CHAPTER II

Cymbidium Rot: Isolation, characterization and identification of the causal organisms

2.1. Introduction

The *Cymbidium* has originated from the Greek word Kymbion, which means boat shaped, since the largest petal (labellum or lip) resembles boat. It is one of the most popular and desirable orchids in the world because of the beautiful flowers. The genus *Cymbidium* comprises approximately 50 species. Geographic distribution extends from the northwestern Himalaya to Japan and south through Indochina and Malaysia to northern and eastern Australia (Yukawa and Stern, 2002). Cymbidiums exhibit distinctive ecological diversification and occur as terrestrial, epiphytic, and lithophytic life forms. One species, *C. macrorhizon*, once thought to be a parasite (Hooker, 1890), lacks foliage leaves and has a strong mycoparasitic existence.

Swarts (1799) established the genus *Cymbidium* with a broad generic delimitation. Circumscription of the genus has been variously defined by later workers: Lindley (1833), Blume (1848, 1849, 1858), Reichenbach (1852, 1864), Hooker (1890), Schlechter (1924), Hunt (1970), Seth and Cribb (1984), and DuPuy and Cribb (1988).

Cymbidiums make great houseplants, and are also popular in floral arrangements and corsages. They have been cultivated for thousand of years, especially in ancient China. One feature that makes the plant so popular is the fact that it can survive during cold temperatures (as low as 7^0C). Orchid hobbyists in temperate climates appreciate the fact that they can bloom in winter, when few other orchids are blooming. Seedling establishment of the genus is distinguished by protocorm or by rhizome (Shimasaki and Uemoto, 1987). In terrestrial *Cymbidium,* a rhizome needs 1-2 year to differentiate to a plantlet as, for example, *C. ensifolium* (Chung *et al.*, 1985; Lu *et al.*, 1992), *C. forrestii* (Paek and Yeung, 1991), *C. goeringii* (Nagashima, 1982; Duan and Xie, 1983; Shimasaki and Uemoto 1991) and *C. sinense* (Chiou and Wang, 1985; Chang and Chang, 2000).

Asian Cymbidiums are often sold in "growths" (Fitch, 1999). Each growth is an independent pseudobulb that was divided from the back bulb or parent bulb. Each pseudobulbs have an independent root system. The stem of the 'growth' is usually very short, 1 inch (2-3 cm) and is narrow 1/2 inch (1.5 cm). Each growth bears three to twelve leaves in two rows. The stem supports many leaves that branch from it in a grass like fashion. The pseudobulb acts as the main water and nutrient storage device. In comparison to other orchids, the pseudobulb is small and cannot store large amounts of water, necessitating frequent watering during their growth phase (Fitch, 1999). Most species have thick roots which are covered in a spongy white velamen and have only a thin core of vascular tissue. The erect stems are usually short and swollen to form a prominent pseudobulb which is often slightly flattened. Many species produce a new growth annually. In one section of the genus the pseudobulbs grow and flower for several years before a new shoot is produced, and in *C. mastersii*, *C. elongatuni* and *C. suave,* each shoot grows continuously for many years producing an elongated stem rather than a typical pseudobulb.

The inflorescence in *Cymbidium* is unbranched and may be erect, arching or pendulous. Each mature pseudobulb usually produces one or two inflorescences from leaf axils near the base. In *C. eburneum* and its allies the inflorescences arise from the leaf axils near the apex of the pseudobulb, as they do in *C. suave* and *C. elongatum*. The inflorescences bear up to 50 flowers in *C. canaliculatum* and only one in *C. goeringii* and *C. eburneum*. Most species bear 10-20 flowers; the flowers are all immediately recognizable as cymbidiums. They comprise a dorsal sepal, two lateral sepals, two free petals and a three lobed lip which is hinged at the base of the column. There is usually a callus of two distinct ridges along the upper surface of the lip. The anther contains two pollinia or four pollinia fused in two pairs. This brief summary can be applied to all or any of the species. Individually they can be much more precisely defined than this, and some species also vary to quite a large extent.

Though *Cymbidium* flowers are long-lasting, both on the plant as well as when cut, large in size, attractive and existing in a wide range of colours but the plant is attacked by number of pest and diseases. This include fungal diseases like Anthracnose Orchid Spot (Ciferri, 1926); *Cymbidium* wilt (Bose and Yadav, 1989); *Cymbidium* tip burn (Vij and Kaur, 1985); Leaf and Stem rot of *Cymbidium* (Ilieva *et al.*, 1998); Leaf spot diseases of *Cymbidium* (Kazunori and Takayuki, 2000), Leaf blotch (Hill, 2004) etc. Among bacterial diseases Bacterial Brown Rot (Bose and Yadav, 1989) and *Cymbidium mosaic virus* is responsible for viral disease of *Cymbidium* (Jensen, 1959a; 1959b; Sherpa *et al.*, 2003).

In Eastern Himalaya especially in the hills of Darjeeling and its adjoining areas *Cymbidium* has great horticultural value and has been cultivated extensively. Majority of orchid growers in this region mainly at Kalimpong, Kurseong, Mirik, Ging suffered from a huge loss due to an epidemic pseudobulb rot since 1995 during monsoon months.

This chapter deals with the isolation and characterization of the causal agents responsible for the disease '*Cymbidium* pseudobulb rot'.

2.2. Materials and Methods

2.2.1. Field survey and collection of samples

During field survey (2002-2005), different nurseries of Darjeeling hills and of its adjoining area were visited. Areas under survey were different polyhouses (ph) of various localities like Kurseong, Kalimpong, Mirik and Ging (Figure 2.1).

Pseudobulbs and roots of *Cymbidium,* showed symptoms of rotting of different stages were collected from different polyhouses and were wrapped in polythene bags and brought to the plant pathology laboratory for further study.

2.2.2. Symptomological study

Pseudobulbs and roots, showing different degree of disease symptoms were examined morphologically and compared with the healthy bulb.

2.2.3. Histopathological study

Different degrees of rotted bulbs were cut into small pieces with sterile razor blade and the pieces were fixed in formalin-aceto-alcohol solution (FAA No.1- 90 ml of 70% ethanol; 5 ml of formalin and 5 ml of glacial acetic acid) (Johansan, 1940) for 3 days. The samples were dehydrated through an ethanol series. To observe the host penetration, longitudinal and transverse sections were mounted on clean glass slide and stained with lactophenol-cotton blue. Sections were examined with Leica DMSL research microscope and photographed on Kodak plus film with Minolta X 300 camera.

Rotted samples were fixed in FAA for 3 days. The samples were dehydrated through ethanol series. To observe the bacterial invasion within the cell lumen sections were mounted on small glass block coated with gold by HITACHI E-1010 Ion Sputter and examined under HITACHI S-2360N Scanning electron microscope and photographed on Kodak academy 200 plus film with Nikon camera.

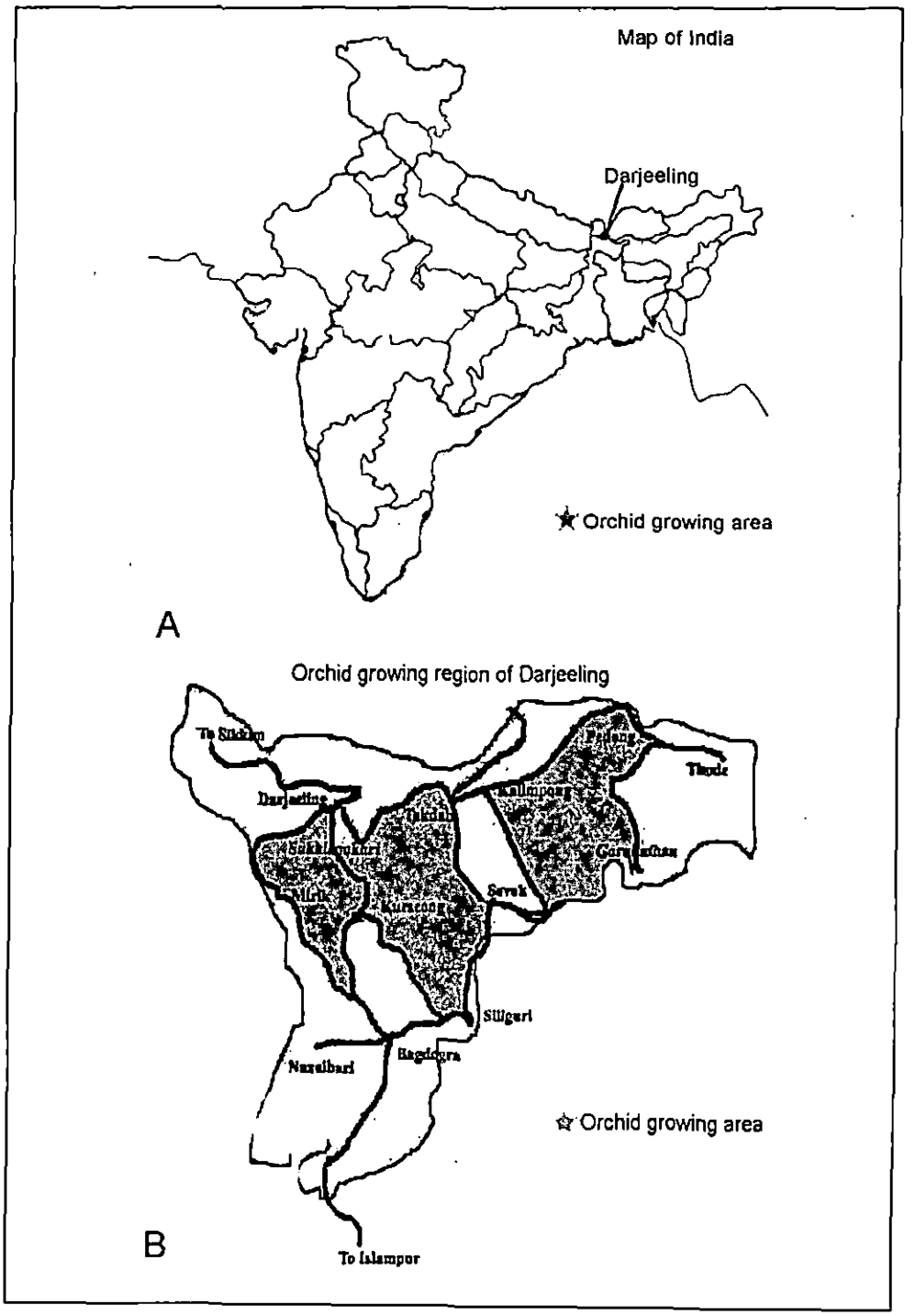


Figure 2.1: Study area: A. Map of India showing the area where the study was undertaken. B. Enlarge view of the area showing different orchid growing zones of Darjeeling and its adjoining area.

2.2.4. *Isolation of pathogens*

The infected samples were rinsed in tap water and rotted portions were cut into small pieces, surface sterilized with 1.5% sodium hypochlorite for 2 min and washed several times with sterile distilled water. Approximately 1 gm of rotted pseudobulb pieces were added to 100 ml of sterile distilled water in flasks and shaken on a rotary shaker at 150 rpm for 30 min. Bulb and root segment as well as a 10 fold dilution series of the resulting water suspension, were plated on various general and selective media (Persson *et al.,* 1997) to recover the *Cymbidium* rot organisms. The plates were then incubated for 4 days at 22^{0}C. Different media were used to isolate the pathogens related to the disease such as Potato Dextrose Agar (PDA) media were used for isolation of fungal pathogen supplemented with antibacterial antibiotic streptomycin (Stevens, 1981); Peptone Glucose Agar (PGA) media were used for isolation of both fungal and bacterial pathogens; Czapex-Dox Agar media for isolation of pathogenic fungi; Malt Extract Agar used for isolation of fungal pathogen (Dreyfusss, 1986; Bills and Polishook, 1994); Nutrient Agar media used for rapid isolation of bacterial pathogen.

2.2.5. *Characterization and Identification of pathogen*

Fungal pathogens were identified through cultural characteristics on agar media and microscopic observation and identified according to Gilman, (1945) and Subramanium (1971). The fungal pathogens were further confirmed by Agharkar Research Institute, Puna, India. These were subcultured and maintained on PDA slant at 4^{0}C for further study.

Cultural, microscopic and biochemical parameters were evaluated to identify bacterial pathogen following Bergy's Manual of Determinative Bacteriology (Holt *et al.,* 1994). The bacterial pathogen was subcultured and maintained on NA slant at 4^{0}C for future reference.

2.2.6. Pathogenicity test

Pathogenicity test was performed by dipping method (Haglund, 1989). Pseudobulbs were surface sterilized with 1.5 percent sodium hypochlorite for 2 minutes and washed by sterile distilled water for several times. Spore / cell suspension were counted on a hemacytometer in order to prepare a concentration of 10^6 cell /spore per milliliter of the suspension. Inoculated bulbs were covered with separate sterilized polythene bags and incubated at 22 $\pm$ 1^0C aseptically upto seven weeks at a relative humidity of 80 percent and disease severity was assayed periodically.

2.3. Results and Discussion

Field trips were done periodically round the year from 2002-2005, at different polyhouses like Kurseong-12 polyhouses; Mirik-3 polyhouses; Kalimpong-4 polyhouses; and Ging-1 polyhouse (Figure 2.2). Disease incidences of *Cymbidium* rot in these polyhouses were recorded (Table-2.1).

Table 2.1. ***Pseudobulbs rot incidence on Cymbidium in different Polyhouses at Growers field at Mirik, Kurseong, Kalimpong, and Ging.***

Location	Poly house	Total number of plants observed	Total number of plants infected	Percentage of infection
Mirik	Poly house 1	300	11	3.6
	Poly house 2	600	495	82.5
	Poly house 3	500	380	76.0
Kurseong	Poly house 1	800	09	1.1
	Poly house 2	700	08	1.1
	Poly house 3	300	300	100
	Poly house 4	1500	1500	100
	Poly house 5	300	300	100
	Poly house 6	250	206	82.4
	Poly house 7	350	03	0.8
	Poly house 8	300	260	86.7
	Poly house 9	600	600	100
	Poly house 10	400	340	85.0
	Poly house 11	300	190	63.3
	Poly house 12	1500	1300	86.6
Kalimpong	Poly house 1	300	170	56.6
	Poly house 2	220	10	4.5
	Poly house 3	1000	800	80
	Poly house 4	1600	1350	84.3
Ging	Poly house 1	300	225	75

From the table it is quite evident that 74% of the visited nurseries showed 50-100% disease severity. In Kurseong cent percent disease incidence were recorded in some polyhouses. The severity of the disease was generally associated with the high humidity, soil moisture and often related to poor drainage system and irrigation water. The disease assumes an epidemic form during wet season and its spread is added by wind and rain. From the table it is also noticeable that some of the polyhouses viz. one from Mirik, three from Kurseong and one from Kalimpong showed less than 50% disease incidence. Even in some polyhouses the rate of disease incidence were negligible. The cause of this insignificant disease incident in such polyhouses has been investigated using various field techniques, such as direct interviews, discussion with the growers and by direct observation. The outcome of such observation is summarized below:-

- These polyhouses are located in isolated places i.e. they are away from other nurseries. No other orchid nurseries were found nearby.
- In these polyhouses proper hygienic conditions were maintained. It has been seen that these houses have proper aeration, light intensity and irrigation system.
- Moreover, growers of these polyhouses propagate the pseudobulb from healthy plants by themselves. They do not procure pseudobulbs from any other sources.

During interviewing with about 25 members of Himalayan Orchid Society of Kurseong a huge loss of *Cymbidium* rot was found to occur. Each member of the society cultivated about 6000 *Cymbidium* clones during the year 1995 and the first appearance of disease occurred after one year of plantation. According to their report, initially the disease symptoms appeared in few plants in the nursery and spreads from one nursery to another during their plant exchange. Disease

Figure 2.2: (a and b). Diseased Cymbidium plants in nurseries; (c and d) Rotted pseudobulbs and roots in view.

severity increases year after year and during 1998 to 2002 it was in its climax when the members lost almost 95-98% of their planted *Cymbidium*.

The severity of the disease stages was assayed symptomatically and considered Disease Severity (DS) as 0 in case of healthy bulbs as 100% when it was completely rotted being hollow, fibrous and dry. Intermediate stages were considered comparing with healthy bulbs. DS was described as earlier middle and later phase.

Earlier phase was considered when bulbs were soft, pulpy followed by oozing of liquid and becomes yellow in colour but there was no apparent change in colour of leaves when compared with the healthy plants.

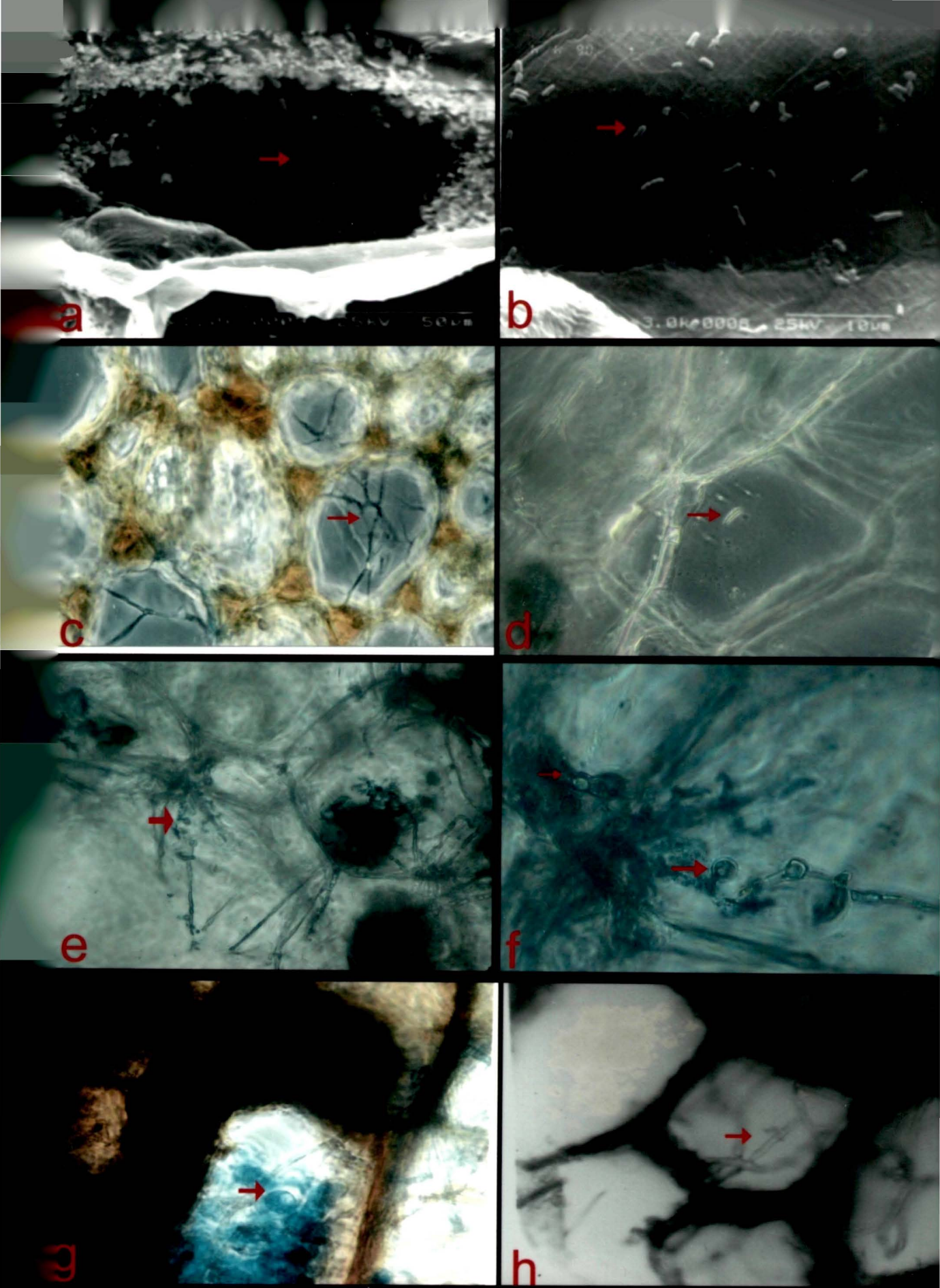
Middle phase was considered when bulbs and roots started to lose weight and disintegration of tissue started infected bulbs become brownish in colour and leaves of the plants become yellow in colour and starts withering.

Later phase was recorded when the internal tissues were completely destroyed, bulbs become hollow, fibrous and dry with dark brown or blackish colour and leaves become dry, black and few in number.

2.3.1. Histological studies

Histological examinations of infected pseudobulb revealed that infection by bacterial and fungal pathogen induced cellular alternations in the epidermal, cortical and vascular parenchymatous tissues.

Scanning electron microscopic study of early stages of rotted pseudobulbs, when the bulbs become soft and pulpy and oozes liquid, it showed the presence of rod shaped, single, sometime paired bacteria, size ranging from 0.9-3.0 × 0.4-0.6 µm within the cortical parenchymatous region (Figure 2.3a and 2.3b). Observation under phase contrast microscope of the same stage of infected bulb showed motile, small, rod shaped bacteria within the parenchymatous ground tissue. The dark brown colour liquid with foul odor which oozes from the



igure 2.3. (a) SEM of early stage of rotted pseudobulb showing abundance of E. carotovora within th ll lumen; (b) Enlarge view of the same; (c) Phase contrast microscopy of septate mycelium of . oxysporum within the cortical region of middle stage of rotted pseudobulb; (d) macroconidia of . oxysporum; (e) Septate mycelium with chlamydospore; (f) Chlamydospore in enlarge view; (g) Phas ntrast microscopy of sporangium of M. hiemalis f. hiemalis within the late stage of rotted pseudobulb nd (h) Coenocytic hyphae of M. hiemalis f. hiemalis within the infected cell.

infected bulb of early stage were also observed under the light microscope and found the abundance of rod shaped, motile gram negative bacteria. This bacterium was isolated and identified as *Erwinia carotovora,* the detail of which is given later in this chapter. Light and phase contrast microscopic observation of transverse and longitudinal section of intermediate stages of infection, when the pseudobulbs lose weight and disintegration of tissue started, it showed that sub-epidermal and cortical parenchymatous tissues were radially colonized by the ramifying intra and intercellular septate hyphae (Figure 2.3c), presence of macroconidia (30-50 µm) (Figure 2.3d) and chlamydospore (6-8 µm) (Figure 2.3e and 2.3f) of the fungi within the sub epidermal and cortical cells which was isolated and identified as *Fusarium oxysporum.* The detailed characterization of this fungus has been discussed later on this chapter. At the later stage of infection when the tissues become more or less dry and fibrous the damaged cortical cells showed coenocytic, hyaline mycelium with globose sporangia within the rotted parenchymatous cells (Figure 2.3g and 2.3h), it was also isolated and identified as *Mucor hiemalis* f. *hiemalis,* the isolation and characterization of the organism discussed later on.

2.3.2. Isolation, characterization and identification of pathogens

Isolation of pathogens were done in different selective and general media as described in detail in materials and methods. At the early stage of infection, a flat, waxy, entire, yellowish bacterial colony found to be present abundantly in Nutrient Agar media. At the intermediate stage of infection a typical white cottony fungal colony found in all selective and general fungal isolation media where as at the later stage of infection a creamy and wooly fungal colony found in abundance both in general and selective fungal isolation media.

At the early stage of infection, an abundance of a bacterium was observed. Different morphological, cultural and biochemical characters were evaluated to identify the bacterium which was as follows:

- **MORPHOLOGICAL**
  - **Size:** 0.9-3.0 × 0.4-0.6µm
  - **Shape:** Rods
  - **Gram Nature:** Gram Negative
  - Aerobic in Nature
- **CULTURAL**

Agar plate character:

Growth: Moderate
Form: Flat
Surface: Smooth and waxy
Elevation: Raised
Edges: Entire
In broth: Turbid with white sediment
Colour: Whitish grey

- **BIOCHEMICAL**

Amylase	: Negative
Pectinase	: Positive
Gelatin hydrolysis	: Positive
Indole Production	: Positive

Acid and Gas formation from different Carbohydrates

Glucose	: Positive
Sucrose	: Positive
Glycerol	: Positive
Manitol	: Positive
Fructose	: Positive

Based on the above-mentioned characters, following Bergy's Manual of Determinative Bacteriology (Holt *et. al.*, 1994) and also by culturing on *Erwinia* specific media (D3 media) which characteristically produced red colouration of the medium (Haskett and Kado, 1970) the bacterium was identified as *Erwinia carotovora* (Figure 2.4a and 2.4d).

At the intermediate stage of infection the infected tissue showed dominance of a fungal pathogen. The pathogen produced typical white cottony colonies on PDA (Figure 2.4e). The asexual reproductive structures consist of conidia and

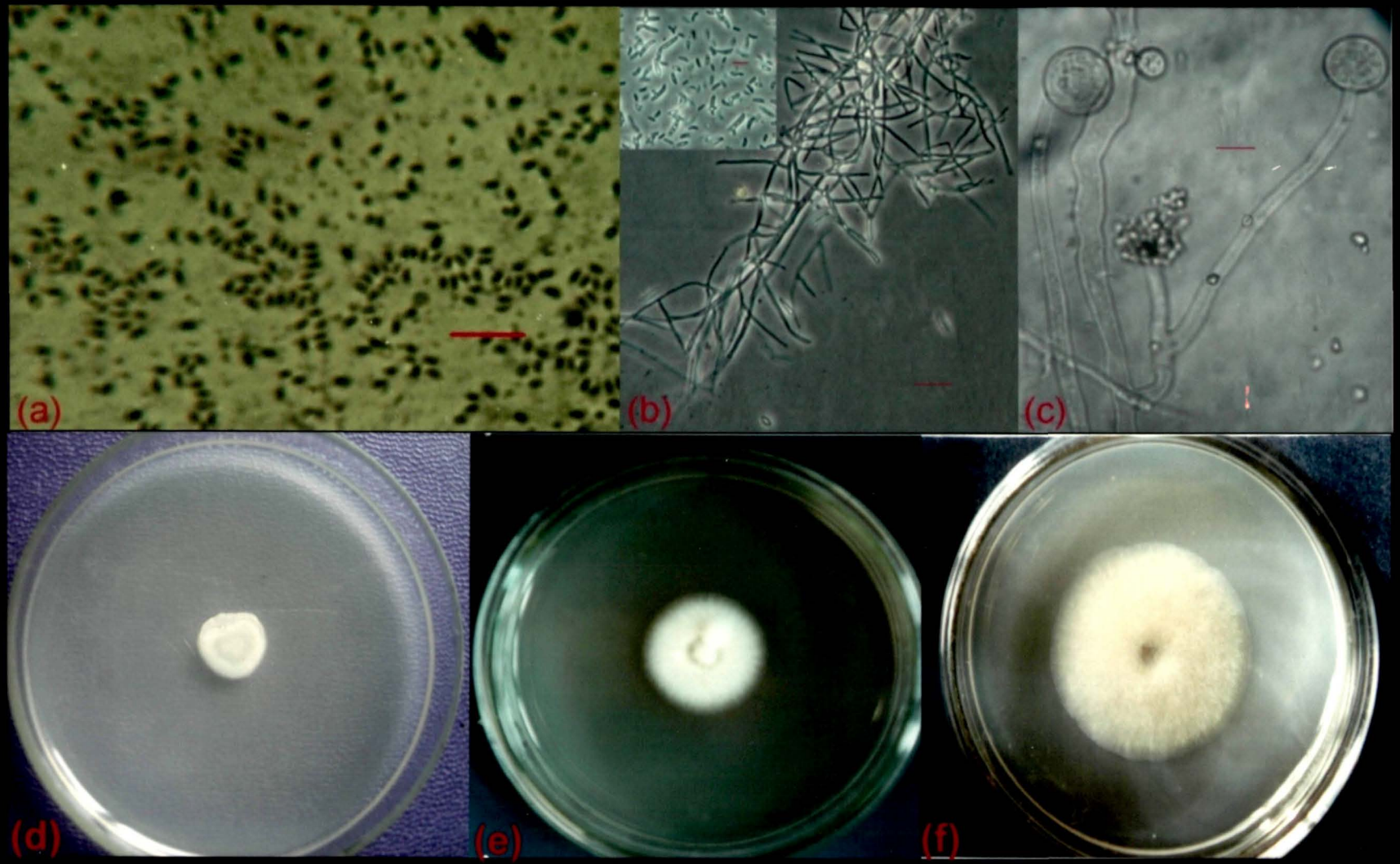


Figure 2.4. Microscopic Photograph of (a) Erwinia carotovora, Bar= 10µm (b) Fusarium oxysporum mycelia with spore in culture, Bar= 50µm (Inset : macro and microconidia, Bar= 30µm), (c) Mucor hiemalis f. hiemalis, Bar= 25µm. (d) Colony of E. carotovora on nutrient agar plate (e) and (f) Colony of F. oxysporum

chlamydospore. Conidia are produced in sporodochia, conidiophore simple; two types of conidia were observed; microconidia and macroconidia (Figure 2.4b). Microconidia mostly one celled with or without septa, hyaline, oval, oblong produced singly from the tip of the phialides, size of microconidia ranges from 4-14 × 2-4 µm.

Macroconidia were long, curved (sickle shaped), cylindrical to sub-cylindrical to fusiform mostly 3-5 septate and pointed at both the ends, size of macroconidia varies from 25-54 × 3-5 µm. Chlamydospores were terminal and intercalary, size of which ranges from 6-10 µm in diameter. Growth rate of the pathogen on PDA plate after five days of incubation ranges from 3.2 to 4 cm. Based on these morphological and cultural characteristics the fungus was identified as *Fusarium oxysporum* and was further confirmed by the Agarkar Research Institute (ARI), Puna, India.

At the later stage of infection, another fungus showed its dominance in the rotted pseudobulbs and roots. A creamy and wooly colony (Figure 2.4f) developed on PDA. Microscopic observation reveled mycelium were slender, coenocytic, sporangiophores unbranched to branched. Sporangia spherical, brownish yellow, visible to necked eye 50-52 µm in diameter (Figure 2.4c). Columella free, spherical 28-48 µm. Spores usually unequal, elongated 7 × 3.2 µm, smooth and hyaline. Growth rate of the fungus on PDA after 72 hours of incubation varies from 7.5 to 7.8 cm. Based on these morphological and cultural characters the fungus was identified as *Mucor hiemalis* f. *hiemalis* and was further confirmed by ARI.

2.3.3. Pathogenicity test

Pathogenicity test was performed by dipping method in two ways; inoculating the pathogens *separately* and *consecutively*. Pseudobulbs were surface sterilized with 1.5 percent sodium hypochlorite for 2 min and washed by sterile distilled water for several times. In the first case sterilized bulbs were dipped separately into

fungal as well as bacterial spore / cell suspension (10^6 cfu / ml) for 1 minute. For control set the healthy bulbs (Figure 2.5a) were dipped in sterilized water. Inoculated and control bulbs were covered with separate sterilized polythene bags and incubated at 20^0C aseptically upto 47 days at a relative humidity of 80 percent and disease severity was assayed periodically.

When the pathogenicity test was performed separately with single pathogen i.e. *E. carotovora, F. oxysporum* and *M. hiemalis* f. *hiemalis* and incubated upto 47 days, *Erwinia carotovora* exhibited maximum tissue disintegration followed by *F. oxysporum* and *M. hiemalis* f. *hiemalis,* but none of the individual pathogens caused 100% tissue disintegration. Maximum of 70% destruction (considered when whole pseudobulbs become dark coloured; soft and pulpy, oozing of liquid with foul odor; epidermal, subepidermal and ground tissue become dark and affected) was recorded in case of *E. carotovora* on 47 days (Figure 2.5d); *F. oxysporum* caused a maximum of 30% tissue disintegration (considered when the bulbs become wrinkled; epidermal and subepidermal cells were affected) on 47 days (Figure 2.5c) and *M. hiemalis* f. *hiemalis* caused 10% destruction (discolouration of pseudobulb) on 47 days (Figure 2.5b).

In the second case i.e. in *consecutive* inoculation, the bulbs were dipped in spore / cell suspension of pathogens according to their natural occurrence. All other parameters were kept as such as in the case of separate inoculation. Bulbs were inoculated firstly by *E. carotovora* and after 12 days of this inoculation (when the symptoms like early phase appear) the same bulbs were inoculated (dipped) with *F. oxysporum* and incubated. After 15 days of second inoculation (when symptoms like middle phase appear) third inoculation was done by *M. hiemalis* f. *hiemalis.* Complete destruction (DS= 100%) was recorded 20 days after third inoculation (Figure 2.5e). Total time period required for complete destruction by consecutive inoculation method was 47 days. When the pathogenicity test was performed in consecutive dipping method symptoms mimicked the natural

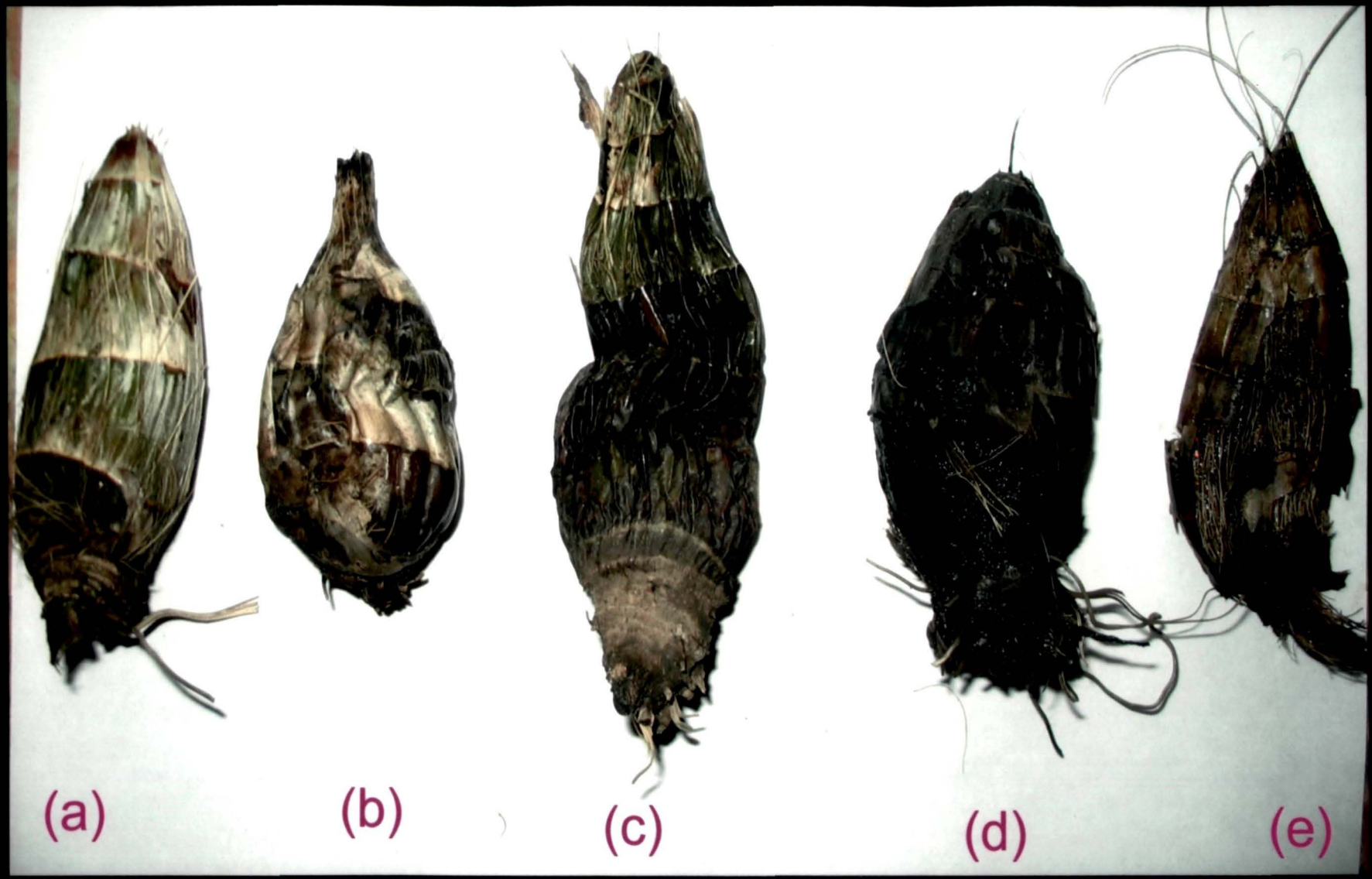


Figure 2.5.Pathogenicity test: (a) Healthy pseudobulb, (b) Inoculated with Mucor hiemalis f. hiemalis.(c) with Fusarium oxysporum , (d) with Erwinia carotovora (e) inoculated all three pathogens.

symptoms found in the field condition. This experiment was done for at least five times and every time they reproduce the same symptoms.

It is an interesting information on host-pathogen combination that three pathogens act synergistically for ultimate demolition of the host. Even from the pathogenicity test it was clear that *Cymbidium* rot is orchestrated by the action of *E. carotovora, F. oxysporum* and *M. hiemalis* f. *hiemalis*. The next step is to isolate a biocontrol agent that can effectively control these three pathogens. Literature survey suggests that bacterial pathogens are unable to control by fungal antagonist or there have been no clear examples regarding the use of fungal antagonist to control bacterial plant pathogens in the rhizosphere or spermosphere (Whipps, 2001) so the objective is to isolate a putative bacterial antagonist that can show strong inhibitory effect against all three pathogen. Although a range of different bacterial genera and species have been studied, the overwhelming number of papers have involved the use of *Pseudomonas* species. Clearly, *Pseudomonas* species must have activity but it begs the question as to the features that make this genus so effective and the choice of so many workers (Whipps, 2001). Pseudomonads are characteristically fast growing, easy to culture and manipulate genetically in the laboratory and are able to utilize a range of easily metabolizable organic compounds, making them amenable to experimentation. In addition, the genus *Pseudomonas* is ubiquitous in nature and can be found readily in ecological habitats as diverse as water, soil, and plant surfaces. (de Weger *et al.*, 1995; Marilley and Aragno, 1999). The next chapter will discuss about the isolation, characterization and screening of potential fluorescent pseudomonads having antagonistic activity against all three pathogens.

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