STUDIES ON LEAF INFECTING AND ENDOPHYTIC FUNGI OF *Mangifera indica* L.

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(Shipra Y. Chaudhary)

STUDIES ON LEAF INFECTING AND ENDOPHYTIC FUNGI OF *Mangifera indica* L.

ABBREVIATIONS

µg/l	Microgram/ litre
ºC	Degree in Celsius
h	Hour
g	Grams
mg	Milligram
mg/ g	Milligram/ gram
ml	Millilitre
mm	Millimetre
m sq	Meter square
kg	Kilogram
PDA	Potato Dextrose Agar
pH	Potentials of Hydrogen Ion
wt	Weight
WLS	Wild Life Sanctuary
ppm	Parts per million

Table Index

Introduction

I. INTRODUCTION

Mango (*Mangifera indica* L.) belonging to the Family Anacardiaceae, is the most important commercially grown fruit crop of the country. It is considered to be the king of fruits. Truly, the mango is India's greatest contribution to the international sweet teeth. It has been held in high esteem by the Hindus. Its blossoms are used for the worship of goddess Saraswati and festoons (Toran) of mango leaves are strung over doorways on auspicious occasions. It finds mention in Vedas too. The mango has been described as 'Kalpaviriksha' or wish-giving–tree. Its common vernacular name 'Aam' means 'The common'. It is not only common throughout India, but it is also the fruit of the common man (Arya, 2004).

The mango had become established in India at a very early stage. The invading armies of Alexander the Great, found it established in the Indus Valley in 327B.C. It is now a common fruit in all the Malay Archipelago, Indonesia, the Philippines, the West Indies and Madagascar. It has also been introduced into many other lands like Brazil, the U.S.A. and Queensland in Australia. It has become established in Somaliland on the eastern coast of Africa before 1331. By the 16^{th} century, it had reached the Persian Gulf. By 1690 it was grown under glass house conditions in England (Arya, 2004).

M. indica is a large evergreen tree, 10-45 m high and requires comparatively low maintenance cost. The tree has a trunk with a girth of 9.6 m and branches upto 24 m long and 3.6 m in circumference. It covers an area of 243 m sq. and average yield of the tree is 18,000kg. The tree also lives long, some trees as old as 300 years are said to be living even today.

Mango is grown in at least 87 countries but nowhere it is so greatly valued as in India, Where, it occupies about 70 percent of the area devoted to fruit crops with a total production of 8.21 million tons (Prakash and Srivastava, 1987). The mango is grown in almost all states of India. Major mango growing states are Uttar - Pradesh, Bihar, Andhra Pradesh, Orissa, West Bengal, Maharashtra, Gujarat, Karnataka, Kerala and Tamil Nadu. Besides well-organized orchards it is grown as road side tree and as isolated tree in backyards of houses and on boundaries of the fields.

Table I.1- State wise production of Mango in thousand metric tons

State	2006-07 Production	2007-08 Production	2008-09 Production
Andhra Pradesh	3865	4157	2522
Bihar	1306	870	1329
Gujarat	834	665	869
Karnataka	1368	1223	1284
Kerala	445	445	821
Maharashtra	646	710	712
Orissa	431	251	449
Tamil Nadu	580	753	821
Uttar Pradesh	2980	3365	3465
West Bengal	549	623	548

Source: Indian Horticulture Database 2009 (with modified the source data)

The main varieties of mango grown in the country are Alphanso, Dashehari, Langra, Fajli, Chausa, Totapuri and Neelum etc. In Western India the chief varieties are Alphonso or Hafus or Badmi, Pairi, Kesar, Borsha, Feradin, Rajapuri, while in Southern

India, Bangalora or Totapuri or Thevadiyamuthi, Neelum, Rumani and Pairi are grown. Although, India is the largest mango producing country, accounting about 60% of world production, the export of fresh fruit is limited to Alphonso and Dashehari varieties. India's share in the world mango market is about 15 percent. Mango accounts for 40 percent of the total fruit exports from the country. There is good scope for increasing the area and productivity of mango in the country.

Taxonomy of *Mangifera indica* Linn.

Division	Magnoliophyta
Class	Magnoliopsida
SubClass	Rosidae
Order	Sapindales
Family	Anacardiaceae
Genus	*Mangifera*
Species	*indica*

The chemical constituents of the different parts of *M. indica* were reviewed by Ross (1999) and Scartezzini and Speroni (2000). Various kinds of chemical compounds have been reported from vegetative and floral parts of *M. indica*. Quercetin, Kaempferol, Mangiferin (1, 3, 6, 7-tetrahydroxy xanthone), gallic acid and m-digallic acid were reported to be present in the leaves of *M.indica* (Elsessi and Satch, 1965). Butin (7,3',4'-tri OH flavonone) have been reported from the stem bark (Ansari *et al.,* 1967). Isoquercetin, Kaempferol and laucoanthocyanins were reported from alcoholic extracts of mango panicles (Bose and Siddiqui, 1948; Singh and Bose, 1961). 1,3,6,7-tetra-oxygenated and 1,3,5,6,7-penta-oxygenated xanthones and polyphenols were reported from various part of *M. indica* (Ghosal *et al.,* 1978). Guha and Chakrabarti (1933) reported vitamins B, B2 and C in Indian mango. The seed fat

of *M. indica* contains 53 percent molecules of mono, di and tri saturated glycerides respectively (Narayanan and Kartha, 1962). Good mango varieties contain 20% of total soluble sugars in which the non-reducing sugars are more than the reducing sugars.

Economic Importance

The mango fruit is one of the most highly prized dessert fruits of the tropics. The fruit is very popular with the masses due to its wide range of adaptability, high nutritive value, and richness in variety, delicious taste in which sweetness and acidity are delightfully blended and excellent flavour. The fruit is consumed raw or ripe. Young and unripe fruits are usually acidic and used in pickles, chutney, amchur and culinary preparations. Mango fruit is utilized at all stages of its development both in its immature and mature state.

The wood is used as timber, and dried twigs are used for religious purposes. Except root every part of the plant is used. Even the kernel inside the stone is not wasted. The mango kernel contains about 8-10% good quality fat which can be used for saponification and also as a substitute for cola in confectionery. Its starch is used in confectionery industry. The mango kernel is also effective against diarrhea and asthama.

Mango also has medicinal uses. The ripe fruit has fattening, diuretic and laxative properties. It helps to increase digestive capacity. The smoke of burning leaves is supposed to cure hiccups and some throat troubles. Baked and sugared pulp is given to patient of cholera and the plague. The bark is a source of resins and gum. The gum and resinous substance exuded by the stem end of the harvested fruit are mixed with lime juice and given in cases of scabies and skin infections. The bark is used in tanning of leather. The wood has many uses; it is used in making and packing

cases and percussion drums. Its leaves are used as fodder. Thus the mango tree has several uses and is fully utilized in India (Anonymous, 1998).

Disease incidence in Mango

The wide range of climatic conditions and environmental situations in which mango grows indicates the nature and the diversity of the associated disease problems. Over 140 fungi and about 12 nematodes and a dozen of phanerogamic parasites and epiphytes are associated with *M. indica.* Yet, there are a few diseases which are of great economic importance (Reddy, 1975). Different parts of *M. indica* are known to suffer from a number of diseases caused by fungi, bacteria and insects. Among the diseases, those affecting flowers and fruits are most destructive ones. The diseases manifest themselves as several kinds of rots, die back, mildew, necrosis, scab, blotch, stem bleeding, wilt, spot, canker, sooty mould and malformation. Some of these diseases take heavy toll of tree and produce alike, and have become a limiting factor in mango cultivation in some regions. Mango fruits both ripe and unripe are also vulnerable to variety of diseases. Disease of fruit occurring in transit and storage results in to great spoilage because of the lack of proper storage facilities. Among the several spoilage organisms, fungi are the most destructive, causing extensive damage during storage and transport of mango fruits.

During the survey conducted by Koti Babu (1998) of various mango orchards in and around Baroda it was found that prevalence of floral malformation in many trees. The percentage occurrence of the disease ranged from 10 to 50% in different orchards. The outcome of survey revealed that floral malformation was encountered in eight varieties of mango trees falling under the age group of 8 to 30 years viz. Rajapuri, Kesar, Ladva, Limdi, Alphonso, Dadamio and a local variety growing in different orchards.

Mango leaves suffered with certain fungal pathogens like *Colletotrichum gloeosporiodes, Diplodia natalensis, Macrophoma mangiferae, Phoma glomerata* and *Botryodiplodia theobromae.* Mango scab was first described in 1943 from specimens from Cuba and Florida. Now it is found in most of the mango growing areas around the world, including South East Asia. It is considered a very minor disease in the USA and the Philippines. It was first identified in Australia in January 1997, near Darwin. It appears to have been in the Northern Territory and Queensland since at least the early 1990s but was thought to be a form of flowering anthracnose (Conde *et al.,* 2007). The cause of the disease is a fungus, *Elsino*ë *mangiferae,* also called *Denticularia mangiferae*. This fungus survives on living plant tissues. Common symptoms are brown spots with haloes, edge lesions, corky lesions on the lower surfaces of leaves and elongated dark lesions along main veins under the leaves. In wet weather, numerous small brown lesions or shot holes may form on young leaves leading to their defoliation. Other symptoms seen on leaves include lesions with centre scabs and numerous small lesions about 0.1 mm in diameter along secondary veins. Leaves often appear distorted due to the effects of marginal or edge lesions.

Another fungal disease of young fruits of mango is mango powdery mildew. It is the primary cause of the widespread problem of poor mango fruit set. Worldwide it is found in most mango growing areas. It is often sporadic in severity but has been reported to cause up to a 20% loss in production (Cook, 1975). Mango powdery mildew is an easily recognizable problem and the symptoms are very apparent and are diagnostic. The disease was first recorded on 1914 in Brazil and the fungus was named and described by Berthet (Briton Jones, 1923; Uppal *et al.,* 1941). *Oidium mangiferae* Berthet causing powdery mildew of mango, is widely distributed

throughout the Pacific region. It infects panicles, fruits, and leaves. Mango is the only known host of the mango powdery mildew pathogen.

What is an Endophytic fungus?

Since the discovery of endophytes in Darnel, Germany in 1904, (Tan and Zou, 2001) various investigaters have defined endophytes in different ways, which is usually dependent on perspective from which the endophytes were being isolated and subsequently examined.

An endophytic fungus lives in mycelial form in biological association with the living plant, at least for some time. Therefore, a minimal requirement before a fungus is termed an 'endophyte' should be the demonstration of its hyphae in living tissue. An endophyte is a "plant living within another plant" (Morris, 2001).

Since the discovery of the world's first billion-dollar anticancer drug, paclitexel (Taxol) from *Pestalotiopsis microspora* (Speg.) G.C. Zhao and N. Li, a fungus that colonises the Himalayan Yew tree *Taxus wallichiana* Zucc., without causing apparent injury to the host plant, interest is growing in symptomless parasitic fungi, termed the 'endophytes'(Bacon and White, 2000). They gave an inclusive and widely accepted definition of endophytes- "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects". While the symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggest they can also be aggressive saprophytes or opportunistic pathogens. Both fungi and bacteria are the most common microbes existing as endophytes. It seems that other microbial forms, *e.g.* Mycoplasmas and Archaebacteria, most certainly exist in plants as endophytes, but no evidence for them has yet been presented. The most frequently isolated endophytes are the fungi. It turns

out that the vast majority of plants have not been studied for their endophytes. Thus, enormous opportunities exist for the recovery of the novel fungal forms, taxa and biotypes (Strobel and Daisy, 2003).

The endophytes are usually Ascomycetes and mitosporic fungi. These colonizes plant tissue and remains within the tissues, except that fruiting structures may emerge through the surface of the plant tissue. The most common endophytes are anamorphic members of Ascomycetes, and they are often closely related to fungi known to cause diseases. Phylogenetic evidence is used to suggest that some endophytes have evolved from pathogens and from others, vice versa. The mechanisms of host recognitions and development of colonization may also be common among closely related endophytic and pathogenic fungi. Some endophytes can colonize multiple species of plants and they can be cultured from their host plant in an appropriate growth medium.

Distribution of Endophytes

Fungal surveys of various hosts during the past 20 years have demonstrated that endophytic colonization of land plants by fungi is ubiquitous. Endophytes are known from plants growing in tropical, temperate, and boreal forest; from herbaceous plants from various habitats, including extreme arctic, alpine (Petrini, 1987; Fisher *et al*., 1995), and xeric environments (Mushin and Booth, 1987; Mushin *et al.,* 1989) and from mesic temperate and tropical forests. Endophytic microfungi may be diverse at an exceedingly small scale; a single conifer needle may harbor several dozen species. Endophytic microfungi typically are present as internal, unseen, microscopic hyphae; their presence is revealed externally only when they sporulate. Sporulation of endophytes is usually a seasonal or ephemeral event.

Diversity of Plants and endophytes

Of the myriad of ecosystem on earth, those having the greatest biodiversity seem to be the ones also having endophytes with the greatest number and the most biodiverse microorganisms. Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. Ultimately, biological diversity implies chemical diversity because of the constant chemical innovation that exists in ecosystems where the evolutionary race to survive is the most active. Tropical rainforests are a remarkable example of this type of environment. Competition is great, resources are limited and selection pressure is at its peak. This gives rise to a high probability that rainforests are a source of novel molecular structures and biologically active compounds (Redell and Gordon, 2000).

How Endophytes live and reproduce?

Endophytes can be transferred either vertically (directly from parent to offspring) or horizontally (from individual to unrelated individual). Vertically transmitted fungal endophytes are asexual and transmit via fungal hyphae penetrating the host's seeds. (*e.g. Neothyphodium*). Since their reproductive fitness is intimately tied to that of their host plant, these fungi are often mutualistic. Conversely, horizontally transmitted fungal endophyts are sexual and transmit via spores that can be spread by wind or insect vectors. Since they spread in a similar way to pathogens, horizontally transmitted endophytes are often closely related to pathogenic fungi (Carroll, 1988).

Endophytes from mycelia (growth structures) grow between the cells of a plant, mainly in the leaf sheaths and reproductive structures. As seed production initiates, the endophytes grow upward in the plant and when the seed forms, the endophytes infects the outer layers of the seed. This is how the endophyte transfers

from a plant to a seed in the field. Hence, endophyte infected turf grasses were born. When the seeds germinated and grew, the endophytes are injected in the new plants (Morris, 2001).

The highest concentration of endophyte occurred in the crown, stems and leaves, while low amounts existed in the roots. This made sense because there was little resistance to insects that feed on roots. Further, Funk (1980) discovered that endophytes could transfer from one plant to another when using conventional plant breeding technique.

Evolution of Endophytes

Acquired chemical defense appears to be a common basis for endophytic association between plants and the fungi, but many higher plants themselves produce potent and multifarious chemical defenses (Bell, 1981; Bailey and Mansfield, 1982; Kuc and Rush, 1985; Harborne 1986).

Taxonomic evidence further supports a close relationship between endophyte and pathogen. Frequently endophytes are sister species to virulent pathogens on the same or closely related hosts. Thus, *Acremonium coenophialum*, an important grass endophyte, is very closely related to *Epichloetyphina*, a pathogen (Clay, 1988).

Taxonomically, endophytes are with few exceptions members of Ascomycetes, but within the Ascomycetes they belong to diverse group *i.e.* Loculoascomycetes, Discomycetes and Pyrenomycetes (Petrini, 1986).

Biology and Ecological roles

Many of the fungi commonly reported as endophytes are regarded as minor or secondary pathogens by forest pathologists. Their common occurrences in both healthy and diseased tissues underscore the uncertainty of boundaries separating endophytes, facultative pathogens, and latent pathogens. Indeed, the behavioral

differences between many fungi considered as “endophytic” and those considered to be “latent pathogens” are slight and simply may reflect differences in the duration of the latent or quiescent phase and the degree of injury sustained by the host during active growth of the fungus (Williamson, 1994).

Fungi described as “endophytic” characteristically exhibit a prolonged, inconspicuous period in which growth and colonization cease temporarily, resuming after a physical, or maturational, change in the host. This episodic growth is a defining feature of endophytes, whether they ultimately are considered commensal saprobes, latent pathogens, or protective mutualists (Jeffrey *et al.,* 2004).

The ecological roles played by endophytic fungi are diverse and varied (Saikkonen *et al.,* 1998). Endophytes offer benefits to their host’s fitness, such as preventing pathogenic infection and possibly making leaves less palatable to herbivores. At the same time the leaves offer a substrate for the fungi to grow upon and provide food substances for their growth and reproduction. Although diverse endophyte produce toxins in culture, such compounds have been difficult to detect in plant host tissue.

One of the natural products obtained from endophytic microbes is antibiotics. It is defined as low molecular weight organic natural products made by a microorganism that is active at low concentration against other microorganisms. *Pestalotiopsis microspora* is common rainforest endophyte, possesses enormous biochemical diversity. Many secondary metabolites produced by a myriad of strains of this widely dispersed fungus. One such secondary metabolite is ambuic acid, an antifungal agent which has been recently described from several isolates of *P. microspora* (Li *et al,.* 2001).

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp. represents the first cytochalasin type compound with a three ring system replacing the cytocalisin macrolide ring. This metabolite mainly exhibits antibacterial activity in disk diffusion assays against *Bacillus subtilis*, *Salmonella enterica* and *Staphylococcus aureus* (Horn *et al.*, 1995). An endophyte *Fusarium* sp. from the plant *Selaginella pallescens* was screened for its antifungal activity (Brady and Clardy, 2000). Colletotric acid, a metabolite of *Collectotricum gloeosporioides*, an endophytic fungus in *Artemisia mongolica* displayed antimicrobial activity against bacteria as well as agaist the fungus *Helminthosporium sativum* (Zou *et al.*, 2000).

The array of alkaloids and other chemicals synthesized by the endophytes endow the plant with more resistance to nematodes, insect herbivores and livestock (Schardl *et al.*, 2004). Therefore, there is a need to investigate the physiology and phylogenetic relationships of the fungus to other fungal endophytes producing bioactive metabolite to optimize their characteristics.

Work has been done on the occurrence of endophytic fungi of mango from Gujarat. The practical work undertaken for the thesis entitled **"Studies on leaf infecting and Endophytic fungi of *M. indica*"** includes:

OBJECTIVES

1. To study the leaf infecting fungi of different varieties of mango
2. To study the endophytic fungi present in mango leaf, stem and bark tissues
3. To study the aeromycoflora in mango orchards
4. To do the histological study of mango leaves
5. To study the effect of fungi on chemicals present in leaves
6. To do the physiological studies for endophytic fungi
7. To study biocontrol of pathogenic fungi

Literature Survey

II. Literature Survey

Disease is an interaction among the host, parasite and environment. Human being, plants and animals are affected by a large number of microorganisms which leads into sickness, malformation and death. Disease resistance is the capacity of an organism to prevent, restrict or retard disease development and occurs at high moderate or low level. All plants have resistance to some pathogen under certain condition (Stakman and Harrar, 1957).

India, being geographically subtropical country with warm and humid climate, provides suitable conditions for development and spread of a number of plant diseases. Many of diseases are caused by different groups of fungi.

Cause of Postharvest Losses (Parasitic, Non Parasitic and Physical)

Parasitic: These are of microbiological origin and may begin at any time during the ontogeny, or after maturity of the product (Latent and Non-latent).

Non Parasitic: These disorders are due to the normal and abnormal physical functions of the host in the absence of infection.

Physical: These are injuries to fresh fruits that occur during cultivation, harvesting and marketing. The most common injuries include mechanical damage, bruising, crusting, cuts, abrasions, etc.

The Infection Process: Knowledge of the time and mode of infection is essential for the development of an effective programme for disease control. Fruits attached to the plant may be infected by direct penetration of a pathogenic fungus through the intact cuticle, by wounds, or by natural openings on the surface of the host. Furthermore,

many postharvest diseases are initiated through injuries to the produce during and subsequent to harvest, such as cut stems and mechanical damage to the surface cells in the courses of handling and transportation. The important postharvest factors responsible for diseases are weather, plant nutrition, cultural practices and chemical sprays that may influence the severity of decay during storage (Prakash, 2009).

In the first type infection remain distinct and separate, the infected hosts are somewhat randomly distributed in the container *viz.* stem end rot. The pathogen spread to neighbouring hosts units in the container *viz.* nesting of *Sclerotinia* sp., *Botrytis* sp., *Rhizopus* sp., *Penicillium* sp., *Aspergillus* sp. Pathogens that contact the fruit in the field may occur as microscopic surface contaminants on the fruit skin or in soil and debris on the skin and stem tissues, or they may be present as symptomless infections within the skin or stem-end of the fruit. Air, water, insects animal vectors, animal excreta, etc. can transmit the inoculum.

Infection and Pathogenesis: Host infection may occur with or without formation of appressorium via direct penetration of the cuticle, or entry through stomata, lenticels, wounds or abscission scar tissues.

Stem-End Infection: Endophytic colonization of the inflorescence is an important mode of infection for mango due to *Dothiorella dominicana*. Colonization by stem-end rot fungi (*L. theobromae* and *Dothiorella dominicana*) of the peduncle or pedicel of fruit is restricted by wound periderm and the cuticle. The only ways of natural infection of the uninjured fruits by spores of *L. theobromae* were the exposed surface of the pedicel or pedicel scar. The pathogen could not invade through the intact rind or the pedicel unless these were injured, even though the fruits had fully ripened (Prakash, 2009).

L. theobromae invaded the mango pedicel through wounds on detached mature green and ripe fruit with invasion of fruit occurring without any apparent quiescent stage but found that the pathogen also penetrated the cuticle directly (Prakash, 1996). *Alternaria* infection of mango fruits started soon after fruit set, and because the fruit growth period is relatively short, continuous protectant treatments were necessary to reduce the latent infection on the surface significantly.

Molecular Aspects of Infection: Little is known about the molecular trigger that includes the germ tube to differentiate of an appressorium. It has been shown that excess nutrients over-ride it and protein synthesis is not required once germination has occurred followed protein synthesis during conidium germination and appressorium formation on cellulose membranes. A specific polypeptide (95 kDa) was synthesized only when appressoria matured (a period lasting from 12-24 h after germination). This molecule is involved in the synthesis of the melanin precursor, Scytalone.

In the broadest sense, the term endophyte applied to fungi (or bacteria) which live within plant tissues for all or part of their life cycle and cause no apparent infection (Hirsch and Braun, 1992). This definition excludes the mycorrhizal fungi but does not imply that endophytic fungi are not cultivable on artificial media and includes virtually the entire spectrum of symbiotic interaction in which fungi and plants participate: parasitism, commensalism, and mutualism.

During 1940s these fungi were first noticed, but only at the turn of the 21st century the ubiquity of these fungi is recognized. Endophytes can colonize virtually hundred percent of the host population, but many infect a far smaller fraction, while some are rare (Caroll and Caroll, 1978; Petrini, 1986). Stone *et al*., (1994) considered that endophytes have common attributes. First, they are internal, at least subcuticular

and have contact with and derive nutrition from the living host tissue. Second, they establish at least a transitory biotrophic nutritional relationship with their host. Third, they remain symptomless *i.e.* disease free during life time. Endophytes are also thought to be latent pathogens (Brown *et al*., 1998). Asymptomatic endophytes have diverse relationship with their host plants and may only produce disease symptoms under certain conditions.

Pathogenic fungi capable of symptomless occupation of their hosts during a portion of the infection cycle, quiescent infections (Williamsons, 1994) and strains with impaired virulence can be considered endophytes (Schardl *et al.*, 1991, 1994; Fisher *et al.*, 1992; Fisher and Petrini, 1992; Freeman and Rodriguez, 1993) as can a variety of commensal, saprobic and mutualistic fungi that have cryptic, non-apparent patterns of host colonization.

All available evidence suggests that endophytes have evolved directly from plant pathogenic fungi. Apparently innocuous endophytes may cause pathogenic symptoms when the host is stressed (Millar, 1980; Andrews *et al.*, 1985) and many endophytes on coniferous hosts show the limited substrate use of parasitic fungi (Carroll and Petrini, 1983; Stone, 1986). Hawksworth (1991) estimated there may be as many as 1.5 million different fungal species, yet only about 75,000 have been described. As more evidence accumulates, estimates keep rising as to the actual number of fungal species. For instance, Dreyfuss and Chapela (1994) estimate there may be at least 1 million species of endophytic fungi alone. Based on the results of the leaves of two distantly related under storey tree species of *Heisteria concinna* (Olacaceae) and *Ouratea lucens* (Ochnaceae) at Barro in Colorado island, in Panama. Arnold *et al.* (2000) suggested that tropical forests are hyperdiverse and they recovered 3000 fungal strains representing more than 418 morpho-species.

In agricultural situations the distinction between latent pathogen and endophyte becomes fuzzy (Nathaniels and Taylor, 1983; Kulik, 1984). Pathogens of crop may exist endophytically in weeds growing in the same fields (Hepperly *et al.*, 1985). If the transition from pathogen to endophyte is easy, modern agricultural practices should encourage pathogenic facets of latent fungal infection. Where pests are controlled chemically, endophyte- tolerant hosts would derive no benefit from the association, and the accommodation between fungus and plant would be disrupted as natural selection favoured endophyte- in tolerant hosts.

In India, various facets of endophytic fungal diversity and biology are being investigated by Suryanarayanan *et al*., (2003). Suryanarayanan *et al.,* (2002) studied tropical forests in the Nilgiri Biosphere Reserve of the Western Ghats for endophyte assemblages based on hosts recurrence and spatial heterogeneity of their endophyte and concluded that the dry tropical forests had much less endophytic diversity compared to wet tropical forests. Recent studies have demonstrated that fungal endophytes are neither passive residents nor a mere assemblage of latent pathogens of their hosts (Sparrow, 1960; Ganley *et al.,* 2004). They possibly represent a storehouse of new species of fungi, especially in the tropics (Rodrigues and Samuels, 1990; Jacob and Bhatt, 2000). Furthermore, molecular evidence shows that certain fungi, such as *Phyllosticta capitalensis* and *Colletotrichum* spp., have a very wide host and geographical range. For example, *P. capitalensis* occurs as an endophyte in countries like South Africa, Japan, Thailand, India and Brazil, which suggests that this fungus could have been described several times as different species, especially since species name in case of the genus *Phyllosticta* is almost invariably based on the host from which it is isolated (Baayen *et al.,* 2002; Okane *et al*., 2003; Pandey *et al*., 2003; Rodrigues *et al.,* 2004).

Different medicinal plants were screened to isolate fungal endophytes in Western Ghats of India by Raviraja (2005), and Raviraja *et al.,* (2006). Fungi like *Curvularia* and *Fusarium* were found in maximum number of cases among 18 different endophytic fungal isolates. Tejesvi *et al*., (2006) studied the fungal diversity of some medicinal trees of southern India. They have isolated 48 fungal species from bark samples of *Terminalia arjuna, T. chebula, Crataeva magna, Azadirachta indica, Hollarrhena antidysenterica* and *Butea monosperma.* Ananda and Sridhar (2002) studied endophytic fungal diversity of mangrove species in west coast of India. and reported 35 fungal species including 4 sterile ones. They have reported highest species richness in *Rhizophora mucronata*. Endophyte assemblage was also reported in another mangrove plant *Rhizophora apiculata* by Kumaresan and Suryanarayanan (2002). Rajgopal and Suryanarayanan (2000) isolated 963 fungi from the bark samples of 10 tropical tree species in southern India. Kharwar *et al.,* (2008) studied endophytic fungal complex of *Catharanthus roseus*. They have isolated 183 endophytic fungi representing 13 fungal taxa from leaf, stem and root tissues of *C. roseus* from two sites representing two different ecosystems in north India and suggested that the endophytes are both host and tissue specific. They also concluded that despite ecological variations, there were little differences in the species richness of fungal endophytes.

Endophytes are increasingly recognized as important mediators of interactions between plants and their competitors, seed dispersers, herbivores, and pathogens (Carroll, 1988; Clay, 1988; Chapela, 1989). They have been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities (Bills and Polishook, 1991). One example of this is the *Claviceps* species,

which are themselves responsible for the production of ergot alkaloids. These fungi produce alkaloids in pure culture (Clay, 1988).

Endophytes have been described as mutualists that protect both grasses (Clay, 1990) and conifers (Carroll, 1991) against insect herbivory, and many of those fungi produce biologically active secondary metabolites (Fisher *et al*., 1984a; Polishook *et al.,* 1993; Pelaez *et al.,* 1998). Fisher *et al.,* (1984b) reported antibacterial or antifungal activity for more than 30% of the endophytic isolates from Ericaceous plants. Dreyfuss (1986) reported antibiotic activity from isolates of the endophyitc *Pleurophomopsis* species and *Cruptosporiopsis* species, as well as from a sterile endophyte from *Abies alba*. Endophytic species of the Xylariaceae frequently produce compounds with high biological activity, including cytochalasins (Dreyfuss, 1986; Brunner and Petrini, 1992) and indole diterpenes (Hensens *et al.,* 1999).

Non grass endophyte produce antifungal (Pelaez *et al*., 2000) or antibacterial substances, as well as insecticidal compounds (Johnson and Whitney, 1992; Hensens *et al.,* 1999). Many of those compounds are produced intra-cellularly and although the substances may have survival value for the endophyte (*e.g.* through interference competition), their general role (if any) in protection of living hosts has not yet been determined (Saikkonen *et al.,* 1998).

Bills *et al*., (2002) described a metabolic distinction between tropical and temperate endophytes through statistical data which compared the number of bioactive natural products isolated from endophytes of temperate origin. Not only did they find that tropical endophytes provided more active natural products than temperate endophytes.

The importance of chemical heterogeneity among plant populations as a defence against herbivorous insects has been repeatedly stressed in the recent literature (Berenbaum, 1981; Denno and McClure, 1983; Strong *et al.,* 1984; Berenbaum *et al.,* 1986). Such heterogeneity seems to be highly heritable (Berenbaun *et al*., 1986) and consequently must arise through the process of meiosis and somatic mutation. Trees such as Douglas–fir may live 1000 years in tracts with favourable soil that escapes fires; even shorter lived trees may easily live 100 years. During a tree's lifespan its genotype is fixed, with little variation possible in its particular mix of allelochemicals and other defences. Conversely, univoltine insects go through a single generation each year, and many insects and pathogens achieve multiple generations annually. In such situations one might expect an insect or pathogen to specialize in overcoming the defences of a single host individual or clone. Edmunds and Alstad (1978) have demonstrated such specialization for a scale insect on pine. Hosts might accommodate different endophyte species in response to challenges from different herbivorous insects.

Another fascinating use of antibiotic products from endophytic fungi is the inhibition of viruses. *Muscoder albus* is a newly described endophytic fungus obtained from small limbs of *Cinnamomun zeylanicum* (Worapong *et al.,* 2001). This Xylariaceaous (non-spore producing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds (Strobel *et al.,* 2001). One of the most commonly isolated endophytes of the world's yews is *Peslatotiopsis microspora,* which produce paclitaxel and its derivatives. Paclitaxel is a highly functionalized diterpenoid, represent the first major group of anticancer agents and found in each of world's Yews (*Taxus wallichiana*) (Suffness, 1995). Strobel *et al.,* (1997) isolated *Peslatotiopsis guepini* from the extremely rare, and previously

thought to be extinct, Wollemi pine (*Wollemia nobilis*) exist in South America and Australia, which was shown to produce paclitaxel. Several other endophytes are known to have anti-insect properties. Nodulisporic acids, a novel indole diterpens, exhibit potent insecticidal properties against the larvae of blowfly. The first nodulisporic compounds were isolated from an endophyte, a *Nodulisporium* sp., from the plant *Bontia daphnoides* (Demain, 2000).

Table II.1-List of some fungal endophytes isolated from tropical and subtropical trees

Endophytic Fungi	**Source Plant**	**References**
Acremonium acremonium	*Crataeva magna, Azadirachta indica, Hollarrhena antidysenterica, Butea monosperma*	Tejesvi *et al.*, 2006
Acremonium strictum, Asteromella andrewsii, Calcarisporium arbuscula	*Butea monosperma*	Tejesvi *et al.*, 2006
Alternaria alternata, Aspergillus fumigatus, A. niger, Cortnespora sp., *Drechslera ellisii, Emericella* sp., *Pestalotia macrotricha, Rhizoctonia* sp., *Stenella agalis*	*Aegle marmelos*	Gond *et al.*, 2007
Aspergillus versicolor	*Coffea arabica*	Sette *et al.*, 2006
Botryodiplodia theobromae	*Terminalia arjuna, H. antidysenterica,*	Tejesvi *et al*., 2006
Calcarisporium arbuscula	*Butea monosperma*	Tejesvi *et al*., 2006
Chaetomium globosum	*T. arjuna, H. antidysenterica,C. magna, Vitex negundo, A. marmelos*	Tejesvi *et al.*, 2006, Raviraja, 2005; Raviraja *et al.*, 2006; Gond *et al.*, 2007
Chloridium sp., *Cladosporium* sp., *Cochlonema* sp.	*T. arjuna*	Tejesvi *et al.*, 2005, 2006
Cladosporium cladosporioides	*T. arjuna, A. marmelos, C. arabica, A. indica,*	Tejesvi *et al.*, 2006, Sette *et al.*, 2006; Gond *et al.*, 2007
Colletotrichum sp.	*Theobroma cacao*	Arnold *et al.*, 2003

Fusarium chlamydosporum	*Acanthus ilicifolius, Rhizophora mucronata, C. magna, A. indica*	Ananda and Sridhar, 2002; Tejesvi *et al.*, 2006
Fusarium verticilloides	*H. antidysenterica , C. magna, A. indica*	Tejesvi *et al.*, 2006
Gliocladium delequescens	*T. arjuna, C. magna*	
Humicola fuscoatra Myrothecium verrucaria, M. cinctum	*H. antidysenterica , T. arjuna*	Tejesvi *et al.*, 2006
Memnoniella sp., *Monocillium* sp., *Phialophora* spp., *Tubercularia vulgaris*	*T. arjuna*	Tejesvi *et al.*, 2006
Stemphylium sp.	*T. arjuna,*	Tejesvi *et al.*, 2006,
Phyllosticta sp.	*A. indica*	Tejesvi *et al.*, 2006
Petriella sordida	*A. officinalis*	Ananda and Sridhar, 2002
Nigrospora oryzae	*T. arjuna , A. indica C. tomentosa, A. marmelos, Bauhinia phoenicea*	Tejesvi *et al.*, 2005, 2006
Trichoderma harzianum	*T. arjuna , H. antidysenterica , C. magna, C. arabica*	Tejesvi *et al.*, 2006, Sette *et al.*, 2006
Trichoderma sp.	*C. robusta, V.negundo, Ocimum sanctum, T. arjuna , A. indica, A. marmelos*	Sette *et al.*, 2006, Banerjee *et al.*, 2006, 2009, Tejesvi *et al.*, 2006
Trichophyton sp.	*V.negundo*	Banerjee *et al.*, 2006
Verticillium sp.	*C. magna , A. indica, A. marmelos*	Gond *et al.*, 2007, Tejesvi *et al.*, 2006
Verticillium albo-atrum	*A. indica*	Tejesvi *et al.*, 2006
Zygosporium masonii	*A. officinalis, A. ilicifolius, R. mucronata,*	Ananda and Sridhar, 2002

Materials and Methods

III. MATERIALS AND METHODS

SURVEY

Survey of various study sites, orchards/ fields and different localities in and around Vadodara district were carried out for the collection of diseased as well as healthy, symptomless leaves of Mango plants. Survey was carried out during the year 2006 to 2008.

PREPARATION OF CULTURE MEDIUM

Potato Dextrose Agar (PDA) medium

Potato (Peeled)	200g
Dextrose	20g
Agar	20g
Distilled Water	1000ml

Two hundred grams of peeled potatoes were sliced into small pieces in 500ml of distilled water and boiled for about 25 min. The broth obtained was then filtered through cheese cloth and the solution was made up to volume of 500 ml. To this 20 g of dextrose was added. Simultaneously 20g of agar was melted in 500 ml of distilled water by heating on a hot plate. The potato broth was poured into agar solution and restored to final volume of 1000 ml with distilled water. The medium was then sterilized after adjusting the pH to 6.0

Only Borosil glasswares and Qualigens and SRL pure reagents (Analytical grade) were used throughout the investigation. Glassware were cleaned first with "Surf" a detergent, after that they were rinsed with dilute chromic acid and finally with distilled water.

Lactophenol- Cotton Blue –Stain for Fungi

Phenol- 20 g

Lactic acid- 20 ml

Glycerol- 40 ml

Distilled water 20ml and then 5 ml of 1% aqueous cotton blue solution was added.

Staining Technique

Some of the fungal mycelium was placed on a slide and stained with cotton blue. Excess stain was removed by lactophenol and a coverslip was placed on the slide and observed under the microscope.

PLANT SAMPLE COLLECTION

Methods for studying patterns of infection and colonization by endophytic fungi are essentially the same as those used in the study of fungal plant pathogens (Stone *et al.,* 1994). For isolation of endophytic fungi mature, healthy, green, asymptomatic plant leaves, stem and bark were collected by sampling trees of *M. indica* growing at different locations of Vadodara district (Gujarat). Infected and diseased leaves of mango plants for the isolation of pathogenic fungi were also collected from different places of the city. It is important that samples be handled carefully and processed as quickly as possible following collection, usually within 48 h. Samples should be air dried to remove any surface moisture before transport and storage. Samples were tagged and labeled. All samples were immediately placed in separate polythene bags, brought to the laboratory and processed.

ISOLATION AND CULTURE OF ENDOPHYTES

The method most commonly used to detect and quantify endophytic fungi is isolation from surface- sterilized host tissue. All the samples were washed thoroughly in running tap water for 10 min. to remove the debris adhered and finally washed with

double distilled water to minimize the microbial load from sample surface. The samples were cut into small pieces. Sample pieces were surface sterilized by sequential washes in 70% (v/v), ethanol (1min) and 3.5 % (v/v) NaOCl (2 min), rinsed with sterile water and allowed to surface dry under sterile conditions. After successive washing the samples were plated on Petri dishes containing potato dextrose agar (PDA) medium supplemented with streptomycin (250mg/L) and incubated for 20 days at 25± 2ºC in BOD incubator. Samples were observed for fungal growth at 2 days intervals for 20 days. Actively growing fungal tips immerging from plant tissues were then subculture on PDA slants.

ISOLATION OF PATHOGENIC FUNGI

The diseased and infected leaves were washed in running tap water. Small pieces (2 x 4mm) were cut and placed in Petri dish containing 0.1% $HgCl_2$ solution. After two minutes these pieces were transferred in Petri dish containing sterile distilled water. After two successive washing in sterile distilled water these pieces were then inoculated in Petri- dish containing PDA. The whole operation was performed in horizontal laminar flow (under aseptic condition). The observation was made everyday and growth of fungi if any was recorded after 7 days.

IDENTIFICATION OF ISOLATED FUNGAL CULTURES

The isolated fungi were identified according to their macro and microscopic structures. The temporary slides of isolated pure fungal cultures were prepared in lactophenol – cotton blue stain for identification purpose. For final confirmation the fungal cultures were also sent to Dr. P. N. Chaudhari's laboratory, at New Delhi and Agharkar Research Institute, Pune.

DATA ANALYSIS

The colonization frequency (%CF) of each isolated fungus was calculated using the formula given by Hata and Futai (1995).

$$\%CF = (Ncol/ Nt) \times 100$$

Where, Ncol= Number of segments colonized by each fungus

Nt = Total number of segments studied

STUDY ON AEROMYCOFLORA

The atmosphere contains all the major groups of microbes. Outdoors air, with changing season of the year, contains a variable of fungal spores. The presence of "air- spora" a term used by Gregory (1961), for the population of air borne particles of plant or animal origin may pose great health hazards to living beings. Fungal spores are an important component of the air spora.

Requirements

For isolation of aeromycoflora the settling plate method was used (Pelczar *et al.*, 1986). In this technique, a Petri dish containing a sterilized Potato Dextrose Agar (PDA) medium was used to trap the fungal species. The petriplates containing PDA were horizontally exposed at a height of 1m at each study areas for 5-10 min. After exposure, these plates were incubated at 25 ± 2ºC for seven days. Number and type of the individual colonies developed on agar plates were counted. Each colony represented a particle or a microorganism which has fallen on the agar surface and the fungi were identified after subculturing them.

Percentage abundance of each fungal isolate was calculated as follows:

$$\% \text{ of abundance} = \frac{\text{Total number of colonies of a particular fungal species in all the replicates}}{\text{Total number of colonies of all the replicates}} \times 100$$

STUDY OF ANTAGONISTIC ACTIVITY OF ENDOPHYTES

The antagonistic activity of endophytic fungi against three fungal pathogenic species *i.e. Gloeosporium mangiferae* Henn., *Fusarium moniliformae var. subglutinans* and *Lasiodiplodia theobrome* (Pat.) Griffon & Moube was studied. Three endophytic fungi, *viz. Robillarda sessalis* Sacc. and *Phomopsis mangiferae* S. Ahmad were isolated from the healthy leaves of mango and *Pestalotiopsis guepinii* (Desm.) Steyaert from barks. The dual culture method was used in order to investigate whether endophytic fungi have any antagonistic activity against these pathogenic fungi. Mycelium discs (diameter, 5 mm) of 4-day-old fungal culture were placed in a potato dextrose agar (PDA) plate. Each of the three endophytic fungi was inoculated at the center of each PDA plate. The plates were incubated at 25±2°C for 5 days.

The percentage of inhibition was calculated from the following equation-

$$\text{Inhibition (\%)} = \frac{\text{growth diameter in the control sample} - \text{growth diameter in the sample with treated endophytes}}{\text{growth diameter in the control sample.}} \times 100$$

The experiment was repeated twice in five replicates.

HISTOLOGICAL STUDY

Collection and Fixation

Diseased leaves were excised from the trees and immediately fixed in formalin -acetic acid –alcohol (FAA), one of the common killing agents. Killing means sudden and permanent termination of life processes.

FAA- 5 ml formalin +5 ml glacial acetic acid + 90 ml of 50% alcohol

Fixing

Keep in FAA for 48 h and in 50% alcohol for 18 h. After fixation, sections were passed in different % of TBA and alcohol.

10 % TBA = 10 ml TBA + 40 ml Ethanol + 50 ml D. W.

20 % TBA = 20 ml TBA + 50 ml Ethanol + 30 ml D. W.

35 % TBA = 35 ml TBA + 50 ml Ethanol + 15 ml D. W.

55 % TBA = 55 ml TBA + 45 ml Ethanol

75 % TBA = 75 ml TBA + 25 ml Ethanol

100 % TBA = 100 ml TBA

After 24 h of fixation, the leaf samples were cut into small pieces and preserved in 70% alcohols till further processing. The fixed samples of leaves were embedded in paraffin and sectioned at 8-10µm thickness in transverse plane using Leica rotator microtome. Ordinary parawax was used for infiltration and for embedding the plant material.

Adhesives- These are fluid used for affixing paraffin section of the slide.

Haupt's Adhesive- Dissolve 1 g gelatin in 100 cc distilled water at 30ºC when it was completely dissolved add 2 g phenol crystals and 15 ml glycerine. Stir it well and filter. Add 3-4 % of aqueous formalin.

Place a drop of adhesive, smear it evenly on slide, and immediately add formalin with pipette. Place section on formalin. Put the slide on warming plate at 40 to 42º C until the section has straightened out. Formalin helps in coagulating gelatin of adhesive.

The sections were stained with toluidine blue and mounted in DPX (Berlyn and Miksche, 1976).

PATHOGENICITY TEST (Koch's Postulates)

Healthy leaves and fruits of the plant were inoculated with the spore suspension or the fungal cultures of the test fungi after surface sterilization with 70% ethyl alcohol. The inoculation was done without making injury and with injury to ensure the entry of pathogen. Plant parts were monitored for the development of

disease symptoms and pathogens were reisolated from the leaves and fruits of test plants after seven days to confirm the pathogenicity according to Koch's postulates.

PHYSIOLOGICAL STUDIES

To find out the suitable media for growth of the 3 organisms, 25 ml of the six different composition of liquid medium was taken in 150 ml cleaned and oven dried Erlenmeyer's conical flasks. The culture media was autoclaved at 15 lbs. p.s.i. for 15 min. whenever, the medium contained complex substances liable to decomposition or denaturalization, fractional sterilization was done which involved exposure of conical flasks containing medium to steam for 30 min on three successive days.

Inoculation was carried out under aseptic condition in Horizontal Laminar Flow. Ten to twelve days old cultures were used for inoculating the flasks containing different media with the help of inoculating needle. Inoculated flasks were incubated at 25 ± 2ºC for 15 days. At the end of incubation period, change in pH of the medium and degree of sporulation of the organisms were recorded. The degree of sporulation was classified into four categories viz. Nil, poor, good and excellent designated as N, P, G and E respectively on the basis of visual and microscopic observations.

In order to assess the growth of organisms, their fungal mats were harvested at the end of incubation period on previously dried and weighed Whatman filter paper No. 1 and washed several times with distilled water. The filter papers were again dried in an electric oven at 60 ºC for 72 h. Dried mats with filter papers were transferred to a desiccator for at least 24 h to bring down to room temperature and finally weighed. The difference between final and initial weights of the filter papers indicated the dry weight of the fungal mat. Dry weight of the mycelial mat and degree of sporulation were considered as a measure of response of the organisms to different treatments. Each set of the treatment was run in triplicate. There is no noticeable

difference among replicates and hence only the average values of three replicates have been recorded and used as quantitative measure for comparing the growth. The change in pH of culture filtrate after incubation period was also recorded.

The dry weight results were statistically analyzed and standard error (S. E.) was calculated by the formula:

$$\textbf{Standard Error (S.E)} = \frac{\sqrt{\textbf{mean square of the error}}}{\textbf{No. of replicates}}$$

Critical Difference was calculated by the formula

C.D = S.E x t x √2,

Where‘t’ represent probability at 5 % level.

Dry weight of the mycelial mats was graded into good, less, very less and poor. The dry weights higher or lower of the moderates have been designated as good or poor respectively except for some preliminary studies dealing with the effect of certain media on growth and sporulation the initial pH was adjusted in subsequent studies by adding adequate quantities of N/10 HCl or NaOH soln. The pH of the medium or filtrate was determined with the help of pH meter.

Utilization of different mono-, oligo- and poly- saccharides was studied. Solutions containing mono-saccharides were autoclaved at 15 p.s.i. for 15 min. while fractional sterilization was carried out for di- and polysaccharides. These solutions were inoculated with test endophytic fungi at a fixed time for 15 days; so that, 1 to 15 days old cultures could be available on 16^{th} day. Dry weights of mycelial mat and pH of the medium were recorded after incubation period of 5, 10 and 15 days to determine the growth and filtrates were chromatographically analyzed every alternate day to detect the presence of various sugars during their utilization.

For chromatographic analysis of sugars Whatman No. 1 paper was used. Depending on the nature of development ascending chromatography is done. The size

of chromatography papers were 20 cm wide and 25 cm long. Drops of known volume (0.005 ml) were taken from the filtrates every alternate day and were placed at about 2 cm height from the base on the chromatograms by a micropipette at a position located for this purpose.

Solvent system-

The running solvent used was BAW (n-butanol- acetic acid- water 4: 1: 5 v\v) Ranjan *et al.,* 1955.

The upper organic layer was used as the running solvent; the lower aqueous layer was used to saturate the chromatographic chambers. The paper chromatograms were run in BAW for about 7 h. When the run is over, the papers were taken out. After drying at room temperature, chromatograms were developed by dipping it in spray reagent. The sprayed paper was then heated in an electric oven at 110º C for 5-10 min. to develop the bands.

Spray Reagent (Aniline Hydrogen Phthalate)

100 ml n-butanol is saturated with water by slow addition of small aliquots and shaking. Approximately 10 ml of water goes into butanol. To 100 ml of this solution 0.91 ml (9.30 mg) of aniline and 1.6 g phthalic acid was added and stirred well.

The position of a spot in a chromatogram is recorded as its Rf value. The Rf values were calculated by the following formula:

$$Rf = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$$

The presence of starch was determined with the help of potassium iodide solution (Ghosh and Tandon, 1965).

CHEMICAL ANALYSIS

Healthy, green and diseased leaves of four varieties, Rajapuri, Totapari, Kesar and Desi of mango were taken and thoroughly washed in tap water followed by distilled water and dried in an oven at 60 ºC. The dried samples were powdered and stored in air tight glass bottles or plastic bags. The method followed for the extraction and characterization of compounds are given below.

FLAVONOIDS

For extraction, isolation and identification of flavonoids the following procedure was followed: 10g powder of leaves was extracted in a soxhlet extractor with methanol for 48h till the plant material became colourless. The Methanolic extract was concentrated to dryness in a waterbath. 50ml of water was added to dry residue and the water soluble phenolic glycosided were filtered out. The filtrate was hydrolysed in a waterbath for one hour using 7% HCl. This hydrolysate was extracted with diethyle ether/ solvent ether, whereby the aglycones got separated into ether fraction (Fraction A). The remaining aqueous fraction was further hydrolysed for ten hours to ensure the complete hydrolysis of all the o-glycosides. Aglycones were once again extracted into diethyl ether (Fraction B) and residual aqueous fraction was neutralized and evaporated for the analysis of glycoflavones.

Ether fraction A and B were combined and analyzed for aglycones using standard procedures (Mabry *et al.*, 1970; Markham, 1982; Harborne, 1984). The combined concentrated extract was banded on Whatman No. 1 paper. The solvent system employed was Forestal (Con. HCl: Acetic acid: Water, 3:30:10) or 30% glacial acetic acid. The developed chromatograms were dried in air and the visibly colored compounds were marked out. The papers were observed under ultraviolet light (360nm) and the bands were noted. Duplicate chromatograms were then sprayed

with 10% aqueous $Na_2 CO_3$ and 1% $FeCl_3$ and the color changes were recorded. The bands of compounds were cut out from unsprayed chromatograms and were eluted with spectroscopic grade methanol. The UV absorption spectra of theses compounds were recorded in methanol using 'Shimadzu UV 240' spectrophotometer.

The aqueous fraction remaining after the separation of aglycones was neutralized by the addition of anhydrous Na_2CO_3 and concentrated to dryness. To the dry residue, methanol was added and then filtered. The Methanolic extract was banded on Whatman No. 1 paper and the Chromatogram was developed with water as the solvent system. Glycoflavones were visualized by their colour in UV and with 10% Na_2CO_3 spray. Further analysis and identification were done using spectroscopic method as explained before.

POISONED FOOD TECHNIQUE

This technique plays a significant role in bioassay methods to evaluate the bio-efficacy of the compound. This involves identifying a synthetic nutrient medium in which the pathogen grows, incorporating the target compound in to the medium, inoculating the test organism, and incubating for the required time. The mycelial growth of the fungus is estimated by measuring the diameter of the radial growth of the mycelial tips. A comparison with the control in which only the nutrient medium is taken indicates the bio-efficacy of the compound. If there is no growth, then the compound is said to be a poison (Nene and Thapliyal, 1979). The details of the experiment are given below.

Aqueous leaf extracts of ten different medicinal plants viz. *Tylophora indica* (Burm f.) Merrill, *Rauwolfia tetraphylla* L., *Withania somnifera* (L.) Dunal, *Strychnos nux vomica* L., *Catharanthus roseus* (L.) G. Don., *Calotropis procera* (Aiton) W.T. Aiton, *Ocimum sanctum* L., *Aloe vera* (L.) Burm.f. *Melia azadirachta* L., and

Adhatoda vesica L. were evaluated for their antifungal activity against *F. moniliformae* var. *subglutinans, G. mangiferae,* and *L. theobromae.*

For this 100g of the leaf samples was taken, washed dried and crushed with distilled water and then filtered and made 100 ml crude extract. Now 2ml and 10 ml aqueous leaf extract of these medicinal plants were added to the molten Potato dextrose Agar (PDA) medium to prepare 2% and 10% conc. of extract and the other without extract which served as control. The pathogen of interest was used from the growing tips (punched in fungal mat grown on PDA medium in sterile Petri plates) was placed at the center and allowed to grow, and all plates were incubated at room temperature. Radial growth in terms of diameter (mm) was examined and readings were taken at an interval of 7 days and 10 days respectively. Five different plates were used as replicates and the entire experiment was repeated three times.

Results and Discussion

A. Pathological Studies

1. Survey of Orchards

Survey of various study sites, orchards/ fields and different localities in and around Vadodara district were carried out. Collection of diseased as well as healthy, symptomless leaves of mango plants was done. Survey was carried out during the year 2006 to 2008. Vadodara district is located at 22°18′N 73°11′E22.30°N 73.19°E in western parts of India, Gujarat, at an elevation of 39 metres. It is the 3rd biggest in Gujarat and 18th largest city in India with an area of 148.95 km². The results of Fungi isolated is recorded in Table 2.1 and 3.1.

Study sites were broadly classified into-

Botanical Garden and Arboratum

They are significant divisions of the Botany department of The M. S. University of Baroda, Vadodara, India. The Botanical garden and Arboratum are spread in approximately 10 acres of land. The total number of plants of *M. indica* in arboretum is 8 and in garden 6.

Outdoor Locations and Gardens

Other localities include some outdoor locations and public gardens like Akota garden, Kamatibaugh, Vaccine Ground, nearby locations of residential area like Sama, Kareilibaugh, Amit nagar, Pratapgunj and road side planted mango trees on Vadodara-Bharuch and Vadodara- Ahemedabad highway. Collection of healthy and diseased leaves were done in all three seasons from these locations.

Orchards

Some mango orchards/ fields in Ranoli village are situated near G.S.F.C. Vadodara (8 Km. away from Baroda Railway Station) and Sama -Savali village on Express highway. Ranoli village is situated at 22º 24’ 10” N latitude and 72º 47’ 36” E longitude.

RWLS

Field survey was undertaken in Ratanmahal Wildlife Sanctuary (RWLS), District Panchmahel in the November 2007. Ratanmahal lies nearly 35 km south-east from Devgadh Baria, the head quarter of Baria taluka. It is situated between 74º 37' to 74º 11' E Longitude and between 22º 32' to 22º 35' N Lat.

2. Isolation and Identification of Leaf Infecting Fungi

The plant is affected by a number of diseases at all stages of its development, right from the seedling in the nursery to the harvesting of fruits. These may be further affected by pathogens in storage or transit. Hardly any plant organ is immune, and almost every part viz., leaves, stem, branch, twig, root, petiole, flower and fruits are affected by various pathogens. All trees are attacked by one or more fungi that can cause scattered, rather definite, round to oval, angular, or irregularly shaped spots on the leaves (PLATE III, Figs. A & B). These spots usually become conspicuous from late June through August. Leaf spots are the most common diseases of fruit and ornamental trees. Most of these diseases are favoured by cool weather, light and frequent rains, fog or heavy dews, high humidity, and crowded or shady plantings.

A few spots on the leaves do little harm to a tree and are far more unsightly than they are injurious. However, leaf spot infections that start early in the growing season can lead to premature defoliation. If it occur over two or more successive years, it can seriously weaken a tree, reduce its growth, and increase its susceptibility to bark borers, winter injury, and other diseases. Leaf spots commonly increase in number and size in late summer and early autumn as the leaves begin to senescence. The occurrence of a leaf spot disease late in the growing season generally does not seriously affect the health of a tree. Certain leaf spots have special names, such as anthracnose, black spot, downy spot or white mold, ink spot, leaf blister or curl, shot-hole, sooty blotch, and tar spot.

From early spring into summer, microscopic spores are produced in tremendous numbers on the surface of the leaves or in speck sized pycnidia, acervuli, and perithecia embedded in the diseased leaf tissue. The spores are spread primarily

by air currents, splashing rains, and insects to newly emerging leaves of susceptible trees where, in the presence of free water, the spores germinate and penetrate the epidermis, and infection begins. Depending on the fungus, there may be one or several cycles (generations) of the pathogen in one growing season. The symptoms were mainly present on the margins and the tip of the leaves.

Disease Symptoms and Isolated Fungi

A. *Alternaria* Leaf Spot

The fungus *Alternaria alternata* (Fr.) Keissler is responsible to produce *Alternaria* leaf spots, a minor disease of mango. Symptoms appear on both the surfaces of leaves as small brownish circular spots. Initially it became apparent on lower surface of the leaf where it shows light brown coloration. The spots gradually enlarged and became irregular black and formed larger patches.

B. *Curvularia* Leaf Spot

During a isolation, another fungus responsible for causing leaf spots was identified as *Curvularia lunata* (Wakker) Boedijn var. *aeria* (Batista, Lima and Vasconcelos) M. B. Ellis. The disease started in the form of small light brown spots at the tips of the leaves. Subsequently the symptoms advanced towards the petiole covering almost the entire leaf surface and changed the colour into dark brown.

C. *Phoma* Leaf Spot

Another pathogen isolated from diseased mango leaf was *Phoma glomerata* (Corda) Woll and Hochapfel. The symptoms of the disease were noticeable only in the old leaves. Initially the lesions were minute, irregular, yellow to light brown, scattered

over the leaf lamina. As the lesions enlarged, their colour changed from brown, to cinnamon and these become irregular. Fully developed spots were characterized by dark margins and dull grey necrotic centers. Under severe infection conditions, infected leaves got defoliated.

D. Vegetative Malformation

The two species of *Fusarium* viz. *F. moniliforme* var. *subglutinans* (Wollenw & Reinking) and *F. oxysporum* were isolated from the vegetative malformed shoot. The pathogens were responsible for causing vegetative malformation. In vegetative malformation the vegetative buds in the leaf axils or at the apical meristem of the younger plants developed abnormally compact rosette- like shootlets, bearing tiny leaf rudiments. The affected new shoots on the old trees became thick, stunted and developed a whorl of small leaves. The intensity of mango vegetative malformation disease was found to be more prevalent during the rainy season. Summanwar *et al.*, (1966) reported the first occurrence of a fungus *F. moniliforme* associated with mango malformation and proved its pathogenicity. Later on the fungus was further identified as *F. moniliforme* var. *subglutinans* (Verma *et al.*, 1974; Chadha *et al.*, 1979).

E. Anthracnose of Mango

Anthracnose, caused by *Colletotrichum gloeosporioides* Penz. & Sacc. (Perfect stage *Glomerella cingulata*), a member of Melanconiales in Fungi Imperfecti is probably the most important disease of mango, wherever, it is grown. The infection of young leaves first appear as small dark spots that enlarged to irregular dark lesions, often with a distinct yellow halo. Under wet conditions, lesions may coalesce into large infected areas, especially along the leaf margins. Older leaves are also susceptible, but the fungus remains latent until the leaves senesce. The fungus usually survived

between seasons primarily on infected and defoliated branches, terminals and mature leaves. Conidia were produced in fruiting bodies, referred to as acervuli, over a wide range of temperatures and especially under rainy or humid and mature leaves. Conidia were spread by splashing rain or irrigation water. Conidia were readily produced on all infected tissues and serve as secondary inoculum to spread the disease. The ascospores did not appear to serve an important role in the spread of the disease.

F. *Lasiodiplodia* Leaf Spot

L. theobromae is a cosmopolitan and causes infection in diverse species. As a disease agent, pathogen is encountered in its anamorphic state, named as *Lasiodiplodia*. *L. theobromae* was detected frequently during isolation from leaves. Domsch *et al.*, (1980) reported that this fungus attacks more than 280 species of plants in different parts of the world. Symptoms produced by it are in the form of twig blight, tip dieback, gummosis and bark splitting (Malik *et al*., 2005). Drying of tip, discoloration and darkening of bark some distance from the tip are common symptoms. Later, it moves downward involving bigger branches as well. As a result, the leaves are shed followed by exudation of gum from the diseased portions. In severe cases, bark splitting or cracking has also been noticed. These symptoms may be found alone or in combination of two or more symptoms in different mango orchards of the world (Ploetz, 1999; Iqbal *et al*., 2007). The fungus also attacked the collar region and severity of symptoms turned the plant to decline. Ploetz and Prakash (1997) mentioned the impossibility of reproducing the symptoms in artificial inoculation conditions, so, it is assumed that this fungus keeps itself as endophyte and intensifies its attack when plants are susceptible or weak.

Result of Isolation Studies

In the recent study total 17 leaf infecting fungi and one black sterile mycelium have been isolated from 4 different varieties of mango leaves. Out of these fourteen organisms belonged to Fungi Imperfecti and three organisms to Ascomycetes. Fungi Imperfecti consisted of a large number of parasitic and saprophytic organisms. It is a major disease causing group. Fungi Imperfecti members were well equipped to complete their life cycle within a very short span and were highly evolved to adapt themselves into changing environment and can survive well even in extreme adverse conditions. They not only inhabited almost all parts of the plant before harvest but also deteriorated fruits and seeds during post harvest phase.

Fungi like *Alternaria alternata* (Fr.) Keissler, *Lasiodiplodia theobromae* (Pat.) Griffon & Moube, *Curvularia lunata* (Wakker) Boedijin, *Drechslera hawaiiensis* (Bugnicourt) Subram. & Jain, *F. moniliforme* var. *subglutinans, Gloeosporium mangiferae* Henn, *Nigrospora sphaerica* Sacc., *Phoma glomerata* (Corda) Woll and Hochapf. and *Thielavia subthermophila* Mouchacca. were frequently isolated from diseased mango leaves. Apart from these fungal pathogens several other fungi including *Acremonium acutatum* W. Gams., *Aspergillus nidulans* (Eidam) G., *Cladosporium cladosporoides* (Fres.) de Vries, *Colletotrichum gloeosporioides* Penz. & Sacc., *F. pallidoroseum* (Cooke) Sacc., *F. solani* (Mart.) Sacc., *Helminthosporium hawaiiensis* Bugnic., *Trichoderma viride* Pers. were also isolated along with them. The percentage colonizing frequency for each isolate was calculated. It was observed that the fungal pathogen mainly responsible for producing symptoms on leaves showed maximum percentage of colonizing frequency as compared to other associated fungi. Table 2.1 revealed that out of 17 isolates *F.*

moniliforme var. *subglutinans*, *Gloeosporium mangiferae* Henn, and *Lasiodiplodia theobromae* (Pat.) Griffon & Moube were the only pathogens isolated from each variety of all the locations and showing maximum colonizing frequency of 16.8, 27.7 and 35.0 respectively. It was also found that maximum 12 fungal pathogens associated with mango leaf infection were isolated from Rajapuri variety as compared to other three varieties. Since the occurrence of these three pathogenic fungi in all the 4 varieties of mango leaf and causing serious diseases which resulted in the reduction of yield and poor quality of fruit, therefore, it was thought to study them and suggest some control strategies for them.

Morphological Description of Leaf Infecting Associated Fungi

***Acremonium acutatum* W. Gams.:**

Colonies, hyaline, slow growing. Sporogenous cells phialidic, mostly simple, awl-shaped, erect, arising from substratum or from fasciculate aerial hypahe. The phialides are separated from hyphae by a septum and taper towards their apices. At the apices of the phialides are the hyaline conidia are present. Conidiophore short, simple, narrowed towards the tip, erect 18-45 X 3-3.6 µm. Conidia one celled, fusiform to ellipsoidal, hyaline, smooth, 4.4-6.5 X 1.5-2.2 µm (PLATE VII, Fig. A).

***Alternaria alternata* (Fr.) Keissler:**

Colonies effuse, gray, dark, blackish brown. Conidiophore short, simple straight, 3-septate, branched or unbranched upto 50 µm long and 3-6 µm wide. Conidia muriform with 3-8 transverse septa and light brown in colour. The length of the conidia varied from 30.99 to 42.47µm. The width of the conidia was varied from 11.90 to 17.37µm. The beak length of conidia varied from 18.7 to 23.81µm (PLATE VI, Fig. F). The number of cells in each conidium varied from 2-9.

***Aspergillus nidulans* (Eidam) G.:**

Colonies somewhat valvety, margins thin, cress green from abundant conidial heads, changing from deep dull to reddish with the formation of abundant cleistothecia. Conidiophores light brown, smooth occasionally septate, ranging from 40-130 μm, mostly 70-95 μm in length, 2.5-3.2 μm in diam. Conidia globose to subglobose, gren in mass, 2.6-3.7 μm (PLATE VII, Fig. C).

***Cladosporium cladosporoides* (Fres.) de Vries:**

Colonies effuse, olive green to olivaceous brown, velvety. Conidiophores smooth, without swellings and sympodial elongations, branching acropleurogenously, producing conidia in chains in chains below septum, 35-340 x 2-6 μm. Conidia formed in long branched chains, asptate, ellipsoidal to limoniform, olive green, smooth or rarely verruculose, scares prominent, 3.5-10.2 X 2.6-3.8 μm (PLATE V, Fig. F).

***Colletotrichum gloeosporioides* Penz. & Sacc.:**

Colonies with dense whitish to dark gray aerial mycelium. Setae abundant, long, rigid, bristle like, septate, dark. Conidia falcate, fusiform, apices, acute, 18-23 X 3.5-4 μm. Appressoria abundant, medium brown, clavate to circular, 9-14 X 6.5-11.5 μm (PLATE IV, Fig. E).

***Curvularia lunata* (Wakker) Boedijin:**

Colonies with profuse aerial mycelium and dark olive brown. Conidiophores arising singly or in groups, branched or unbranched, erect, straight, septate, variable in length and 3-5 μm wide. Conidia acropleurogenous, solitary, smooth walled, 3-septate,

obovoidal to broadly clavate, curved at the subterminal cell. The subterminal cell swollen and distinctly larger than the remaining cells. Three transverse septa present. The size varied from 20 – 28 X 8 - 14µm (PLATE VI, Fig. B).

***Drechslera hawaiiensis* (Bugnicourt) Subram. & Jain:**

The fungus produces colonies, which are dark gray with abundant aerial mycelium. Conidiophores simple, slightly geniculate, pale to mid- brown, septate, 42-145 X 2-7 µm. Conidia straight, ellipsoidal, oblong or cylindrical, pale brown, smooth, 3-7 distoseptate, 12-37 X 5-11 µm (PLATE VII, Fig. B).

***Fusarium moniliforme* var. *subglutinans* (Wollenw & Reinking):**

Fusarium is most common soil fungi. It is reported to cause wilt and mango malformation, a disease of flowers. Three different types of *Fusarium* were obtained during isolation.

Colonies white at first becoming pink vinaceous to violet. Microconidiophores single, lateral, subulate, phialids form from aerial hyphae. Microconidia agglutinated in chains and remain joint or cut off in false heads, later scattered in clear yellowish to rosy white aerial mycelium as a dull, colourless powder, 1-2 celled, fusiform, ovate, 0-septate mostly 8.4 X 2.4 (4-18 X 1.5-4) µm, 1-setate mostly 17 X 2.9 (9-30 X 2-5) µm. Conidiophores producing macroconidia consists of basal cell having 2-3 apical phialides, macroconidia formed in clusters called sporodhochia , which are delicately awl shaped, slightly sickle shaped or almost straight mostly 3-septate 36 X 3 (20-60 X 2-4.5) µm. Chlamydospores are absent (PLATE V, Fig. D).

***F. pallidoroseum* (Cooke) Sacc.:**

Colonies of *F. pallidoroseum* were rose to livid red to crimson. Aerial mycelium

floccose, somewhat lighter coloured and becoming brown, sporulation often scarce. Conidiophores densely branched along with solitary phialides. Phialides doliform 10-14 X 3.5-4.5 µm. Conidia slender, falcate, moderately curved with pointed curved apical cell, basal cell pedicillate, mostly 5-6 septate, 41-60 X 4-5.5 µm.

***F. solani* (Mart.) Sacc.:**

Colonies of fungus are green to bluish brown. Mycelium sparse and floccose. Conidiophores unbranched and branched, monophialidic. Phialides bearing microconidia long, slender, 15-40 X 2-3 µm. Microconidiophore subcylindric, obclavate to doliiform, 10-25 X 3-4 µm. Microconidia not abundant and not in chains, 4-14 X 2.5-5 µm. Macroconidia produced on shorter flask-shaped phialides, abundant, falcate, curved with bluntly beaked apical cell, mainly 5-7 septate and 50-85 X 5-7 µm in size.

***Gloeosporium mangiferae* Henn.:**

A form genus of several hundred Imperfect Fungi (family Melanconiaceae) having no setae around the acervuli and often causing anthracnose of plants. Conidia are 1-celled, hyaline, ellipsoidal, straight or slightly curved, usually embedded in a gummy substances. Conidiophore is short packed in a palisade underneath the host epidermis which is ruptured by the developing mass of conidia. Conidia are hyaline, 1-celled, ovoid to oblong, 3-6 X 8-15 µm (PLATE V, Fig. A).

***Helminthosporium hawaiiensis* Bugnic.:**

Colonies dark gray with abundant aerial myceelium. Conidiophores simple, slightly geniculate, pale to mid- brown, septate, 42-145 X 2-7 µm. Conidia straight,

ellipsoidal, oblong or cylindrical, pale brown, smooth, 3-7 distoseptate, 12-37 X 5-11 µm.

***Lasiodiplodia theobromae* (Pat.) Griffon & Moube. [syn: *Botryodiplodia theobromae*]:**

Culture was initially white to smoke grey, with fluffy, aerial mycelium on PDA. Colonies soon became grey or black and fast spreading with immersed, superficial and branched septate mycelia. The upper surface gradually developed prominent fruiting bodies. Shiny black pycnidia were produced on the surface. Conidiophores absent. Conidia were initially hyaline, unicellular and subovoid to ellipsoid, with a granular content. Mature conidia were bicelled, dark brown, thick walled and ellipsoidal finally developing a median septum with dark brown pigmentation and longitudinal striations. The size varied from 20-30 X 10-15 µm. Paraphyses upto 50 µm long (PLATE IV, Fig. C).

***Nigrospora sphaerica* (Sacc.) Mason:**

The fungus produces colonies spreading broadly, at first white with small shining black conidia later turns brown to black when sporulation is abundant. Conidiophore branched, smooth and brown in colour, 4-8 µm wide. Conidiogenous cells solitary, monoblastic, subspherical, colourless 6-11 µm diam. Conidia solitary, acrogenous, simple, spherical, 1-celled, black smooth, 10-22 µm in diameter (PLATE IV, Fig. B).

***Phoma glomerata* (Corda) Woll & Hochapfel:**

Colony growing fast 5-7.5 cm diam. in 7 days on PDA. Pycnidia abundant, dark brown, globose to subglobose 155-465 µm. Conidia 1-celled, cylindrical or

ellipsoidal, straight or slightly curved, hyaline to light olivaceous, rounded at both ends, biguttulate, 5-9 X 2.5-3.5 µm (PLATE V, Fig. E).

***Thielavia subthermophila* Mouchacca.:**

It produces dark grey colonies with a dense aerial mycelium and a black reverse. Conidia (aleuriospores, chlamydospores), formed laterally or terminally on the hyphae or on short branches, are broadly clavate or pyriform, with a truncate base, 1-celled, hyaline or light brown and measure 4-7 x 3-4 µm. The ascomata develop in the mycelial mat and are spherical, black, 90-200 µm in diameter. The thin, dark wall of the ascomata is composed of *textura epidermoidea* or of flattened, in outline irregular, 5-8 µm sized cells and is often covered with dark hyphae. The ascospores are fusiform or ellipsoidal, 1-celled, brown, 14-19 x 8-10 µm, with a distinct, subapical germ pore (PLATE IV, Fig. A).

***Trichoderma viride* Pers.:**

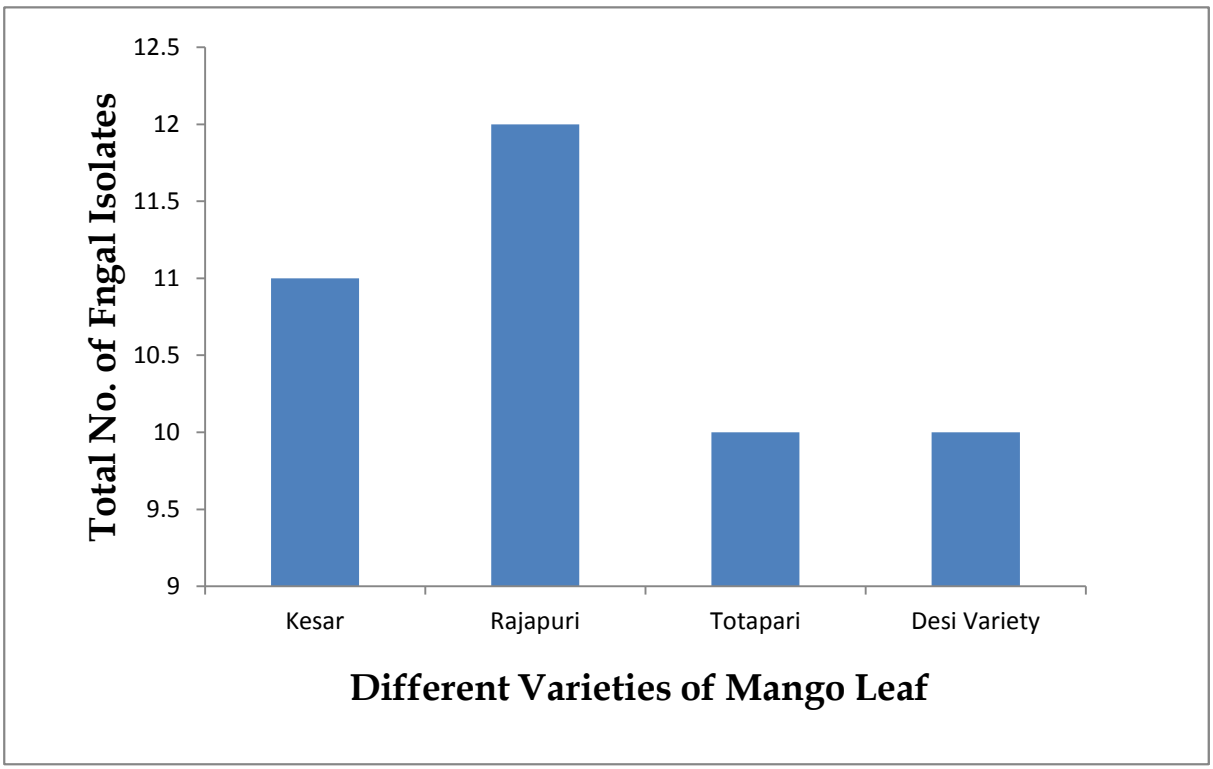
Colonies growing rapidly upto 9cm in 4 days on PDA. Watery white becoming hairy from the formation of loose scanty aerial mycelium. Conidiophores hyaline, upright and much branched. Phialides formed in false whorls beneath each terminal phialide occasionally arise singly or in opposite pairs along the branches. Conidia globose or short ovoid, 1-celled, borne in small terminal clusters, bluish green to dark green, 4-4.8 X 3.5-4µm.

Table 2.1- Showing colonization frequency of fungal organisms isolated from diseased leaves of four varieties of *Mangifera indica*

Sr. No.	Fungi Isolated	Kesar	Rajapuri	Totapari	Desi variety
1.	*Acremonium acutatum* W. Gams.	4.4	2.1	-	-
2.	*Alternaria alternata* (Fr.) Keissler	-	1.4	1.8	-
3.	*Aspergillus nidulans* (Eidam) G.	1.4	3.7	-	-
4.	*Cladosporium cladosporoides* (Fres.) de Vries	2.9	1.4	-	2.0
5.	*Colletotrichum gloeosporioides* Penz. & Sacc.	5.8	-	2.9	-
6.	*Curvularia lunata* (Wakker) Boedijin	-	2.9	-	5.8
7.	*Drechslera hawaiiensis* (Bugnicourt) Suram. & Jain	-	5.8	8.8	-
8.	*Fusarium moniliforme* var. *subglutinans* (Wollenw. & Reinking)	1.4	9.0	4.4	2.0
9.	*F. pallidoroseum* (Cooke) Sacc.	-	7.3	-	-
10.	*F. solani* (Mart.) Sacc.	2.9	-	3.7	-
11.	*Gloeosporium mangiferae* Henn.	4.4	10.2	7.3	5.8
12.	*Helminthosporium hawaiiensis* Bugnic.	1.4	-	-	2.9
13.	*Lasiodiplodia theobromae* (Pat.) Griffon & Moube	7.3	11.7	5.8	10.2
14.	*Nigrospora sphaerica* (Sacc.) Mason	-	3.1	-	3.7
15.	*Phoma glomerata* (Corda) Woll & Hochapfel	2.9	-	5.8	-
16.	*Thielavia subthermophila* Mouchacca.	-	1.4	3.1	5.8
17.	*Trichoderma viride* Pers.	-	-	2.9	7.3
18.	Sterile black mycelium	1.4	-	-	2.1

Note: Data based on 3 replicates of 3 leaves of a mango tree.
(-) = Absence of colony

Fig. 2.2 Total no. of fungal isolates from four different varieties of mango leaves



3. Occurrence of Endophytic Fungi on Different Plant Parts

Fungal endophytes reside in healthy tissues of all terrestrial plant taxa studied to date and are of diverse groups. These are abundant in tropical woody angiosperms. Several factors have stimulated interest in endophytes in tropical forests, including the contribution of endophytes to fungal diversity (Hawksworth, 2001), in shaping plant community dynamics (Arnold *et al.,* 2001), as a source of novel bioactive compounds (Strobel and Long, 1998).

Endophytic fungi are unique among the many plant microbial symbionts because they are the only microbial group thought to benefits their hosts through direct defence against natural enemies of the plant (Carroll, 1988; Clay, 1988; Faeth and Bultman, 2002). Being mutualists, these endophytic microbial populations are known to enhance the absorption of soil nutrients such as phosphorus, and other inorganic elements, nitrogen fixing efficiency (Saito and Minamisawa, 2006; Shrestha *et al.,* 2007) and plant growth with the production of hormones like auxins, abscisins, ethylene, gibberelins, and kinetins (Hallmann *et al.,* 1997; Pepper, 2000).

Various workers have studied distribution patterns of endophytes within plant tissues, and in most cases foliar endophytes were examined (Lodge *et al.,* 1996; Rodrigues *et al.,* 1997 and Wilson *et al.,* 1994). The species composition of the endophytic assemblage and frequency of infection varied according to host species, site characteristics such as elevation, exposure, associated vegetation, tissue type and tissue age. On the other hand, for large woody perennials growth stage and position in the canopy may also affect the distribution of endophytes. Endophytes colonized plants often grow faster than non-colonized ones (Cheplick *et al.,* 1989). An endophyte in one plant could be a pathogen of the other depending on the balance

between pathogenicity and endophytism of the microorganism in different hosts. Lots of researches show that in some occasions the saprophyte condition of the endophyte fungi that live in arboreal species transmute in negative effects when the host has nutritional disorders or hydric stress making it more sensitive to the attack (Schulz *et al.,* 1999).

The current study was undertaken to get the knowledge about the fungal endophytes from living symptomless leaves, stem and bark tissues of four varieties of *M. indica.* The isolation and characterization of fungal communities was intended to get an estimate of the diversity of the endophytic fungi from the economically important fruit crop mango.

It is evident from Tables 3.2, 3.3 and 3.4 that the distribution of endophytic fungi present in various parts of the plant was not uniform. The leaf, stem and bark samples differed in their endophytic fungal colonization. Most species isolated during this study belong to the genera which have already been described as endophytes from different hosts at different locations. In addition, some of them are cited as pathogens of many hosts. A total of 23 different endophytic fungi representing 17 genera were obtained from healthy leaf, stem and bark segments of this plant. Of the total species isolated from different locations, 3.3% were belonging to Ascomycetes, and rest to Fungi Imperfecti. The population of Hyphomycetes was more, 80% Hyphomycetes and 13.3% Coelomycetes members were isolated. However, 11 isolates did not produced spores hence remained unidentified therefore; they have been listed under the sterile mycelia.

Leaf samples from each location harbored the greatest number of endophytes as compared to the stem and bark samples of the same location. Out of 23 endophytic

fungi maximum 20 endophytes were isolated from leaves only. This finding was in accordance with the report of endophytic diversity in leaves, twigs and roots of *Gynoxis oleifolia* (Fisher *et al.*, 1995). To find out the presence of fungal endophytes in leaves total of 3,649 leaf segments of four varieties from 10 different locations were studied. Out of which maximum 15 species of fungi were recovered from locality 9. Total 62.5% leaf endophytes were recovered from locality 9 while the least, at 41.6% were recorded from localities 3, 4 and 5. The mango leaves from ten different localities were highly colonized by *Alternaria alternata* (PLATE VI, Fig. F) showing 255.2 total percentage colonization frequency and stood number one among all fungal taxa recovered from this host. *A. alternata* has been routinely isolated as endophytes of leaves from wheat and other plants (Sieber, 1985; Petrini, 1986) like maize (Wellacher, 1991) and *Aegle marmelos* (Gond *et al*., 2007). In addition, *Alternaria* has also been isolated as leaf infecting pathogen of mango during this study.

It is evident from the Table 3.1 that in leaves of *M. indica*, other than *Alternaia alternata,* two species of *Aspergillus i.e. A. niger* and *A. flavus* , *Cladosporium cladosporioides*, *Penicillium citrinum* and *Fusarium roseum* were also dominating genera. The occurrence of *Cladosporium cladosporoides* (PLATE V, Fig. F) in mango leaves was recorded in 80% samples collected from all evaluated samples from different localities. There are no such reports available for *Cladosporium* species, as an important pathogens of mango, but as an opportunist. In fact, it is known that the presence of *Cladosporium* spp. in different organs of the saprophytic plants as a parasite or a saprophyte is being recognized as an important contaminant of indoor environment and crop environment. *C. cladosporiodes* Fresen. is a species that has already been reported as a mango endophyte in Australia

(Johnson *et al.*, 1992). As an endophytic fungi *A. flavus* and *A. niger* showed 79.5 and 91.1% colonizing frequency respectively. Although *Aspergillus* is ubiquitous and usually reported to occur as an epiphyte but it has also been known to grow endophytically (Shulthess and Faeth, 1998). For the first time fungus *A. fumigatus* CY018 was isolated as an endophytic fungus in *Cynodon dactylon* (Liu *et al*., 2004). Cabral *et al.*, (1993) showed that epiphytic fungi can sometimes invade the substomatal chambers of living leaves and Phytopathologist Petrini (1986) speculated that such fungi might be able to enter living leaves of plants under stress.

Chaetomium globosum (PLATE VI, Fig. A) was one of the basic components of endophytic fungi isolated from the leaves and constituted 37.7% of the total count. Although *Chaetomium globosum* is reported to be a coprophilous fungus and a common biodeterioration agent of cotton and other plant tissues, it is also recorded as an endophyte from *Rhizophora apiculata* a mangrove in India and from Kandelia candel as an endophyte from roots of *Alnus glutinose* (Suryanarayan *et al*., 1998).

Phomopsis mangiferae (PLATE VI, Fig. D) was isolated as an endophyte showing 34% of colonizing frequency from all the analyzed samples of leaf. Species of *Phomopsis* are widespread plant pathogens and endophytes (Boddy and Griffith, 1989). *Phomopsis* has been reported in both cultivated plants such as mango (*Mangifera indica*), papaya (*Carica papaya*), avocado (*Persea americana*) (Ploetz *et al*., 1994), coconut (*Cocos nucifera*) (Mariano *et al.*, 1997), blueberry and cranberry (Farr *et al.*, 2002) and *Stylosanthes* sp. (Pereira *et al*., 1993) and in wild plants in the families *Sapotaceae* (Lodge *et al.*, 1996; Bayman *et al*., 1998), *Ericaceae* (Bills *et al.*, 1992; Okane *et al.*, 1998), *Arecaceae* (Rodrigues, 1994) and *Meliaceae* (Gamboa, 1998; Gamboa and Bayman, 2001). *Phomopsis* has traditionally been considered to be highly pathogenic (Rehner and Uecker, 1994), but some *Phomopsis* species are

thought to be mutualistic endophytes (Webber and Gibbs, 1984; Carroll, 1986). According to Farr *et al.,* (1999) some species of *Phomopsis* appear to be restricted to one plant host genus or family, while other species can be isolated from diverse plant hosts. Conversely, strains of *Phomopsis* isolated from one host species are not necessarily closely related and may represent more than one taxon.

The total nine isolates belonging to seven different genera were recovered from 980 stem pieces. The maximum 88.8% were recorded from Location 5 and minimum 55.5% from Locations 2 and 4. *Monascus ruber* with 45.1% colonization frequency was the dominant endophyte of stem. *F. pallidoroseum* showed the 19.6% colonizing frequency which was minimum among all.

It is clear from observation presented in Table 3.3 that *Monascus ruber* (PLATE V, Fig. B), *Nigrospora sphaerica* (PLATE IV, Fig. B) and *Trichoderma piluliferum* (PLATE VII, Fig. F) were host part specific and showing dominancy in stem tissues and these were not observed colonizing other tissues of the mango plant. In the year 2009 Sharma *et al.,* reported nine isolates of *Trichoderma* species among several other endophytes from *Ficus religiosa* as an effective endophytic bioagent against soil borne pathogens viz. *R. solani* and *S. rolfsii.* Fungus *N. sphaerica* was also isolated as a leaf infecting fungi from diseased mango leaves of Rajapuri and desi variety. Apart from these the other fine example of tissue specificity was showed by *Robillarda sessilis* (PLATE IV, Fig. D), *V. albo-atrum, Microascus cirrosus* (PLATE VII, Fig. E) and *Epicoccum purpurescens* (PLATE VI, Fig. C) which were isolated from leaf tissues only as endophyte. Other than that *A. alternata* and *F. roseum* was reported in the leaves and stem tissues of *M. indica* but not from its bark.

It is evident from Table 3.4 that a Basidiomycetous member and *Pestalotiopsis guepinii* (Desm.) Steyaert. (PLATE V, Fig. C) were isolated as an endophytes from

bark pieces. A fungal mycelium showed the clamp connection so it was identified as a member of Basidiomycetes. These were confined to the bark tissues and showed 23.9% of colonizing frequency. Earlier It has also been isolated as an endophyte through leaves, young branches, inflorescences and pedicels in mango plants (Johnson *et al*., 1992) . It has been found as an endophyte in Yew plantations, where it produces a cancer preventive product called taxol. Non identified species of *Pestalotiopsis* have been reported as causal agent of the grey dots in leaves and branches of mango and cashew, even though these diseases are not so important for the plants (Mabbett, 1998).

The results presented in Table 3.4 showed that the maximum 55% endophytes were recorded from bark tissues of the trees situated in Location 2 and minimum 30.5% from Location 5. No representative member from Zygomycotina was recorded in this experiment from any part of the host.

Morphological description of three endophyitc fungi selected for physiological studies-

1. *Robillarda sessilis* Sacc.

Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Ascomycetes
Subclass	Xylariomycetidae
Family	Amphisphaeriaceae

The genus *Robillarda* described by Saccardo (1884) in *Sylloge Fungorum*. It is found in Africa (Angola), Asia (India), Central America (West Indies), Europe (Hungary, Italy and Russia), North America (USA) and Oceania (Solomonls) and has been recorded from many trees and herbs. Conidial appendages and conidiogenesis are key characters in the identification of Coelomycete genera and species. Pycnidia brown to pale, in spots, erumpent to sub superficial, globose to flattened, with small ostiole, 120-200 μm diameter, 110-135 μm high, filled with numerous conidia. Conidiophores hyaline and ampuliform. Conidia hyaline, 2-celled with a central, transverse septum, thick walled, 10-12.5 x 2.5-3.2 μm (11.1x 2.7 μm average, lb=4.1), furnished with 2-3 apical appendages. Appendages are hyaline, straight or curved, persistent and 13-26 μm in length (18.7 μm average).

2. *Phomopsis mangiferae* S. Ahmad.

Phylum	Ascomycota
Class	Sordariomycetes
Subclass	Sordariomycetidae
Order	Diaporthales
Family	Diaporthaceae

Species of *Phomopsis* are widespread plant pathogen and endophyte (Boddy and Griffith, 1989). The Coelomycetous genus corresponds to the imperfect state of Diaporthe (Pyrenomycetes: Ascomycotina). It was characterized by dark pycnidia at the periphery of the culture, often with long, black setae up to 5 mm, and abundant comma-shaped β-conidia, and ovoid α-conidia, immersed in a white creamy liquid (Sutton 1980). Mycelium was sparse, often white-yellowish, sometimes brown, wrinkled in appearance, and septate. Antagonism between colonies (as evidenced by pigmented zones of interaction) was common. Abundant dark pigments were deposited on the bottom of the plate.

3. *Pestalotiopsis guepini* (Desm.) Steyaert

Phylum	Ascomycota
Class	Sordariomycetes
Subclass	Xylariomycetidae
Order	Xylariales
Family	Amphisphaeriaceae.

A fungus with white mycelia producing numerous acervuli with black droplets of spore masses was observed on PDA. Conidia were produced in ink-like fruiting bodies. After two weeks, acervular conidiomata developed. The isolates had 5-celled smooth walled conidia. Apical and basal cells were hyaline, conical. The three intermediate cells were dark brown, with the two upper ones sometimes darker. Conidia measured 18-24 x 9.0-10.5 µm. There were typically 3 apical appendages 17.3-28.7 µm long and a basal appendages 4.3-7.5 µm long. In old cultures the conidia became shrunken. These were seen as dark black bodies.

Table 3.1- Showing percentage occurrence of endophytic fungi from leaves of *M. indica* present in ten different locations in and around Vadodara

Sr. No.	Endophytic Fungi	LOCATIONS									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1.	*Acremonium p*ersicinum (Nicot) W. Gams.	-	-	-	3.7	-	5.8	-	6.7	-	-
2.	*Alternaria alternata* (Fr.) Keissler	29.6	31.1	27.4	28.7	-	19.4	21.9	30.3	-	26.8
3.	*Aspergillus awamori* Nakazawa	-	7.7	-	-	6.7	-	4.2	5.8	3.1	-
4.	*A. flavus* Link.	13.2	25.0	-	-	19.4	11.4	-	-	8.3	2.2
5.	*A. fumigatus* Fres.	-	-	2.7	22.2	-	-	-	10.0	14.1	-
6.	*A. niger* van Tieghem	-	17.2	-	16	9.2	-	12.3	20.6	5.8	10.0
7.	*A. tamari* Kita.	11.7	-	-	-	-	5.2	-	-	7.0	-
8.	*Chaetomium globosum* Kunze ex Steud	-	-	6.1	-	11.0	-	13.2	7.4	-	-
9.	*Cladosporium cladosporoides* (Fres.) de varies	33.3	-	12.0	5.5	-	8.3	-	21.0	-	-
10.	*Curvularia lunata* (Wakker) Boedijin	10.4	-	19.4	-	-	-	4.6	-	8.2	14.5
11.	*Drechslera hawaiense* (Bugnicourt) Subram & Jain	14.8	-	-	16.6	5.8	-	4.3	-	1.2	-
12.	*Epicoccum purpurascens* Ehrenb.	-	11.7	-	-	-	5.2	-	5.2	7.1	8.6
13.	*F. pallidoroseum* (Cooke) Sacc.	-	19.4	5.8	-	8.6	-	5.2	-	-	1.1
14.	*F. roseum* Link.	27.2	-	-	15.1	-	6.3	-	8.3	2.0	-
15.	*Microascus cirrosus* Zukal.	-	7.1	-	16.3	-	-	3.7	-	-	-
16.	*Penicillium citrinum* Thom.	25.0	-	18.8	-	-	12.0	-	9.5	5.2	8.9
17.	*Phomopsis mangiferae* S. Ahmad	7.1	1.0	-	-	5.2	-	-	8.3	5.8	6.7
18.	*Robillarda sessilis* Sacc.	-	-	3.2	-	11.4	-	5.5	-	8.0	-
19.	*Trichoderma viride* Pers.	19.4	-	-	11.3	-	8.3	-	3.7	4.9	8.3
20.	*Verticillium albo-atrum* Reinke & Berthold	8.3	-	-	-	2.1	-	-	-	3.2	-

1= Ranoli Village, 2= Bharuch, 3= Kamatibaugh, 4= Akota Garden, 5= GSFC,

6= Vaccine Ground, 7=Pratapgunj, 8= Sama, 9= Ratanmahal, 10= Botanical Garden/Arboratum

Table 3.2- Showing percentage occurrence of endophytic fungi from the leaves of four different varieties of mango

Sr. No.	Endophytic Fungi	Kesar	Rajapuri	Totapari	Desi Variety
1.	*Acremonium p*ersicinum (Nicot) W. Gams	5.8	3.7	-	-
2.	*Alternaria alternata* (Fr.) Keissler	19	8.3	-	17.2
3.	*Aspergillus awamori* Nakazawa	-	-	3.1	7.7
4.	*A. flavus* Link.	2.2	-	-	8.3
5.	*A. fumigatus* Fres.	-	2.2	2.7	-
6.	*A. niger* van Tieghem	-	-	5.2	-
7.	*A. tamari* Kita.	5.8	-	-	9.2
8.	*Chaetomium globossum* Kunze ex Steud	-	5.5	7.0	-
9.	*Cladosporium cladosporoides* (Fres.) de Vries	7.4	-	-	6.1
10.	*Curvularia lunata* Wakker) Boedijin	-	4.6	8.2	2.2
11.	*Drechslera hawaiense* (Bugnicourt) Subram & Jain	4.3	2.7	-	-
12.	*Epicoccum purpurascens* Ehrenb.	5.2	-	6.0	-
13.	*F. pallidoroseum* (Cooke) Sacc.	3.1	5.2	-	-
14.	*F. roseum* Link.	-	3.7	2.2	1.0
15.	*Microascus cirrosus* Zukal.	3.7	-	-	3.1
16.	*Penicillium citrinum* Thom.	4.3	-	5.5	2.2
17.	*Phomopsis mangiferae* S. Ahmad	-	2.2	4.6	-
18.	*Robillarda sessilis* Sacc.	10.1	3.1	1.0	-
19.	*Trichoderma viride* Pers.	2.2	-	2.9	-
20.	*Verticillium albo-atrum* Reinke & Berthold	-	-		3.7

(-) = Absence of colony

Table 3.3- Showing colonizing frequency of endophytic fungi from Stem of *M. indica* present in ten different locations in and around Vadodara

Sr. No.	Endophytic Fungi	LOCATIONS									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1.	*Alternaria alternata* (Fr.) Keissler	8.1	13.0	-	-	1.9	11.0	-	-	2.9	6.0
2.	*Aspergillus flavus* Link.	4.6	-	7.0	9.1	5.1	-	-	4.5	-	-
3.	*A. niger* van Tiegham	-	4.6	-	7.2	-	5.5	-	6.0	-	3.5
4.	*Chaetomium globosum* Kunze ex Steud	8.6	-	5.2	-	5.8	4.3	7.3	-	-	4.2
5.	*F. roseum* Link.	9.2	11.1	-	8.8	5.2	-	4.5	-	6.8	-
6.	*Monascus ruber* van Tiegham	-	8.1	10.7	-	4.3	-	-	-	4.1	-
7.	*Penicillium citrinum* Thom.	1.2	1.4	-	-	2.0	9.0	-	12.0	-	-
8.	*Nigrospora spahaerica* Sacc.	-	-	8.8	4.0	-	7.2	5.1	-	12.9	-
9.	*Trichoderma piluliferum* J. Webster & Rifai	3.3	-	1.3	8.8	5.7	-	-	3.3	-	9.0

Note: Observations based on 41 isolations from each location. 980 explants were used from each plants. Minimum 3 plants were studied.

Table 3.4- Showing colonizing frequency of endophytic fungi from Bark of *M. indica* present in ten different locations in and around Vadodara

Sr. No.	Endophytic Fungi	LOCATIONS									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1.	*Aspergillus awamori* Nakazawa	6.4	-	7.4	2.1	8.9	3.2	-	4.1	-	4.4
1.	*A. flavus* Link.	-	5.4	-	5.6	-	-	3.4	-	2.4	-
2.	*A. fumigatus* Fres.	-	3.1	3.9	-	4.3	2.2	-	7.1	-	-
3.	*A. niger* van Tiegham	7.4	-		-	-	-	-	1.2	-	7.8
4.	A member of Basidiomycetese	-	4.1	-	-	3.4	-	2.5	-	-	-
5.	*Fusarium pallidoroseum* (Cooke) Sacc.	5.0	11.6	-	8.3	3.4	-	-	3.3	8.2	-
6.	*Penicillium citrinum* Thom.	3.2	-	-	-	6.6		3.5	-	-	-
7.	*Pestalotiopsis guepinii* (Desm.) Steyaert	-	5.1	2.3	-	-	2.9	-	-	13.1	-
8.	*Trichoderma harzianum* Rifai.	-	-	1.2	4.9	6.6		11.2	-	-	3.9
9.	*T. piluliferum* J. Webster & Rifai	2.2	9.1	-	11.0	-	-	-	5.8	-	1.2
10.	Greyish sterile mycelium	2	2	-	-	1	2	-	-	-	1

Note: Observations based on 41 isolations from each location. 980 explants were used from each plants. Minimum 3 plants were studied.

4. Histological Studies

The mango leaves are characterized as dorsi- ventral, because the epidermis is followed by Parenchyma layer. Microtome sections of diseased leaves showed the presence of fungal hyphae in inter and intracellular spaces. It runs from one cell to adjacent cell through pits and also between the two cells (through middle lamella). Branching was also seen in fungal hyphae. Transverse section of leaves revealed the histological condition of infected leaves. A relatively thick cuticle covered the epidermis of infected leaves (PLATE VIII, Fig. A). The cuticle often extended into the radial walls of the adjacent epidermal cells. The mid rib epidermis is covered with a tick cuticle measuring 2.7- 3.6µm in thickness. Whereas in healthy leaves cuticle measuring 2.0-2.5µm thickness covers the epidermis on both the sides of leaf. In healthy leaves sub epidermal region was occupied by 3-4 layered thick walled lignified sclereids while in diseased leaves such regular arrangement of sclereids was not found (PLATE VIII, Fig. B). Most of the cortical cells are filled with phenolic contents. In lamina region the palisade tissues were compactly arranged and short in length as compared to the palisade tissues of healthy plants.

The primary duct lumen is often noticed with gum-resin accumulation. Vessels appear in radial multiples with relatively less lumen diameter. A relatively thick walled sclerenchymatous perivascular fiber covered the gum resin duct in the cortical region of the infected leaves. Gum resin duct lumen was filled with secretary material whereas in healthy leaves it was free from any such deposition (PLATE VIII, Fig. E). Dark deposits were commonly found in the intercellular spaces and in phloem parenchyma. Such deposition was not noticed in healthy leaves. The vessel element walls were relatively thick and lignified and the lumen showed accumulation of granular darkly stained material. The frequency of vessels was found to be more but

there diameter was less in case of diseased leaves. The lumen diameter of vessels becomes reduced due to the presence of fungal hyphae. The xylem vessel lumen sowed the presence of fungal hyphae (PLATE VIII, Fig. G). Similar observations along with decrease in the number of vascular elements have been reported by Raafat *et al.,* (1995). The relatively more number of lignified sclereid layers in cortex and relatively thick walled (lignified) vessels could be attributed to the lignifications role played by peroxidase enzyme. Vessels in the Xylem tissues were highly lignified. In healthy leaves vessels were relatively thin walled with no dark deposition in lumen.

Table 4.1- Showing the Histological details of Vessels and Gum resin ducts in healthy and diseased mango leaves

S. No.	Histological Details of Leaf Components	Diseased Leaf	Healthy Leaf
1	Vessel lumen diameter (μm)	23.3 ±4.0	34.3 ±5.6
2.	Vessel frequency (in 1 sq. mm)	13.1 ±4.7	12.7 ±2.5
3.	Gum resin duct Lumen diameter (μm)	130.5 ±45.0	188.3 ±77.5

5. Antagonistic Activity of Endophytic Fungi against Pathogenic Fungi

The word antagonism means hostility that results in active resistance, opposition, or contentiousness. In terms of phytopathology it is the action of any microbe that suppresses the activity of a plant pathogen due to an opposition in physiological action. Antagonistic microorganisms play an active role in micro environment. If a plant surface is there can compete with the pathogens for nutrients, inhibit pathogen multiplication by secreting antibiotics or toxins, or reduce pathogen population through hyperparasitism.

This kind of interaction between microorganisms leads to effective control of several diseases. The intensive use of fungicides has resulted in the accumulation of toxic compounds potentially hazardous to humans and the environment, and also in the build-up of resistance of the pathogens. In view of this, investigation and the application of biological control agents (BCAs) seems to be one of the promising approaches (Cook, 1985). Biocontrol involves the use of naturally occurring nonpathogenic microorganisms that are able to reduce the activity of plant pathogens and thereby suppress plant diseases. In this direction fungal endophytes may prove one of the most appropriate biological control agents (BCA).

The activities of endophytes in suppressing fungal pathogens have been confirmed by Poling *et al.,* (2008); Salehpour *et al*., (2005); as well as Muhammad and Amusa (2003). Various methods through which these endophytes inhibit fungal pathogens in the rhizosphere include competition for available nutrients, oxygen and space; parasitism and physical destruction of fungal cell walls by the action of hydrolytic enzymes produced by the endophytes (Taechowisan *et al*., 2009).

Scientists have found that carposphere, phyllosphere, phylloplane, flowers and in few cases other matrixes have provided the major source for antagonist (Sharma, 2003; Belve *et al.,* 2006; Sharma and Awasthi, 2010). Smolka (1992) suggested probability of more antagonists in an orchard having natural population of trees as compared to a chemically managed orchard. Natural mycoflora maintains a balance among the microbe normally present and inhibits the growth of newer arrivals. Taking this clue, efforts have been made to use antagonistic yeast to control post harvest food decay (Sharma and Awasthi, 2010).

The antagonistic activity of three endophytic fungi viz. *R. sessilis, P. mangiferae* and *P. guepinii* isolated from *M. indica* was examined using the dual culture method. The plant pathogenic fungi, *Gloeosporium mangiferae, Fusarium moniliforme* and *Lasiodiplodia theobromae* which cause leaf spot diseases in mango were used for the study. Discs (5 mm) from the margins of actively growing cultures of antagonists and pathogens were placed 40 mm apart in petri dishes containing PDA medium (Mortan and Strouble, 1955). There were five replicates of each treatment. Growth and sporulation were observed in all the isolates and in control. The zone of inhibition of mycelial growth of *G. mangiferae, F. moniliforme* and *L. theobromae* induced by *R. sessilis, P. mangiferae* and *P. guepinii* were measured. The measurements were taken after 3d and 5d.

In control Petri dishes (without pathogen) *P. mangiferae* and *P. guepinii* grew at a faster rate and covered the whole Petri dish within 72h, whereas, *R. sessilis* showed comparatively a slower growth. It was found that *P. guepinii* was effective in suppressing the growth of *L. theobromae* and *F. moniliforme.*

The observation based on the comparative analysis of the radial growth of three pathogens revealed that *L. theobromae* showed maximum inhibition of 68% as

compared to the control in the presence of *P. guepinii* at 5th day. *P. guepinii* had higher antagonistic activity for *L. theobromae* than other two endophytes. In the beginning, pathogen grew very fast as compared to antagonists and covered almost the entire Petri dish, but after 3rd d the *P. guepinii* started covering the area around the pathogen and checked its further growth (PLATE I, Fig. D).

F. moniliforme showed the highest percentage inhibition in radial growth in presence of *P. guepinii* followed by *R. sessilis* and *P. mangiferae.* The diameter of radial growth recorded was 33.3, 42.1 and 46 mm respectively on 5th day.

The highest mycelial growth and less inhibition of *G. mangiferae* was favored by *R. sessilis i.e.* 54.7 mm (40% inhibition) and the lowest mycelial growth and high inhibition by *P. mangiferae i.e.* 32.2 mm (64% inhibition) on 5th d (PLATE I, Fig. C). The overlapping mycelial growth was seen in between *R. sessilis* and *L. theobromae,* which was higher as compared to others. From the results it can be concluded that the fast growing antagonist like *P. guepinii* caused more inhibition of pathogen than slow growing antagonists. It may be probably due to mycoparasitism and competition for nutrients. These observations are in accordance with those made by Lugtenburg *et al.,* (1991).

Antagonists have been frequently used as biocontrol agents. Important constraints being adverse environmental conditions such as extreme dryness, heat and cold limited shelf life and inability to control latent infections. For suitability of an antagonists to be used as a commercial product, Hofstein *et al.,* (1994) has outlined following criteria-

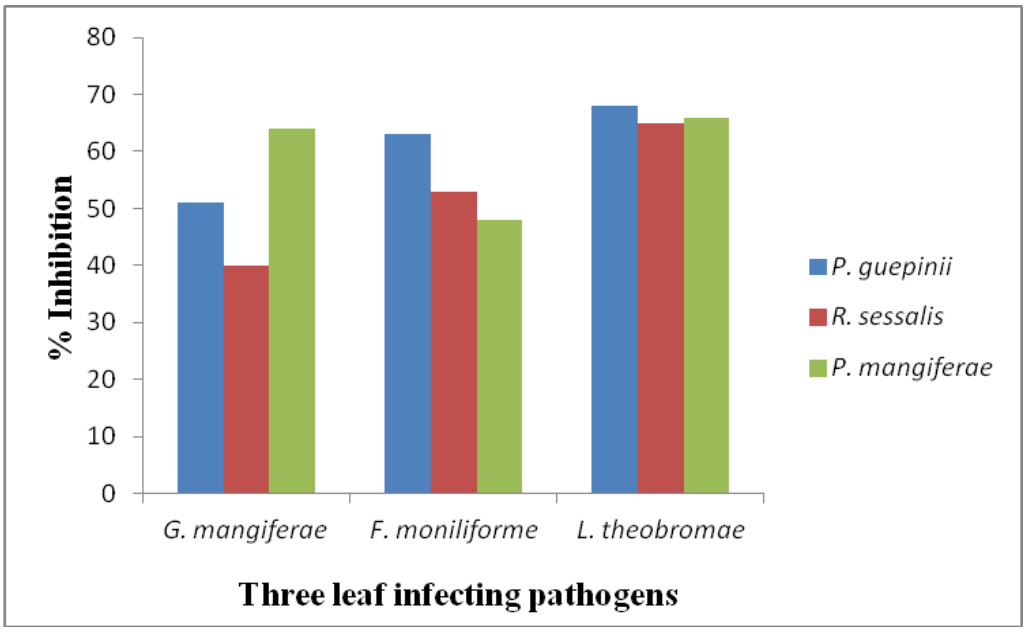
1. It should be genetically stable.
2. Effective at low concentration.

3. Effective against wild rang of pathogens.
4. Does not produce metabolites that are deleterious or harmful to human health.
5. Compatible with commercial processing procedures.

Table 5.1-Effect of three endophytic fungi on growth of *G. mangiferae, F. moniliforme* and *L. theobromae* in dual culture

Endophytes	Radial growth (mm)						Growth inhibition (%) over control					
	G. mangiferae		*F. moniliforme*		*L. theobromae*		*G. mangiferae*		*F. moniliforme*		*L. theobromae*	
	3d	5d	3d	5d	3d	5d	3d	5d	3d	5d	3d	5d
R. sessilis	64.3	54.7	62.8	42.1	55.2	31.2	29.0	40.0	30.0	53.0	38.0	65.0
P. mangiferae	57.7	32.2	64.8	46.0	46.7	30.1	35.0	64.0	28.0	48.0	48.0	66.0
P. guepinii	55.0	44.0	57.0	33.3	41.5	28.2	38.0	51.0	36.0	63.0	53.0	68.0

Fig. 5.1 Showing percentage inhibition in radial growth of three leaf infecting pathogens in presence of three endophytes



6. Study of Aeromycoflora

Fungal spores play a significant role in plant pathology and human respiratory allergy. Gregory (1961) termed the air borne pollen grains and fungal spores as "aerospora". Spore concentration in atmosphere is the result of complex interaction between biological and environmental factors. Airborne fungal spores are ubiquitous in nature (Burge, 1985) and are considered to act as indicator of the level of atmospheric bio pollution. The presence of fungal propagules, volatiles and mycotoxins in the air can cause a health hazards in all segments of the population (Kakde *et al.,* 2001). They were also responsible for deterioration of organic objects. Tilak and Kulkarni (1972) studied the indoor aeromycolflora of caves of Aurangabad. The paintings of Ajanta and Ellora caves have deteriorated due to fungal growth (Dhawan *et al.,* 1991). Mycological analysis of indoor air was done in Bharat Kala Bhawan, Varanasi (Singh and Tandon, 1991) and Libraries in Lucknow (Srivastava, 1991). Arya and Arya (2007) reported aeromycoflora of different vegetable and fruit markets of Baroda. Arya *et al.,* (2001) used gravity fall method to detected the aeromycoflora while occurance of fungal flora in Ajanta and Elora caves was studied by Rotorod sampler modified by Harrington (Harrington, 1959).

The present study deals with the aerobiological survey of different orchards, public gardens and residential houses having *M. indica* plantations with respect to seasonal variation (summer, rainy and winter). The study was carried out during May 2008 to April 2009. It was also observed that out of total 24 fungi recorded, *Aspergillus flavus* and *A. niger* showed maximum percentage frequency.

Aeromycoflora in Different Localities

During the present investigation a well- marked variation in aeromycoflora of

three different areas was recorded. In spite of showing variation, some fungal populations were common to all the three places. *Aspergillus flavus, A. niger, Alternaria alternata, F. roseum* and *Cladosporium herbarum* were dominant in the air of all the three areas. The species of *Curvularia lunata* and *Alternaria alternata*, members of dematiaceous hyphomycetes were more prevalent than other fungal genera. The fungal genera like *Aspergillus tamarii, A. parasiticus, Penicillium* sp. and *Curvularia* sp. were present only in the air of residential areas *i.e.* Locality 1, which is thickly populated area while *Monascus ruber* was recovered from the air of orchards *i.e.* Locality 3, which is less populated area.

The variation in composition of aeromycoflora of different areas has been reported by many workers (Barth, 1981; Pasanen, 1990). The distinct variation in aeromycofora of different residential environments has been investigated earlier by Pasanen in 1990. Studies undertaken by Verma (1990) revealed that composition of fungal flora was different in urban and rural areas of India. He also observed the differences in the outdoor and indoor environments. Chauhan and Kulshrestha (2006) studied fungal flora in vegetable markets, parks, gardens, temples in Dayalbagh, Tajmahal and Sikandara areas of Agra city. Maximum fungal population was recorded in Dayalbagh followed by Tajmahal areas and fruit markets. They concluded that the maximum number of fungal spores from Dayalbagh and Tajmahal were due to high vegetation concentration. Dayalbagh has cultivation of crops like Wheat, Bajra, Maize and vegetables in the nearby villages and the fungal spores produced in the crops were present in the outdoor air of the area. The number of fungi recorded by them was less in Ravali and Mankameshwar temples situated in thickly populated area. Scientists have found that air pollution also affects the aeromycoflora of a locality (Treshow, 1980; Agarwal *et al.*, 1987).

Further it was also observed that out of 24 fungal species and 2 sterile colonies, 15 fungal spore types were recovered from Locality 1 of residential area, 18 fungal types from Locality 2 of public garden and the maximum 19 number of fungal spore types from Locality 3 *i.e.* orchard. These variations in component of aeromycoflora of different areas may be due to specific characteristics of these localities regarding local vegetation, population, passing vehicles, air pollution, industrialization and amount of organic waste and decaying material present in and around these localities which may directly or indirectly influence the composition of aeromycoflora of an area.

Changes in Fungal Flora in Different Seasons

Air carries innumerable spore, mycelia of fungi and pollen. Fungal spores contribute to a major portion of air spora. The study of air borne fungal spores is important for both plants and human health. The seasonal fluctuation in occurrence and concentration of fungal spores varies from place to place, depending on the local flora, topography of the landscape and human interference. Seasonal and diurnal variations of air borne fungal spores have been studied earlier in cities like Jabalpur (George and Verma, 2000), Burdwan (Chakraborty *et al.,* 2000), Nagpur (Kakde *et al.,* 2001), Lucknow (Khandelwal, 2001) and Vadodara (Arya *et al.,* 2001).

A detailed study was concluded to record season change in the fungal flora of the entire three localities. Data presented in Table 6.1 and Fig. 6.2 revealed that the fungal population was not homogenous throughout the year and it showed seasonal variation. Seasonal variation in the concentration of fungal species may takes place due to changes in the meteorological conditions. Out of 24 fungal types, 8 fungal organisms along with 1 sterile colony were recorded during summer of 2008 while in winter season the number of colonies increased to 13 in localities 1 and 3. Seasonal

variation in the number of fungal colonies is highly influenced by the environmental factors, especially temperature. Decrease in temperature during winter, increased numbers of fungal colonies, while the numbers of fungal colonies were less during hot summer which might be due to unfavourable environmental condition. Decrease in waste organic matter also retards the growth of fungi hence environmental fungal loads is reduced. Same observations were recorded by Verma and Khare (1987) in winter season in Jabalpur; Jadhav (1996) over rice field; Tiwari (1999) from Raipur; Tiwari and Sharma (2008) on leaves of *Ocimum sanctum*. Tiwari and Saluja (2009) working with *Catharanthus roseus* have also reported highest fungal incidence during winter season, moderate during rainy season and minimum number of fungal types in summer season. Chauhan and Kulshrestha (2006) working in Agra found that moderate temperature, high relative humidity and mild rain fall form fungal population. An average of two years data indicates that the spore population of fungi follows a definite periodicity in the weather conditions prevailing over Agra. In general the, the fungal spores were observed in considerable concentrations from September to March, with peak incidence during November and February. The results of the present study are also supported by observations by Agarwal and Shivpuri (1974) who have reported that extreme heat and cold are unfavourable for the sporulation of fungal spores. The study further found the role of local crop patterns and vegetables around the sampling sites (living and dead) in concentration of air spora of a particular sampling site.

Experimental results presented in Table 6.1 and Fig. 6.2 indicate that *Aspergillus niger, A. flavus, Alternaria alternata, Cladosporium* sp. and *Fusarium roseum* were most frequently occurring fungi on the leaf surface of *M. indica*. Shah *et al.,* (1996) had earlier reported presence of these phytopathogens in indoor

aeromycoflora of different museums in Vadodara city. According to Thaware *et al.*, (2011) total 11 *Aspergillus* species were found to be associated with spices in different seasons of the year.

Although a seasonal variation was recorded in outdoor aeromycoflora yet certain fungi like *A. awamori*, *C. herbarum* and *F. roseum* were present in all the seasons in locality 1. Two species of *Aspergillus i.e. A. flavus and A. niger* were present in locality 2 and *A. niger, A. alternata, C. lunata* and *T. viride* were present in locality 3, throughout the year.

It could be concluded from the present study that aeromycoflora varied in 3 different localities having mango trees. Occurrence of aeromycoflora showed seasonal variation. This may be due to high sensitivity of fungi to environmental factors such as high temperature.

Fig. 6.1- City map of Vadodara, India showing 3 experimental locations

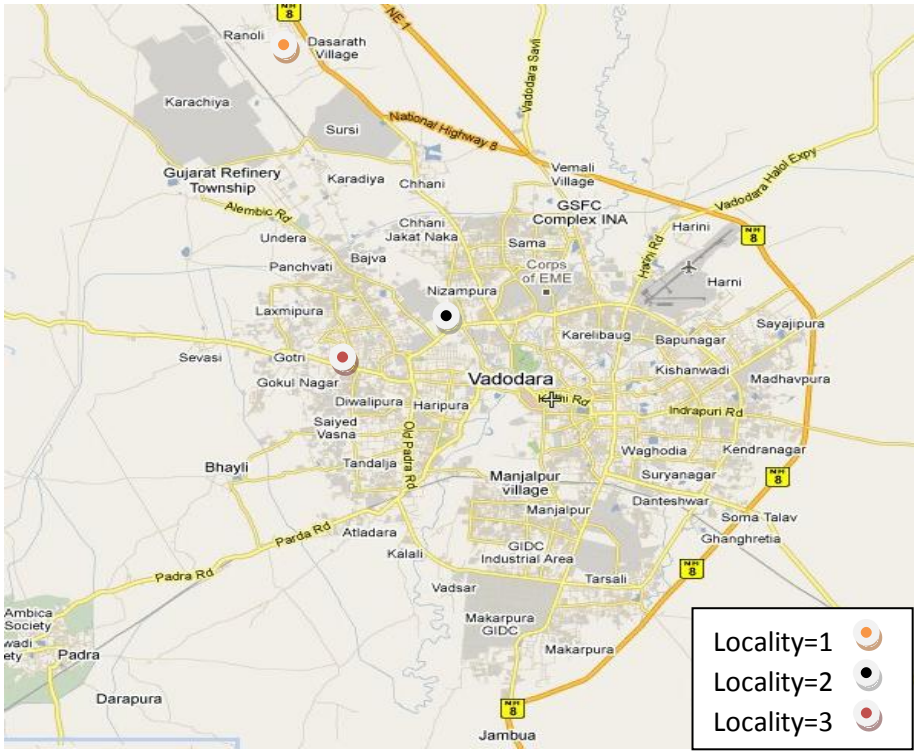


Fig. 6.2 Total fungal colonies present in atmosphere of 3 different localities in 3 different seasons

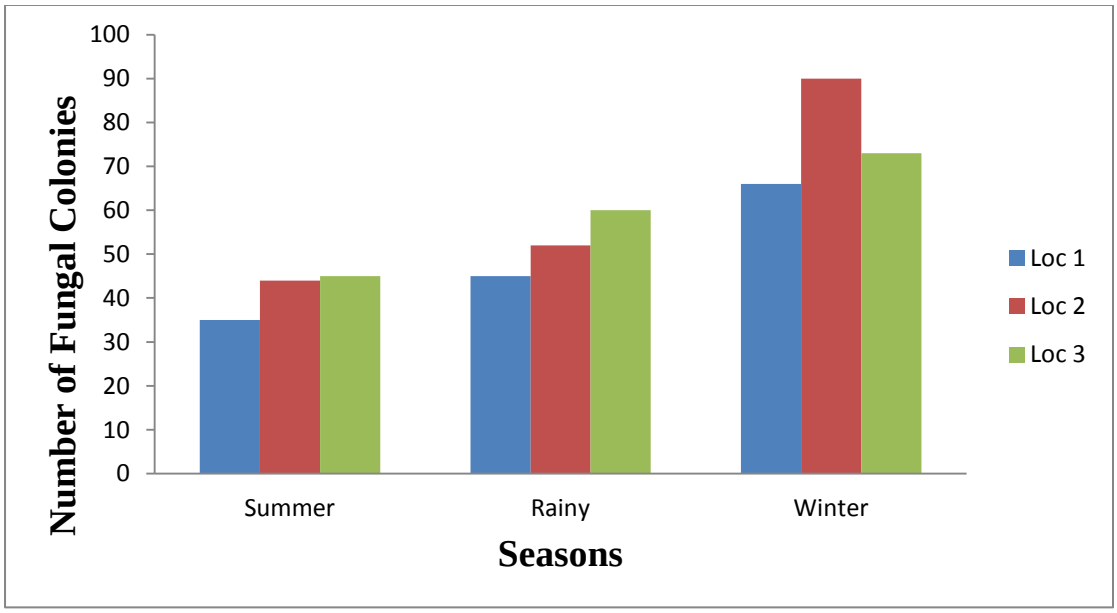
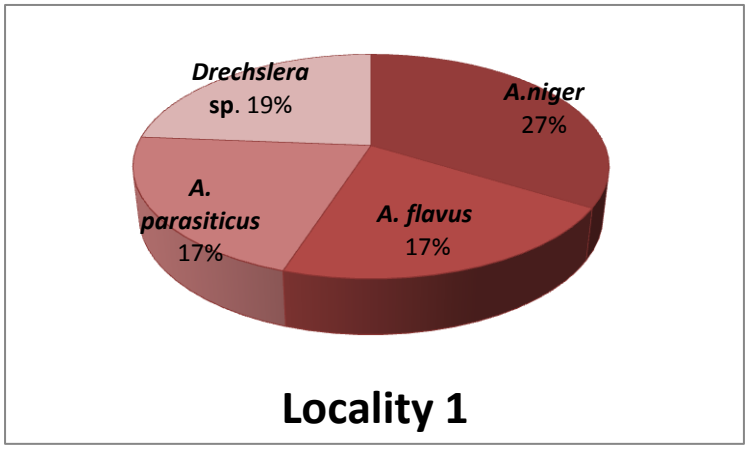
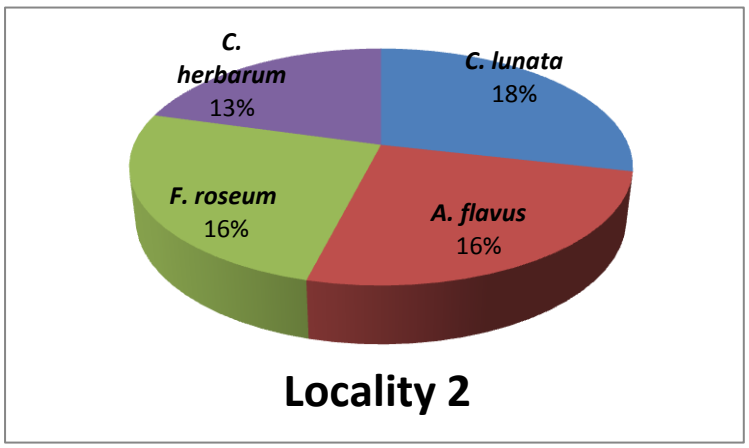


Fig. 6.3 Distribution of major fungal colonies isolated from three Localities





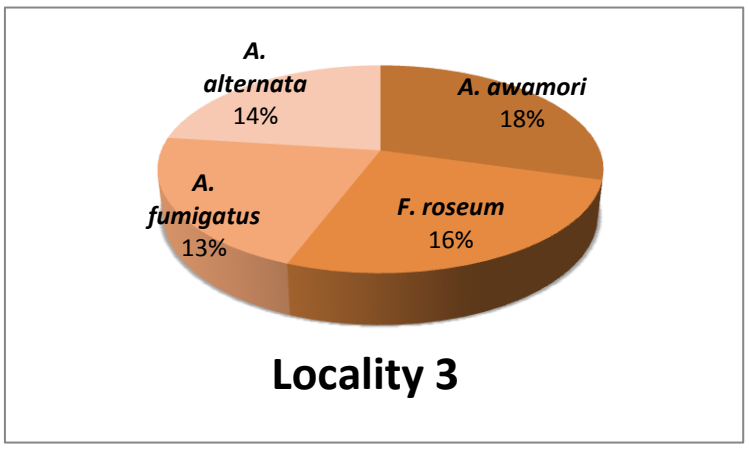


Table 6.1- Total fungal colonies present in three different localities in three different seasons

S. N	NAME OF FUNGI	LOCALITY 1						LOCALITY 2						LOCALITY 3					
		Summer		Rainy		Winter		Summer		Rainy		Winter		Summer		Rainy		Winter	
A.	**Zygomycotina**	**TC**	**%CF**	**TC**	**%CF**	**TC**	**%CF**	**TC**	**%CF**	**TC**	**%CF**	**TC**	**%CF**	**TC**	**%CF**	**TC**	**%CF**	**TC**	**%CF**
1	*Mucor hiemalis*	4	10.8	-	-	2	2.7	3	6.2	2	3.3	-	-	3	6.6	-	-	1	1.2
2	*Rhizopus stolonifer*	-	-	3	6.3	1	1.3	4	8.3	2	3.3	-	-	-	-	1	1.6	3	3.8
B.	**Ascomycotina**																		
3.	*Aspergillus awamori*	2	5.4	4	8.5	5	6.9	-	-	-	-	6	7.0	-	-	11	18.3	7	9.0
4.	*A. flavus*	-	-	8	17.0	4	5.5	5	10.4	10	16.6	7	8.2	5	11.1	-	-	9	11.6
5.	*A. fumigatus*	-	-	-	-	-	-	-	-	3	5.0	4	4.7	2	4.4	8	13.3	-	-
6.	*A. niger*	10	27.0	-	-	7	9.7	6	12.5	7	11.6	10	11.7	5	11.1	4	6.6	8	10.3
7.	*A. tamarii*	-	-	7	14.8	10	13.8	-	-	-	-	-	-	-	-	-	-	-	--
8.	*A. parasiticus*	-	-	8	17.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	*Aspergillus* sp.	-	-	-	-	-	-	1	2.0	-	-	7	8.2	-	-	5	8.3	-	-
10.	*Penicillium* sp.	3	8.1	-	-	7	9.7	-	-	-	-	-	-	-	-	-	-	-	-
11.	*P. citrinum*	-	-	-	-	-	-	5	10.4	7	11.6	-	-	3	6.6	-	-	-	-
12	*Monascus ruber*	-	-	-	-	-	-	-	-	-	-	-	-	5	11.1	-	-	-	-

Contd.

Contd.

C.	Fungi imperfecti	Locality 1						Locality 2						Locality 3					
		Summer		Rainy		Winter		Summer		Rainy		Winter		Summer		Rainy		Winter	
13.	*Alternaria* sp.	-	-	-	-	-	-	-	-	3	5.0	4	4.7	-	-	-	-	-	-
14.	*Alternaria alternata*	-	-	6	12.7	7	9.7	5	10.4	-	-	9	10.5	6	13.3	4	6.6	11	14.2
15.	*Cladosporium herbarum*	2	5.4	4	8.5	9	12.5	-	-	2	3.3	11	12.9	-	-	9	15	3	3.8
16.	*Curvularia lunata*	-	-	-	-	-	-	-	-	11	18.3	8	9.4	4	8.8	7	11.6	11	14.2
17.	*Curvularia* sp.	-	-	2	4.2	6	8.3	-	-	-	-	-	-	-	-	-	-	-	-
18.	*Drechslera* sp.	7	18.9	-	-	3	4.1	3	6.2	-	-	6	7.0	-	-	-	-	7	9.0
19.	*Fusarium roseum*	3	8.1	3	6.3	2	2.7	8	16.6	3	5.0	-	-	7	15.5	5	8.3	-	-
20.	*F. pallidoroseum*	-	-	-	-	3	4.1	-	-	-	-	-	-	-	-	-	-	2	2.5
21.	*F. oxysporum*	-	-	-	-	-	-	-	-	-	-	6	7.0	-	-	3	5.0	7	9.0
22.	*Trichoderma viride*	4	10.8	-	-	-	-	4	8.3	3	5.0	-	-	5	11.1	1	1.6	2	2.5
23.	*T. harzianum*	-	-	-	-	-	-	-	-	2	3.3	4	4.7	-	-	2	3.3	-	-
24.	*Lasiodiplodia theobromae*	-	-	-	-	-	-	-	-	-	-	2	2.3	-	-	-	-	2	2.5
25.	Sterile white mycelium	-	-	2	4.2	2	2.7	3	6.2	1	1.6	1	1.1	-	-	-	-	1	1.2
26.	Sterile black mycelium	2	5.4	-	-	4	5.5	1	2.0	4	6.6	-	-	-	-	-	-	3	3.8

Data represents the total no. of colonies of viable spores present in the air that appeared on three PDA plates after seven days of incubation.

(-) = absence of colony, TC= total colony, %CF= percentage colony frequency

Locality 1= Residential area, Locality 2=Public Garden, Locality 3= Orchard

7. Estimation of Flavonoid Contents in Healthy and Diseased Leaves

Different plant parts of mango have been analysed for their chemical constituents by various workers. Number of investigations has been conducted on the chemical and physical characteristics as well as utilization of various parts of mango like mango seed kernel and mango peel. The current study was undertaken to estimate the flavonoid content in four different varieties of healthy and diseased mango leaves.

Table 7.1- Showing the distribution of Flavonoids in healthy and diseased leaves of four varieties of *M. Indica*

S. No.	Mango Leaf Variety	Healthy	Diseased
1.	Rajapuri	Quercetin Aglycons	High conce. of quercetin and aglycons
2.	Totapari	2'-quercetin 3' 4' di methoxy quercetin 3'o methoxy quercetin	Quercetin absent High conce. of Mangiferin
3.	Kesar	Quercetin 3 prime methoxy quercetin	Quercetin absent High conce. of Mangiferin
4.	Desi variety	3'o methoxy quercetin/ rhamnetin/isorhamnetin (Derivatives of quercetin)	High conce. of quercetin and Mangiferin

The outcome of the Thin Layer Chromatography study based on the UV spectra resulted that all the four varieties of leaf showed variation in the peak. The healthy leaves of Rajapuri variety contained quercetin aglycon whereas in Kesar variety 3'-3 prime methoxy quercetin being the predominant compound along with quercetin. The diseased leaves of Rajapuri variety showed high concentration of all the compounds. In case of diseased leaves of Kesar variety peak for quercetin was not

found but UV spectra showed very high concentration of Mangiferin. The Totapari variety also showed the same compounds in diseased mango leaves. The healthy leaves of Totapari variety contained 2'-quercetin, 3' 4' di methoxy quercetin, 3'o methoxy quercetin where as in diseased leaves quercetin was absent and very high amount of Mangiferin was present. Diseased leaves of desi variety of mango showed high concentration of quercetin and Mangiferin both, whereas, UV spectra of healthy leaves showed peak of quercetin.

On the basis of the comparative analysis it is clear that concentration of Mangiferin was found to be very high in diseased leaves. In 1999, Chauhan, reported the same results during her researches on histopathological studies in mango affected by certain fungi.

8. Eco-Friendly Approach to Control Leaf Infecting Fungi

Botanical pesticides can be recommended as an ecochemical and sustainable strategy in the management of plant diseases. Because of their biodegradable nature, systemicity after application, capacity to alter the behavior of target pests and favorable safety profile, it is supposed that plant–based pesticides play a significant role in achieving evergreen revolution. Traditionally, different fungal diseases are controlled by synthetic fungicides, which increase agricultural costs at the same time contaminate the environment with toxic and carcinogenic substances (Carvalho, 2004). A possible alternative to solve such problems is the use of such plants or plant based products having antifungal properties (Miranda, 2003).

Plants generally produce many secondary metabolites which constitute an important source of microbiocides, pesticides and many pharmaceutical drugs. Interest in a large number of traditional natural products has increased in last few years (Taylor *et al.,* 1996). Plant extracts of many higher plants have been reported to exhibit antibacterial, antifungal and insecticidal properties under laboratory trails (Bouamama *et al.,* 2006; Ergene *et al.,* 2006; Kiran *et al.,* 2006; Mohana *et al.,* 2006; Okigbo *et al.,* 2006; Satish *et al.,* 1999; Shariff *et al.,* 2006). Extracts from plants such as *Azadirachta indica* Juss., *Allium sativum* L., *Lippea javanicum* L., *Urtica massaica* Mildbr., *Satureia biflora* L., *Warburgia ugandensis* Pott. and *Zingiber officinale* L. are reported to possess antimicrobial activity against a wide range of phytopathogens (Makeredza *et al.,* 2005; Oniang'o, 2003; Singh *et al.,* 1995). Antimicrobial activity of allicin present in extracts of *Allium sativum* (garlic bulbs) and *Bignonia* leaves has been reported against *F. pallidoroseum* and *A. niger* (Arya *et al.,* 1995).

Mode of Action of Natural Fungicides

Though the chemical nature of several natural fungicides is available, very few attempts have been made to determine the mechanisms operating to control the fungal pathogens. Based on available findings we can conclude that any one or more than one of the following mechanisms are responsible to restrict (fungistatic) or kill (fungicidal) the phytopathogenic fungal agents.

A) Inhibition of fungal metabolic pathways

Chemical fistulosin (Octadecyl 3-hydroxyvindole) isolated from the roots of *Allium fistulosum*, inhibits the protein synthesis of *Fusarium oxysporum.* Eugenol (4-allyl-2-methoxy phenol), a major component of several medicinal and aromatic plants, inhibits the microbial growth and is involved in free radical scavenging, lipid peroxidation and maintenance of redox potential, which together reduces the aflatoxigenecity of the fungus (Jayshree and Subramanyam, 1999).

B) Alteration in cell wall composition and structure

The cell wall protects the fungi against external agents including antifungal metabolites. Many antifungal agents target at cell wall composition and affect the integrity of cells resulting in fungal cell death.

C) Changes in membrane permeability

Membrane acts as barriers between the cell and its external environment an also separate various organelles of the cell. Natural fungicides, particularly essential oils and their monoterpenoid components affect the structure and function (Knobloch *et al.*, 1989). This happens due to inhibition of membrane enzymatic reactions such as respiratory electron transport, proton transport and coupled phosphorylation steps. Essential oils can degenerate hyphal tips and promote cytoplasmic retraction (de Billerbeck *et al*., 2001).

D) Alteration in Hyphal stucture

Treatment with natural fungicides results in microscopically detectable and often macroscopically visible hyphal structure. The hyphal deformations are mainly due to altered or lysed cell wall, and vacuolization or evacuation of the cytoplasm. Trypsin and Chymotrypsin inhibitors from cabbage foliage cause leakage of intracellular contents of *Botrytis cinerea* and *Fusarium solani.* Kaemoferol (3-O-b-D-apiofuranosyl-12 b-D-glucopyranoside), a flavonol diglycoside from the leaves of *Phytolacca americana*, lyse the cell walls diverse pathogenic fungi such as *B. cinerea*, *Magnaporthe grisea*, *Penicillium italicum*, *Diaporthe actinidiae*, *Botryosphaeria dothidea* and *Colletotrichum gloeosporiodes* (Bae *et al*., 1997).

E) Inhibition of fungal cell wall degrading enzymes

Pathogenic fungi produce cell wall degrading enzymes that degrade the plant cell wall polymers and facilitate the pathogen penetration and further colonization. Production of cell wall degrading enzymes (CWDE) is of significance of pathogenesis of necrotrofic fungal pathogens. Important CWDE involved in the pathogenesis of necrotrophic fungi is polygalacturonases, pectinlyase, pectimethylesterase, β-1,4-glucanase and cellulase. The virulence of several necrotrophic is often related to the differences in their production of CWDE (Carder *et al*., 1987).

Effect of Leaf Extracts on Fungal Pathogens

In the present study 10 different aqueous leaf extracts were used against three mango leaf spot fungi viz. *Fusarium moniliforme* var. *subglutinans* Woll. & Rein., *Gloeosporium mangiferae* Henn. and *Lasiodiplodia theobromae* Pat. Antifungal activity of aqueous leaf extracts of *Tylophora indica* (Burm f.) Merrill, *Rauwolfia tetraphylla* L., *Withania somnifera* (L.) Dunal, *Strychnos nux vomica* L., *Catharanthus roseus* (L.) G. Don., *Calotropis procera* (Aiton) W.T. Aiton, *Ocimum sanctum* L., *Aloe vera* (L.)

Table 8.1- Percentage inhibition of three leaf spot fungi of mango against aqueous leaf extracts of ten medicinal plants

S.No.	Medicinal plants	*F. moniliformae* var. *subglutinans*		*G. mangiferae*		*L. theobromae*	
		2%	10%	2%	10%	2%	10%
1.	*Tylophora indica*	10 ±3.5	30 ±2.30	18 ±1.50	33 ±3.0	8 ±1.2	20 ±2.51
2.	*Rauwolfia tetraphylla*	25 ±2.0	49 ±1.52	11 ±2.0	28 ±2.41	13 ±1.56	19 ±2.0
3.	*Withania somnifera*	38 ±3.05	48 ±1.52	35 ±3.05	61 ±1.73	14 ±1.4	37 ±3.4
4.	*Strychnos nux vomica*	35 ±2.08	69 ±1.7	26 ±2.0	43 ±1.5	16 ±1.0	35 ±3.4
5.	*Catharanthus roseus*	21 ±2.51	34 ±2.3	23 ±2.51	31 ±2.30	14 ±1.0	21 ±4.52
6.	*Calotropis procera*	42 ±3.05	52 ±3.5	41 ±3.05	72 ±1.7	12 ±2.08	26 ±1.56
7.	*Ocimum sanctum*	12 ±0.57	20 ±2.51	12 ±2.08	29 ±2.4	7 ±0.80	18 ±4.61
8.	*Aloe vera*	5 ±2.0	10 ±0.57	8 ±1.0	16 ±2.0	5 ±2.2	12 ±1.56
9.	*Melia azadirachta*	22 ±2.05	49 ±1.56	26 ±1.4	79 ±1.62	12 ±2.04	32 ±2.30
10.	*Adhathoda vesica*	10 ±3.5	34 ±3.5	12 ±2.06	30 ±2.30	13 ±1.56	19 ±3.4

Each treatment value is based on observations of three replicates

Results were significant at P≤0.05 level by one way ANOVA

Burm.f., *Melia azadirachta* L., and *Adhatoda vesica* L. was assayed by poisoned food technique (Nene and Thapliyal, 1979) and the results are present in Table 8.1.

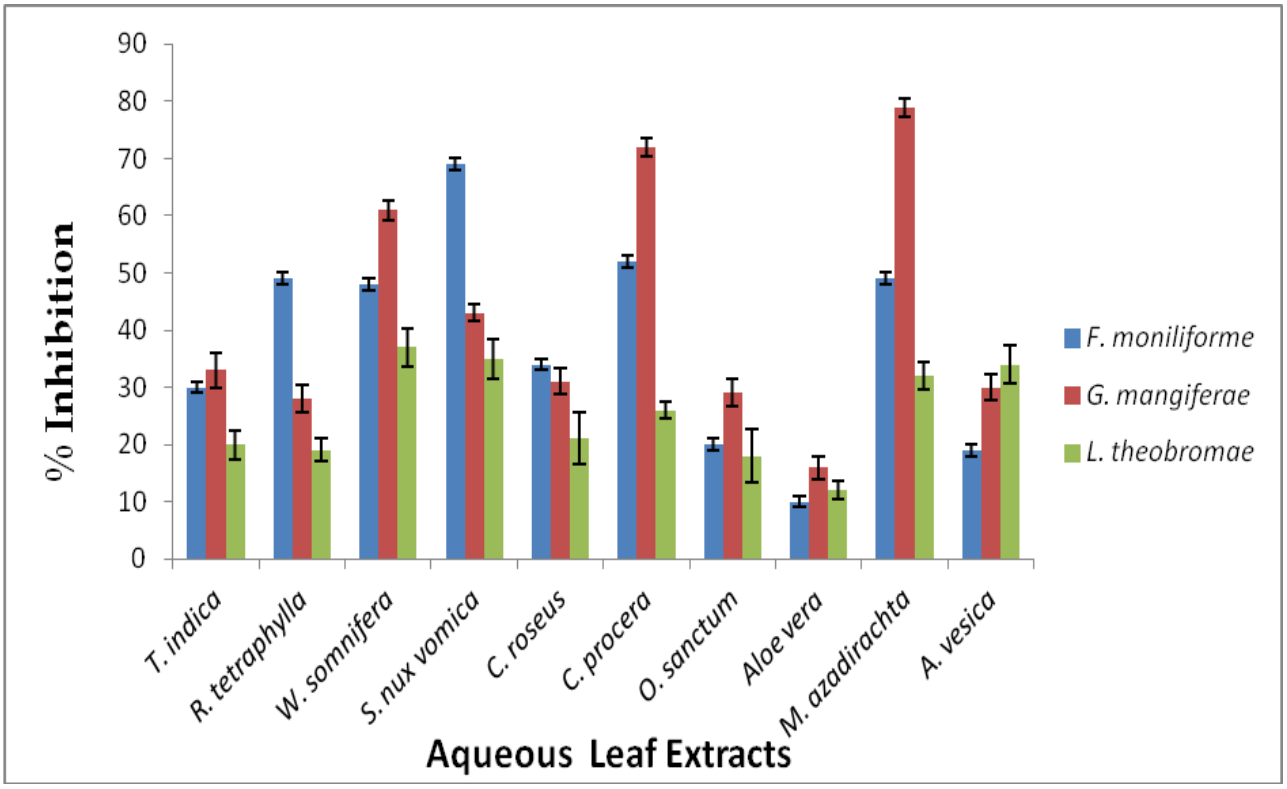
The data from Table 8.1 revealed that the 10% aqueous extract of *S. nux-vomica* showed maximum inhibition of *F. moniliforme* (69%), whereas *C. procera* showed 52% activity, which is the second highest inhibition. Significant reduction in growth of *G. mangiferae* was observed with extracts of two medicinal plants and the extracts showed significant differences in their efficacy. Among all the ten plants

extracts tried aqueous leaf extract of *M. azadirachta* showed remarkable inhibition of 79% in the growth of *G. mangiferae* at 10% concentration followed by *C. procera* at 10% concentration.

From the Table 8.1 it is evident that performance of *S. nux-vomica, W. somnifera* and *C. procera* was comparatively better than other leaf extracts tried. The antifungal nature of *S. nux-vomica* was earlier reported by Arya (1998). The leaf extract of *S. nux-vomica* at 75% concentration showed maximum inhibition (88.8%) of *Phomopsis psidii* causing fruit rot of guava (Arya, 1998).

It was also observed that all the ten leaf extracts were less effective against *L. theobromae* as compared to other two pathogens. The maximum inhibition was shown by extract of *W. somnifera* (37%) for *L. theobromae* at 10% concentration. *In-vtir*o study revealed that *O. sanctum* and *A. vera* aqueous leaf extract showed very poor inhibition against all the three pathogens as compared to other plants extracts. Earliar studies have revealed that aqueous extract of *O. sanctum* inhibited the production of pectolytic and cellulolytic enzymes of *Rhizopus arrhizus* and *Botryodiplodia theobromae* (Patil *et al*., 1992).

Fig. 8.2 Showing percentage inhibition of three pathogenic fungi in by aqueous leaf extracts of ten botanicals



B. Physiological and Biochemical Studies

9. Selection of a Suitable Culture Media

Microorganisms need nutrients as a source of energy and certain environmental conditions to grow and reproduce. In the environment, microbes have adapted to the habitats most suitable for their needs. In laboratory, however these requirements must be met by a culture medium. A wide range of media are used for growing the fungi. Most mycologists develop preferences for certain types of media based on experience and peculiarities of the type of fungi that are routinely grown. Media will affect colony morphology and color, whether particular structures are formed or not, and may affect whether the fungus will even grow in culture. For example some fungi lack the necessary enzymes to utilize different carbon sources. Thom and Raper (1945) suggested use of Czapex's medium for the culture of *Aspergillus* and *Penicillium* spp.

All fungi require several specific elements for growth and reproduction. The requirements for growth are generally less stringent than for sporulation, so it is often necessary to try several types of media when attempting to identify a fungus in culture. Most fungi thrive on Potato Dextrose Agar (PDA), but this can be too rich for many fungi, so that excessive mycelial growth is obtained at an expense of sporulation.

Media generally contain a source of carbon, nitrogen and vitamins. Glucose (dextrose) is the most widely utilizable carbon source, and hence is the most commonly used in growth media. Fructose and mannose are the next most commonly utilized sugars by fungi and are found in media from natural sources. Sucrose (table sugar) also used in some media. Nitrogen sources include peptone, yeast extract, malt extract, amino acids, ammonium and nitrate compounds. Salts including Fe, Zn and Mn, are often added to ‘defined’ media, but are usually not added to the common

media used for routine culture. Fungi have natural deficiency for vitamins that are satisfied at μM to nM concentrations. The most common naturally occurring vitamin deficiencies are thiamin and biotin. Deficiency of both is quite common among the members of Ascomycota. It is therefore, necessary to provide all these essential elements, in utilizable form, in a medium for their successful growth.

The knowledge achieved through these nutritional studies has resulted into a number of natural, semi synthetic and synthetic culture media. However, "There is no universal natural substrate or artificial medium upon which all fungi will grow" (Lilly and Barnett, 1951). Even closely related forms may differ considerably in their nutritional requirements qualitatively as well as quantitatively (Arya, 1982). This selective nature of different fungal isolates has compelled the investigators to perform some preliminary studies and design suitable culture media. A suitable culture media supporting good growth and sporulation of fungi will be of immense value not only for conducting detailed nutritional studies but will also be helpful in maintaining their stock cultures.

Surveying the nutritional capabilities of fungi is an endless task since every chemical found in living organism and a wide variety of manufactured and inorganic material are potentially useful for satisfying the need of fungus (Griffin, 1966).

Exhaustive physiological studies have not been undertaken so far on the present endophytic fungi, *viz.*, *Pestalotiopsis guipinii, Robillarda sessalis* and *Phomopsis mangiferae.* It was therefore, desirable to grow the above endophytic fungi in a number of synthetic culture media in order to select the most suitable basal medium for subsequent studies on the basis of their mycelial growth and sporulation.

The following synthetic culture media were employed-

1. Coon's Medium

Maltose	:	3.5 g
L- asparagine	:	0.25 g
KH_2PO_4	:	1.25 g
Mg $SO_4.7H_2O$	:	0.50 g
Distilled water	:	1000 ml

2. Elliott's Medium

D-glucose	:	5.0 g
L- asparagine	:	1.0 g
KH_2PO_4	:	1.36 g
Na_2CO_3	:	1.06 g
Mg $SO_4.7H_2O$	:	0.50 g
Distilled water	:	1000 ml

3. Richard's Medium

Sucrose	:	50.0 g
KNO_3	:	10.0 g
KH_2PO_4	:	5.0 g
Mg $SO_4.7H_2O$	:	2.5 g
$FeCl_3$	:	0.02 g
Distilled water	:	1000 ml

4. Czapek's Medium

Sucrose	:	30.0 g
$NaNO_3$	:	2.0 g
K_2HPO_4	:	1.0 g

KCl	:	0.5 g
Mg $SO_4.7H_2O$	:	0.5 g
$FeSO_4.7H_2O$	:	0.01 g
Distilled water	:	1000 ml

5. Asthana and Hawker's Medium 'A'

D-glucose	:	5.0 g
KNO_3	:	3.5 g
KH_2PO_4	:	1.75 g
Mg $SO_4.7H_2O$	:	0.75 g
Distilled water	:	1000 ml

6. Modified Asthana and Hawker's Medium 'A'

D-glucose	:	10.0 g
KNO_3	:	3.5 g
KH_2PO_4	:	1.75 g
Mg $SO_4.7H_2O$	:	0.75 g
Distilled water	:	1000 ml

The results obtained have been summarized in Table 9.1 and Fig.9.2.

A perusal of Table 9.1 reveals that Richard's medium supported best growth of *P. guipinii*. It was followed by Czapek's, Modified Asthana and Hawker's Medium 'A', Elliott's, Asthana and Hawker's Medium 'A' and Coon's media. The growth of *R. sessalis* was maximum on Richard's medium followed by Czapek's, Elliott's, Modified Asthana and Hawker's Medium 'A', Asthana and Hawker's Medium 'A' and Coon's media. In the case of *P. mangiferae* growth was also maximum on Richard's medium followed by Czapek's, Modified Asthana and Hawker's Medium 'A', Coon's, Asthana and Hawker's Medium 'A' and Elliott's media.

Out of the six synthetic media tried Richard's medium exhibited best growth of *P. guipinii, R. sessalis* and *P. mangiferae*. Excellent sporulation of all the three endophytic fungi under study was obtained in Czapek's and Asthana and Hawker's Medium 'A'. It varied from good to fair in all others media. At the end of incubation period pH of the media shifted towards acidic side in both the cases. On the contrary for *R. sessalis* pH of the Asthana and Hawker's Medium drifted towards the neutral side.

Complex sugars get hydrolysed during the process of autoclaving of the medium. Acidity of the medium facilitates this phenomenon more. Medium should not have its constituents in high concentration for general use (Leonian and Lilly, 1940). Cochrane (1958) also suggested use of more dilute media than those often used in the studies of morphology and reproduction. Czapek's and Richard's media possess sucrose while maltose was present in the Coon's medium as a source of carbon. In the rest three media viz. Elliott's, Modified Asthana and Hawker's Medium 'A', and Asthana and Hawker's Medium 'A', glucose was present as carbon source. A slight drift in pH of Richard's medium was noticed in case of *Pestalotiopsis* and *Phomopsis*. It became more acidic 4.7 from initial pH of 5.4 in case of Richard's medium.

Though, in Czapek's medium the amount of sucrose is considerably large yet it did not supported excellent sporulation as compared to Asthana and Hawker's Medium. It supported sufficient growth and good sporulation of all the endophytic fungi under study. Further, the medium is easy to handle with regards to the expected need for modifications and substitutions of its constituents. It was, therefore, decided to use Czapek's medium for all subsequent cultural studies.

Table 9.1- Average dry wt., sporulation and change in final pH of three endophytes recorded on different culture media after 15d of incubation

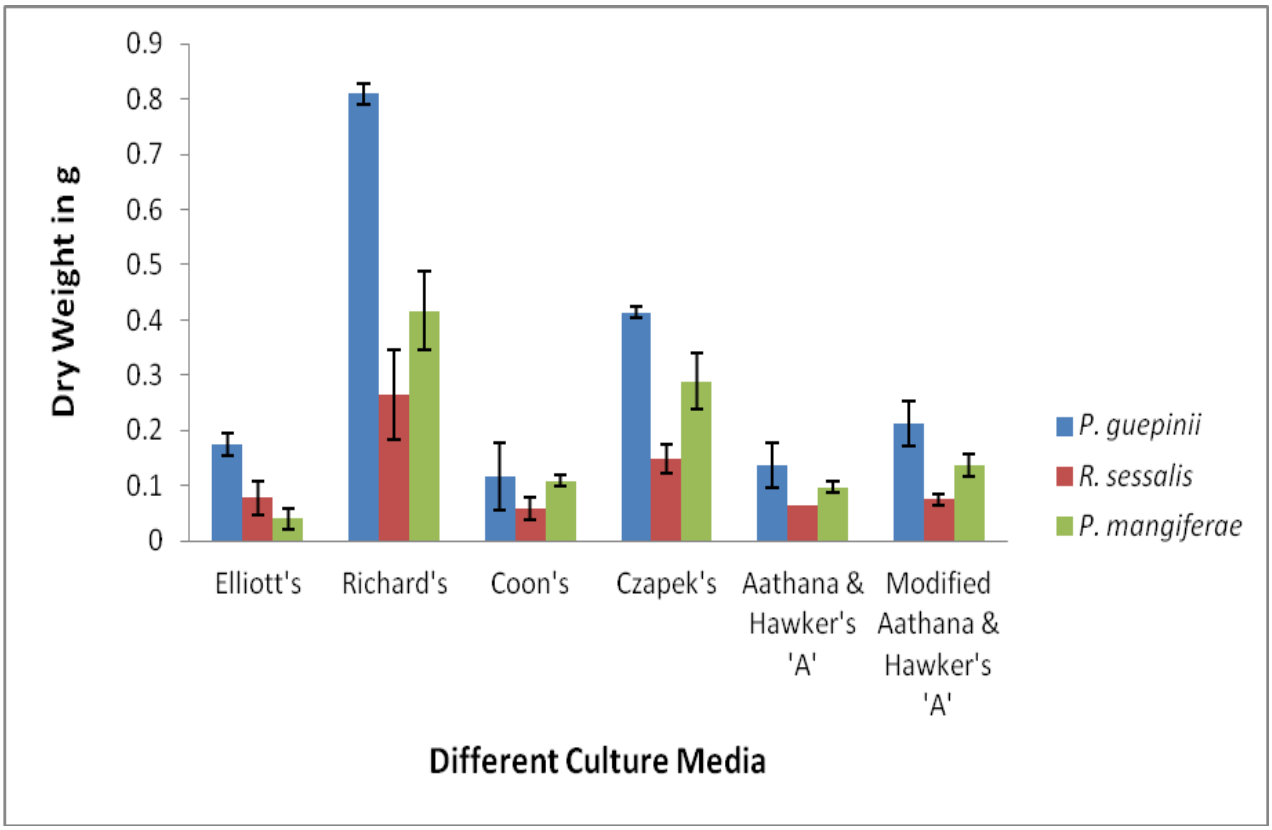
Different Culture Media	Initial pH	*Pestalotiopsis guepinii*			*Robillarda sessalis*			*Phomopsis mangiferae*		
		Dry Wt. (g)	Sporulation	Final pH	Dry Wt. (g)	Sporulation	FinalpH	Dry Wt. (g)	Sporulation	Final pH
Elliott's	7.3	0.174 ±0.02	+	6.2	0.078 ±0.03	+	6.2	0.040 ±0.02	+	6.7
Richard's	5.4	0.809 ±0.02	+	5.7	0.265 ±0.08	+	4.7	0.417 ±0.07	+	5.2
Coon's	5.7	0.117 ±0.06	++	5.6	0.059 ±0.02	+	4.5	0.109 ±0.01	++	5.6
Czapek's	7.5	0.414 ±0.01	+	6.3	0.149 0.025	++	6.6	0.289 ±0.05	++	6.2
Asthana & Hawker's medium 'A'	5.6	0.137 ±0.04	+++	5.7	0.065 ±0.00	+++	6.5	0.104 ±0.01	+++	6.1
Modified Asthana & Hawker's medium 'A'	6.1	0.212 ±0.04	++	5.9	0.076 ±0.01	++	5.2	0.136 ±0.02	++	7.1

Sporulation grade: - = Absent, + = Fair, ++ = Good, +++ = Excellent

Values reported are based on average of three replicates

Results were significant at $P \leq 0.05$ level by one way ANOVA

Fig. 9.2 Showing average dry weight of 3 endophytic fungi on different culture media



10. Influence of Different Hydrogen –Ion Concentrations

Growth and reproduction of fungi are known to be profoundly influenced by the H-ion concentration of the substrate. Fungi generally utilize substrates in the form of solution only if the reaction of solution conducive to fungal growth and metabolism Kiryu (1939). This brings out importance of suitable hydrogen ion concentration for better fungal growth. The pH of the host may play a determining role in the severity of infection in any disease. According to Midha and Chohan (1968) the incipient infection of *Gloeosporium psidii* in the young green guava fruits was due to interaction of pH with other factors. Similarly the damage caused by *Gloeosporium album* varied considerably on different varieties of apples. Cashmary (1946) reported that this was directly related with the varying acidity of the different varieties of the fruits.

The change in growth and sporulation patterns of an organism is accomplished by altered acidity of enzymes- the organic catalysts, or enzyme systems- governing various vital processes. According to Lilly and Barnett (1951) "The chemical changes in media due to alteration of pH, whether imposed from outside or caused by the fungus, affects metabolic processes. The pH of a culture medium changes during the growth of a fungus and these changes may affect the composition of the medium and thus the response of the fungus".

Microorganisms have a wide range of pH optima depending upon the group or species. Each is also having an optimum pH at which growth is at its highest. If the culture medium's environment pH is beyond the range, then denaturation can occur. Denaturation is a process when the proteins folding properties are destructed and that

usually leads to loss of biological activity due to the protein's active site no longer suitable for biological activity.

The pH or hydrogen ion concentration, [H+], of natural environment varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes. Some microorganisms are living at the extremes, as well as extreme point between the extreme. The range of pH over which an organism grows is defined by three cardinal points: the minimum pH, below which the organism cannot grow, the maximum above which the organism cannot grow, and the optimum pH, at which the organisms grow best. Microorganisms which grow at an optimum pH well below neutrality (7.0) are called acidophiles. Those which grow best at neutral pH are called neutrophiles and those grow best under alkaline conditions are called alkaliphiles.

The hydrogen ion concentration of the substrate influences not only mycelial growth and sporulation, but also processes like production of pigments, vitamins, antibiotics and other metabolites. It also affects permeability of protoplasmic membrane, uptake of minerals, entry of essential vitamins and organic acids into the cell, synthesis and stability of proteins and other life processes.

Growth of fungi usually takes place over a fairly wide range of hydrogen ion concentration, *Phyllosticta cycadina* (Bilgrami, 1956) grew between pH 3-9, *Lasiodiplodia* (*Botrydiplodia*) *ananassae* (Tandon and Bhargava, 1962) had a pH range of 2.5 to 11.0. Three isolates each of *B. theobromae* (Srivastava and Tandon, 1970) and *Phoma destructiva* (Grover *et al.*, 1971) grew between pH 3.5 and 8.5. However, a slightly narrow pH range (3.0 to 7.2) was observed for *Phoma herbarum* (Chung, 1967).

In general the pH range for sporulation was found some what narrower than that for growth (Cochrane, 1958). Hawker (1957) stated, even where fruit bodies were formed over a comparatively wide range of pH they may not produce viable spores over the whole range.

For many fungi a pH range of 5.5 to 6.5 has been found to be quite suitable for their maximum growth and sporulation. Similarly, the pH of most plants ranges between 5.0 to 6.5, which obviously favours the establishment of parasites in their hosts. Nevertheless, the minimum, optimum and maximum pH values for the growth and sporulation of individual fungi may very considerably.

The effect of the hydrogen and hydroxyl-ion concentration upon the growth of organisms, in general, has received consider- able attention in late years, but the effect of these ions upon the individual processes produced by an organism has not been so thoroughly studied. It has been repeatedly shown that certain fungi require an acid medium to produce maximum growth, while others require alkaline conditions. These diverse relations would seem to indicate a difference in the production, accumulation, and activation of the various enzymes concerned in the growth of these organisms.

In view of the above mentioned facts, it was considered desirable to study the effect of different pH of the medium on the growth and sporulation of the three endophytic fungi. The initial pH values of Czapek's medium were adjusted from 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0 and 8.0. The results obtained, are recorded in Table10.1 and Fig.10.2.

Results presented in Table 10.1 indicate that all the three endophytic fungi could not grow at lower pH *i.e*. 3.0, 3.5 and 4.0. It is also evident from the table that growth of *P. guepinii* was excellent at pH 5.0, good growth at pH 5.5, 6.0 and 7.0 and

poor at pH 8.0. The behavior of *P. mangiferae* was similar to *P. guepinii* at above pH values. *R. sessalis* grew from 4.5 to 8.0. Its growth was excellent at pH 7.0 whereas, its growth was poor at pH 4.5, 5.0, 5.5, and 8.0 and at pH 6.0 moderate growths was observed. According to Hawker (1950) most fungi grew best at neutral reaction that is at pH 7 or slightly towards acidic side. The same result was observed in case of *R. sessalis.* Lilly and Barnett (1951) were of the opinion that fungi generally tolerate more acid than alkali which was exactly true for the twenty plant pathogenic fungi reviewed by Tandon (1961).

All the three endophytic fungi failed to sporulate between pH 3.0 to 4.0. *P. guepinii* and *P. mangiferae* could not sporulate also at pH 4.5. *P. guepinii* produced excellent sporulation at 5.0 pH. It was good at pH 5.5 and fair at 6.0, 7.0 and 8.0 pH values. *R. sessalis* sporulated excellently at pH 7.0. Its sporulation was good at 5.5, 6.0 and 8.0 pH values while fair at pH 4.5 and 5.0. Excellent fruiting bodies of *P. mangiferae* were recorded at pH 5.0. Its sporulation was good at pH 5.5 while fair sporulation was recorded at pH 6.0 and 8.0. At the end of the incubation period the final pH of the medium drifted towards the neutrality.

The change in pH of the medium as a result of fungal metabolism is a common phenomenon. According to Lilly and Barnett (1951) "Four metabolic processes operate to change the pH of a culture medium: (1) utilization of cations, (2) utilizations of anions, (3) formation of acids from neutrals metabolites (especially Carbohydrates) and (4) formation of bases (especially ammonia) from amino acids and proteins. The net change in pH is the result of the interaction of all of these processes. The net change in pH is the result of the interaction of all these processes". The rise in pH of the culture medium has been attributed to the metabolic activities

during growth resulting in adsorption of anion or production of ammonia from nitrogenous compounds. Lowering of pH in case of media with higher initial pH was, possibly due to absorption of carbon dioxide produced by the fungus during the process of respiration (Singh, 1977).

It is clear from the above results that the best sporulation and maximum yield was recorded for *P. guepinii* and *P. mangiferae* at pH 5.0 and for *R. sessalis* at pH 7.0. Therefore, in all the subsequent studies the initial pH of the medium was adjusted to these pH values.

Table 10.1- Average dry wt., sporulation and final pH of 3 endophytes grown in Czapek's medium at different pH values

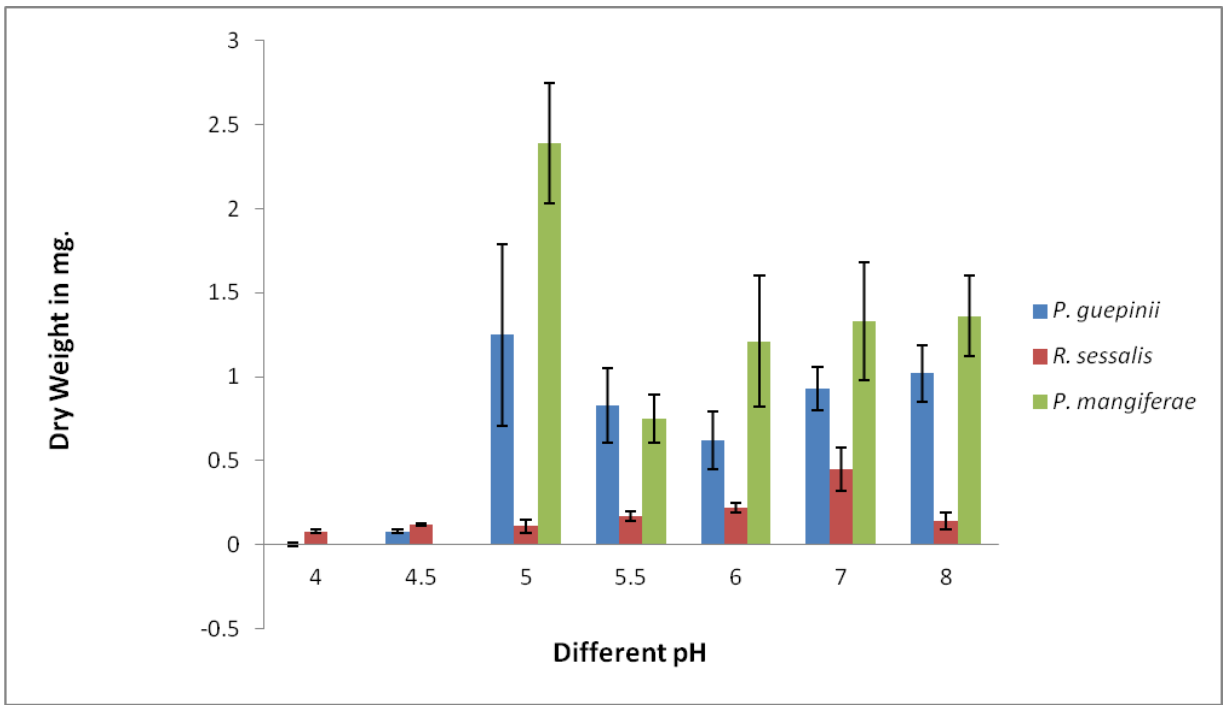
Sr. No.	Initial pH	*Pestalotiopsis guepinii*			*Robillarda sessalis*			*Phomopsis mangiferae*		
		Dry Wt. (g)	Sporulation	Final pH	Dry Wt. (g)	Sporulation	Final pH	Dry Wt (g)	Sporulation	Final pH
1.	3.0	0.00 ±0	-	3.0	0.00 ±0	-	3.2	0.00 ±0	-	5.5
2.	3.5	0.00 ±0	-	3.5	0.00 ±0	-	3.1	0.00 ±0	-	5.5
3.	4.0	0.00 ±0.01	-	4.0	0.08 ±0.01	-	3.5	0.00 ±0	-	6.0
4.	4.5	0.08 ±0.01	-	4.5	0.12 ±0.01	+	4.0	0.00 ±0	-	6.5
5.	5.0	1.25 ±0.54	+++	6.5	0.11 ±0.04	+	4.5	1.39 ±0.36	+++	5.5
6.	5.5	0.83 ±0.22	+	6.4	0.17 ±0.03	++	6.5	0.75 ±0.41	++	6.0
7.	6.0	0.62 ±0.17	+	6.6	0.22 ±0.03	++	7.5	1.21 ±0.39	+	7.0
8.	7.0	0.93 ±0.13	+	6.4	0.45 ±0.13	+++	7.2	1.33 ±0.35	+	6.5
9.	8.0	0.80 ±0.17	++	6.6	0.14 ±0.05	++	7.5	1.10 ±0.24	+	7.0

Sporulation grade: - = Absent, + = Fair, ++ = Good, +++ = Excellent

Values reported are based on average of three replicates

Results were significant at $P \leq 0.05$ level by one way ANOVA

Fig. 10.2 Showing average dry weight of 3 endophytic fungi at different pH



11. Influence of Different Temperatures

A variety of factors affect microbial growth and their sporulation. Temperature is one of the most important environmental factors, which play a significant role in governing various metabolic activities of fungi. Growth can be summarized as the net result of a large number of enzymatic reactions. Thus, the rate of growth would have a direct relationship with the rate of reactions, or to the sum rate of all reactions. It is well known that the reaction is a direct function of temperature. Hence, growth can also be related with temperature, with an increase in temperature, the growth rate will increase but falls abruptly at both the upper and the lower limits. The abrupt fall in the growth rate at high temperature is caused by the thermal denaturation of enzymes.

The range of temperature which favors mycelial growth varies considerably, depending upon the organism concerned. Moreover, in nature, under prevailing humidity conditions of hot or cold air and soil in which a crop and its parasites are growing may be decisive in determining the presence and severity of diseases. According to Deverall (1965), "Temperature affects all the fungal cellular activities and it also cause shift in metabolism".

Long back two American Scientists Wolf and Wolf (1947) have mentioned that only a few fungi were active at 42ºC. Usually fungi do not grow above 40ºC or below 0ºC. However, certain fungi are reported to grow even below the freezing point. Bidault (1921) as well as Brooks and Hansford (1923) reported that *Cladosporium herbarum* could grow slowly at a temperature as low as -6ºC and even feebly at -10ºC. Similarly Pehrson (1948) found that *Phacidium infestans* was capable of growing at -3ºC and the mycelium remained still viable after 138 days at -21ºC. Contrary to this certain organisms have been found to grow well at higher temperatures. Even

plants under snow may be infected by parasitic "snow mould"- *Fusarium nivale* (Dahl, 1934). La Touch (1948) observed the growth of the species of *Chaetomium* on straw at 62ºC. Its maximum development was recorded between 40ºC and 50ºC. However, fungal mycelium is easily killed by elevated temperatures and many fungi die slowly when held in culture at a temperature just above the maximum for growth. Thus, fungi are more tolerant to lower than to higher temperature since the latter coagulates cell proteins (Panasenko, 1967).

Temperature optima (one of the three cardinal points at which mycelial growth of a fungal colony is best) for plant pathogenic fungi have been collected by Togashi (1949). According to which most members have optima in the region between 20ºC and 30ºC and about a half have optima between 26ºC and 30ºC.

Optimum temperature for same fungus may vary on different media for example, *Diplodia natalensis* (Tandon, 1960; Lal and Pathak, 1970) showed maximum growth at 30ºC on PDA, while it was at 27.5ºC on corn meal agar medium (Fawcett, 1921). He further observed that optimum temperature for the average rate of growth of a fungus on the medium used was found to vary with the length of the period of the growth, and with the age of culture. The optimum temperature for second 24 h was found to be 36.5ºC.

Optimum temperature permitting growth may not be always similar to that permitting good sporulation. Cochrane (1958) concluded, "Only two generalizations can be formulated with respect to temperature and sporulation. First, the temperature range permitting growth. Second, each spore form has its own temperature optimum, which may or may not coincide with that of vegetative growth or of other spore forms of the same species".

The effect of small differences in temperature are often great enough to cause zones of intense sporulation altering with zones of sparse and no spore production in colonies growing

under conditions of fluctuating temperatures, as with *Ascochyta rabiei* (Hafiz, 1951).

From the above mentioned facts it is evident that before starting any physiological study, it is necessary to have a thorough knowledge about the temperature requirements of the organisms concerned. Moreover, this would also provide an idea about the environmental conditions which would be most suitable for propagation and survival of the pathogens in nature. Therefore, it was decided to determine the cardinal temperature, especially the optimum temperatures for the better growth and sporulation of *Pestalotiopsis guepinii*, *Phomopsis mangiferae* and *Robillarda sessalis* by dry weight method. Hence, different temperatures such as 5 ºC, 10 ºC, 15 ºC, 20 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC, and 50 ºC were maintained in BOD incubators and the results are summarized in Table 11.1and Fig.11.2.

An appraisal of the results clearly indicate that all the 3 endophytic fungi failed to grow at low temperature (5ºC) as well as high temperature (50ºC). They all could grow a temperature range between 10ºC and 45ºC. In *P. guepinii*, there was a very weak growth at 45ºC. Higher temperature like 35ºC supported moderate growth as compared to the lower temperature (10ºC). Best growth was obtained in the range of 20ºC to 35ºC in all the 3 endophytic species.

R. sessalis produced excellent growth at 30ºC to 35 ºC and good growth at 15 ºC to 25 ºC and it was poor at 10ºC, 40ºC and 45ºC.

The mycelia yield of *P. mangiferae* was excellent at 25ºC and good at 20ºC and 30ºC while it was moderate at 15ºC, 35ºC and 40ºC and poor at 10ºC and 45ºC. There is no growth was observed at 50ºC.

P. guepinii developed excellent growth at 25ºC just like the *P. mangiferae* and at 20ºC and 40ºC it produced good growth, while it showed moderate growth at 30ºC and 35ºC. It produced poor growth between 10, 15 and 45ºC.

Temperature range for the sporulation of the 3 endophytes varied considerably. Excellent sporulation was observed in case of *P. guepinii* and *P. mangiferae* at 25ºC whereas in case of *R. sessalis* it was at 30ºC.

The final pH of the medium showed a drift towards neutral side after 15 days of incubation. This drift was proportional to the growth rate. In the present study, both sub optimal and supra optimal temperatures support only slow growth.

Since the results are indicative of good vegetative and reproductive stages in *P. guepinii* and *P. mangiferae* at 25ºC, *R. sessalis* at 30ºC, it was selected for all subsequent experiments.

Table 11.1- Average dry weight, sporulation and final pH of 3 endophytes grown in Czapek's medium at different temperatures

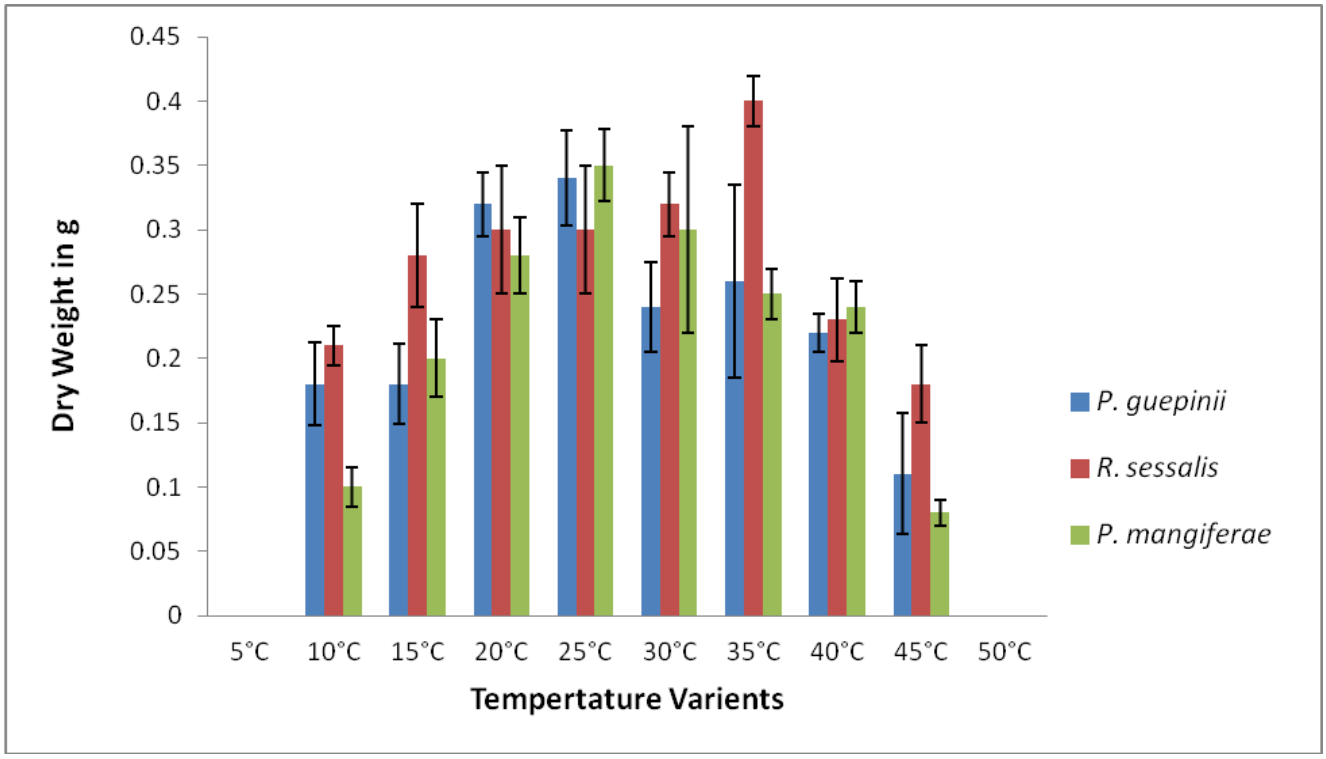
Sr. No.	Temperature in ºC	*Pestalotiopsis guepinii*			*Robillarda sessalis*			*Phomopsis mangiferae*		
		Dry Wt. (g)	Sporulation	Final pH	Dry Wt.(g)	Sporulation	Final pH	Dry Wt.(g)	Sporulation	Final pH
1.	5	00 ±0.0	-	5.2	00 ±0.0	-	7.0	00 ±0.0	-	5.3
2.	10	0.18 ±0.032	-	6.5	0.21 ±0.015	-	7.3	0.10 ±0.015	-	6.4
3.	15	0.18 ±0.031	+	6.2	0.28 ±0.04	-	7.0	0.20 ±0.030	-	6.0
4.	20	0.32 ±0.025	++	7.0	0.30 ±0.050	-	5.7	0.28 ±0.030	+	7.5
5.	25	0.34 ±0.037	+++	6.3	0.30 ±0.050	+	6.1	0.35 ±0.28	++	6.5
6.	30	0.24 ±0.035	++	7.4	0.32 ±0.025	++	6.0	0.30 ±0.08	+	7.3
7.	35	0.26 ±0.075	+	6.4	0.40 ±0.020	+	6.0	0.25 ±0.02	+	6.5
8.	40	0.22 ±0.015	+	6.1	0.23 ±0.032	+	5.9	0.24 ±0.02	+	6.2
9.	45	0.11 ±0.047	-	6.2	0.18 ±0.03	+	7.1	0.08 ±0.01	-	6.2
10.	50	00 ±0.0	-	5.8	00 ±0.0	-	7.0	00 ±0.0	-	5.4

Sporulation grade: - = Absent, + = Fair, ++ = Good, +++ = Excellent

Values reported are based on average of three replicates

Results were significant at $P \leq 0.05$ level by one way ANOVA

Fig. 11.2 Showing average dry weight (g) of 3 endophytic fungi at different temperatures



12. Influence of Different Carbon Sources

Amongst nutritional requirements carbon is of fundamental importance for fungal growth. Whittaker (1951) reported that dry mycelium of a fungus contains carbon between 40 and 50%. It serves two important purposes in the metabolism of fungi. Firstly, carbon compounds are used in the synthesis of cell walls, proteins, nucleic acids and reserve food materials etc. and secondly, they are the sources of CO_2 which is produced by their oxidation.

In nature carbon is mostly available in the complex form, but generally fungi convert them into simpler water soluble sugars of low molecular weight, before utilization. Fungi are so specific in utilization of different carbon sources, that a carbon source may be utilized by a fungus while another source of almost similar chemical nature may prove useless for it. Different workers have found dextrorotatory sugar more preferable than levorotatory of same type. Lilly and Barnett (1956) reported that *Sporobolomyces salmonicolor* could grow on D- arabinose but not on L-arabinose. Shreemali (1973) observed that not only different species but different isolates of same species also have specificity in their choice of carbon compounds. This difference may be due to the permeability of cell wall or due to the presence or absence of specific enzymes, necessary for the respiratory steps followed during the assimilation of that compound. Structural variation and configuration of molecules also affect their utilization (Lilly and Barnett, 1951).

Carbon compounds, other than carbohydrates, such as the higher alcohols, organic acids and organic nitrogen substances are not usually suitable for spore production. This is almost certainly owing either to the inability of the fungus to use them at all or, when they are used to the rapid development of unfavorable pH values in the medium," (Hawker, 1957). Mathur *et al.,* (1950) found that xylose produced

excellent sporulation in *Colletotrichum lindemuthianum*. On the contrary, Grewal (1957) observed that xylose not at all supported sporulation in *Gloeosporium musarum*.

As mentioned above, carbohydrates are the first choice of pathogenic fungi as chief source of carbon (Arya, 1982). Lilly and Barnett, (1951) clearly stated that "there is no single sugar which supports the maximum amount of growth for all these fungi and not all carbon sources are equally suitable for fruiting of fungi. Some which are favorable for mycelial growth do not favor sporulation". Papavizas and Ayers (1964) reported that glucose is preferred by many fungi than any other sugars. But Schade (1940) and Schade and Thimann (1940) reported that the organism like *Leptomitus lacteus* was unable to utilize glucose, fructose, galactose or sucrose.

Unless and until all conditions are absolutely similar comparative work is not possible in fungi because, they are highly sensitive to their environment. So any generalization in their behavior may be wrong in lack of sufficient data. In order to understand the physiology of the fungal organisms, knowledge of carbon nutrition is essential. It was, therefore, decided to study the influence of different carbon sources on the growth and sporulation of three endophytic fungi under investigation.

Following carbon sources were used and the results are recorded in Table 12.1and Fig.12.2.

Carbohydrate Sources

A Monosaccharides

(i) Pentoses ($C_5 H_{10}O_5$)

L- Arabinose, D-Xylose

(ii) Hexoses ($C_6H_{12}O_6$)

D glucose, Fructose

B Oligosaccharides

(i) Disaccharides ($C_{12}H_{22}O_{11}$. H_2O)

Sucrose, Maltose

(ii) Trisaccharide ($C_{18}H_{32}O_{16}$. $5H_2O$)

Raffinose

C Polysaccharide ($C_6H_{10}O_5)_n$

Soluble starch

Considering the different growth and sporulation patterns and the time required to deplete a particular sugar or polysaccharide an effort was made to study two pentose, two hexoses, two disaccharides, one trisaccharide and one polysaccharide.

Utilization of Monosaccharides

Monosaccharides usually are easily assimilable forms of carbohydrate, among which glucose has been reported to be the most efficient source of carbon and energy for most of the fungi (Bilgrami and Verma, 1978). Some fungi like *Polychytrium aggregatum* (Agello, 1948) exhibit exclusive choice for this sugar and fail to grow on other hexoses.

It is evident from the Table 12.1 that all the three endophytes were capable of growing on all the carbon sources used in the present study, but the amount of growth varied considerably upon different substrates. Carbon was found essential for the growth of three organisms since none of them could grow in its absence.

D-xylose (R_f 0.28)

D-xylose (a pentose sugar) was found to be a good source of carbon for the growth of *Phomopsis mangiferae*. It induced excellent sporulation of *P. mangiferae* with the growth rate of 2.2 g/ day. Due to the excellent mycelial growth this

endophyte also showed the maximum dry weight of 0.32g at pH 7.5. Xylose was not completely utilized by all 3 endophytes and its presence was detected up to 15 days on chromatograms. Although there are several reports on complete and fast utilization of this sugar. Arya (1982) reported that D-xylose was also preferred by two another species of *Phomopsis.* *P. gulabia* utilized it in five days in comparison to ten days by *P. viticola*. Cochrane (1958) has also concluded that amongst the pentose's xylose is generally the most utilizable sugar. On the contrary this carbon source could not support the good growth of *R. sessalis*. The sporulation was also poor. Another endophyte *P. guepinii* showed the moderate growth as well as sporulation in the presence of D-xylose.

L – arabinose (R_f 0.21)

Arabinose is an aldopentose – a monosaccharide containing five carbon atoms, and including an aldehyde (CHO) functional group. This aldopentose occurs in nature in the form of arabans, as common constituent of plant polysaccharides and various gums specially gum Arabica. L-arabinose is in fact more common than D-arabinose in nature and is found in nature as a component of biopolymers such as hemicellulose and pectin. Arabinose is utilized by several fungi but as a carbon source its value is generally inferior to both glucose and xylose (Lilly and Barnett, 1956). Moreover, it has been observed that some fungi distinguish between D- and L- enatiomorphs of this pentose. Steinberg (1942) found that *A. niger*, though grew well on L-arabinose, it did not grow at all on its D-isomer. Thus, generally it is the L-isomer which is more conducive to fungal growth. Arya (1991) also found that L-arabinose was preferred source of carbon for *Phomopsis viticola* and *P. sidii* than its D isomer. Due to the importance of this isomer, in present study it is taken as a one of the carbon source. It is evident from the Table 12.1 that L-arabinose is not completely utilized by all the

three endophytes upto 15th days. Both *P. mangiferae* and *P. guepinii* showed excellent mycelial growth and sporulation at the pH 7.6. The dry weight of fungal mass of both endophytes was measured upto 0.33g and 0.28g respectively.

D-glucose (R_f 0.18)

Glucose ($C_6H_{12}O_6$, also known as D-glucose, dextrose, or grape sugar) is a simple sugar (monosaccharide) and an important component of complex carbohydrates like starch and cellulose. This aldohexose is found in various parts of the plants. Sucrose, lactose, maltose, cellobiose and raffinose are made up of glucose moieties. Glycogen the most common reserve carbohydrate of fungi is composed of glucose units.

The Table 12.1 showed that among all the endophytes *P. mangiferae* showed the excellent mycelial growth as well as sporulation in the culture medium supplemented with glucose. There is a gradual increase in dry weight in culture medium having three endophytes. The dry weight of mycelia weighed 0.32g and final pH was 7.4. This result obtained for *P. mangiferae* was almost similar to that found in D-xylose. The dry weight of *R. sessalis* weighed 0.23g and final pH was 7.7. The fungal dry weight obtained for *P. guepinii* was 0.20g. The presence of glucose was detected up to 12 days only in broth medium containing *P. guepinii* while it could be detected up to 14 days in the broth medium of both *R. sessalis* and *P. mangiferae*. Irani and Ganpathy (1960) reported that mycelium of *Penicillium chrysogenum* exhibited consistent presence of glucose, which they attributed to the fact that glucose may be formed due to breakdown of polysaccharides and other sugars. Hasija and Wolf (1969) reported that in *A. niger* glucose was always present as a constituent of mycelium irrespective of the carbon source.

D-Fructose (R_f 0.23)

Fructose, or fruit sugar, is one of the three dietary monosaccharides, along with glucose and

galactose, that are absorbed directly into the blood stream during digestion. Fructose was discovered by French chemist Augustin-Pierre Dubrunfaut in 1847. Pure, dry fructose is a very sweet, white, odorless, crystalline solid and is the most water-soluble of all the sugars. From plant sources, fructose is found in honey, tree and vine fruits, flowers, berries and most root vegetables. In plants, fructose may be present as the monosaccharide and/or as a component of sucrose. Commercially, fructose is usually derived from sugar cane, sugar beets and corn and there are three commercially important forms. Crystalline fructose is the monosaccharide, dried and ground, and of high purity. High-fructose corn syrup (HFCS) is a mixture of glucose and fructose as monosaccharides. Sucrose is the third form. All forms of fructose, including fruits and juices, are commonly added to foods and drinks for palatability, taste enhancement and improved browning of some foods, such as baked goods.

Chemically fructose is a 6-carbon polyhydroxyketone. It is an isomer of glucose *i.e.* both have the same molecular formula ($C_6H_{12}O_6$) but they differ structurally. Crystalline fructose adopts a cyclic six-membered structure owing to the stability of its hemiketal and internal hydrogen-bonding. This form is formally called D-fructopyranose. In solution, fructose exists as an equilibrium mixture of 70% fructopyranose and about 22% fructofuranose, as well as small amounts of three other forms, including the acyclic structure.

The Table 12.1 indicates that all the three endophytes produced excellent amount of dry weight and mycelial growth in the culture medium having fructose as one of the carbon sources in comparison to glucose. The dry weight recorded for all

three *i.e. P. guepinii, R. sessalis* and *P. mangiferae* was 0.26, 0.28 and 0.37g respectively.

Utilization of Oligosaccharides

Sucrose (R_f 0.14)

Sucrose is commonly known as table sugar and sometimes called saccharose. A white, odorless, crystalline powder with a sweet taste is a disaccharide molecule derived from glucose and fructose with the molecular formula $C_{12}H_{22}O_{11.}$ Sucrose is a common oligosaccharide associated with higher plants particularly in fruits along with fructose and in stem of *Saccharum officinarum* (sugarcane) and in roots of *Beta vulgaris* (Beet root). In general it is a good source of carbon for fungi. Many workers while their studies related to fungal physiology have found that fungi are capable to hydrolyse sucrose in to glucose and fructose and thus this disaccharide is assimilated through a hydrolytic pathway. However, Mandels (1954) in *Myrotheicium verrucaria* reported that these organisms are able to consume this sugar through non-hydrolytic pathway.

It is evident from Table 12.1 that *P. guepinii* showed maximum growth in the culture medium containing sucrose as one of the carbon source. This endophyte not only shows the excellent mycelial growth of 0.43g but also an excellent sporulation. It was found to be a good source of carbon for the growth of *P. guepinii* among all the test endophytes. The breakdown of sucrose in to glucose could be detected up to 12 days while in to fructose upto 10 days only in the culture medium containing *P. guepinii*.

In case of *R. sessalis* sucrose was present up to 10 days, glucose up to 14 days and fructose could be detected up to 12-14 days. In the culture medium containing *P.*

mangiferae presence of sucrose was detected up to 10 days, glucose up to 2-14 days while fructose only up to 8 days. The final pH of the medium remained towards acidic side except in *R. sessalis* where it was 7.5 *i.e.* basic.

Maltose (R_f 0.40)

Also known as malt sugar, maltose is formed by uniting two units of glucose that provide the first link in a process that eventually results in the creation of starch. Adding in a third unit of glucose produces a sugar that is known as maltotriose, while further units make it possible to produce maltodextrins. The two glucose units are joined by an acetal oxygen bridge in the alpha orientation *i.e.* by α- 1,4 glucoside linkage. Maltose or malt sugar is the least common disaccharide in nature. It is present in germinating grain, in a small proportion in corn syrup, and forms on the partial hydrolysis of starch. It is a reducing sugar.

It does not usually occur in the free form in chlorophyllous plants, But this disaccharide is obtained as an intermediate product during the digestion of starch to glucose. It consists of two glucose units which are held together. Maltose is utilized by a majority of fungi through a hydrolytic pathway. It yields two molecules of glucose when hydrolysis is accomplished by the enzyme α glucosidase.

It is evident from Table 12.1 that the presence of this sugar was detected up to 15 days on chromatograms in all the culture mediums. It was also observed that mycelial dry weight increased up to 15 days of incubation period in case of *R. sessalis* while mycelial dry weight was more on 10^{th} day in case of *P. guepinii* and *P. mangiferae* as compared to 15^{th} day. Final pH in all the three cases remained acidic on 15^{th} day. Maltose exhibited best growth of *P. guepinii.* It produced maximum dry weight of 0.37g after 10 days as compared to other two endophytes.

Raffinose ($C_{18}H_{32}O_{16}$)

Raffinose is a trisaccharide composed of galactose, fructose, and glucose. It can be found in beans, cabbage, brussels sprouts, broccoli, asparagus, other vegetables, and whole grains. Raffinose can be hydrolyzed to D-galactose and sucrose by the enzyme α-galactosidase (α-GAL), an enzyme not found in the human digestive tract. α-GAL also hydrolyzes other α-galactosides such as stachyose, verbascose, and galactinol, if present. The enzyme does not cleave β-linked galactose, as in lactose. Raffinose has a melting point of 80°C and decomposes at 118°C.

The experiment showed that the *P. guepinii* produced dry weight of 1.43g at 10 days in the presence of this trisaccharide, which is the maximum from all the sugars. After 10 days there was a remarkable reduction in the dry weight. The final pH of the medium remained acidic throughout the study. In case of *R. sessalis* and *P. mangiferae* the increase in dry weight was also recorded up to 10 days and then up to 15 days it got reduced. The final pH was recorded basic for *R. sessalis,* whereas, was acidic in case of *P. mangiferae.*

Utilization of Polysaccharide

The compounds having polymeric structures consisting of large number of monosaccharide units- homopolysaccharides or comprising of two or more different types of sugar units are called heteropolysaccharide. Such carbohydrates are grouped as-

1) Structural polysaccharides, which are found mainly in cell walls and other extra protoplasmic inclusions.

2) The reserve or nutrient polysaccharides are stored in the plant tissue, to be used during the period of active metabolism. Cellulose, pectic substances and chitin are

structural polysaccharides, while starch, dextrin and glycogen are the common reserve polysaccharides. Polysaccharides are not preferable source of nutrition for fungi but they induce good growth and sporulation of various fungal organisms. The utilization of soluble starch was observed in the present investigation and results are summarized in Table 12.1 and Fig.12.2.

Starch

Starch consists of glucose residues joined through α glycosidic linkages and thus may be thought of consisting as of repeating units of Maltose. Starch consists of two types of molecules called amylase and amylopectin. Enzymatic hydrolysis of starch may be represented schematically a follows -

Starch → Dextrin → Maltose → D-glucose

It is evident from Table 12.1 that starch was depleted from medium of *P. guepinii* up to eight days, while it was consumed after eight days in *R. sessalis* and *P. mangiferae.* It was also observed that a gradual increase in dry weight was recorded in culture medium having endophytes *R. sessalis* and *P. mangiferae*. Interestingly all the 3 endophytes produced maximum mycelial dry weight. Final pH in all the three cases remained acidic on 15^{th} day.

Table-12.1 Average dry wt. (g.), growth rate (g/day), final pH and utilization of different monosaccharides, oligosaccharides and polysaccharides by 3 endophytes upto 15 days of incubation

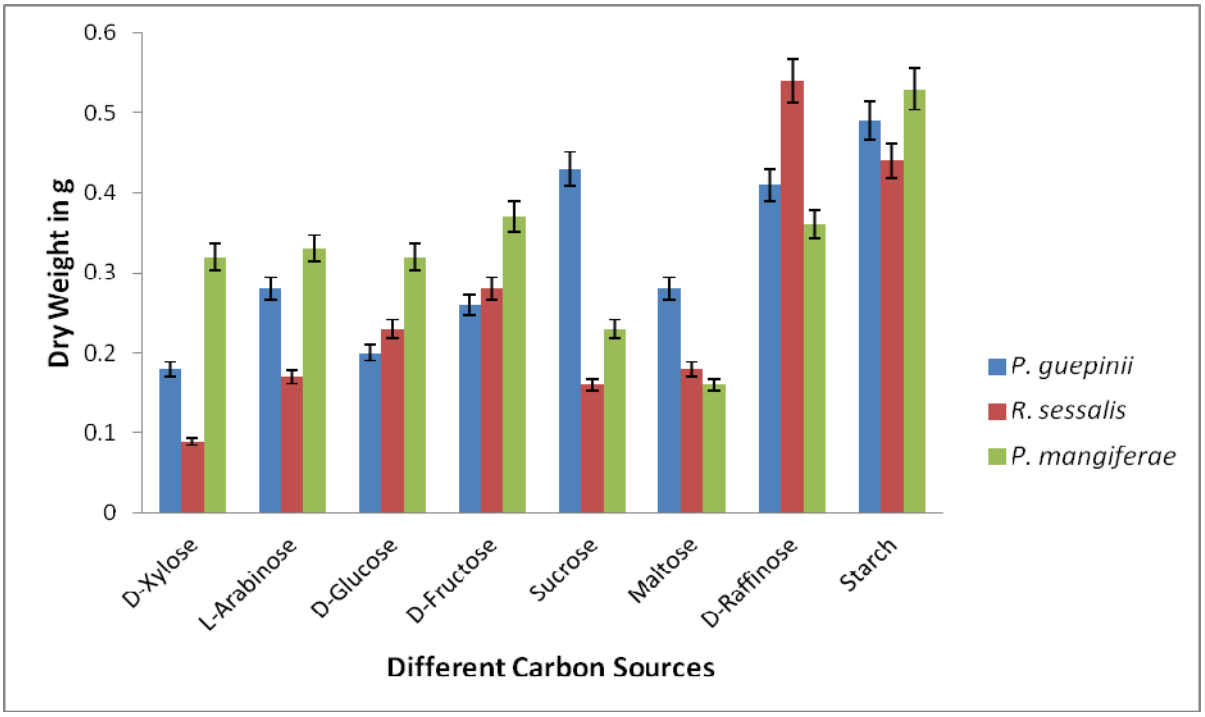
Monosaccharides	Days of incubation	*Pestalotiopsis guepinii*				*Robillarda sessalis*				*Phomopsis mangiferae*			
		Dry Wt. (g)	Rate of growth	Final pH	Presence	Dry Wt. (g)	Rate of growth	Final pH	Presence	Dry Wt. (g)	Rate of growth	Final pH	Presence
PENTOSES													
D-Xylose	5	0.11	.02	6.4	not utilized upto 15 d	0.10	.02	6.3	not utilized upto 15 d	0.09	0.02	5.4	not utilize dupto 15 d
	10	0.20	.02	7.1		0.12	.004	6.8		0.21	.024	7.1	
	15	0.18	-.004	6.6		0.09	-.006	6.2		0.32	.02	7.5	
L(+) Arabinose	5	0.16	.03	6.3		0.24	.05	6.0		0.19	.04	5.2	
	10	0.31	.035	7.0		0.14	-.02	6.4		0.34	.03	7.2	
	15	0.28	-.005	7.4		0.17	.006	6.2		0.33	-.002	7.6	
HEXOSES													
D- Glucose	5	0.16	.03	7.0	Up to 12^{th} d	0.08	.02	7.1	Upto 14 d	0.18	.04	6.3	Upto14d
	10	0.25	.02	6.9		0.14	.01	7.5		0.24	.03	6.7	
	15	0.20	-.01	7.4		0.23	.02	7.7		0.32	.02	7.4	
D- Fructose	5	0.09	.02	6.3	Up to 10d	0.06	.01	6.6	12-14^{th}d	0.08	.02	5.6	Only in 8^{th} d
	10	0.19	.05	6.5		0.13	.014	7.6		0.23	.03	7.1	
	15	0.26	.014	6.7		0.28	.03	7.7		0.37	.03	7.5	
DISACCHARIDES													
Sucrose	5	0.26	.05	6.6	Up to 12^{th}d glu-12, Fru-14	0.13	.03	7.1	Up to 10^{th}d glu-10, Fru-12	0.15	.03	5.6	Up to 10^{th}d glu-10, Fru-12
	10	0.37	.02	6.9		0.12	-.002	7.4		0.24	.02	5.8	
	15	0.43	.01	6.6		0.16	.008	7.5		0.23	-.002	5.7	

contd.

Maltose	5	0.10	.02	4.8	Up to 15thd	0.13	.03	6.3	Up to 15thd	0.12	.024	4.7	Up to 15thd
	10	0.37	.054	6.9		0.12	-.002	7.4		0.24	.024	5.8	
	15	0.28	-.02	6.5		0.18	.01	6.5		0.16	-.02	4.7	
TRISACCHARIDE													
D- Raffinose	5	0.26	.05	6.5	Up to 15thd	0.56	.112	7.6	Up to 15thd	0.26	.05	6.3	Up to 15thd
	10	1.43	.234	6.6		0.60	.01	7.6		0.40	.1	6.6	
	15	0.41	-.2	6.6		0.54	-.012	7.5		0.36	-.01	6.6	
POLYSACCCHARIDE													
Starch	5	0.44	.1	6.4	Up to 8th d	0.22	.044	6.4	Up to 15thd	0.34	.07	5.9	Up to 15thd
	10	0.50	.01	6.7		0.38	.03	6.7		0.48	.03	6.3	
	15	0.49	-.002	6.5		0.44	.012	6.7		0.53	.01	6.6	

The readings are based on the average of three replicates
Results were significant at $P \leq 0.05$ level by one way ANOVA

Fig. 12.2 Showing utilization of different carbon sources by 3 endophytic fungi



Summary and Conclusions

V. Summary and Conclusions

A: Pathological Studies

1. Survey of Orchards

Survey of various Outdoor Locations, Ratanmahal Wildlife Sanctuary (RWLS), Botanical garden and Arboratum of the M. S. University of Baroda, Orchards/ fields and different localities in and around Vadodara district were carried out. Vadodara district is located at 22°18′N 73°11′E22.30°N 73.19°E in western parts of India, Gujarat, at an elevation of 39 metres. It is the 3rd biggest city of Gujarat and 18th largest in whole of India with an area of 148.95 km². Collection of diseased as well as healthy, symptomless leaves of mango plants was done. Survey was carried out during the year 2006 to 2008.

2. Isolation and Identification of Leaf Infecting Fungi

The plant is affected by a number of diseases at all stages of its development, right from the seedling in the nursery to the harvesting of fruits. These may be further affected by pathogens in storage or transit. Mango leaves suffered with certain fungal pathogens like *Colletotrichum gloeosporiodes, Diplodia natalensis, Macrophoma mangiferae, Phoma glomerata* and *Botryodiplodia theobromae.*

In the present study total 17 leaf infecting fungi and one black sterile mycelium have been isolated from 4 different varieties of mango leaves. Out of these, fourteen organisms were of Fungi Imperfecti and three organisms belonged to Ascomycetes. Fungi like *Alternaria alternata* (Fr.) Keissler, *Lasiodiplodia theobromae* (Pat.) Griffon & Moube, *Curvularia lunata* (Wakker) Boedijin, *Drechslera hawaiiensis* (Bugnicourt) Subram. & Jain, *F. moniliforme* var.

subglutinans, *Gloeosporium mangiferae* Henn, *Nigrospora sphaerica* Sacc., *Phoma glomerata* (Corda) Woll and Hochapf. and *Thielavia subthermophila* Mouchacca. were frequently isolated from diseased mango leaves. Apart from these fungal pathogens several other fungi including *Acremonium acutatum* W. Gams., *Aspergillus nidulans* (Eidam) G., *Cladosporium cladosporoides* (Fres.) de Vries, *Colletotrichum gloeosporioides* Penz. & Sacc., *F. pallidoroseum* (Cooke) Sacc., *F. solani* (Mart.) Sacc., *Helminthosporium hawaiiensis* Bugnic., *Trichoderma viride* Pers. were also isolated along with them. The percentage colonizing frequency for each isolate was calculated. It was observed that the fungal pathogen mainly responsible for producing symptoms on leaves showed maximum percentage of colonizing frequency as compared to other associated fungi. Data revealed that out of 17 isolates *F. moniliforme* var. *subglutinans*, *Gloeosporium mangiferae* Henn, and *Lasiodiplodia theobromae* (Pat.) Griffon & Moube were the only pathogens isolated from each variety of all the locations and showing maximum colonizing frequency of 16.8, 27.7 and 35.0 respectively. It was also found that maximum 12 fungal pathogens were formed associated with mango leaves of Rajapuri variety as compared to other three varieties.

3. Occurrence of Endophytic Fungi on Different Plant Parts

An endophytic fungus lives in mycelial form in biological association with the living plant, at least for some time. Therefore, a minimal requirement before a fungus is termed an 'endophyte' should be the demonstration of its hyphae in living tissue.

The present study was undertaken to get the knowledge about the fungal endophytes obtained from living symptomless leaves, stem and bark tissues of four

varieties of mango. The isolation and characterization of fungal communities was intended to get an estimate of the diversity of the endophytic fungi from the economically important fruit crop mango.

The results show that the distribution of endophytic fungi present in various parts of the plant was not uniform. The leaf, stem and bark samples differed in their endophytic fungal colonization. Most species isolated during this study belong to the genera which have already been described as endophytes from different hosts at different locations. In addition, some of them are cited as pathogens of many hosts. A total of 23 different endophytic fungi representing 17 genera were obtained from healthy leaf, stem and bark segments of the mango plant. Of the total species isolated from different locations, 3.3% were belonging to Ascomycetes, and rest to Fungi Imperfecti. The population of Hyphomycetes was more, 80% Hyphomycetes and 13.3% Coelomycetes members were isolated. However, 11 isolates did not produced spores hence remained unidentified.

Leaf samples from each location harbored the greatest number of endophytes as compared to the stem and bark samples of the same location. Out of 23 endophytic fungi maximum 20 endophytes were isolated from leaves only.

The total nine isolates belonging to seven different genera were recovered from 980 stem pieces. The maximum 88.8% were recorded from Location 5 and minimum 55.5% from Locations 2 and 4. *Monascus ruber* with 45.1% colonization frequency was the dominant endophyte of stem. *F. pallidoroseum* showed the 19.6% colonizing frequency which was minimum among all.

It is evident that a Basidiomycetous member and *Pestalotiopsis guepinii* (Desm.) Steyaert. were isolated as an endophytes from bark pieces and these were not

observed colonizing other tissues of the mango plant. A fungal mycelium showed the clamp connection so it was identified as a member of Basidiomycetes. The results showed that the maximum 55% endophytes were recorded from bark tissues of the trees situated in Location 2 and minimum 30.5% from Location 5. No representative member from Zygomycotina was recorded in this experiment from any part of the host.

4. Histological Studies

Microtome sections of diseased leaves showed the presence of fungal hyphae in inter and intracellular spaces. It runs from one cell to adjacent cell through pits and also between the two cells (through middle lamella). Branching was also seen in fungal hyphae. Transverse section of leaves revealed the histological condition of infected leaves. A relatively thick cuticle covered the epidermis of infected leaves. The cuticle often extended into the radial walls of the adjacent epidermal cells. The mid rib epidermis is covered with a tick cuticle measuring 2.7- 3.6µm in thickness. Whereas, in healthy leaves cuticle measuring 2.0-2.5µm thickness covers the epidermis on both the sides of leaf. In healthy leaves sub epidermal region was occupied by 3-4 layered thick walled lignified sclereids while in diseased leaves such regular arrangement of sclereids was not found. Most of the cortical cells are filled with phenolic contents. In lamina region the palisade tissues were compactly arranged and short in length as compared to the palisade tissues of healthy plants.

5. Antagonistic Activity

The antagonistic activity of three endophytic fungi viz. *R. sessilis, P. mangiferae* and *P. guepinii* isolated from *M. indica* was examined using the dual culture method. In control Petri dishes (without pathogen) *P. mangiferae* and *P.*

guepinii grew at a faster rate and covered the whole Petri dish within 72h, whereas, *R. sessilis* showed comparatively a slower growth. It was found that *P. guepinii* was effective in suppressing the growth of *L. theobromae* and *F. moniliforme.*

F. moniliforme showed the highest percentage inhibition in radial growth in presence of *P. guepinii* followed by *R. sessilis* and *P. mangiferae.* The diameter of radial growth recorded was 33.3, 42.1 and 46 mm respectively on 5^{th} day.

The highest mycelial growth and less inhibition of *G. mangiferae* was favored by *R. sessilis i.e.* 54.7 mm (40% inhibition) and the lowest mycelial growth and high inhibition by *P. mangiferae* ie. 32.2 mm (64% inhibition) on 5^{th} d. The overlapping mycelial growth was seen in between *R. sessilis* and *L. theobromae,* which was higher as compared to others. From the results it can be concluded that the fast growing antagonist like *P. guepinii* caused more inhibition of pathogen than slow growing antagonists. It may be probably due to mycoparasitism and competition for nutrients.

6. Study of Aeromycoflora

Present investigation focuses on seasonal variation in outdoor fungal population of different mango growing places in the Vadodara city of Gujarat, India. The study was carried out during May 2008 to April 2009 by gravity fall method. During the present investigation it was observed that fungal population was not homogenous throughout the year and it varied from season to season and place to place. This may be due to high sensitivity of fungi to environmental factors such as high temperature. It was further observed that maximum 234 fungal colony forming units were observed in winter season, moderate in rainy season 167 and minimum number of fungal population *i.e.* 130 were recorded in summer season, from all the three localities. It was possibly due to unfavourable temperature and relative

humidity. It was also observed that out of total 24 fungi recorded, *Aspergillus flavus* and *A. niger* showed maximum percentage frequency.

7. Estimation of Flavonoid Contents

The current study was undertaken to estimate the flavonoid content in four different varieties of healthy and diseased mango leaves. The outcome of the Thin Layer Chromatography study based on the UV spectra resulted that all the four varieties of leaf showed variation in the peak. The healthy leaves of Rajapuri variety contained quercetin aglycon whereas in Kesar variety 3'-3 prime methoxy quercetin being the predominant compound along with quercetin. The diseased leaves of Rajapuri variety showed high concentration of all the compounds. In case of diseased leaves of Kesar variety peak for quercetin was not found but UV spectra showed very high concentration of Mangiferin. The Totapari variety also showed the same compounds in diseased mango leaves. The healthy leaves of Totapari variety contained 2'-quercetin, 3' 4' di methoxy quercetin, 3'o methoxy quercetin where as in diseased leaves quercetin was absent and very high amount of Mangiferin was present. Diseased leaves of desi variety of mango showed high concentration of quercetin and Mangiferin both, whereas, UV spectra of healthy leaves showed peak of quercetin.

8. Eco-Friendly Approach to Control Leaf Infecting Fungi

In the present study 10 different aqueous leaf extracts were used against three mango leaf spot fungi viz. *Fusarium moniliforme* var. *subglutinans* Woll. & Rein., *Gloeosporium mangiferae* Henn. and *Lasiodiplodia theobromae* Pat. Antifungal activity of aqueous leaf extracts of *Tylophora indica* (Burm f.) Merrill, *Rauwolfia tetraphylla* L., *Withania somnifera* (L.) Dunal, *Strychnos nux vomica* L., *Catharanthus roseus* (L.)

G. Don., *Calotropis procera* (Aiton) W.T. Aiton, *Ocimum sanctum* L., *Aloe vera* (L.) Burm.f., *Melia azadirachta* L., and *Adhatoda vesica* L. was assayed by poisoned food technique.

Obtained data revealed that the 10% aqueous extract of *S. nux-vomica* showed maximum inhibition of *F. moniliforme* (69%), whereas *C. procera* showed 52% activity, which is the second highest inhibition. Significant reduction in growth of *G. mangiferae* was observed with extracts of two medicinal plants and the extracts showed significant differences in their efficacy. Among all the ten plants extracts tried aqueous leaf extract of *M. azadirachta* showed remarkable inhibition (79%) of *G. mangiferae* at 10% concentration followed by *C. procera* at 10% concentration.

It was also observed that all the ten leaf extracts were less effective against *L. theobromae* as compared to other two pathogens. The maximum inhibition was shown by extract of *W. somnifera* (37%) for *L. theobromae* at 10% concentration. *In-vtir*o study revealed that *O. sanctum* and *A. vera* aqueous leaf extract showed very poor inhibition against all the three pathogens as compared to other plants extracts.

B: Physiological and Biochemical Studies

9. Selection of a Suitable Culture Media

Out of the six synthetic media tried, Richard's medium exhibited best growth and excellent sporulation all the three endophytic fungi under study was obtained in Asthana and Hawker's Medium 'A'. Though, in Czapek's medium the amount of sucrose is considerably large yet it did not supported excellent sporulation as compared to Asthana and Hawker's Medium but it supported sufficient growth and good sporulation of all the endophytic fungi under study. Further, the medium is easy to

handle with regards to the expected need for modifications and substitutions of its constituents. It was, therefore, decided to use Czapek's medium for all subsequent cultural studies.

10. Influence of Different Hydrogen –Ion Concentrations

Experiment on the effect of hydrogen ion concentration indicated that pH 5.0, 5.0 and 7.0 were best for both growth and sporulation of *P. guepinii*, *P. mangiferae* and *R. sessalis* respectively. Therefore, the initial pH of growth media in all further studies was adjusted at the above mentioned levels for the respective fungus.

11. Influence of Different Temperatures

An appraisal of the results clearly indicate that all the 3 endophytic fungi failed to grow at low temperature (5ºC) as well as high temperature (50ºC). They all could grow a temperature range between 10ºC and 45ºC. Since the results are indicative of good vegetative and reproductive stages in *P. guepinii* and *P. mangiferae* at 25ºC, *R. sessalis* at 30ºC, it was selected for all subsequent experiments.

12. Influence of Different Carbon Sources

Considering the different growth and sporulation patterns and the time required to deplete a particular sugar or polysaccharide an effort was made to study two pentose, two hexoses, two disaccharides, one trisaccharide and one polysaccharide. It is evident from the results that Xylose and L-arabinose were not completely utilized by all 3 endophytes and their presence were detected up to 15 days on chromatograms. Among all the endophytes *P. mangiferae* showed the excellent mycelial growth as well as sporulation in the culture medium supplemented

with glucose. *P. guepinii* showed maximum growth in the culture medium containing sucrose as one of the carbon source. It is evident from the results that presence of maltose sugar was detected up to 15 days on chromatograms in all the culture mediums. It was also observed that mycelial dry weight increased up to 15 days of incubation period in case of *R. sessalis* while mycelial dry weight was more on 10th day in case of *P. guepinii* and *P. mangiferae* as compared to 15th day. The starch was depleted from medium of *P. guepinii* up to eight days, while it was consumed after eight days in *R. sessalis* and *P. mangiferae.* It was also observed that a gradual increase in dry weight was recorded in culture medium having endophytes *R. sessalis* and *P. mangiferae.* Interestingly all the 3 endophytes produced maximum mycelial dry weight.

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*** Original not referred.**

Plates

Illustration of Photographs

PLATE I

Fig. A. Colony morphology of endophytic fungi isolated from leaves of *M. indica.*

Fig. B. Floccose, ochre mycelia of *R.sessalis* Sacc. showing black, round spores.

Fig.C. Competitive interactions between *P. mangiferae* and *G. mangiferae* showing deadlock with mycelial contact.

Fig. D. Partial replacement of *L. theobromae* by *P. guepinii* after initial deadlock at a diatance.

Fig. E & F. Petridish showing isolations of aeromycoflora having Totapuri and Kesar var. of mango on PDA.

PLATE II

Fig. A & B. Showing variation in the length of 4 varieties of mango leaves.

Fig. C. Leaves and fruit of Langda variety.

Fig. D. Leaves and fruit of Neelum variety.

Fig. E. Leaves and fruit of Totapari variety.

Fig. F. Leaves and fruit of Kesar variety.

PLATE III

Fig. A. Leaves of mango showing scab disease.

Fig. B. Mango leaf showing Anthracnose.

Fig. C. Mite attack on a mango leaf.

Fig. D. *Alternaria* leaf spot in mango leaf.

PLATE IV

Fig. A. Ascospores and cliestothecia of *Thelavia subthermophila* (400x)

Fig. B. Black coloured fungal conidia of *Nigrospora sphaerica* (1000x)

Fig. C. Fungal conidia of *Lasiodiplodia theobromae* (400x)

Fig. D. Fungal conidia of *Robillarda sessilis* (1000x)

Fig. E. Conidia of *Colletotrichum gloeosporoides* (400x)

Fig. F. Conidiophore and conidial head of *Aspergillus niger* (400x)

PLATE V

Fig. A. Conidia of *Gloeosporium mangiferae* (1000x)

Fig. B. Cleistothecia of *Monascus ruber* (400x)

Fig. C. Old conidia of *Pestalotiopsis guepinii* (1000x)

Fig. D. Sickle shaped macroconidia of *Fusarium moniliforme* (400x)

Fig. E. Picnidia of *Phoma glomerata* (400x)

Fig. F. Fungal spores of *Cladosporium cladosporoides* (1000x)

PLATE VI

Fig. A. Perithecia of *Chaetomium globosum* with ascospores (400x)

Fig. B. Conidia of *Curvularia lunata* (1000x)

Fig. C. Spores of *Epicoccum purpurascens* (400x)

Fig. D. Conidia of *Phomopsis mangiferae* (1000x)

Fig. E. Conidia of *Penicillium citrinum* (400x)

Fig. F. Conidia of *Alternaria alternata* (1000x)

PLATE VII

Fig. A. Conidia of *Acremonium acutatum* (1000x)

Fig. B. Conidia of *Drechslera hawaiiensis* (400x)

Fig. C. Conidiophore and conidial head of *Aspergillus nidulans* (400x)

Fig. D. Conidia of *Verticillium albo-atrum* (1000x)

Fig. E. Conidia of *Microascus cirrosus* (1000x)

Fig. F. Conidia of *Trichoderma piluliferum* (1000x)

PLATE VIII

Fig. A. Transverse section of leaf showing thick cuticle and sclereids.

Fig. B. Intercellular fungal hyphae between cortical cells.

Fig. C. In mid rib region parenchymatous cells shows presence of spores.

Fig. D. Xylem cells showing the presence of spores.

Fig. E. Resin duct filled with some secretory materials.

Fig. F. Degenerating cell wall of xylem vessels.

Fig. G. Enlarge view of vessels shows fungal mat.

Fig. H. Xylem parenchyma with mycelial growth within the cell.

PLATE IX

Effect of aqueous leaf extracts on three fingi.

Fig. A. Growth inhibition of *L. theobromae* in *Withania somnifera*.

Fig. B. Growth inhibition of *G. mangiferae* in *Calotropis procera*.

Fig. C. Growth inhibition of *F. moniliforme* in *Strychnos nux vomica.*

PLATE X

Plants selected for botanical control experiment in order to find a suitable biopesticide

Fig. A. Plant of *Rauwolfia tetraphylla* L.

Fig. B. Plant of *Aloe vera* (L.) Burm.f.

Fig. C. Climber of *Tylophora indica* Burm.f.

Fig. D. Plant of *Withania somnifera* (L.) Dunal,

Fig. E. Plant of *Catharanthus roseus* (L.) G. Don

Fig. F. Plant of *Calotropis procera* (Aiton) W.T. Aiton,

Appendices

List of Papers Published

1. **Occurrence of endophytic fungal flora from leaves, stem and bark of *Mangifera indica* L. from Vadodara (Gujarat).** In: Phytotechnology: emerging trends 2012.

2. **A New Record of *Pestalotiopsis guepinii* Causing Fruit Rot of Mango from Gujarat, India.** *J Mycol Pl Pathol.* 2010.

2. **Postharvest handling and eco-friendly management of diseases of *Litchi chinensis* (Gaertn.) Sonn.** In: Diseases of fruit trees: recent researches and eco-friendly management 2009.

3. **Fungitoxic effect of systemic resistance inducing protein (CA-SRIP) in ginger under storage by *Fusarial* rot.** *Indian Phytopathology* 2007.

List of Papers Presented in Seminars

1. **Prevention and control of fungal infections on ginger and chilli plants by using *Clerodendrum aculeatum*-systemic resistance inducing protein (CA-SRIP)-** National Conference on Biodiversity and Applied Biology of Plants, Lucknow University 8-10 October 2003.

2. **Endophytic mycoflora of the leaves of *Mangifera indica* L.-** National Symposium on Microbial Diversity and Plant Health Problems, Gorakhpur University 18- 19 December 2006.

3. **Occurrence of endophytic fungal flora from leaves, stem and bark of *Mangifera indica* L. from Vadodara (Gujarat)-** National Seminar on Emerging Areas in Plant Sciences, organized by Botany Dept. The M. S. University of Baroda, Vadodara 22 February 2009.

4. **Amla Cultivars-** Seminar on Amla: An indigenous Tree With Enormous Potentialities, organized by Botany Dept., MSU and Co-ordinated by State Medicinal Plants board, Gandhinagar 4th August 2009.

5. **Ecofriendly management of two fungal pathogens-** National Seminar on Air Pollution Management, organized by Botany Dept., M. S. University of Baroda, Vadodara 7 February 2010.

6. **Use of botanical pesticides for the control of three leaf spot fungi of Mango-** Fourth International Conference on Plants and Environmental Pollution, National Botanical Research Institute, Lucknow 8-12 December 2010**.**

7. **Growth performance and utilization of monosaccharides by three endophytic fungi-** 34th All India Botanical Conference, Lucknow University 10- 12 October 2011.

Papers Accepted for the Publication

1. **Seasonal Variation in Aeromycoflora of Different Places having Mango (*Mangifera indica* L.) Trees in Vadodara, India-** in Indian Journal of Aerobiology.

2. **Use of Botanical Pesticides for the Control of Three Leaf Spot Diseases of *Mangifera indica* L.-** in Proceedings of the National Academy of Sciences, India Section B: Biological Sciences.