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°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°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;).

Pathogenesis 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°C). The culture was deposited in microbiological 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 hemocytometer 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 calipere.

**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 sterile 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 10^6 spores/ml were optimum thresh hold limits to cause 100% incidence of diseases. Thereafter, 0.1 ml of the spore suspension (10^6 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°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 constituents 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°C and the fruits were inoculated with 10⁵ 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 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 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 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 microbiological collection center of the Institute.
**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

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**Fig.15:**

*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. paradoxa* (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 old 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 condiospores 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
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 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 in a 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 conidiospores 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 as $10^5$ spores/ml. This concentration appears to be higher when compared to Mauritius and other varieties as reported by (Sridhara 1975).
This may be due to difference in inherent quality of pineapple varieties and difference in strains of *C. paradoxa*

<table>
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<tr>
<th>Treatment</th>
<th>Inoculation period after SD water wash</th>
<th>Incidence (%)</th>
<th>Severity (%)</th>
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<tr>
<td>Sterile distilled H₂O</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>2</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57&lt;sup&gt;f&lt;/sup&gt;</td>
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<td></td>
<td>48</td>
<td>97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30&lt;sup&gt;a&lt;/sup&gt;</td>
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Table 1: Effect of surface moisture on incidence and severity of SER in pineapple fruit inoculated with *C. paradoxa*

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

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

isolated. Successful infection was found to be dependent on 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 to minimize infection due to *C. paradoxa*. Since *C. paradoxa* depends 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 availability or Relative humidity on the fruit surface plays an important role in fungal infection, mainly through germination of conidiospores. 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>
<thead>
<tr>
<th>Chemical characteristics</th>
<th>Green (% maturity)</th>
<th>Ripe fruits (% ripening)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>80 Physiological</td>
<td>100 Commercial</td>
</tr>
<tr>
<td>T.S.S (° Brix)</td>
<td>10.1± 1.2</td>
<td>11.0 ± 1.5</td>
</tr>
<tr>
<td>Acidity (%)</td>
<td>1.0 ± 0.9</td>
<td>0.87 ± 1.0</td>
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<tr>
<td>pH</td>
<td>3.1 ± 1.3</td>
<td>3.37 ± 0.4</td>
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<tr>
<td>Total sugar (mg/100gm)</td>
<td>6.0 ± 1.5</td>
<td>6.8 ± 2.2</td>
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<tr>
<td>Vitamin C (mg/100gm)</td>
<td>40.0 ± 1.8</td>
<td>39.4 ± 1.3</td>
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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%) 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)

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 (Table 1.2) 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 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 parenchymatous 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 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 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 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 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.