbic acid content was determined by HPLC method, modified from Wimalasiri & Wills (1983) as follows: About 5g of pomegranate arils were blended in 3% (w/v) metaphosphoric acid, centrifuged at 10,000 rpm for 10 min at 4°C and the volume was made up to 10 ml. HPLC analysis was carried out on an analytical liquid chromatograph LC-10A (Shimadzu, Singapore), equipped with a Rheodyne 7725i injection valve, fitted with a 20µl sample loop and a 250 x 4.6 mm, i.d. 5 µm, SS Excil Amino column (SGE, Australia). The sample (10 µl) was eluted with an isocratic solvent mixture comprising 0.1M citrate–phosphate buffer (pH 2.6): acetonitrile (1:3 v/v) with a flow rate at 1.5 ml/min. The UV detection was carried out at 254 nm with a Shimadzu diode array detector, series SPD-M10 Avp, Shimadzu (Singapore).

Total Carotenoids

Total carotenoid content was determined by spectrophotometric method described earlier by Ranganna (2001). In brief, about 10 g of pineapple pulp was blended with 100 ml cold acetone in a pestle and mortar and filtered over cotton pad. Extraction was repeated until residue was colorless. Acetone extract was placed in the separating funnel

and agitated with 25 ml petroleum ether and 5 ml water. The mixture was left to stand for 30 min. The yellow colored petroleum ether extract was collected and filtered over anhydrous sodium sulphate on a Whatman filter paper No. 1. The extract was made up to 25 ml and the color intensity of carotenoid extract was measured at 450 nm in a UV-Visible spectrophotometer (UV-160A, Shimadzu Co. Japan). The total carotenoid content was calculated on the basis of the calibration curve of β-carotene and expressed as β-carotene equivalents mg/ 100 gm FW pineapple.

Total protein content

Concentration of the protein was determined by dye binding method (Bradford 1976) using Coomassie Blue G250. The reagent was prepared by dissolving 100 mg of Coomassie Blue G250 in a mixture of 50 ml of 95% ethanol and 100 ml of 85% (w/v) phosphoric acid. After the dye was completely dissolved, the final volume was made up to 1000 ml using double distilled water. Protein was quantified by adding known quantity of the sample to 2.0 ml of the reagent and making up the volume to 3.0 ml with double distilled water. Optical density was measured at 595 nm. The weight of the protein was determined from the standard graph. Bovine serum albumin (BSA, SRL Chemicals, India) was used as a standard.

Phenolic content of pineapple fruit

Free phenolics were isolated according to the method followed by (Subba Rao and Murlikrishna (2002)). 10 gram each of pineapple (in triplicate, n ¼ 3) was extracted (1:50, w/v) in 70% ethanol (350mL, 2 h each), and the supernatants were obtained by centrifugation (Sigma 3-16K, USA) at 3000g for 15 min and concentrated by flash evaporation (Buchi 011, Switzerland); the pH was adjusted to 1.5 with 4 N hydrochloric

acid. Phenolic acids were separated by ethyl acetate phase separation (450 mL) and the pooled fractions were treated with anhydrous sodium sulphate, filtered and evaporated to dryness. Total phenolic acid was estimated spectrophotometrically by Folin-Ciocalteu method with gallic acid as the reference standard and expressed as gallic acid equivalent (GAE) in milligrams per gram dry weight (dw) of sample.

Isolation of bound phenolic acids

Pineapple fruit samples (10 g, each) were defatted with petroleum ether and chloroform (1:1, v/v) and extracted with 70% ethanol (4x50 mL) to extract free phenolic acids. The dried samples were extracted with 1M sodium hydroxide (2x100 mL) containing 0.5% sodium borohydride under nitrogen atmosphere, and the clear supernatants were collected by centrifugation (Nordkvist *et al.,* 1984). The combined supernatants (bound phenolics) were acidified with 4N hydrochloric acid to pH 1.5 and the phenolic acids were extracted and quantified colorimetrically in the same way as free phenolic acids.

The composition of phenolic components in free and bound fractions of pineapple was characterized by HPLC by (model LC-10A. Shimadzu Corporation, Tokyo, Japan), analysis on a reverse phase Shimpak C18 column (4.6x250 mm) using a diode array UV-detector (operating at 280 nm). A solvent system consisting of water: acetic acid: methanol (isocratic; 80:5:15, v/v) was used as mobile phase at a flow rate of 1 mL/min. Standard phenolic acids such as caffeic, pcoumaric, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acid along with ascorbic acid and curcuminwere used for identification of phenolic components present in both free and bound phenolic fractions of pineapple fruit.

Carbohydrate composition of pineapple fruit

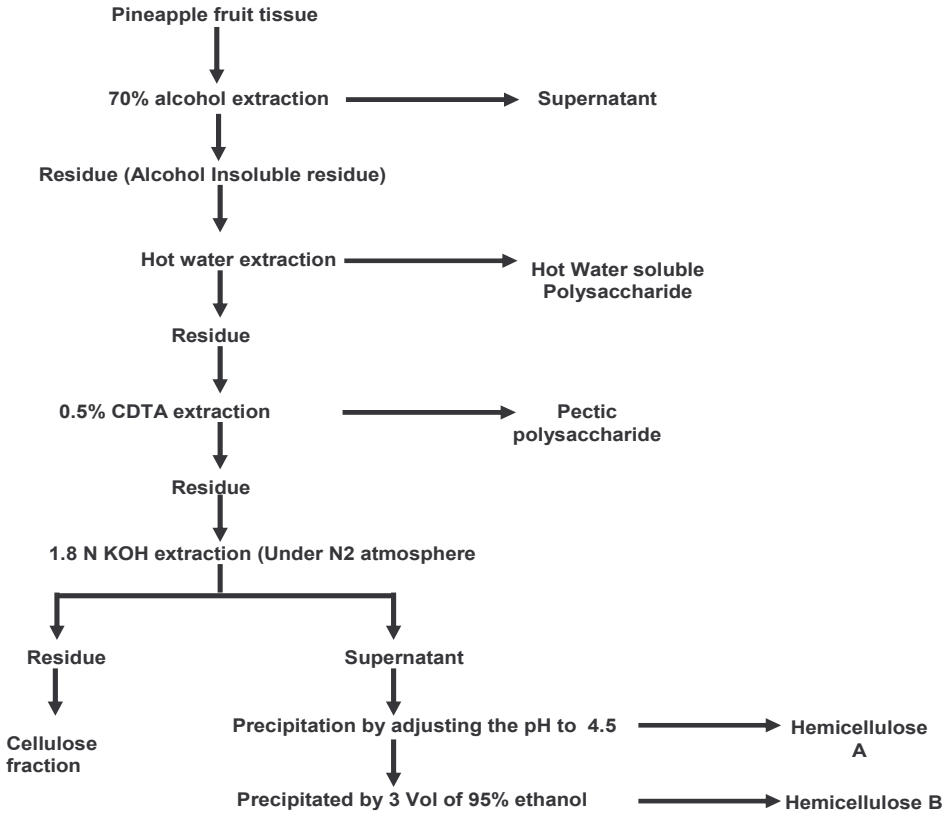
Pineapple fruits at different ripening stage were selected to make alcohol insoluble residues (AIR). A known weight of fruit was separately soaked in three volumes of 95% ethanol. Slicing the tissue and plunging them into alcohol were done instantaneously in order to avoid endogenous metabolic activity due to cutting. They were homogenized in a Sorvall omni mixer and the resulting slurry was kept at 60°C for 40 min to arrest endogenous enzyme activities and to facilitate protein coagulation (Carrington, Greve, & Labavitch, 1993). The slurry was cooled to room temperature. The concentration of the ethanol was adjusted to 70% and filtered through two layers of nylon cloth. The residue was repeatedly extracted in 70% ethanol (four to five times) to remove all the free soluble sugars. The alcohol extract was pooled, and concentrated by rotary evaporator at 40° C. The concentrate was centrifuged and washed with petrol. The aqueous layer was sequentially passed through Dowex(H^+) and Dowex (OH^-) resins (Shashirekha and Patwardhan 1976) and suitably concentrated for total sugar (Dubois), reducing sugar (Miller) estimation and HPLC.

Soluble sugar separated from ethanol extract was analyzed by HPLC using an amynopropyl column (Waters) with a RI detector using the mobile phase, Acetonitrile: water (80:20) at a flow rate of 1ml/min. The respective peaks were identified by running the appropriate standards under identical conditions and relative concentration of the individual sugar was calculated based on the peak area compared to the fixed area of the peak produced by corresponding standard sugar.

The residue after ethanol extraction is washed with acetone and diethyl ether, and air dried. Based on differential solubility, the AIR thus obtained was sequentially extracted with (1) hot water for HWS, (3) 0.5% EDTA for pectins, and (4) 1.8 N KOH

(under N2 atmosphere for hemicellulosic fractions). The alkali extract was adjusted to pH 4.5 to precipitate out hemicellulose A, whereas the supernatant on precipitation with ethanol (3 vol.) gave hemicellulose B (Yashoda, Prabha, & Tharanathan, 2006). The final alkali-insoluble residue was designated as cellulosic fraction (Salimath & Tharanathan, 1992). In each case of the fractions, the obtained fractions are precipitated using 95% alcohol, centrifuged to remove alcohol and the residue was dried in solvent exchange (ethanol -ether) weighed and kept in a desiccators.

Scheme for extraction of cellulose



Enzyme activity

Preparation of pineapple enzyme source

Extraction of the enzyme from the healthy, diseased and treated pineapple fruit tissues was carried out by adopting the procedure developed by Sanches-Ferrer *et al.* (1990). A 100 g sample of the pineapple fruits was homogenized in 100 ml of 50 m M cold acetate buffer (pH 5.5) containing 6 % (W / V) Triton X - 114, 2 m M EDTA, 1 m M $MgCl_2$ and 1 m M PMSF as a protease inhibitor in a blender for 10 min and then filtered. The homogenate was kept at 4° C. The supernatant was collected and stored at – 20° C until use. The conditions were modified whenever specific extraction and assay condition required for the following enzymes. The enzyme activities were determined spectrophotometrically in UV-visible spectrophotometer, Cintra 10A instrument. unless otherwise stated

Protein estimation

Protein content of the enzyme extracts was determined by the Bradford method (1976), using bovine serum albumin (BSA) as standard protein.

Polyphenol oxidase (EC 1.10.3.2 or 1.14.18.1) enzyme activity

As a crude enzyme of PPO contains interfering substances, the enzyme was extracted with acetone as described by (Flurkey and Jen 1978; Das et al 1997). The reaction mixture (1 ml) was composed of 0.1 ml of substrate, 0.8 ml of buffer and 0.1 ml of enzyme extract. Reactions were carried out at room temperature unless specified. Substrate blank and enzyme blanks were used as reference blanks, which were prepared by mixing all components except substrate or enzyme source respectively. The

enzymatic activity was calculated from the slope of the linear portion of the curve. Four replicates were maintained for each assay. Enzyme activity is expressed as Units/ Kg/ s

Kinetics of PAL

1) pH

Optimum pH studies were carried out using Citrate and phosphate buffer between pH 3 and 8. Optimum pH was found to be pH 5 and assays were carried out at pH 5 with citrate buffer. Enzymatic activity of the crude enzyme extract was determined as described above. All assays were performed with three replicates.

2) Temperature

PPO activity of the crude enzyme extract was measured between 15 and 60°C. All assays were performed in triplicate, and the enzymatic activity under each temperature condition was expressed in relative form as the percentage of the highest activity reached. Assays were carried out at optimum temperature 30°C

3) Evaluation of substrate specificity

PPO activity was tested using crude enzyme source against four substrates: Dopa, Dopamine, Tyrosine, and Catechol. Catechol was used for the assay. The concentration used for all the substrates was 5 mM. All assays were done with four replicates.

Peroxidase (EC. 1.11.1.7) activity

Peroxidase enzyme activity was standardized as described by (Mayer, 1987) The reaction mixture (1 ml) was composed of 0.05 ml of substrate, 0.05 ml of H_2O_2, 0.85 ml of buffer and 0.05 ml of enzyme extract. Reactions were carried out at 25°C. Substrate

blank and enzyme blanks used as reference blanks were prepared by mixing all components except substrate or enzyme extract respectively. The enzymatic activity was calculated from the slope of the linear portion of the curve. Four replicates were maintained for each assay. Enzyme activity is expressed as Units/ Kg/ s

Kinetics of POD

1) pH

Optimum pH studies were carried out using Citrate and phosphate buffer between pH 3 and 8. Optimum pH was found to be pH 5 and assays were carried out at pH 5 with citrate buffer. Enzymatic activity of the crude enzyme extract was determined as described above. All assays were performed with three replicates.

2) Temperature

POD activity of the crude enzyme extract was measured between 15 and 60°C. All assays were performed in triplicate, and the enzymatic activity under each temperature condition was expressed in relative form as the percentage of the highest activity reached. Assays were carried out at optimum temperature 25°C

3) Evaluation of substrate specificity

POD activity was tested using crude enzyme source against four substrates: Hydrogen peroxide and Catechol. Catechol was used for the assay. The concentration used for all the substrates was 5 mM. All assays were done with four replicates.

Phenylalanineammonia lyase (EC 4.3.1.5)

The assay of PAL is standardized as modified method of Martinez, Chaves, and Anon (1996). Fruit tissue (10 g) was homogenized in 20 ml 0.1 M borate buffer (pH 8.8) with 5 mM b-mercaptoethanol, 2 mM EDTA and 1% (w/v) PVPP. After stirring for 1 h at 4^{o}C, the solution was filtered through two layers of muslin cloth and centrifuged at 10,000 rpm for 20 min. The supernatant was used for PAL assay. For PAL activity assay, the reaction mixture contained 2.50 ml 50 mM l-phenylalanine in 0.1 M borate buffer (pH 8.8) and 0.50 ml supernatant extract. The substrate was pre-incubated at 40^{o}C for 20 min before mixing with the enzyme extract. The reaction was at 40^{o}C for 1 h and was stopped by adding 0.1 ml 6 N HCl. The change in OD at 290 was measured against a blank without substrate. PAL activity was expressed as Units/ Kg/ s

Kinetics of Phenylalanineammonia lyase

1) pH

Optimum pH for Phenylalanineammonia lyase activity of the crude enzyme extract of pomegranate arils was studied using citrate-phosphate buffer between pH 2.8 and 7. Enzymatic activity of the crude enzyme extract was determined as described above. Optimum pH was used to carry the assay with the samplesAll assays were performed with four replicates.

2) Temperature

Effect of temperature on Phenylalanineammonia lyase activity of the crude enzyme extract pineapple tissue was measured between 30 and 55°C. All assays were performed in triplicate, and the enzymatic activity under each temperature condition was

expressed in relative form as the percentage of the highest activity reached. Optmum temperature was found to be 40°C and used for the assay.

3) Evaluation of substrate concentration

Phenylalanineammonia lyase activity of the crude enzyme extract was tested against two substrates: l-phenylalanine. Both, the reaction medium and the determination of enzymatic activity accorded with the procedures described above. The concentration used for all the substrates was 2 mM. All assays were done with four replicates.

Bromelain activity

Enzyme activity was determined by casein method, measuring the absorbance at 280 nm with tirosine as standard (Murachi 1976 and Baldini et al, 1993). Spectrophotometer UV-Vis) was used for total protein and enzyme activity determination.

Kinetics of Bromelain

1) pH

Optimum pH studies were carried out using Citrate and phosphate buffer between pH 3 and 8. Optimum pH was found to be pH 5 and assays were carried out at pH 5 with citrate buffer. Enzymatic activity of the crude enzyme extract was determined as described above. All assays were performed with three replicates.

2) Temperature

Bromelain activity of the crude enzyme extract was measured between 15 and 60°C. All assays were performed in triplicate, and the enzymatic activity under each

temperature condition was expressed in relative form as the percentage of the highest activity reached. Assays were carried out at optimum temperature 25°C

3) Evaluation of substrate specificity

Bromelain activity was tested using crude enzyme source against four substrates: Casien and BSAl. Casein was used for the assay. The concentration used for all the substrates was 5 mM. All assays were done with four replicates.

Cell wall degrading enzymes

Pectinase

Assay for pectinolytic activity was determined at 45 °C by viscometry for endo-polygalacturonase (endo-PG) and by the release of reducing sugars, expressed as galacturonic acid, for exo-polygalacturonase (exo-PG). For endo-PG, 2.5 ml of sample was mixed with 7.5 ml of 1% (w/v) pectin in 0.1 M acetate buffer, pH 4.5 maintained at 30° C in a water bath. Reduction in viscosity was followed with an Ostwald viscometer (Technico, BS/U, England). One endo-PG unit (U) was defined as the amount of enzyme that reduces the viscosity of the solution by 50% per minute under the conditions mentioned above. For exo-PG, 0.25 ml of sample was added to a solution containing 0.25 ml of 1% pectin in 0.1 M acetate buffer, pH 4.5. Samples were incubated at 45 °C for 30 min and reducing sugars were determined by the dinitrosalicylic acid (DNS) method [25]. One exo-PG unit (U) was defined as the amount of enzyme that liberates one micromole of galacturonic acid per minute under the conditions mentioned above. All measurements were made in triplicate.

Diseased sample showed a slight pectinase activity while the culture filtrate does not showed any activity with the above said substrates and assay condition. Therefore further studies with pectinase were not carried out.

Cellulase

Qualitative assay (Gel diffusion assay)

Preparation of gel plates: CMC is dissolved in the buffer in which the enzyme was tested by clarification by centrifugation. 0.1% CMC and 1.7% agarose gel is prepared by boiling. The mixture was autoclaved and poured into Petri plates to form 3mm thickness gels. After solidification of the gel wells (8mm dia.) were punched using cork borer. The culture filtrate 200ml was poured into the wells and plates were incubated for 24hrs. Later the wells were washed with distilled water and Congo red stain (1mg/ml of water) was flooded to cover the gel and incubated for ten minutes. The stained are removed by rinsing in water. Gel was destained by 1N NaCl solution. The sample containing endoglucanse activity showed decolouration against red back ground of CR stain (Wood and Weisz 1987).

Cellulase activity assays

Assays for Endo-glucanase

Cellulase enzyme activities are carried out as described by (Badal C. Saha, 2004) Endo-glucanase (EC 3.2.1.4), activity was assayed in a reaction mixture (0.5 ml) containing 1% (w/v) boiled CMC solution, 50mM acetate buffer, pH 5.0, and appropriately diluted enzyme solution. After 30 min incubation at 30°C, the reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid method

(Miller, 1959), one unit (U) of EG or CMCase activity is defined as the amount of enzyme which produces 1 μ mole reducing sugar as glucose per min in the reaction mixture under the specified conditions.

Assay for Cellobiohydrolase and β-glucosidase

Cellobiohydrolase (EC 3.2.1.91), and β-Glucosidase (EC 3.2.1.21) activities were assayed in a reaction mixture (1 ml) containing 5mM p-nitrophenyl-d-cellobioside and 5mM p-nitrophenyl-d-glucoside, respectively, 50mM acetate buffer, pH 5.0, and appropriately diluted enzyme solutions. After incubation at 50 °C for 30 min, the reaction was stopped by adding 1ml of ice-cold 0.5M Na_2CO3 and the colour that developed as a result of p-nitrophenol (pNP) liberation was measured at 405 nm. One unit (U) of each enzyme activity is defined as the amount of enzyme that releases 1 mole pNP per min in the reaction mixture under these assay conditions.

Activity assay was carried out for all three constituent enzymes of cellulases viz endo-glucanase, exoglucase or cellobiohydrolase and glycosidase. We found highly enhanced endoglucanse activity compared to glycosidase activity both in samples extracted from diseased fruit and extracellular culture filtrate. Cellobiohydrolase activity was not found in the above samples; hence the ivestgation was concentrated only on endoglucanase activity principle in SER disease process.

Results and Discussion

Firmness

The force required for penetration into the fruit gradually decreased in raw green fruits over a period of eight days, with the advance of ripening. A significant ($p<0.05$) 20 N decrease in firmness was recorded in 50% ripened pineapple when compared to raw fruits. More than 50% decrease in firmness was recorded in 100 % ripe fruits when compared to matured green fruits (Fig 2. 1). Diseased SER pineapple tissue offered least resistance and exhibited lowest firmness when compared to healthy green and ripe fruts. Highest force of 90 N was recorded in green fruits, while lowest force of 44 and 17 N was observed in the 100 % ripe and diseased fruit respectively. This is due to maceration of disease tissue (Fig 1.15) by *C. paradoxa.* Decrease in firmness of raw fruits as a function of ripening was well documented in different fruits (Douglas *et al.,* 2003).

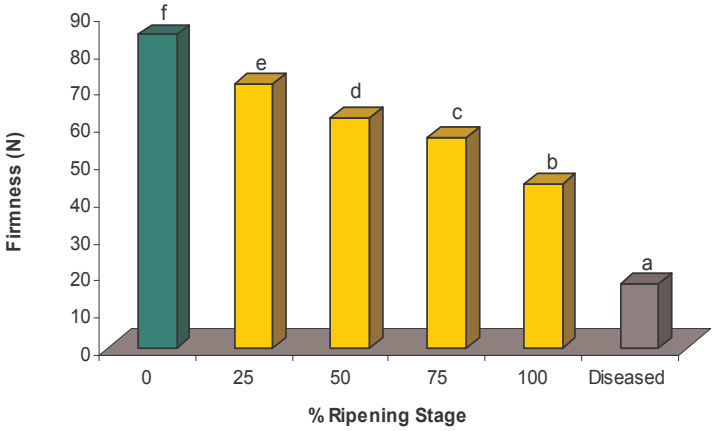


Fig.2.1:

Firmness of pineapple at different stages of ripening and in diseased fruit

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

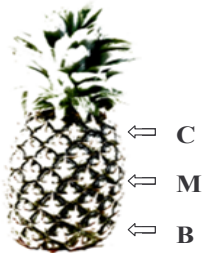
Colour

Visual characterization of intensity of change in colour of pineapple fruit from raw green to characteristic orange yellow at different stages of ripening has been subjected to instrumental color measurement to provide numerical scale. In the present

study Hunter L a b color measuring system was employed for the same. Where L indicates lightness, (white =100 to black = 0), whereas 'a' indicates red (+a) to green (-a) and 'b' indicates yellow (+b) to blue (-b).

The change in a* value showed a nonlinear relationship with L* value with advance of time. The change in a* value reflect the reduction of green colour of the surface shell colour which in turn govern the degree of ripening of the fruit. The b* value of surface colour showed linearity with the duration of ripening of fruit (Table 2.1). Individual colour values such as 'a', decreased with fruit ripening in contrast with increase in of the colour value such as 'b'. Lightness 'L' values were associated with the

Table 2.1:
Colour reflectance (L, a, b values) at different regions and ripening stages of pineapple fruit



		L	a	b	DE
Matured green	C	30.63	-2.07	11.28	60.73
	M	32.21	-077	9.46	59.16
	B	33.69	-0.22	8.84	55.34
25 %	C	32..38	-1.64	10.92	55.28
	M	34.26	-1.17	16.65	53.60
	B	37.20	-2.61	13.64	55.08
50 %	C	33.19	-2.10	12.44	53.46
	M	38.38	-0.96	13.92	53.99
	B	40.86	-1.23	13.04	54.58
75 %	C	36.54	-1.68	13.37	55.65
	M	45.14	7.42	17.16	48.84
	B	46.35	9.59	20.70	49.85
100 %	C	45.98	9.28	20.11	49.57
	M	47.10	3.68	20.31	48.08
	B	49.97	6.19	21.83	46.50

accumulation of carotene pigments. The increase in L* value with increase in degree of ripening of fruit can be correlated to increase in carotene pigment. Pineapple fruit skin changes its colour of from green to yellow during normal ripening. The change begins at peduncle/stem end and progress towards crown. It was used as an indicator of fruit

ripening in the present study. This phenomenon of ripening has been commercially exploited as an index to measure degree of ripening (Collins. 1968) and accumulation of carotene (Douglas *et al.,* 2003).

Physilogical Loss of Water (PLW)

The cut portion of pineapple forms the main avenue for increased loss of water due to increased rate of respiration and transpiration that are governed directly to storage temperature and time. Rapid loss of water at a rate of 1 % per day occurred in fruits during the period of ripening and storage at RT. In SER pineapple tissue about 8-9 % of water loss after 10th day of infection was observed (Fig 2.3), which coincides with manifestation of visible shriveling followed by gradual shrinking of the pineapple that leads to a commercially objectionable level of shriveling.. Many fruits become unsalable and manifest shriveling after loosing 7-20 % of their weight (Ben-Yehoshua and Rodov, 2003). The pivotal physiological role of water in pineapple is maintenance of freshness and quality of fruit as is the case for most perishables (Herppich *et al.,* 2000). Water stress affects many of the fruits in many ways since water is the medium for most of biochemical reactions (Landrigan *et al.,* 1996). The decreased

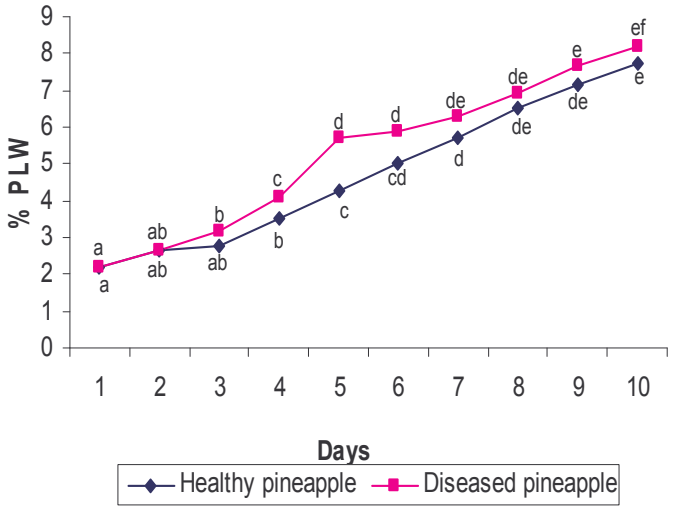


Fig.2.3:

PLW of pineapple fruits at different maturity stages of ripening and in inaoculated fruits

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

turgidity of the cell, which further leads to the loss of membrane integrity (Landrigan *et al.,* 1996*;* Jiang, & Fu, 1999) resulting in efflux of organic solutes that may increase the susceptibleness of pineapple fruit to *C. paradoxa.*

Respiration

The main factor affecting the quality of fresh fruit pineapple was respiration. Pineapple being a non climacteric fruit does not show a respiratory peak after harvest. Whole fruit showed an intermediate respiration rate.. The highest quantity of carbon dioxide was measured on 6th day of storage at ambient temperature (Fig 2.4). However,

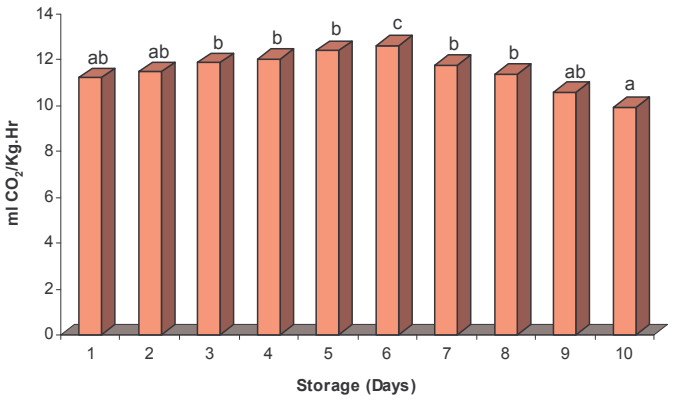


Fig.2.4:

Respiratory pattern of pineapple fruits during storage and ripening

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

there was no significant difference between thes of Co_2

output during these storage periods. The interesting observations being drop in rate of respiration after 6th day of storage (Fig 2.4). The elevated respiration rate on 6th day correlates with attainment of 50% ripening of fruit. In the absence of climacteric patteren of respiration an elevated respiration rate with concomitant increase the out put of carbon di oxide on 6th day may indicate induction of an array of biochemical changes, which may be responsible to impart a better color flavour and taste. Also, elevated CO_2

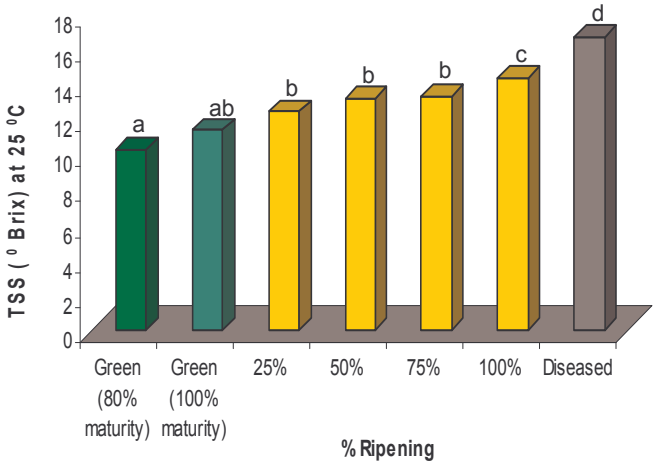
of internal fruit atmospheres may have delayed fungal growth. (Antonio M. and Adel A.K., 2006). Change in quality beyond this point may favour C.pardoxa infection. Interstingly elevated respiration may also indicate the end of physiological resistance to *C. paradoxa* infection.

Total soluble solids

The pineapple fruit showed a gradual increase in total soluble solids (TSS) from 10 to 15% at the end of ripening. (Fig 2.5), indicating the increase in sugar, minerals, organic acids content. Increase in TSS content in pineapple at different stages of ripening is attributed to the conversion of stored carbohydrates to sugars. It is interesting to observe significant (P<0.05) increase in TSS in diseased fruit tissue (Fig 2.5). This may be due to accumulation of cellulosic polysaccharides due to degradation of cell wall from *C. paradoxa.* In addition, progressive growth and colonization of mycelia in the diseased pineapple tissue, that may also contribute to the increased TSS **(**Smith and Harris 1995**).**

Fig.2.5:

TSS of pineapple fruits at different stages of maturity, ripening and in diseased fruits

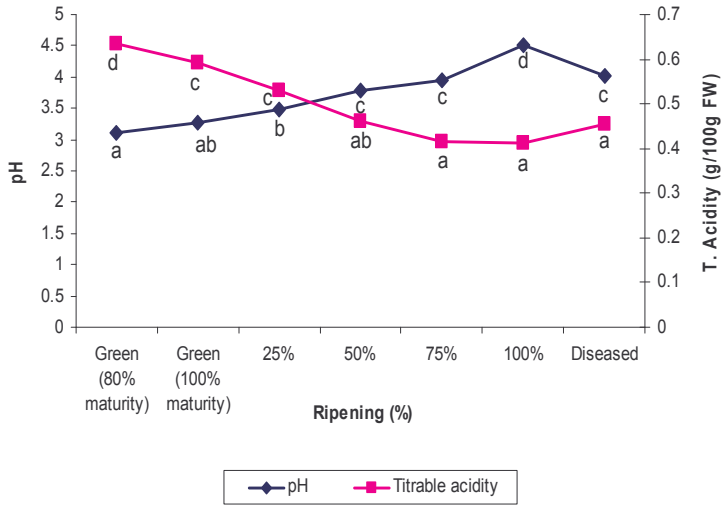


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

pH and titrable acidity

Pineapple fruit acidity is a result of the total nonvolatile acids that occurs as free organic acids (Chan *et al.,* 1973; Teisson and Combres, 1979), mostly stored in the vacuoles of cells. Organic acids found in the pineapple include citric, malic, oxalic acids; however, the major acid accounting for titrable acidity in pineapple is citric acid (Ref Yamaki, 1984). The highest titrable acidity (0. 65 as % citric acid) was recorded in raw green fruits. This was followed by a continuous, but significant decrease in titrable acidity to the lowest concentration of 0.46 (as % citric acid), which was recorded in 100% ripe fruit (Fig 2.6). The changes in juice pH agreed with the changes in titrable acidity. The ratio between acid and pH declined during ripening of pineapple. The change in acids during ripening was attributed to change in citric acid in pineapple. The difference in various acids has been reported in different variety of pineapple.

Decrease in acid content is usually associated with the respiration process, since acids forms the necessary respiratory substrate. This kind of decrease in titrable acidity was observed during normal ripening of most of the fruits (Bashir *et al.,* 1989). The increase in the pH in pineapple fruit is due to the decreased acid content.



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Fig.2.6:

pH and Titrable acidity of pineapple fruits at different stages of maturity, ripening and in diseased fruits

The measurement of pH of pineapple juice extracted from different stages of ripening revealed that, there is a gradual and significant ($P \leq 0.05$) increase from 3.2 to 4.5 from raw green to 100% ripe fruits (Fig 2.6). Coinciding to this a drastic decrease in titrable acidity was also recorded from raw green to 100% ripe fruits (Fig.2.6). In terms of quality of fruit, acids and sugars are important components, which provide characteristic taste and flavor and may also influence the colour of the carotene rich fruits (Bashir *et al.,* 1989). The magnitude of pH gives immediate or actual acidity (actual hydrogen ion concentration) of sample. On the other hand, titrable acidity gives the total or potential acidity, i.e. total number of acid molecules, both protonated and unprotonated.

It is interesting to note that the point of intersection between pH and acidity coincides with 25 % ripening (Fig 2.6) of pineapple fruit. In terms of SER disease, the point of intersection may indicate the decline of resistance in pineapple tissue against *C. paradoxa* infection. Alternative explanation would be the point of intersection may signify the predisposition stage of susceptibleness in pineapple fruit. The reverse trend in pH and acidity ratio at 50% ripe fruit, as a consequence to ripening may be a critical parameter that may influence the resistance of the fruit. Change in acid content plays important role in incidence of infection of pineapple fruit by *C. paradoxa*. The absence of citric acid from pineapple was recorded in fruits infected by *C. paradoxa* (Adisa, 1985)

Ascorbic acid content

Ascorbic acid present abundantly in all plant cells and perform many biological functions. As an antioxidant, it is known to inhibit oxidative browning directly or indirectly (Smirnoff, 1996). Rapid decrease in ascorbic acid content during ripening of

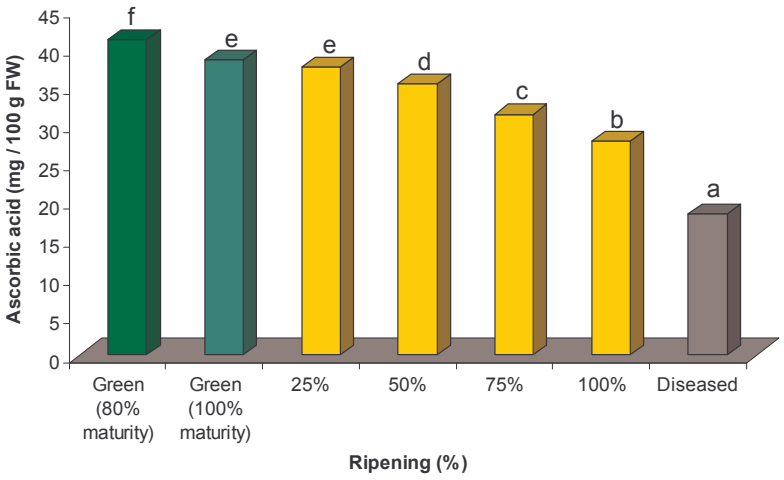


Fig.2.7:

Ascorbic acid content in pineapple fruits at different stages of maturity, ripening and in diseased fruits

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

fruits have often been reported (Mercado-Silva *et al.,* 1998; Yahia *et al.,* 2001;). Pineapple fruits also showed a similar trend with rapid and significant ($p \leq 0.05$) depletion in the ascorbic acid content during ripening and in tissue infected with *C. paradoxa* (Fig 2.7). It has been suggested that. *C. paradoxa* might use organic acids for respiration, thus decrease in its concentration. A similar trend of decrease in ascorbic acid content during ripening and infection was recorded in many fruits (Bashir & Abu-Goukh, 2003). Complete loss of Ascorbic acid in *Botrydia theobramae* infected orange fruits has been reported (Srivastava and Tandon, 1968). However, no complete loss of ascorbic acid in pineapple fruits, loss of ascorbic acid upto 90% was recorded in *C. paradoxa* infected fruits (Adisa, 1985).

Ascorbic acid as such is a very unstable molecule and mechanism(s) for ascorbic acid losses are still not fully understood. Multiple factors such as oxidation, pH, relative humidity and temperature were ascribed to its degradation in the cells. Decrease in

ascorbic acid add up to the decrease in acidity, increase in pH which has positive impact on *C. paradoxa* infection. The loss in ascorbic acid content in many fruits has been reported to increased incidence of diseases (Veltman *et al.,* 1999).

Carotenoids

A rapid and significant (p<0.05) increase in the carotene pigment concentration was observed in pineapple fruit between 25-50 % ripening (Fig. 2.8). The highest concentration of carotene pigments (500 mg/100 g) were recorded in 100 % ripe fruit,

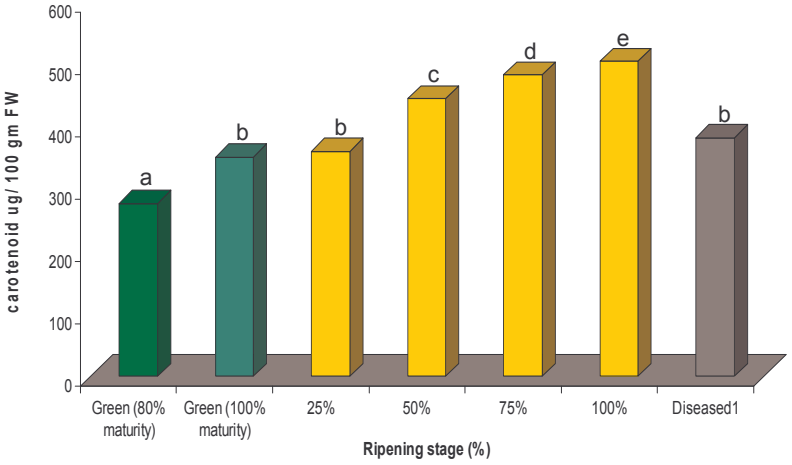


Fig.2.8:

Carotenoid content in pineapple fruits at different stages of maturity, ripening and in diseased fruits

Each value is a mean of three different observations.
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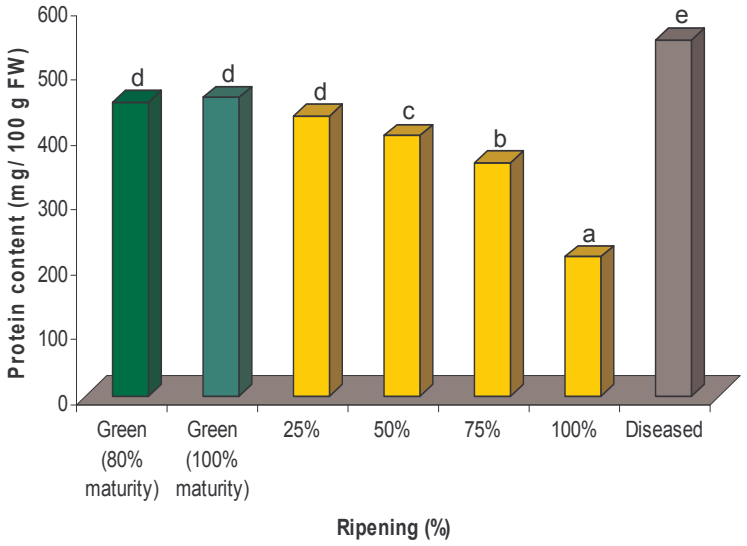
Significant (P<0.05) decrease in diseased tissue was recorded (Fig.2.8). The increase in carotene content coincides with 50% ripening of fruits. Steady increase in carotene pigments was observed till complete ripening of pineapple fuits. Decrease in the carotene content in infected tissue may also influenced by decrease in acidity, total organic acids as observed in diseased tissue.

Total protein

The total protein content showed a negative trend during ripening. Significant ($P<0.05$) decrease was observed in with increase in ripening of fruit. In contrast significant ($P<0.05$) increase in protein content was recorded in diseased pineapple tissue (Fig 2.9). This may be due to increased fungal biomass inside the fruit tissue (Barkai-Golan 1992).

Fig.2.9:

Total protein content in pineapple fruits at different stages of maturity, ripening and in diseased fruits



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Biochemical changes

Total free phenolic content change during ripening of pineapple fruit

Pineapple fruit showed a rapid and significant ($P<0.05$) depletion in free phenolics during ripening from raw green fruit to 100 % ripe fruit over a period of 7-8 days. Significant ($P<0.05$) reduction of free phenolics was recorded in 50% ripe fruits. The decrease was rapid and significant in 75 and 100% ripe fruits also (Fig 2.10). Infected pineapple tissue showed significant loss in free phenolics and recorded lowest 0.8 mg

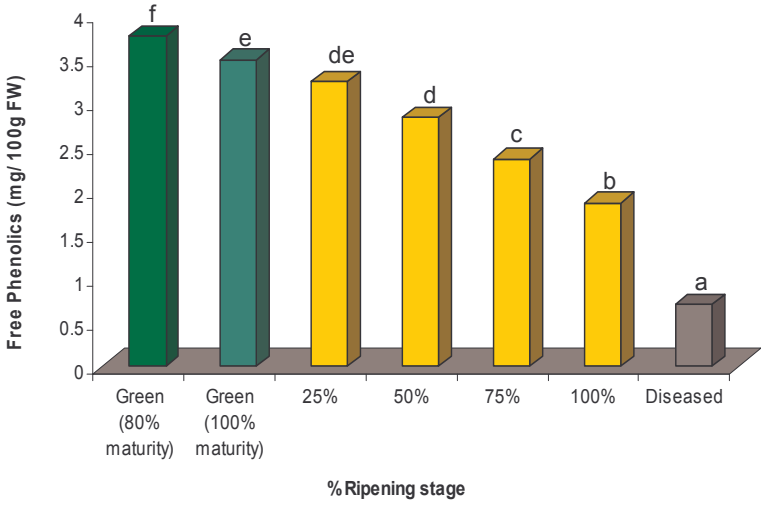


Fig.2.10:

Total free phenolic content in pineapple fruits at different stages of maturity, ripening and in diseased fruits

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

/100g tissue. The highest phenolic content is responsible for resistance in green and 25% ripe fruits may to *C. paradoxa*. Bound phenolics showed a decreasing trend with the advance of ripening of fruit (Fig 2.11). Significant reduction of free and bound phenolics in natural ripening process of pineapple has been attributed to involvement of phenolic

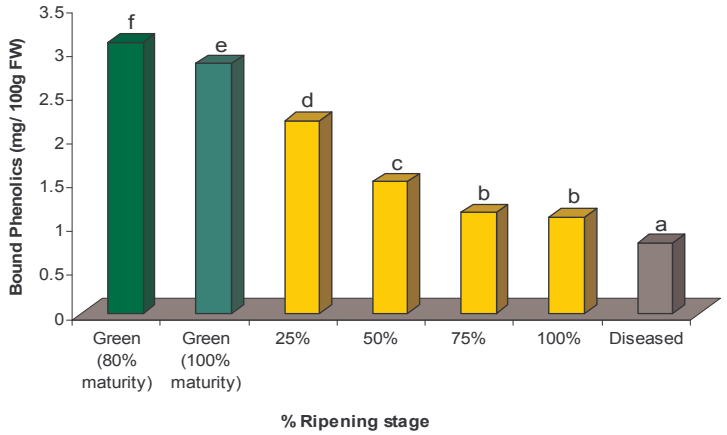


Fig.2.11:

Total bound phenolic content in pineapple fruits at different stages of maturity, ripening and in diseased fruits

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

derivates in various interactions with oxidative enzymes and its contribution in lignification of cell wall. While significant (P<0.05) reduction of both free and bound phenolics in SER diseased tissue may be due to altered membrane permeability or disruption of membrane integrity to intercellular and intracellular hemibiotrophic invasion of pineapple tissue by *C. paradoxa* (Fig 1.14).

Table 2.2

Free phenolic content of pinepple at different stages of ripening

Free Phenolics	0%	25%	50%	75%	100%
Gallic acid	1.7 [a]	1.68 [a]	0.91 [a]	0.44 [a]	-
Protocatechuic acid	2.09 [a]	1.32 [a]	0.57 [a]	0.25 [a]	-
Gentisic acid	24.11 [c]	21.45 [c]	3.64 [b]	2.72 [a]	3.05 [a]
Ferulic acid	18.43 [b]	14.12 [b]	1.34 [a]	1.22 [a]	0.34 [a]
p-coumaric acid	2.42 [a]	-	-	-	-
Synapic acid	-	-	-	7.32 [b]	6.52 [b]

Each value represents mean of three different observations ± S.D. Mean values with different superscripts [[a, b] and [c]] differ significantly at P < 0.05

Gentisic acid and Ferulic was observed to be the major components of free phenolic contents in pineapple. With advance of ripening there was significant decrease in all the constitution phenolic acids **(Table 2.2)**. Ferulic acid and other phenolic acids are

known to offer significant microbial resistance to the fruits (Benner, 1993). Significant ($P<0.05$) decrease of phenolic acids in 50% ripe fruits may render the fruits susceptible for *C. paradoxa* infection. The phenolic acid contents in bound phenolics of pineapple fruit

Table 2.3

Bound phenolic content of pinepple at different stages of ripening

Bound Phenolics	0%	25%	50%	75%	100%
Gallic acid	25.16 [c]	15.14 [b]	13.42 [c]	11.13 [b]	7.67 [a]
Protocatechuic acid	19.47 [b]	-	-	-	-
Gentisic acid	16.12 [a]	13.15 [b]	9.42 [b]	-	-
Ferulic acid	77.44 [e]	59.60 [d]	15.60 [c]	4.16 [a]	9.12 [a]
p-coumaric acid	15.16 [a]	7.42 [a]	6.49 [a]	-	-
Synapic acid	29.59 [d]	25.45 [c]	16.45 [d]	4.44 [a]	-

Each value represents mean of three different observations ± S.D. Mean values with different superscripts [a, b and c] differ significantly at $P < 0.05$

were 50 times higher than that of the free phenolics except Gentisic acid. Ferulic acid was found to be the major phenolic acid in bound phenolics (Table 2.3). High content of phenolics may be an essential component for defense against various pathogens that are constantly challenging the fruits, as evident from the antimicrobial activity of phenolics in various fruits (Ndubizu, 1976; Mulvena *et al.,* 1969). Phenolics from various fruits and their contribution to antimicrobial and other biochemical responses are well documented (Benner, 1993; Bennett and Wallsgrove, 1994). Decrease in phenolics may be attributed to strengthening of the plant cell walls by polymerization into lignans and lignins (Randhir, Shetty, 2005). Alteration in membrane permeability of cell organelles and

disruption of membrane integrity due to intercellular and intracellular hemibiotrophy invasion of pineapple tissue by *C. paradoxa* results in interaction between phenols and polyphenol oxidase which are generally found in separate compartments in the cell (Crisosto *et al.,* 1999). This may result in rapid reduction in the concentration of total phenolic content in the invaded tissue.

Total sugars and reducing sugars

Pineapple showed an increase in the concentration of total sugars during ripening of fruit (Fig 2.12). Marginal increase in total sugar, from green mature fruit to 100% ripe fruit during ripening was recorded. However, diseased fruits showed significant increase in total sugars. This may be attributed to the hydrolysis of cell wall into simple

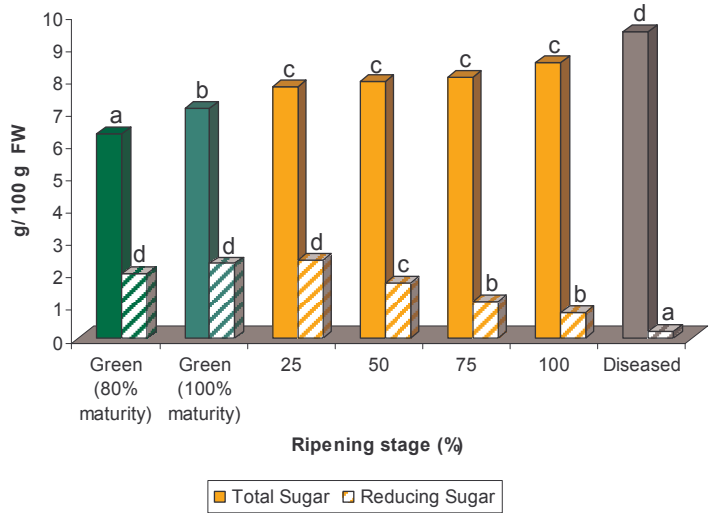


Fig.2.12:

Total and reducing sugar content in pineapple fruits at different stages of maturity, ripening and in diseased fruits

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

cellulolytic and pectolytic polysaccharides along with mycelia of *C. paradoxa*. Reducing sugar content was three times less in raw fruits. With the advance of ripening, there was a decrease in the ratio of total and reducing sugars (Fig 2.12). The decrease in reducing sugars ratio is attributed to their utilization in respiration and other metabolic activities. Pineapple fruit infected with *C. paradoxa* results in the significant decrease or total

disappearance of reducing sugars (Adisa, 1985), similar trend was recorded in other diseased fruit tissues (Cohen and Schiffmann-Nadel, 1972).

Cell wall components in pineapple fruit tissue

Cell wall carbohydrate profile revealed increase in concentration of free sugar, WSP and ASP with advance of ripening of pineapple. While, An opposite trend, decrease in concentration of Hem A, Hem B and AIR was observed (Table 2.4). Increased soluble sugar in the pineapple fruit cell wall fraction may be due to hydrolysis of cell wall constituents mainly cellulose, pectin, hemicellulose and other constituents by cellulose and pectinase enzymes produced during normal fruit ripening (Coughlan,1985). This forms the major reason for loss in firmness of the fruit and more susceptibility to decay. The derivatives of cellulose may be stimulatory for cellulase production of *C. paradoxa* as observed in many pathogens (Davis *et al.,* 1986).

Table 2.4

Cell wall carbohydrate composition (percent dry weight) at different stage of ripening of fruit

Ripening Stage (%)	Free sugar	WSP	Pectic	Hem A	Hem B	AIR
0	7.75 ^{c}	0.90 ^{a}	0.46 ^{a}	1.40 ^{a}	1.30 ^{a}	2.05 ^{b}
25	7.93 ^{b}	1.08 ^{a}	0.53 ^{a}	1.02 ^{a}	0.93 ^{a}	1.83 ^{a}
50	8.04 ^{b}	1.12 ^{a}	0.85 ^{a}	1.10 ^{a}	0.80 ^{a}	1.24 ^{a}
75	8.52 ^{b}	1.19 ^{a}	1.12 ^{a}	0.83 ^{a}	0.71 ^{a}	1.37 ^{a}
100	9.46 ^{b}	1.50 ^{a}	1.49 ^{a}	0.70 ^{a}	0.68 ^{a}	1.01 ^{a}

WSP= Water soluble polysacchardes; ASP= Alchol soluble polysacchardes; Hem= Hemicellulose

Each value represents mean of three different observations ± S.D. Mean values with different superscripts [^{a,b} and ^{c}] differ significantly at $P < 0.05$

Identification of sugars in pineapple fruit were carried out by using HPLC

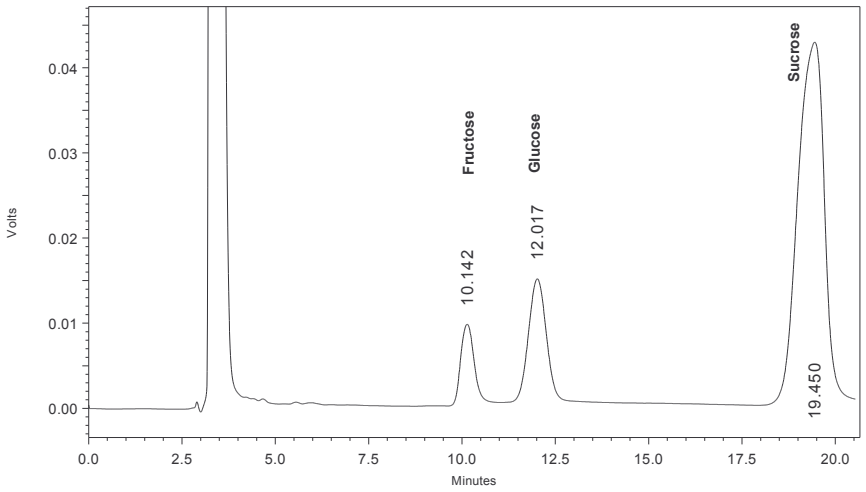


Fig.2.13:

HPLC profile of sugars in ripe healthy pineapple fruit

revealed the presence of three major sugars namely, fructose, glucose and sucrose (Fig 2.13). They were identified by using standard sugars. Presence of more monosacchardes favour C. *paradoxa* for their growth as observed in ascomycetus fungi.

Cellulose and pectin content

Cellulose forms a major component of cell wall of Pineapple fruit tissue. With the advance of ripening there was less or no significant reduction in cellulose content **(Fig.2.14).** Pectin content was found to be very less compared to cellulose, a common feature observed in monocotyledons.

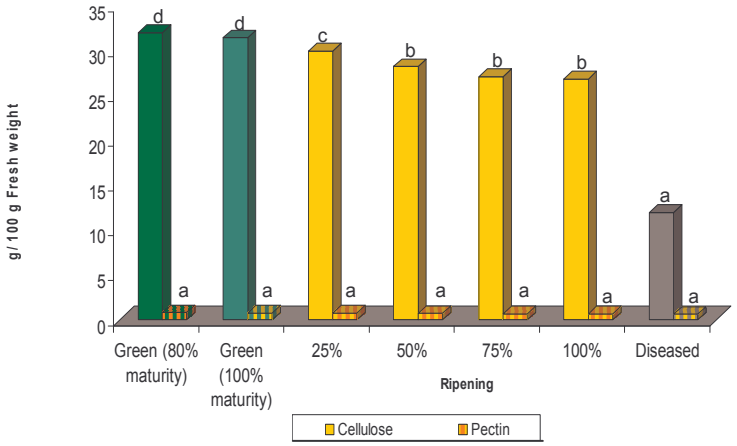


Fig.2.14:

Cellulose and pectin content in pineapple fruits at different stages of maturity, ripening and in diseased fruits

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Enzyme activity assays

Polyphenol oxidase activity

Statistical analysis of polyphenol oxidase enzyme activity content has shown that there was a negative significant (P<0.05) effect of ripening, i.e., the activity was lower

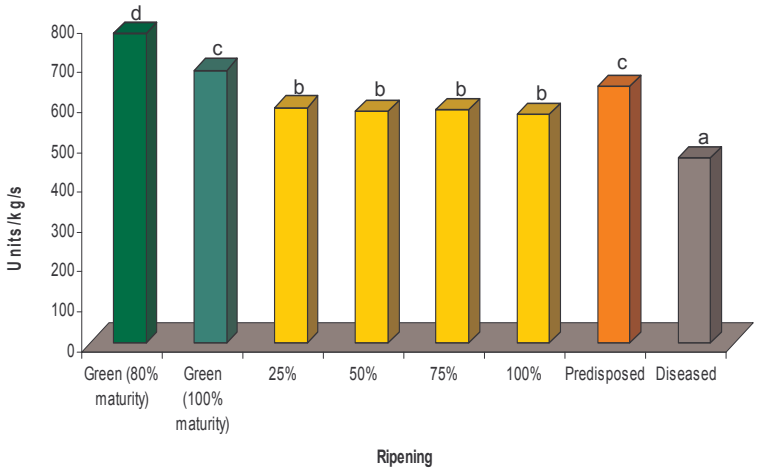


Fig.2.15:

PPO activity in pineapple fruits at different stages of maturity, ripening and in diseased fruits

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in more ripe fruits (Fig 2.15). Polyphenol oxidase is an oxidative enzyme, and its activity is reported to be the major contributing factor in browning of many fruits and vegetables. Enzyme extract of pineapple fruits revealed an increase in PPO activity during fruit development and maturation. With advance in ripening decrease in ppo activity was exhibited in pineapple tissue. The (13%) decrease in its activity observed in 50% ripe. In contrast, a sudden significant (P≤0.005) increase by about 12.7% was observed in

predisposed zone tissue. While, a significant (P<005) decrease in activity was observed in black rot tissue of pineapple (Fig 2.15). The interesting feature appear to the greatest polyphenol oxidase activity was found in tissues adjacent to SER diseased zone. The browning of tissue proceeds with discolouration and transluceny of tissue surrounding the infected region, which is termed as predisposition zone. The predisposion of pineapple tissue has been found to be the result of *C. paradoxa* mycelial intracellular invasion (Fig 1.14). The analysis of polyphenol oxidase has shown that there was 200units increase in diseased tissue. Interestingly amount of polyphenol oxidase was higer (400 units) in predisposed tissue. Infection showed a positive significant effect on PPO activity.

The increase in the activity in predisposed zone tissue seemed to be associated with pineapple resistance to C.paradaxa infection. This result supports the earlier observation that free phenolic acids may be induced by infection and removed as they are converted into lignin or cross linked cell walls (Ray and Hammerschmidt 1998).

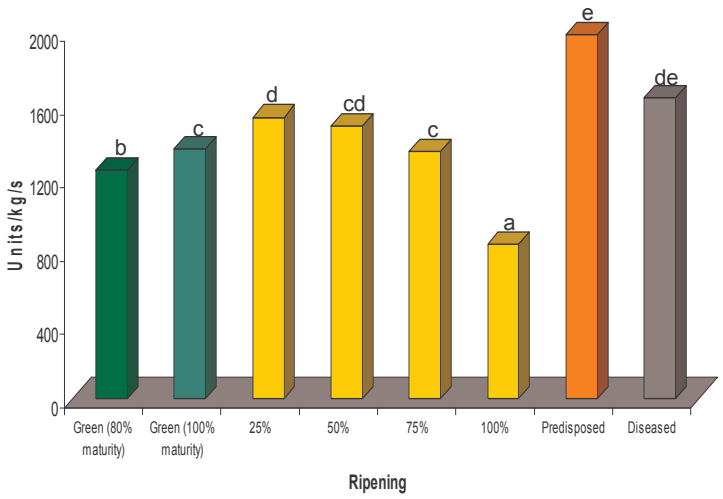
Its activity in *in-vivo* condition would be influenced by many endogenous enzyme inhibiting factors. Most important being, which makes it inactive, is a well-defined cellular compartmentation in healthy fruits. which prevents the enzyme from the contact with substrates, whereas in the pineapple fruit infected with *C. paradoxa* the cellular compartmentation is known to be disrupted due to its intracellular mycelial invasion and hemibiotrophic colonization (Fig 1.14) bringing it in contact with endogenous substrate. Disruption of cellular membrane may also observed during ripeinging and senescence (Wade & Bishop, 1978). Other than cellular compartmentation, ascorbic acid, anthocyanins, and low pH are also known to inhibit PPO activity (Vamos-Vigyazo, 1981).

Peroxidase activity

Peroxidase activity was high in the green mature fruit, remain constant or slight increase till 50% ripening of fruit. Later decline steadily reaching a minimum at 100% ripe fruit (Fig 2.16). Similar to PPO, peroxidase is an oxidative enzyme, and also reported to involved in defense reactions of fruits in relation to infection. Its activity is usually observed in ripening or senescing tissues of fruits. Lowest POD activity (852 Units/mg protein/min) was observed in 100% ripe fruits. whereas its highest activity (945 Units/mg protein/min) was observed in the green matured fruit (Fig 2.16). With advance of ripening decrease in POD was observed 75 and 100% fruits. In contrast a significant (P<005) increase by 800 Units/mg protein/min., was observed in predisposed zone of pineapple tissue (Fig 2.16). Increase in POD acitivity has been correlated with Pineapple resistance

Fig.2.16:

POD activity in pineapple fruits at different stages of maturity, ripening and in diseased fruits



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against *C. paradoxa* infection. Peroxidase constitutes the first lines of dense process which include the accumulation of lilgnin, wound healing, phenolic and aliphatic compounds (Mohan and Kolattududy 1990).

Phenylalanine ammonia lyase

It has been found associated with SER disease of pineapple. The activity normally occurs around infected/necrotic zone of pineapple fruit tissue with development of predisposed tissue, which sandwiches between the healthy and infected/necrotic pineapple tissue. PAL has been reported to play an important role in the browning process of many fruits and vegetables (Ke & Saltveit, 1989). Accumulation of PAL with advance of maturity during development of pineapple fruit has been demonstrated. In healthy pineapple,

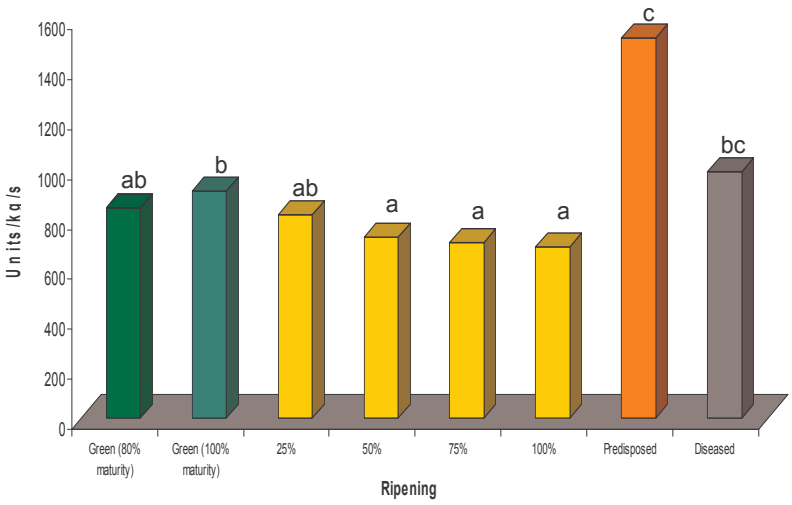


Fig.2.17:

PAL activity in pineapple fruits at different stages of maturity, ripening and in diseased fruits

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Highest PAL activity was observed in 100% green matured fruits. Significant ($P<0.05$) decrease in PAL activity with initiation of ripening as observed in 25 % ripe fruits (Fig 2.17). Further ripening of pineapple fruits exhibited little or insignificant decrease in PAL activity. In contrast, significant ($P<0.05$) and rapid increase in PAL activity during infection process of *C. paradoxa* pathogens is evident (Fig 2.17), in order to render resistance to invading pathogens. Induced resistance due to increased activity of PAL in pineapple tissue may be attributed to its contribution to the synthesis of phenolic compounds, some of which are know to have antioxidant properties (Rice-Evans *et al.,* 1997). Being a key enzyme in phenolic biosynthesis, phenylalanine ammonia-lyase has

also been considered to be associated with browning (Dixon and Pavia, 1995, Saltveit, 2000) as observed in predisposed disease zone of pineapple. Further oxidation reaction of these polyphenols leads to browning and cell death which renders intercellular invasion and colonization of *C. paradoxa* in pineapple tissue. A significant reduction in PAL activity in necrotic/SER tissue of pineapple has been recorded (Fig 2.17). The result support that de novo synthesis after infection rather than pre existing PPO and PAL is important in SER disease of pineapple.

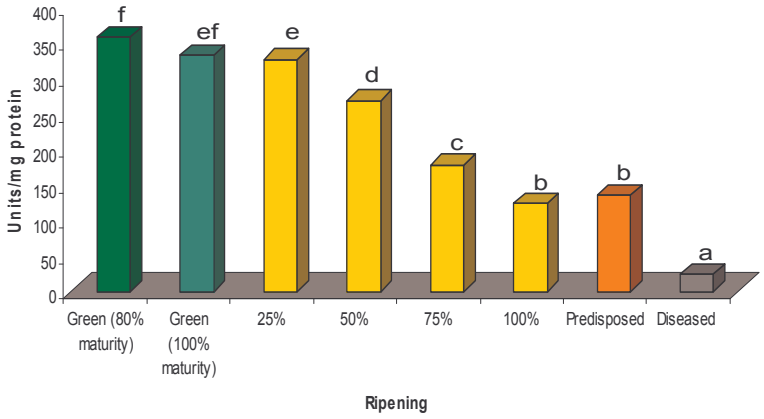
Role of PPO, POD and PAL in Pineapple in context with C. paradoxa infection

Internal damage has been widely linked to stress-induced polyphenoloxidase (PPO, EC 1.10.3.2 or 1.14.18.1) and peroxidase (POD, EC. 1.11.1.7; Mayer, 1987). It may be similar to PPO has been proposed to be related to SER development. Recently Stewart, *et al.,* (2001) indicated that PPO mRNA can be induced, in pineapple fruit. However, the significance of the involvement of this enzyme to SER development has not been adequately documented. While POD has been implicated in discoloration of numerous fruits and vegetables, its significance in SER development is unresolved. Higher POD activity in predisposed fruit tissue with translucent symptoms may suggest its involvement. Being a key enzyme in phenolic biosynthesis, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) has also been considered to be associated with browning of predisposed pineapple tissue around *C. paradoxa* invaded or necrotic tissue. The accumulation of PPO (Fig 2.15), POD (Fig 2.16) and PAL (Fig 2.17), in predisposed tissue of pineapple at increased level compare to uninfected healthy fruit, may indicate that they are induced by infection process, to play an important role as first line of defense system against *C. paradoxa*. The possible mechanism of overcoming this chemical defense by *C. paradoxa* may be; 1) as a consequence of continued ripening

induced changes; 2) due to virulence pathogen factors and 3) simultaneous or synergistic action of both, is still unknown. On the other hand, the cell protective system in pineapple comprises a variety of antioxidant constituents like carotene, bromelain, organic acids, phenols and their enzyme including ascorbate peroxidase. There is little information to correlate pineapple SER disease with these cell protective systems. Given its significance in other species, it would be of value to examine, the relationship of the antioxidant constituents and their enzymes, to SER development in pineapple fruit in context with its ripening changes.

Bromelain

In healthy pineapple highest bromelain activity was observed in 100% green matured fruits. Bromelain enzyme remained at a high level during fruit development, dropped only during the final stage of ripening. Decrease in bromelain activity with the advance of ripening



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Fig.2.18:

Bromelain activity in pineapple fruits at different stages of maturity, ripening and in diseased fruits

achieved significance ($P<0.05$) level in 75 and 100% ripe fruits (Fig 2.18). The lowest concentration of bromelain was observed in necrotic tissue. In contrast, little increase in bromelain activity in predisposed tissue during infection process of *C.*

paradoxa pathogens was evident (Fig 2.18). This may be due to contribution of fungal protease as observed in initial pathogenesis of many diseases of fruits (Billon-Grand *et al.,* 2002).

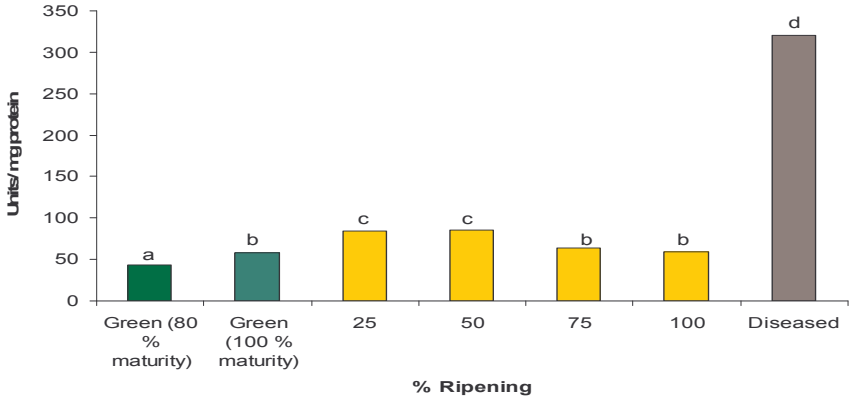
Cell wall degrading enzymes

Pectinase

The pectolytic enzymes are not all constitutive and they may need inducers for their production (Bateman and Bhasham, 1976); the inducing substrate could be supplied to the pathogen only as the fruit ripens. Since Pectin content is very less in pineapple, the pectinase activity was undetectable level (data not shown).

Cellulase

An increase in cellulase activity with advance of ripening was observed in the pineapple fruit tissue. The increase in activity was significant ($P<0.05$) in 25 and 50 % ripe fruits, when compared with raw green fruits. Interestingly there was a reduction in



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Fig.2.19:

Cellulase activity in pineapple fruits at different stages of maturity, ripening and in diseased fruits

cellulase activity after achievement of 50% ripe in fruits. In contrast an increase of 6-7 times cellulase activity was observed in SER disease tissue (Fig 2.19). *C. paradoxa* appeared to be highly efficient in utilizing the cellulose from pineapple tissue for its growth. The virulence of the pathogen may be related to the production of high cellulase by *C. paradoxa*. Most fungal cellulase is induced, the inducing substrate being cellulose or its degradative constituents during normal ripening in the fruits. However, *C. paradoxa* may secrete cellulase independent of cellulose as in *R. solanifer, P. digitatum and P. italicum* (Spalding, 1963, Barkai-Golan and Karadavid, 1991). For the first time it was apparently evident that *C. paradoxa* produce cellulase to facilitate process of infection on pineapple fruit.

Summary

Taken together, the results suggest that SER in pineapple fruit is a consequence of structural and functional reorientation of cell, their organels and constitutional substrates, due to dynamics of fruit ripening process. Lowering of pH, acidity, reducing sugars and ferulic acid concentration in cell wall and increases in TSS and cellulase activity with advance in ripening, are the major host factors that positively influence the invasion of *C. paradoxa*. Concomittantly, high cellulase enzyme production and decompartmentation of host cell organelles, due intercellular and hemibiotrophic colonization of *C. paradoxa*. Consequently it appears that to de novo synthesis of PPO, POD and PAL, lead to discoloration, translucency of surrounding tissues subsequently resulting in browning. This chain of biochemical and pathological events as a consequence of ripening and pathogen invasion may leads to plasmolysis and ultimately death of host cells. The maceration of infected host tissue may be attributed to cellulase, while blackening due to *C. paradoxa*'s black mycelium and chain of spores. The results clearly indicated that pathogenesis due to *C. paradoxa* stimulates the biosynthesis of polyphenol compounds by enhancing PAL activity. For the first time role of cellulase enzyme in pathogenesis of *C. paradoxa* was demonstrated.

Production of extracellulase enzyme and their activity in *C. paradoxa* has been demonstrated. This has been achieved by design and development of bioreactor to produce extracellular enzyme from *C. paradoxa*. The details are presented in the next chapter.

Chapter - 3

Cellulase production and its activity in *Ceratocystis paradoxa*

Introduction

Studies on different enzymes involved in predisposed diseases and SER necrotic tissue clearly indicated the cellulase was the key enzyme produced by *C. paradoxa*, which enabled the fungus for intracellular invasion and hemibiotrophic colonization of pineapple fruit tissue. Moreover, a correlation was found between cellulase activity of the pathogen in the fruit tissue and the severity of the disease symptoms. Understanding the nature of cellulase produced by *C. paradoxa* fungi is of interest, to device the novel method, to deactivate the fungal invasion and thus to control incidence of infection on pineapple fruit. High account of cellulase enzymatic activity during incubation period of the disease, before the appearance of symptoms may help in pre-symptomatic disease identification of SER disease in pineapple fruits. This may enable to undertake the corrective control measures. The major constraint experienced during the investigation was to obtain cellulase in sufficient quantity from a single source or a batch culture of the pathogen. This chapter encompasses successfully designing and development of lab scale reactor to obtain sufficient quantity of cellulase from a single batch and demonstration of endoglucanse activity of enzyme.

Materials and Methods

Cellulase activity in cultural filtrate of *C. paradoxa*

Designing and development of lab scale broth culturing reactor for production of extracellulase enzyme from *C. paradoxa*.

Fungal culture

C. paradoxa strain used was freshly sub cultured on PDA plates and incubated for 2 days. While inoculating, one tablet of agar containing the growing margin region of the pure culture was removed from the culture plate by using a sterile cork borer and inoculated the reactor aseptically inside the laminar air flow hood.

Materials

All the glass wares were procured from Borosil, Mumbai, Teflon tubes were obtained from Hi media.

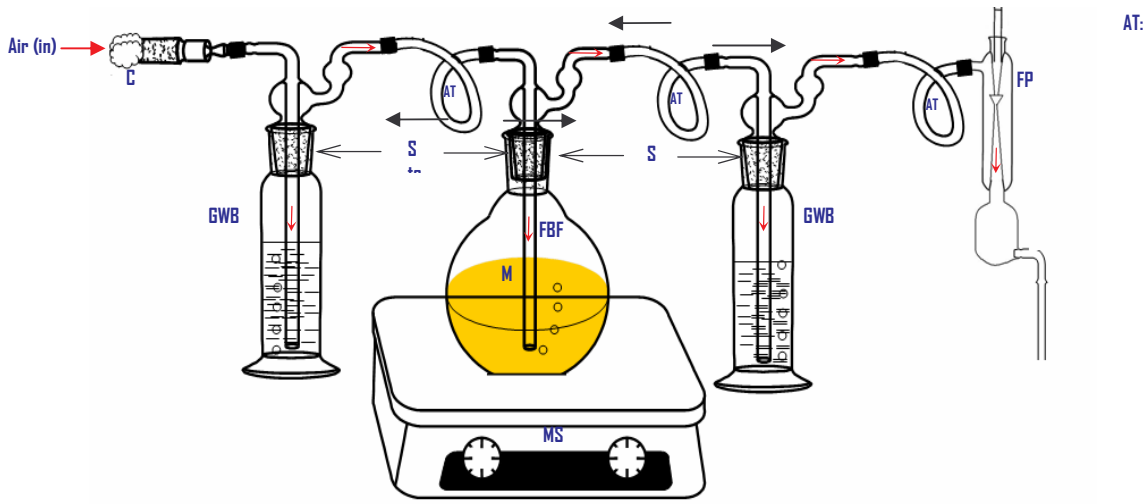
Glass Apparatus

The reactor contains 500 ml capacity 'Gas wash bottle' (1), 1L 'Flat bottomed flask' and 500 ml 'Gas wash bottle' (2). The mouth of these apparatus were fitted with air tight two holed rubber stopper fitted with long bent inlet glass tube and small bent outlet glass tube. The out let GWB (1) is connected to 'Flat bottomed flask' while the outlet of FBF was connected GWB (2), the outlet of which was connected to water trap. These were inter-connected by using by autoclavable Teflon pipes as shown in the figure. The

first gas washing bottle inlet is plugged tightly with sterile cotton gas filtration unit. GWB 1 & 2 was filled with 250 ml of distilled water while FBF was filled with 500 ml of PDA broth media. The whole apparatus were autoclaved for twenty minutes with120 lbs pressure. The GWB (2) outlet pipe was connected to a filter pump, which inturn connected to a water tap. The air tightness was ensured by applying petroleum jelly or food grade wax at all the connections.

Fig.3.1:

Configuration of bioreactor for the production of extracellular cellulase from *C. paradoxa*



Autoclavable Teflon tube

C: Cotton (sterilized)

FBF: Flat bottom flask

FP: Filter pump

GWB: Gas washing bottle

M: Potato Dextrose Broth media

MS: Magnetic stirrer with heater

S: Stopper

Working principle

When suction was created by water trap the atmospheric air pass through the inlet of GWB1 through sterile cotton plug. Cotton plug acts as filter to remove the dust particles and spores present in the air. The filtered air pass through the water present in GWB1 which further purifies or settles the suspended particles. The purified air gets into FWB which contains culture broth inoculated with *C. paradoxa.* The broth was continuously agitated through magnetic stirrer beads. The air after passing through the culture broth connects in the head space provided in FBF which will be suctioned out through water filter pump. The bioreactor was run continuosly for 25 days. The culture broth was collected and filtered by using four layered muslin cloth. The fungal biomass was dried in oven at 60° C till it become dry. The dry weight was recorded. The filtrate was centrifuged at 20,000 rpm for 30 min. sodium azide (0.02%) was added to the culture filtrate and stored under refrigeration, which was used for various experiments.

Enzyme recovery from reactor and partial purification

After a precise period of incubation the working of the reactor was stopped. The *C.paradoxa* biomass is carefully separated by filtration through 2 layers of nylon cloth. The filtrate was tested for endoglucanase activity and protein content by Gel diffusion and Bradford method respectively. The culture filtrate was centrifuged at 4°C at 20,000 rpm. to remove any suspended material and stored in -20°C, by aliquoting by adding 0.02% Sodium azide.

A known quantity of the culture filtrate was concentrated 30-40 fold by lyophilization. The concentrate was dialysed against buffer at 4°C with 3 changes and concentrated by lyophilization. The sample was tested for EG activity and protein

content. The resultant enzyme fraction was used to investigate its activity in in-vivo and in-vitro condition. The composition of the enzyme concentrate was determined by SDS-PAGE and activity was fixed in the gel as described by

Enzyme Assays

Qualitative assay (Gel diffusion assay)

Preparation of gel plates: CMC is dissolved in the buffer in which the enzyme was tested by clarification by centrifugation. 0.1% CMC and 1.7% agarose gel is prepared by boiling. The mixture was autoclaved and poured into Petri plates to form 3mm thickness gels. After solidification of the gel wells (8mm dia.) were punched using cork borer. The culture filtrate 200ml was poured into the wells and plates were incubated for 24hrs. Later the wells were washed with distilled water and Congo red stain (1mg/ml of water) was flooded to cover the gel and incubated for ten minutes. The stained are removed by rinsing in water. Gel was destained by 1N NaCl solution. The sample containing endoglucanse activity showed decolouration against red back ground of CR stain (Wood and Weisz 1987).

Endo-glucanase activity assay

Cellulase enzyme activities are carried out as described by (Badal C. Saha, 2004) Endo-glucanase (EC 3.2.1.4), activity was assayed in a reaction mixture (0.5 ml) containing 1% (w/v) boiled CMC solution, 50mM acetate buffer, pH 5.0, and appropriately diluted enzyme solution. After 30 min incubation at 30°C, the reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid method (Miller, 1959), one unit (U) of EG or CMCase activity is defined as the amount of

enzyme which produces 1 μ mole reducing sugar as glucose per min in the reaction mixture under the specified conditions.

Temperature and pH

The optimum temperature for the hydrolysis of CMC was measured between 40 and 80 °C by incubating each reaction mixture in 50 mM sodium acetate buffer, pH 4.5, for 30 min. For determination of the pH dependence, reaction mixtures in buffer (pH 3.0-9.0) were incubated for 30 min at 40 °C. To study pH and temperature stabilities, samples containing purified endoglucanase in buffer (pH 2.0-10.0) were incubated at 40 °C for 30 min. Following the specified treatments, samples were diluted 10-fold in 100 mM sodium acetate buffer, pH 4.5, and incubated at 40 °C for 30 min with CMC to determine remaining enzyme activity. The activity of enzyme solutions pre-incubated at neutral pH at different temperatures for increasing periods of time was also tested.

Polyacrylamide Gel Electrophoresis

Samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gels (Laemmli 1970) with a Miniprotean II unit (Pharmacia). Proteins separated in the gel were stained with Coomassie Brilliant Blue, and their molecular masses were estimated with standard markers (Sigma). For endo-glucanase activity stain, gels were washed immediately after electrophoresis with 20% 2-propanol (two washes of 20 min each) to remove SDS (27) and then incubated for 4 h at 37 °C in 100 mM sodium acetate buffer (pH 5.0) plus 1% CMC (28). After a 5 min wash with distilled water, the CMC adsorbed to the polyacrylamide gel was stained for 15 min

with 0.1% Congo red. Bands of hydrolyzed substrate became visible as clear zones on a red background after a washing with 1 M NaCl.

Effect of Temperature and pH on Enzyme Activity and Stability

The optimum temperature for the hydrolysis of CMC was measured between 4 and 60 °C by incubating each reaction mixture in 50 mM sodium acetate buffer, pH 5.0, for 30 min. For determination of the pH dependence, reaction mixtures in Britton and Robinson buffers (pH 3.0-9.0) were incubated for 30 min at 40 °C. To study pH and temperature stabilities, samples containing purified endoglucanase in Britton and Robinson buffers (pH 3.0-9.0) were incubated at different temperatures (28-70 °C) for 30 min. Following the specified treatments, samples were diluted 10-fold in 100 mM sodium acetate buffer, pH 5.0, and incubated at 40 °C for 30 min with CMC to determine remaining enzyme activity. The activity of enzyme solutions preincubated at neutral pH at different temperatures for increasing periods of time was also tested.

Cellulose fractions from pineapple cell wall

Cellulose fraction for the demonstration of in-vitro action of endoglucanase was prepared from pineapple cell wall. The fraction was prepared by sequential extraction of pineapple cell walls as desribed in Chapter-2. Before assay the aliquot of the fraction was swelled overnight by immersing in suitable buffer and then performed the assay.

Results and Discussion

Production of extra cellulase enzyme from the Bioreactor

The bioreactor (Fig 3.2) was run continuously for a period of 10 to 25 days. Endo-

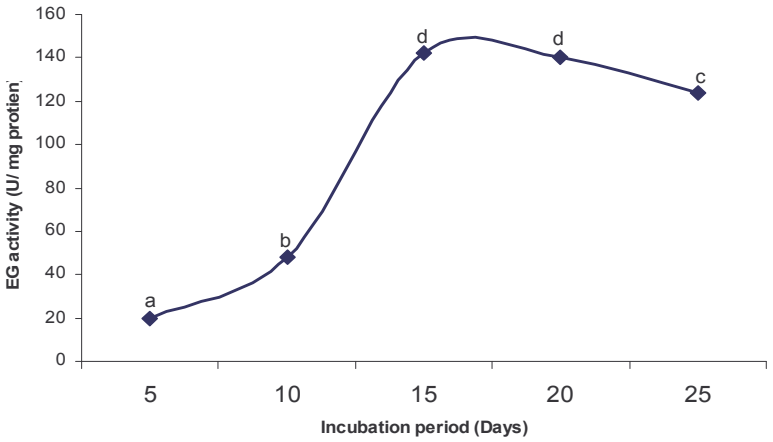
Fig.3.2:

Bioreactor for the production of extracellular cellulase from *C. paradoxa*

glucanase activity was tested in cultured broths at 10, 15, 20 and 25th day interval. The result indicated that, there was an increase in endoglucanse activity with increased incubation period. However, highest activity (142 U/mg protein) was observed on 15th day (Fig 3.3). Subsequently, decline in

Fig.3.3:

EG activity during broth culture of C.paradoxa in bioreactor



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

EG activity from 20 to 25 days old culture filtrate was observed. Hence 15th day culture filtrate was selected for further studies.

Optimization of broth volume

The standardization of media volume with bioreactor volume was carried out. The results indicated that, broth culture quantity of 50% (1000 ml) to the flask volume was found to be optimum to obtain high bio-mass of 8 gm from *C. paradoxa* (fig 3.4). This

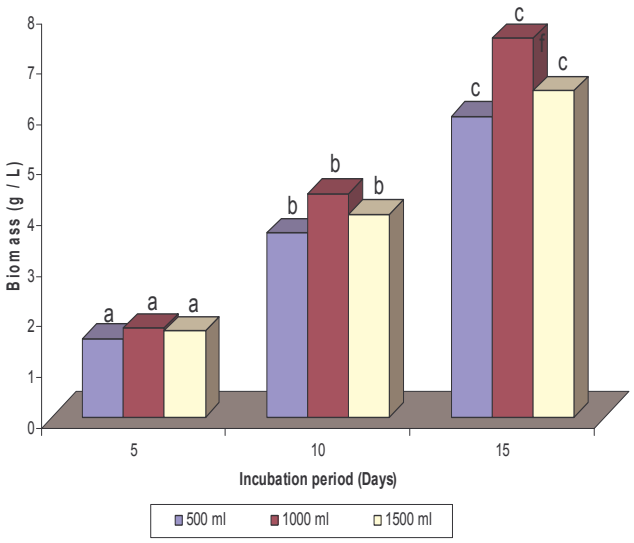


Fig.3.4:

Effect of media volume on biomass production during broth culture of C.paradoxa

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

may be due to available larger surface area at the equatorial region in the flask. The other interesting observations made were; the lesser or higher the volume of broth culture resulted in less biomass, earlier sporulation in the culture resulting low quantity of cellulase production.

Enzyme Production in Liquid Cultures

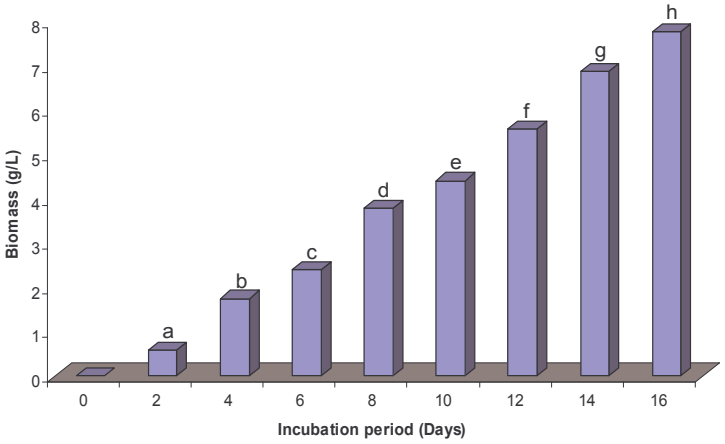


Fig.3.5:

Rate of biomass production during broth culture of C.paradoxa

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

There was moderate increase in endo-glucanase activity in the culture filtrate till 8th day. The levels of activity increased significantly (P<0.05) from 9th day onwards (Fig 3.5). It appears that there is a correlation between production of fungal biomass, glucose depletion in media and endoglucanase activity. Fungal

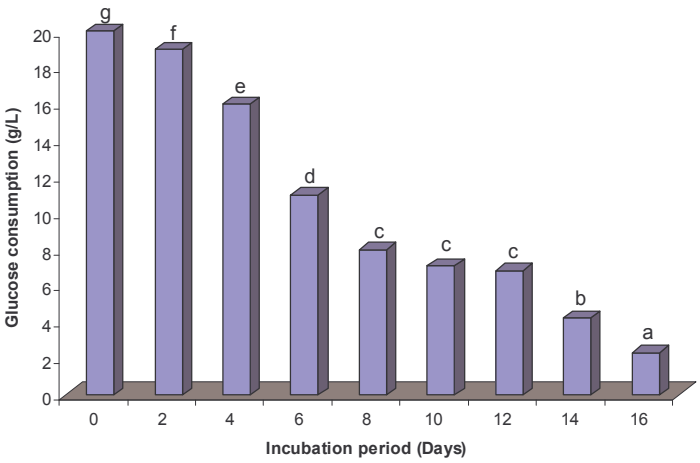


Fig.3.6:

Utilization of glucose in broth culture during biomass production

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

biomass increased markedly from day 3 to day 15 of cultivation (Fig 3.5), concomitantly there was a decrease in glucose level during early stages of cultivation and more rapidly

from 12 to 16 days (Figure 3.6). It appears that most of the glucose from the growth medium was exhausted by 16th day onwards. Glucose exhaustion coincided with highest production of fungal biomass in the reactor.

Endoglucanase activity of culture filtrate

The extracellular cellulase enzyme obtained from culture filtrate of *C. paradoxa* was tested for endoglucanase activity. The radial diffusion of enzyme in CMC bearing gel slab showed higher diffusion zone (Fig 3.7A) compared with sterilized phosphate buffer pH 7.2. (Fig 3.7B) and denatured enzyme (Fig 3.7C) by autoclaving for 20 minutes in 115 lbs. Diffusion zone diameter was proportional to logarithm of enzyme concentration as specified by many workers (Martin and Bamforth, 1983; Edney *et al.,* 1986).

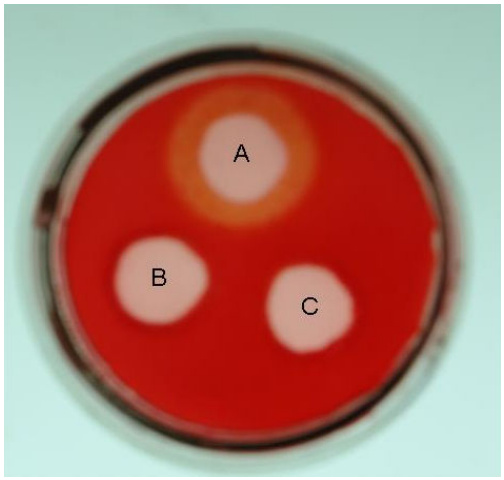


Fig.3.7: Endoglucanase activity of culture filtrates of *C. paradoxa*

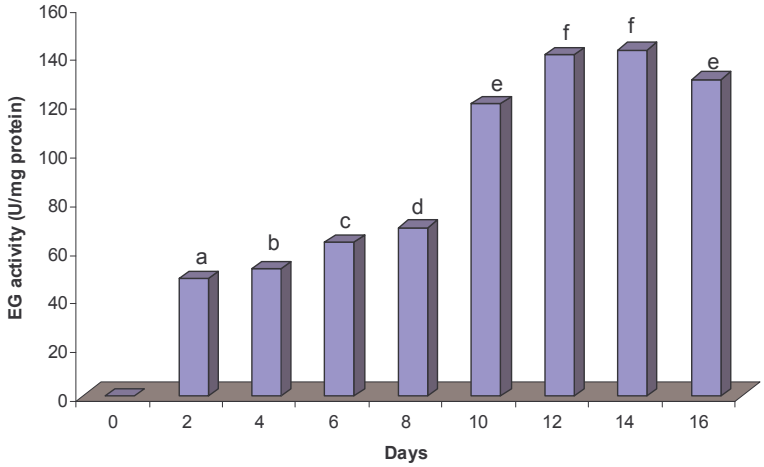
A-Endoglucanase, B- Denatured EG, C- Control

Endoglucanase activity was detected in cultured broths from second day onwards. However, the activity was found to be less than 60 U/mg protein. Rapid significant ($P<0.05$) increase in EG activity was observed from 10th day onwards with maximum EG activity on 12-15th day of culture filtrate (Fig 3.8). At this point, production of endoglucanase ceased, and the levels of activity decreased from 16th day. It appears that the relationship exist between formation of *C. paradoxa* biomass, glucose consumption

and EG production. There was a inverse relationship between biomass with glucose depletion in culture broth. Glucose exhaustion coincides with highest EG activity, similar to other fungi like *Aspergillus niger*. *A. Niveou, Trichoderma viridae, T. reesei* (Ruijter and Visser, 1997).

Fig. 3.8

Endoglucanase activity of C.paradoxa during broth culture at different duration



Each value is a mean of three different observations.
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Production of endoglucanse activity in liquid cultures may takes place during the late trophophase, coincidently with glucose depletion. This behavior seems to be in agreement with the general observations that cellulase systems are repressed in the presence of more easily metabolizable carbon sources like glucose (Bisaria and Mishra, 1989). Endoglucanase formation in various fungi starts only when the repressing carbohydrate glucose is completely metabolized (Ronne, H. 1995). Strains of *C. paradoxa* also produce other extracellular enzymes. Amylase production by *C. paradoxa* isolated from the pith of the sago palm was reported (Kainuma, 1985), and the glucoamylase component was purified and characterized (Ishigami, 1985; Ishigami, 1985; Monma, 1992) and tested for semicontinuous hydrolysis of sweet potato raw starch (Noda, 1992). More recently, several strains of *C. paradoxa* isolated from olive mill wastewater disposal ponds were found to produce laccase activity (Robles, *et al.,* 2000). Among them, the strain *C. paradoxa* CH32 also produced *â*-glucosidase activity (Lucas, 2000). This is the first report on endoglucanse production by *C. paradoxa* in context with

SER disease of pineapple. The production of cellulolytic enzyme is reported in other plant pathogenic fungi such as *Macrophomina phaseolina* (Wang, and Jones, **1995**), *Phytophthora infestans* (Bodenmann, *et al.,* 1985), or *Sclerotium rolfsii* (Sachslehner, *et al.,* 1985). Cellulolytic enzymes should play a role in the penetration of plant cell walls (Albersheim, *et al.,* 1965; Bateman, 1976). It appears that, the cellulolytic enzyme is a key pathogenic factor for *C. paradoxa* in establishment of SER disease in pineapple.

pH and Temperature optima for EG activity

The pH and temperature dependences of endoglucanase activity and stability are shown in Figure 3.9 and 3.10. Highest activity was detected in the pH interval of 5.0-9.0, the pH optimum being 5.0. Enzyme activity decreased markedly at pH <3.0 and >9.0 (Fig 3.9). Though the enzyme optimum activity was 5.0, there is no decrease in alkaline pH up to 9.0. and maintained >90% activity at pH range 6-9. A significant decrease was observed in pH 10.0. However, the enzyme was inactivated under very low acidic pH<2 conditions (Fig 3.9). The endoglucanase enzyme from *C. paradoxa* showed optimum activity under acidic pH values and under moderate temperatures of incubation. In this respect it resembles other endoglucanases produced by mesophilic fungi, although

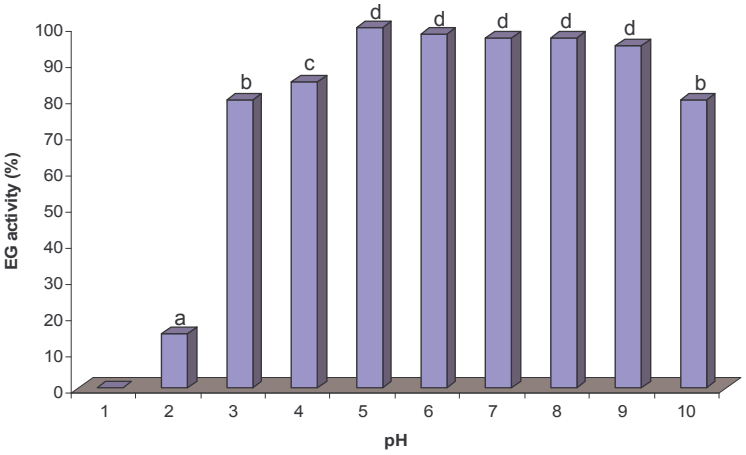

Fig. 3.9:

Endoglucanase activity of C.paradoxa at different pH

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.

endoglucanases differ markedly in their pH and temperature optima for activity (Schülein, 1997).

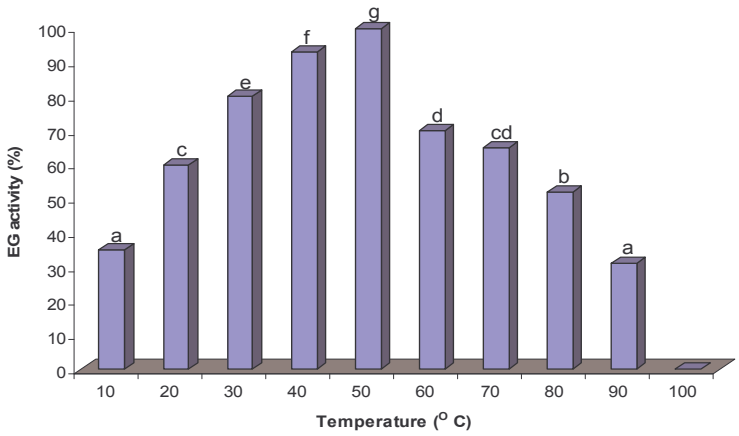

Fig. 3.10:

Endoglucanase activity of *C. paradoxa* at different temperature

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Endoglucanase activity was higher in the temperature interval of 30-50 °C, and the optimum temperature was 50 °C under the described assay conditions (Fig 3.10). A significant ($P<0.05$) decrease in enzyme activity above 50 °C was recorded. Enzyme preparations were very stable and showed activity above 90% at 40 and 50°C after being incubated for 24 h at this temperature (Fig 3.11). However, the activity was lost after 30 min of incubation at 100 °C.

The extracellular cellulase enzyme obtained from culture filtrate of *C. paradoxa* was also tested for endoglucanase activity by gel diffusion method. The radial diffusion of enzyme in CMC bearing gel slab showed higher diffusion zone in cellulase incubated for 30 min (Fig 3.11A) at ambient temperature. While, cellulase enzyme incubated at 50°C (Fig 3.11B) and 70°C (Fig 3.11C) showed relatively less activity. Thus, the endoglucanase activity was found to be function of temperature.

Effect of celluase enzyme of culture filtrate on cellulose of pineapple tissue

Celulose fractions obtained by sequential fractionation of pineapple fruit tissue was incubated with enzyme concentrate from cultural filtrate showed EG activity. The EG enzyme activity steadily increases with time upto 60 minutes of incubation, later the activity achievied the stationary phase (Fig 3.12).

The interesting observation being the high endoglucanse activity at acidic pH ranging from 4.0 -5.0 coincides with pH range of 50-100 % ripe pineapple fruits. The resistance for SER disease incidence recorded in raw green and 25 % ripe fruits may be correlated due to pH <4.0. Thus, pH may be one of the key factors for pathogenesis of *C. paradoxa*. The physiological pH of 50% ripe pineapple fruits recorded were more than pH 4 that seems to be favourable for production extracellular cellulose enzyme and endoglucanse activity of *C. paradoxa*. The

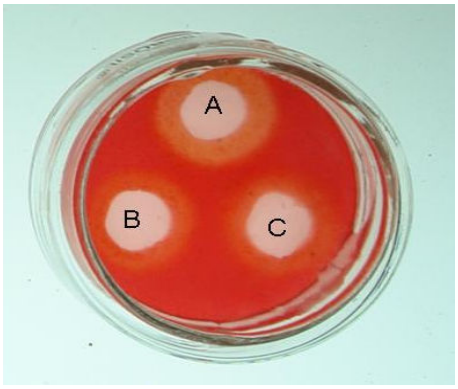

Fig. 3.11:

Endoglucanase activity of crude enzymes subjected to different temperature

A- Endoglucanase from cultural filtrate
B- EG incubated at 50° C for 30 minutes
C- EG incubated at 70° C for 30 minutes

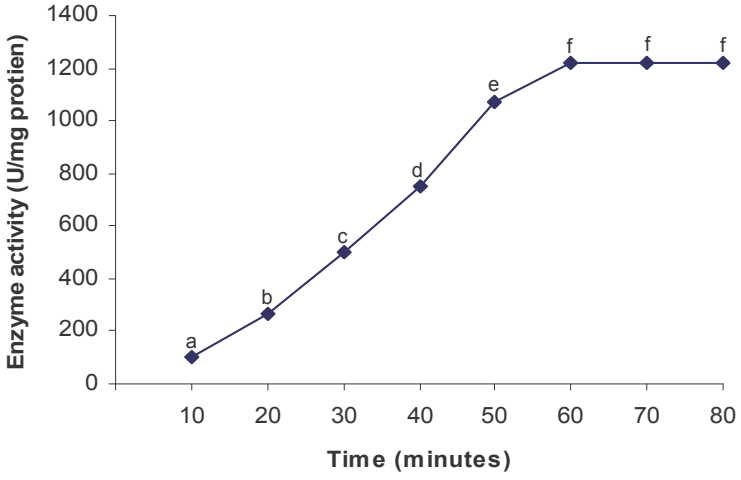

Fig. 3.12:

Endoglucanase activity of culture filtrates of *C. paradoxa*

Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

endoglucanse activity of *C. paradoxa* at wide range of temperature may be a key virulent factor that can be attributed to high postharvest spoilage due to SER disease under tropical conditions. For the first time role of Cellulase enzyme of *C. paradoxa* in pathogenesis of SER disease of pineapple was demonstrated.

SDS-PAGE and EG activity of culture filtrate of *C. paradoxa* and in SER diseased pineapple fruit tissue

The fungal biomass from the culture filtrate was separated by filtering it through two layered nylon cloth. The fungal biomass was washed thrice with distilled water. It was dried over a pre weighed filter paper in an oven maintained at 60°C until attaining uniform weight. The culture filtrate was centrifuged at 20,000 rpm for 15 min at 4° C to remove spores and mycelial fragments. The clear cultural filtrate thus obtained was tested for endoglucanase activity and protein content. The enzyme was concentrated by lyophilization and stored in-20° C.

SDS PAGE of endo-glucanase isolated from diseased fruit

When non-denatured samples of cellulose enzmes from both culture filtrate concentrate and from diseased tissue sample were analyzed by SDS-PAGE, four distinct protein separations were observed (Fig 3.13). All the four bands were tested for endoglucanase activity. Their zymographic detection of EG activity corresponds to 37 kDa protein (Fig 3.13 A, B & C). Using the standard assay procedure for determination of free reducing ends with the dinitrosalicylic acid reagent, we found a high activity against carboxymethylcellulose, but no activity on crystalline cellulosic substrates. This procedure involves separation of solids before boiling with Miller's reagent. Enzyme-treated samples were boiled with Miller's reagent before separation of

solids did not show any difference in their activity. Thus we were able to detect a large increase in the concentration of reducing equivalents formed for all of the crystalline substrates tested. We suspect that reducing ends formed by endoglucanase action remained bound to the solid substrate. Boiling with Miller's reagent would facilitate release of endoglucanase-cleaved fragments into the medium. Furthermore, the increased activity found on acidswollen Avicel strongly suggested that the cellulase in culture filtrate concentrate cleaved within the amorphous regions of cellulosic materials. Endoglucanases have been reported to act preferentially on amorphous substrates (Wood, 1991). However, degradation of crystalline cellulose by the low molecular mass endoglucanase S from Streptomyces sp. LX has also been reported (Li, X, *et al.,* 1998). Strains of *C. paradoxa* also produce other extracellular enzymes. Amylase production by *C. paradoxa* isolated from the pith of the sago palm was reported (Kainuma, *et al.,* 1985). More recently, several strains of *C. paradoxa* isolated from olive mill wastewater disposal ponds were found to produce laccase activity (Robles, *et al.,* 2000). Among

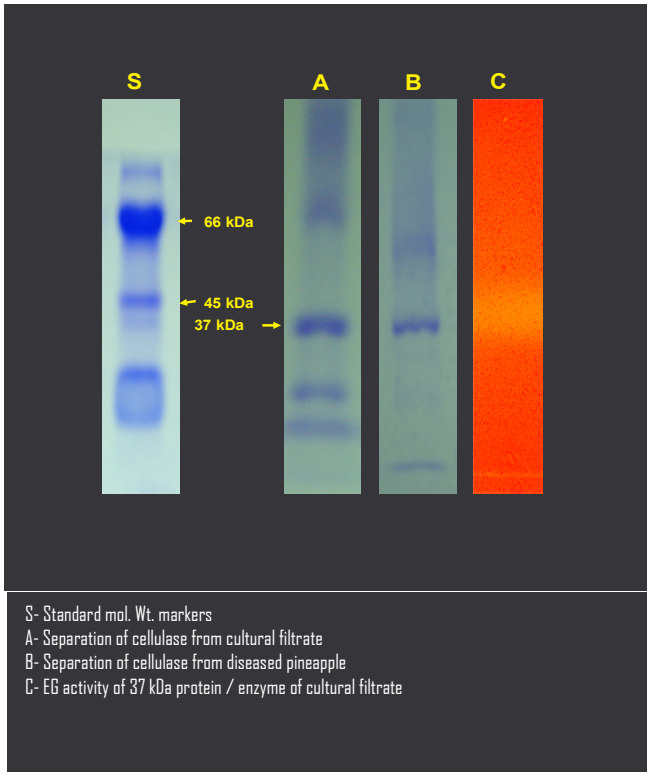

Fig. 3.13:
SDS-PAGE of SER diseased pineapple fruit tissue, and culture filtrate of *C. paradoxa*, and thier EG activity

them, the strain *C. paradoxa* CH32 also produced â-glucosidase activity (Lucas, *et al.,* 2000). The SDS-PAGE and zymographic studies showed the involovement of pathogen extracellular cellulase enzyme in pathogenic process of SER disease.

Summary

Since, cell wall degrading enzyme was found to be one of the major virulent pathogenic factors, *Ceratocystis paradoxa* isolate was tested for production of pectinase and cellulase enzymes in pure culture. To overcome the difficulty to obtain single batch broth culture extracellulase in sufficient quantity a bioreactor was successfully designed and commissioned. This is essential to ward of both quantitative and qualitative variation in production of extracellulase cell wall degrading enzymes due to nutritional and cultural conditions. The configuration of bioreactor mechanism of working was illustrated. The important conditions optimized for bioreactor for continuous production of cellulase were viz., broth volume, yield and duration of culture growth. Maximum yield of 8 g was obtained over a period of 15 days at the ratio of 1:2 (v/v) of broth and reactor. The culture filtrate of bioreactor was tested for extracellulase activity. The crude enzyme was tested for cellulase (endoglucanase) activity.

In the present investigation the crude enzyme concentrate were subjected to SDS-PAGE., followed by protein staining to purify and differentiate the patterns of cellulase enzyme of *C. paradoxa*. It exhibited presence of four distinct bands. Each band was tested for endoglucanse activity by CMC stain. Of the four, only the 37 kDa protein showed the endoglucanse activity. Cellulase isolate from diseased tissue also showed similar patteren of protein bands. It appears cellulase produced by C. *paradoxa* is a mixture of four proteins/enzymes. The different components of the crude mixture might be responsible for cellulase's biological effects. This can also form as a taxonomic criterion to study the extent of biochemical and pathogenic variation among and with in the species of *C. paradoxa* from different host, which is worthwhile to be explored.

Chapter - 4

Physical and chemical measures to control SER disease

Introduction

Control of postharvest diseases of fruit is mostly dependent on controlled atmosphere storage, refrigeration and fungicides. Among these, fungicides are widely used to reduce postharvest decay and extend the shelf-life of pineapple. However, fungicides are becoming less effective because of the development of pathogen resistance, along with consumer concerns about possible risks associated with the use of chemicals. Among a number of new strategies being investigated to control postharvest decay without the pollution of the environment and risk to public health, use of physical measures and preservatives are promising. They are also known to induce systemic acquired resistance (SAR), a defense mechanism that plays a central role in disease resistance. The work carried out in this regard is presented in this chapter.

Materials and Methods

Fruit samples

Pineapple fruit (*Ananas comosus* cv. Queen) with shell green in colour were obtained from Devaraja Fresh Fruit Market Mysore. Fruit were either used immediately or held at room temperature (25 °C) and experiments installed within 24–48 h. The peduncle was cut with sharp sterilized knife leaving 2 cm length from the fruit base before inoculation

Cultures

C. paradoxa was isolated from naturally stem end rot infected fruit. Cultures of *C. paradoxa* were maintained on PDA at 4 °C. The pathogen was periodically inoculated and re-isolated from pineapple fruit to maintain pathogenicity (El-Neshawy and Wilson, 1997 and Piano *et al.,* 1997). A spore suspension of 1×10^5 spores ml^{-1} was prepared from 7 day old cultures grown on PDA at 27 °C. All inoculations were done by atomizing 1 ml of prepared spore suspensions on the cut peduncle end of the pineapple fruit.

Irradiation experiments

UV irradiation

UV irradiation experiment was carried out as described by (Droby et al., 1993) Different stages of C.paradoxa cultures in Petri plates were exposed to UV-C light in a UV chamber provided with a UV bulb at 15 cm height. While exposing the upper cover of the plate was removed and the culture was exposed to various period of time. Lethal dose was determined by replica plating on PDA media and observing the survival of the fungi after a given dosage of UV. The experiment is done in triplicate to check the results. After recording the results all cultures were carefully discarded by autoclaving.

Gamma Irradiation

Gamma irradiation was carried out as described by (A. Kader et al., 1968) Different stages of C.paradoxa cultures in Petri plates covered with plastic papers and were irradiated keeping in the gamma chamber (Model GC-5000), loaded with Co-60 and dose rate at 6. 401 K. Gy. /Hr under ambient condition. The lethal dose for each of the stage was determined by culturing the culture in a replica plates and incubating them in 27°C for 3 days. The temperature raise in the chamber during the dosage 25-40°C have no effect on survivability of the culture without gamma irradiation up to 8 K.Gy. After recording the results all cultures were carefully discarded by autoclaving.

Screening of fungicidfal activity of chemicals

Poisoned Food Technique or radial growth (RG) test

Poisoned Food Technique was determined as described by Golembiewski, *et al.,* (1995). Different concentration of fungicide and chemicals in sterile distilled water were prepared and mixed with about 20 ml of molten PDA at about 60° C to give a final concentration of 500 ppm (v/v) poured the mixture of medium into Petri plates and allowed the medium to set. Agar disc (8 mm) of five day old culture in which pathogen is cultivated was transferred to agar disc of the plates containing media amended with fungicide or chemicals. Petri plates were incubated at 27 °C for 5 days. After 5 days, colony diameter was measured at two positions at right angle to each other and calculated the mean diameter of the colony. The relative growth in different treatments was calculated with the following formula.

$$\frac{\text{Mean colony diameter in fungicide amended medium}}{\text{Mean colony diameter in un-amended medium (control)}} \times 100$$

Spore germination test

Spore suspension ($1x10^5$ spores/ml) was prepared using sterile pineapple juice filtrate. The pineapple juice filtrate was prepared by extracting the juice from field-ripened fruit using a juice press. The juice was filtered through six layers of cheesecloth and an equal amount of distilled water was added. The juice filtrate was filtered through sterile Whatman No. 1 filter paper then with a 0.22 micron syringe filter (Micron Separations, Inc., Westboro, MA. The juice was diluted with sterilized distilled water in the ration of 1:1 vol. The solution was inoculated with spores in sterilized cavity slides and incubated at 27°C. The germination of spores was determined microscopically after 12 hrs (Wilson Wijeratnam *et al.,* 2005)

In vitro test for hot water treatment

A conidial suspension of 10^5spores/ml was prepared by serial dilution and a separate set of three sterile screw cap test tubes, each containing 10 ml of the spore suspension was used for each temperature tested. Temperature regulated water baths (Grant W6–KD) were pre-heated where necessary and held at 28±°C (ambient temperature)., 50, 53 and 55°C, respectively. Sets of spore suspensions were held in respective water baths for 5 min including the 2 min required for the 10 ml suspension to reach the required temperature. Each set of tubes was removed from the water bath after the prescribed heat treatment and cooled in cold water (22±2°C). Spore germination on PDA Petri plates after heat treatment was determined via a spread plate technique for each treatment temperature. Colony counts were recorded 48 h after incubation at 28±2°C. Spore suspensions (10^5spores/ml) from plates of controls, as well as heat-treated spores showing sporulation and mycelial growth were prepared. Respective spore suspensions were inoculated onto the peduncles of healthy pineapples. Post-heat

treatment virulence of the pathogen was tested by examining nine pineapples inoculated with 0.1 ml of 10^5 spores/ml of the control spore suspension. Further sets of nine pineapples were similarly inoculated with cultures of the respective heat-treated spores. Incidence of disease on fruit was recorded after 6 days incubation at 28±°C.

In vivo test for hot water treatment

For in vivo experiments, the inoculum of *C. paradoxa* spores was prepared as described previously in the in vitro studies. Peduncles of nine fruit were inoculated for each spore concentration 10^5pores/ml of *C. paradoxa*. A set of nine non-inoculated fruit served as controls. Peduncles were trimmed to a length of 3–4 cm before inoculation. Fruit were incubated at 28±2°C for 6 days, and examined for incidence and severity of disease. The experiment was repeated twice. Results presented in Fig. 1(a and b). With non-inoculated treatments, 0.1 ml of sterile distilled water was placed on the cut surface of the peduncle.

The hot water dip treatment was carried out in a 70 cm x145 cm rectangular metal tank of depth 45 cm, fitted with a detachable rectangular rack with circular rings of 10 cm diameter. Pineapples placed on the rings in the crown up position in batches of 18 fruit, were subjected to the 3 min dip treatment. The detachable rack ensured that only peduncles of fruit were exposed to hotwater and prevented scorch damage to other areas of the fruit during the dip treatment (Wilson Wijeratnam *et al.,* 2005).

The water in the tank was preheated and maintained at 50, 53, 55°C via a programmable thermostat. Temperature gradients were avoided by means of a 0.5 hp pump, which ensured circulation of water inside the tank.Water temperature was monitored using a temperature probe (Omega USA, Type T, Model EP1 6114).Water in the tank was changed after each treatment. Pineapples used as controls were dipped in tap

water at 28°C for 3 min. The above experiment was repeated twice. Severity of black rot disease (no infection; SER disease symptoms) was observed via longitudinal dissection of the fruit, were scored after periods of storage. A completely randomized design was adopted for all in vivo treatments.

Results from fruit quality trials were subjected to analyses of variance and mean separations conducted via DMRT at the 5% level of significance. Statistical analysis of data was carried out with the Statistical Analysis System (SAS) computer package– Version 6.

Results and Discussion

Physical treatment

UV treatment

Because the UV wavelengths employed to elicit hormetic effects are strongly absorbed by the DNA of living organisms, consideration has been given to the possibility that the UV wave length delivered to fruit can directly inactivate fungi or fungal spores that may be present at the surface of the fruit. Indeed, the wavelength range in question is sometimes referred to as 'germicidal'. In the present investigation effect of UV under different duration was tried on both vegetative and reproductive structure of *C. paradoxa*.

Table 4.1

Effect of different duration of UV exposures on mycelia and spores of *C. paradoxa*

Ceratocystis paradoxa	**UV (280 nm) exposures (min)**				
	10	20	30	40	50
Spores germination	+	+	+	+	+
Mycelia - 1 day	+	+	+	-	-
Mycelia - 3 days	+	+	+	+	+
Mycelia - 14 days	+	+	+	+	+

Inactivation of one day old mycelia occurred once a sufficiently high UV dose had been accumulated by the organism for a period of 50 minutes. Inactivation of one day old mycelia growth was the only achieved in the present investigation (Table 4.1). But the spores and mycelia of 3rd and 14th day were found to be unaffected by the UV exposure, even for a prolonged period of 50 minutes. Similar limitation of UV in arresting the growth of fungal mycelia and spores has been reported (Gardner and Sharma, 2000).

However illumination of fruits and vegetables for UV for short duration is in practice. This may be due to induction of resistance in the UV treated fruits against pathogens by accumulation of antifungal phytoalexins or phenolic compounds (Rodov, *et al.,* 1992; Merceier, *et al.,* 1993).

Table 4.2
Effect of different dose (Wide range) of Gamma Irradiation on *C. paradoxa* spores/ mycelia

Ceratocystis paradoxa	Gamma irradiation Dosage in K.Gy				
	1	3	7	8	9
Spores germination	+	+	+	+	-
Mycelia - 1 day	+	+	+	+	-
Mycelia - 3 days	+	+	+	+	-
Mycelia - 14 days	+	+	+	+	-

Table 4.3
Effect of different dose (Narrow range) of Gamma Irradiation on *C. paradoxa* spores/ mycelia

Ceratocystis paradoxa	Gamma irradiation Dosage in K.Gy					
	8.0	8.1	8.2	8.3	8.4	8.5
Spores germination	+	+	+	-	-	-
Mycelia - 1 day	+	-	-	-	-	-
Mycelia - 3 days	+	+	+	-	-	-
Mycelia - 14 days	+	+	+	-	-	-

The initial screening of Gamma irradiation Dosage (K.Gy) was carried out between 1-9 K.Gy. The results revealed that, effective control of spores and mycelia at different stages of growth was achieved at 9 K.Gy dosages (Fig 4.2). Based on this result,

the effective dosage range between 8.0-8.5 K.Gy was tried. The result indicated that, 1 day old mycelia were effectively controlled at 8.1 K.Gy, while, a dosage of 8.3 K.Gy was necessary to control spore germination and 3 and 10 day old mycelia (Fig 4.3). Permitted dosage of irradiation range 1-3 K.Gy for fresh fruit and vegetables. Further high dose of irradiation may induce various degrees of injury to healthy succulent tissues of fruits and vegetables. Since high dosage was recorded to control both vegetative and reproductive stages of *C. paradoxa*. ***In vivo experiment was not carried out on*** **pineapple.**

Under suitable condition, radiation is known to reduce the population or eliminate microbial pathogens and retard the physiological process such as ripening, senescence and sprouting. Since radition systems are highly expensive, Difficulty in uniform exposure of the pineapple fruit and Long duration of treatment of UV restrained to undertake the in-vivo studies. The present study serve academic interest.

Since hot water treatment is non-toxic and preferred, it was tested both in vitro and in vivo.

Table 4.4

Effect of hot water treatment on *C. paradoxa* spores (10^5 spores/ ml)

Heat treatment (°C)	Duration of treatment (Min)	Mean Colony Count
45	5	356±15
	10	120±2
50	5	55±10
	10	26±3
55	5	10±7
	10	1±1

Deactivation of conidiospres of *C. paradoxa* by hot water treatment was found be the function of temperature and time of treatment. With increase in temperature there was decrease in mean colony count. The lowest, single colony count was achieved in hot water treatment at 55°C for 10 minutes (Table 4.4). With in the temperature increase in time of treatment resulted in 80-90% decrease in colony count. Colony counts were not possible in control plates spread with non heat-treated spores.

Table4.5

Effect of hot water treatment on incidence of SER disease in pineapple fruits

Heat treatment (°C)	Duration of Time (Min)	Incidence of Infection
45	5	+
	10	+
50	5	+
	10	+
55	5	-
	10	-
Inoculated fruits with Sterile distilled water dip (at room temperature)	5	+
	10	+
Un-inoculated fruits Sterile distilled water dip (at room temperature)	5	-
	10	-

hot water treatment at 45 and 50°C showed ineffective in controlling incidence of SER disease in pineapple. In contrast at 55°C all fruit showed no incidencse of SER disease (Table 4.5).

Fruit maintained as controls with spore inoculation and cold water treatment showed symptoms of the SER disease. While, control un-inoculated fruits showed no SER disease symptoms. There was no significant difference in ripening behavior and quality of hot water treated fruits with normal fruits. Hence, data was not shown. Hot water treatment has been effectively used to control various post harvest diseases in many fruits (Barkai-Golan and Phillips, 1991; Coats, *et al.,* 2003).

The increasing concern of the public regarding the health hazards and environmental pollution, fungicide residues on fruits pose a great risk to consumer, lead to the search for safe alternative to synthetic fungicide. A fact that, the effectiveness of synthetic fungicides has been reduced by the frequent development of resistance by the pathogens further highlighted the need for new natural substances for alternative strategies for the control of post harvest pathogens like *C. paradoxa*. In the present investigation selected natural defensive constitutive and inducive chemicals of fruits and vegetables were screened against SER disease causing organism viz. *C. paradoxa*. Different type of chemicals namely, GRAS, preservatives, antioxidant, were screened against positive standard benomyl, a post harvest fungicide.

Among the different GRAS chemical acetic acid showed more promising in reducing the incidence of SER disease on pineapple, the order of their effectiveness in inhibition of fungi both in-vitro and in-vivo as follows; acetic acid >hydrogen peroxide > propionic acid (Table 4.6). Among preservatives, benzoic acid exhibited a significant ($P<0.05$) reduction in germination of spores and in mycelia growht in vitro. Benzoic acid displayed complete inhibition of infection of SER disease incidence. Use of benzoic acid to *C. paradoxa* infection in pineapple has been reported by earlier workers (Aradhya et al 1982).

Table 4.6

In vitro and In vivo effect of different chemicals to control *C. paradoxa* growth, spore germination and infection on piinepple fruit.

Chemicals (500 ppm)	*In vitro*		*In vivo*
	Spore germination (%)	Radial growth (%)	Infection (%)
GRAS			
1. Hydrogen peroxide	61 [g]	68	+++
2. Acetic acid	52 [f]	51 [f]	+
3. Propionic acid	84 [k]	79 [l]	+++
Preservatives			
1. Benzoic acid	02 [b]	10 [b]	--
2. Sodium carbonate	49 [e]	51 [f]	+
3. Potassium carbonate	53 [f]	55 [g]	+
4. Sodium bicarbonate	59 [g]	63 [h]	++
5. Potassium bicarbonate	65 [h]	69 [i]	++
6. Calcium propionate	71 [i]	76 [k]	+++
7. Potassium sorbate	76 [j]	81 [l]	+++
Natural compounds			
1. Ethanol (20 %)	40 [d]	47 [e]	+
2. Salisylic acid	60 [g]	63 [h]	++
3. Chitosan	66 [h]	68 [i]	++
Antioxidant			
1. Ferulic acid	01 [a]	06 [a]	--
2. Chlorogenic acid	26 [c]	19 [c]	-
3. Cinnamic acid	42 [d]	43 [d]	++
4. Tannic acid	53 [f]	57 [g]	++
5. Ellagic acid	65 [h]	71 [j]	
Fungicide			
Benomyl	00	00	--

Note: - No infection; + infection restricted to peduncle only; ++ < 12% infection; +++ >50% infection

Each value represents mean of three different observations ± S.D. Mean values with different superscripts [a, b and c] differ significantly at P < 0.05

Benzoic acid and its derivatives have been shown to be the best inhibiotors of some of the major post harvest pathogens, such as Alternaria spp., Botrytis cinerea, Penicillium digitatum, sclerotinia sclerotiorium and Fusarium oxyspoum (Lattanzio *et al.,* 1995). Sodium and potassium carbonate also showed reduction in percentage germination of *C. paradoxa* spore and inhibition of mycelial growth, concomitantly less incidence of disease in vitro. The effectiveness of preservatives both in vitro and invivo control of SER disease, in order were; Benzoic acid > Sodium carbonate > Potassium carbonate > Sodium bicarbonate > Potassium bicarbonate > Calcium propionate > Potassium sorbate (Table 4.6). Among the different natural chemicals tried, the order of the effectiveness in inhibition of fungi both in-vitro and in-vivo is as follows; Ethanol > Salisylic acid > Chitosan. Ethanol showed fewer incidences of disease symptoms in in-vivo.

Among five natural constitutive antioxidant of fruit and vegetables, ferulic acid was found to be as effective as benomyl in complete control of SER disease both invitro and invivo. In addition chlorogenic acid also showed complete control of SER disease invivo. The order of effectiveness of antioxidants in control of SER disease is as follows; Ferulic acid > Chlorogenic acid >Cinnamic acid > Tannic acid > Elagic acid (Table 4.6).

Inhibitory effect of ferulic acid and chlorogenic acid on fungal pathogens like Fusarium and sclerotinia sclerotiorium has been demonstrated by Lattanzio *et al.,* 1995. Ferulic acid is the principal phenols identified in pineapple (Table 2.2, 2.3). The concentration of these phenols decline as fruit ripens with a corresponding increase in fruit susceptibility to the SER disease. The level of ferulic acid in raw green and 25% ripe fruit was high with high level of resistance to *C. paradoxa* infection, than 50% and above ripened fruits (Table 2.2, 2.3). The threshold concentration of ferulic acid appears to be >50% in pineapple, which is essential to induce resistance to *C. paradoxa* infection. Below which it renders the fruit susceptible for infection. Our results support this

phenomenon. Presence of ferrulic acid in high concentration can be regarded as constitutive antimicrobial barrier against *C. paradoxa* infection. In pineapple fruit Ferrulic acid along with other phenolic acids may contribute to resistance through their antimicrobial properties or by enhancing resistance of the host (Friend 1981; Mohan and Kollatadudy). Among the 18 different classes of natural compounds screened, Benzoic acid among preservatives, ferulic acid and chlorogenic acid among antioxidants showed direct effects on the pathogen and complete control of SER disease on pineapple. When compared with plethora of synthetic substances these chemical compounds offer the prospects of an effective, nontoxic and zero-residue antimicrobial compounds for the control of post harvest disease of fruits, in particular to SER disease of pineapple.

Significant ($P<0.05$) decline in ferulic acid level in 50% ripe fruits may be one of the reason for its susceptibility for *C. paradoxa* infection. In vitro experiments also confirm the above observations. It appears ferulic acid can directly inhibit the growth of mycelia and spore germination of *C. paradoxa*. In addition being a cell wall constitutent its accumulation may induce defense mechanism that plays a central role in disease resistance. Systemic acquired resistance (SAR) is an inducible defense mechanism that plays a central role in disease resistance. There is evidence that accumulation of ferulic acid, salicylic acid and chlorogenic acid are necessary for the induction of Systemic acquired resistance (SAR) in plants (Yang *et al.,* 1997). In addition, SAR can also be activated by exogenous treatments like hot water and chemical inducers (Wilson *et al.,* 1994; Gorlach *et al.,* 1996). In the present investigation use of ferulic acid, and chlorogenic acid has been developed as a novel crop protection agent, which not only have anti-microbial properties on *C. paradoxa*, but also may increase pineapple resistance to disease.

Summary

Various physical and chemical treatments were tried to control the SER disease of pineapple. Among the physical treatments tried, UV and irradiation evoked a little response in arresting the pathogenic growth in-vitro. In the present investigation inhibition of *C. paradoxa* spore germination and arrest of mycelical growth were achieved by gamma irradiation at high dosage. This serves only the academic interest. Hot water treatment (55° C for 5-10 minutes) was found to be highly effective in total inhibition of *C. paradoxa* infection both in in-vitro and in-vivo. It offers a practical approach to control SER in pineapple since it kills *C. paradoxa* that causes surface decay, while maintaining pineapple fruit quality during ripening and storage. It also is relatively easy to use, cost effective, has a short time and is efficient in uniform heat transfer.

Limitation to use synthetic fungicide due to consumer's safety and increasing resistance of postharvest pathogen to synthetic fungicide was the major concern behind screening constitutive and/or inductive chemical compounds from biological sources against *C. paradoxa.* Among 18 various chemical compounds screened, benzoic acid, ferulic acid and chlorogenic acid were effective in complete control of SER disease in pineapple. Further research on combined effect of hot water with above chemicals, a better understanding of the pathology, biochemistry and quality of treated produce may enable the development of more precise and effective post harvest treatments against *C. paradoxa* infection on pineapple, in particular.

General Summary and Conclusion

General Summary and Conclusion

The research undertaken towards understanding of intricate relationship between host and pathogenic factors of SER disease in pineapple var.'queen' caused by *Ceratocystis paradoxa,* which is essential to devise a nontoxic, zero residue control measures, is briefed below.

Chapter 1

As a first step, to be familiar with decaying agent, the fungus has been isolated from naturally infected fruit, identified and established its pathgenicity, nature of invasion and colonization. The pathogen was deposited in culture collection center of the institute with Acc.No. 3695. The contribution of pathogen to severity of the disease in fresh fruit is dependent on the the level of inoculum, spore germination and rate of vegetative growth. Minimum threshold concentration of spores, enhanced spore germination and rate of growth of mycelia of *C. paradoxa* in the presence pineapple fruit juice was

demonstrated. A consistent symptom of SER disease of pineapple by *C. paradoxa* is the production of soften peduncle that typically involve the death and maceration of infected and surrounding tissue, which makes the host tissue predispose for fungal invasion and colonization. Most revealing account exhibited in diseased tissue was rapid spread and colonization of pathogen with advance of ripening. Wheither such progress relates to the difference in the chemical compositions of fruit in context with maturity and ripening were thoroughly investigated.

Chemical factors that govern two stages of maturity viz. 1) physiological and 2) commercial maturity stages were defined. The susceptible stage of pineapple fruit to *Ceratocystis paradoxa* infection has been recognized. The green and 25% ripe fruit exhibited resistant to SER pathogen. The chemical factors like pH above 4, less acidity and more sugars associate with 50% ripe fruits were found be the major chemical factors that favour the *Ceratocystis paradoxa* for infection.

"Stem end rot disease manifestation is the ultimate expression of its final display of all its complex interrelationships with its pathogen. Histological account of SER disease has been worked out to elucidate cellular and structural changes in the host (pineapple fruit) tissues with invasion of pathogen (*C. paradoxa*). Scanning Electron Microscopic observations of infection process of *C. paradoxa* revealed a range of colonization status. For the first time, direct penetration of fruit tissue by *C. paradoxa's* infected hyphae and their hemibiotrophic colonization were demonstrated. Extensive disorganization and disintegration of diseased tissues of cell wall were found to be responsible for collapse of fruit tissue. The most striking features of host reaction were viz., plasmolysis of cells. Blackening of tissue was due to excessive production black mycelium and single or chain of chlymydospores of *C. paradoxa.*

A detail investigation on constitutional and inductive chemical factors of pineapple and pathogenic factors of Ceratocystis paradoxa was carried out and presented in second chapter.

Chapter 2

There is little information to correlate SER infection with the cell protective system of pineapple. Given its significance in other species, it would be of value to examine the relationship of the antioxidant enzymes to blackheart development in pineapple fruit. In this chapter, the role of PPO, POD and PAL were investigated along with total phenol, and cell wall constitutive chemicals in pineapple fruit. These changes were examined at physiological, commercial maturity stage, at different stages of ripening and also in SER disease tissue of pineapple.

The present investigation demonstrated the possible role of antioxidant enzymes like PPO, POD and PAL in pre symptomatic biochemical changes like softening and translucency of tissue surrounding infection loci. Excessive or denovo synthesis of these enzymes in disease mainly involve in predisposition of tissue by discoloration subsequently browning of tissue. The other constitutional changes in pineapple tissue, as a consequence of *C. paradoxa* infection were also emphasized.

It appears that there are two major influencing factors that determine the resistance of fruit against *C. paradoxa* infection. Among the parameters more suitable to grade pineapples into classes of maturity and ripe stage were (in the case of the intact fruit), skin colour (shell colour) and (in the case of the flesh), flesh colour. The selected parameters to predict resistance in pineapples were: components of cell wall, polyphenol, total proteins, total soluble solids (TSS), titrable acidity, TSS/acid (also known as the

Brix/acid ratio), pH, colour and translucency. Shell clour, Brix/acid ratio, pH, ferulic acid content of cell wall showed a definite role as fruit resistance index. The changes of these chemicals as a function of ripening provided the suitable host-index parameters for disease resistance against *C. paradoxa* infection.

The cut end of peduncle provides an avenue for entry of pathogen. A state-of-the-art relation between host and pathogenic factors regarding physical, biochemical and pathological status, revealed that pineapple fruit start ripening immediately after attainment of physiological matuity. Inordinate delay in disease expression in intact, green fruit may well coincides with the firmness, high ferulic acid content in cell wall, high pH, low Brix/acid ratio. Ripening changes like softening of tissue, rapid decrease in moisture, TSS, Ascorbic acid, pH, concomitant increase in total sugars, and cellulase enzymes were found to be the key factors for susceptibility of the fruit for infection. A delay in these changes in pineapple may be responsible for extended period of latency of *C. paradoxa.*

Though pineapple produces cell wall degrading cellulase enzyme during ripening the concentration may be insufficient or qualitatively different. The cell wall derivatives may induce production of cellulase enzyme in *C. paradoxa* In the present investigation 10 to 15 time increase in cellulase enzyme in disease tissue may indicate that *C. paradoxa* not only contributes its enzyme to the host, but also induced increase production of this enzyme. Cellulase proved as a virulent factor of *C. paradoxa* against defense system of the pineapple fruit. It is also responsible for massive maceration of host tissue and decompartmentation of cell organelles. For the first time role cellulase enzyme in pathogenesis of SER disease in pineapple was distinctly demonstrated. The failure to produce cellulase enzymes may be responsible for avirulence of the pathogen.

Chapter 3

Cellulase being key virulent pathogenic factor in *C. paradoxa* infection, the pathogen isolate was tested for production of cellulase and pectinase enzymes in pure culture. To overcome the difficulty to obtain single batch, broth culture extracellulase in sufficient quantity a bioreactor was designed and successfully commissioned. This is essential to ward of both quantitative and qualitative variation in production of extracellulase cell wall degrading enzymes due to nutritional and cultural conditions. The configuration of bioreactor was illustrated. The important conditions optimized for bioreactor function for continuous production of cellulase were viz., broth volume, yield and duration of culture growth. Maximum yield of 8 gm per litre of broth was achieved for a period of 15 days. The culture filtrate of bioreactor was tested for extracellulase (endoglucanase) activity. C.paradox's cellulase enzyme found to be highly potent even at high temperature (50°C) and both in acidic and alkaline pH range (5-9). This pathogenic adaptability may be the underlying reason to cause heavy postharvest SER disease in pineapple at tropical, arid conditions like India.

In the present investigation the crude enzyme concentrate was subjected to SDS-PAGE., followed by protein staining was used to identify the protein pattern of extracellulase enzyme of *C. paradoxa*. It exhibited presence of four distinct bands. Each band was tested for endoglucanse activity by CMC stain. Of the four protein bands the 37 kDa only showed the endoglucanse activity. It appears cellulase produced by *C. paradoxa* is a mixture of four proteins/enzymes. The other components might be responsible for cellulase's biological effects. This can also form as a taxonomic criterion to study the extent of biochemical and pathogenic variation among and with in the species of *C. paradoxa* from different host, which is worth to be explored.

Chapter 4

Non toxic or zero residues, effective post harvest control of SER disease in pineapple fruit was tried by dual methods viz. 1) Use of physical method like UV, irradiation and hot water treatment and 2) use of constitutive or inductive chemicals of host along with FDA recommended preservatives.

Hot water treatment (55^{o}C for 5-10 minutes) to cut stem end of pineapple were effective in inhibiting spore germination and also the incidence of SER disease in pineapple fruit. Among an array of eighteen chemicals tried, Benzoic acid, ferulic acid and chlorogenic acid at the concentration of 500 ppm were found to be effective for significant inhibition of spore germination and also induced significant reduction in infection and incidence of SER in pineapple fruit. The combination of these two treatments can be a new strategy to provide non-toxic, zero residue control measure to SER disease in pineapple.

Futuristic note

The investigation provides a comprehensive account of SER disease of pineapple caused by *C. paradoxa*, hitherto not available in any published form. The work envisaged in this thesis, provides an insight into the array of pathological and biochemical factors associated with ripening of pineapple fruit. The knowledge base generated on constitutive or inductive chemicals to control SER disease of pineapple may warrant further studies to understand the mechanism of inhibition pathogen or towards inducing resistance to pineapple fruit, which can be effectively used for devising new generation,

host friendly, effective, zero toxic control measures, targeted to specific host and / or pathogen.

Interesting thing observed being production of four cellulase enzymes with less or no pectinase enzymes indicating that cellulase is the major cell wall degrading enzyme responsible for pathogenicity of *C. paradoxa*. It is also interesting to enumerate the role of potent, multiple cellulase enzymes of *C. paradoxa* in pathogenesis. It is worth while to exploit its application in biomass degradation of agri-horticultural waste.

List of Publications and Presentations

Pulications

1) Paper entitled "A Low Cost Commercial Technology for Pineapple handling and Transportation to Boost Rural Economy of Northeastern States" is published in Journal of Rural Technology. Vol. no. 1, Issue 3 April 2004.

Authors: S.M. Aradhya, **H.M. Swaroop Kumar,** R.S. Matche, M.N. Keshava Prakash, S.M. Pandian, K.R. Kumar and K.V.R. Ramana.

2) Paper entitled "Effect of Ambient and Low Temperature Storage Conditions on Shelf life and Quality of Pineapple Fruits **(**Ananas comosus. Var. Queen) was published in Conference proceedings of "National symposium on Food & Nutritional Security" held in Palampur, H.P. on Sept.18-19, 2003

Authors: **Swaroop Kumar H.M.,** K.V.R. Ramana and S.M. Aradhya.

3) Paper entitlaed "Accumulation of Bioactive Compounds during Growth and Development of Mango Ginger (Curcuma amada Roxb.) Rhizomes" is published in Journal of Agricultural and Food Chemistry 2007. 55, 8105-8111

Policegoudra, R.S**., Swaroop Kumar H.M.** and Aradhya, S.M.

Presentations

a) Poster paper "Effect of Ambient and Low Temperature Storage Conditions on Shelf life and Quality of Pineapple Fruits **(**Ananas comosus. Var. Queen) was presented in National symposium on Food & Nutritional Security…, held in Palampur, H.P. on Sept.18-19, 2003 & awarded **First Prize**.

Swaroop Kumar H.M., K.V.R. Ramana and S.M. Aradhya.

b) Poster paper entitled "Postharvest Disease Management in Pineapple" was presented in 44th annual conference of the association of microbiologists of India, University of Agricultural Sciences, Dharwad. November 12 - 14, 2003. Authors : **Swaroop Kumar H.M.,** K.V.R. Ramana and S.M. Aradhya.

c) Poster paper entitled "Antibacterial effect of Punicalagin- a high molecular weight polyphenol from pomegranate fruit waste on food borne human pathogens," was presented in 44th annual conference of the

association of microbiologists of India. University of Agricultural Sciences, Dharwad. November 12 - 14, 2003. Authors: Anand P. Kulkarni., **Swaroop Kumar H.M.** and Aradhya S.M

d) Poster paper "Changes in free sugar composition during ripening of Pineapple fruit" was presented in international conference, IFCON-2003 at CFTRI, Mysore, 5-8th Dec. 2003. and awarded **Third Prize**. Authors**: Swaroop Kumar H.M.,** K.V.R. Ramana and S.M. Aradhya.

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