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BIOCHEMICAL, IMMUNOLOGICAL AND PHYSIOLOGICAL STUDIES ON SOME PLANT PATHOGENIC FUNGI

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By

VAISHALI C. PAWAR

Registration No. 2998 dated 23rd August 2003

Department of Biosciences,
Saurashtra University
Rajkot – 360 005

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SAURASHTRA UNIVERSITY

DEPARTMENT OF BIOSCIENCES

University Campus
RAJKOT - 360 005
Gujarat (INDIA)
Tel : (O.) 2578501-12
Ext. : 409 / 410
Direct line : 2586419
Fax : 2577633/ 2586419

Ref. : Su / Bio /145

Date : 01/06/07

CERTIFICATE

This is to certify that the data embodied in this thesis entitled "BIOCHEMICAL, IMMUNOLOGICAL AND PHYSIOLOGICAL STUDIES ON SOME PLANT PATHOGENIC FUNGI" is an original piece of research work carried out by Ms. Vaishali C. Pawar in my laboratory and interpreted by her under my guidance at the Department of Biosciences, Saurashtra University, Rajkot.

It is further certified that she has put more than six terms of research work and that this work has not been submitted to any other University / Institute for the award of any degree.

(Vrinda S. Thaker)
Supervisor and Guide

Forwarded through

$\frac{1}{c}$ Head
Department of Biosciences
Saurashtra University
Rajkot – 360 005

Acknowledgements

(Vaishali C. Pawar)

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Plant pathogens are the organisms that cause diseases of plants. They exhibit a parasitic relationship with the plant and thus harm them. They typically weaken or destroy cells and tissues of plants, reduce or eliminate their ability to perform normal physiological functions, reduce plant growth and result in disease symptoms or may cause death. Fungi, bacteria, viruses, algae, nematodes, phanerogamic parasites and unfavorable environmental conditions are the most important agents of diseases in plants (Pandey & Trivedi, 1977).

A disease is any abnormal physiological or morphological condition that causes a marked disturbance in the plant as a whole, or any part of it and is induced by certain external agencies. Plant diseases result from the interaction of a pathogen with its host and vice versa. Both influence each other, the host providing nutrition to pathogen and the latter causing disease in the former. Pathogen influences environment through effects such as defoliation, addition of dead crop residue to soil, changing the host physiology and thereby, host root exudation, etc. (Chaube & Singh, 2000). Although these years extensive research on various aspects of plant pathology has contributed enormously to the development of agriculture in India, food crises caused by plant disease epidemics have resulted in starvation and displacement of millions of people (Mayee & Chakrabarty, 2005).

Plant disease is responsible for significant losses of global crop production every year, and thus has major impact on the world's agricultural productivity (Nölke *et al.*, 2004). The percent of crop losses caused by plant pathogens, insect pests and weeds worldwide has been estimated to 42%, accounting for 500 billion worth of damage (Oerke *et al.*, 1994). Out of this, global losses caused by pathogens alone are estimated to be 12% of the potential crop production.

Fungi are one of the agents causing diseases in plants. They are heterotrophic microorganisms having chlorophyll-less, nucleated, unicellular or multicellular filamentous thallus and reproduce by asexual and sexual spores. The body of a fungus is usually composed of distinctive elongated cells called "hyphae" which aggregate together to form a "mycelium". They have halophytic or absorptive type of nutrition. They produce extracellular enzymes to degrade insoluble substrates into smaller fragments and finally into soluble units which are then absorbed by hyphae (Chaube & Singh, 2000). They destroy many agricultural crops, fruits and nut plants. When a disease spreads in epidemic form, it wipes out the whole crop and causes severe losses (Pandey & Trivedi, 1977).

There are numerous diseases of cultivated plants, and each agricultural crop is generally subject to over 100 different diseases. Black or stem rust, brown or leaf rust, yellow or stripe rust and loose smut of wheat, powdery mildew, red rot of sugarcane, wilting, early and late blight of potato, onion smut, anthracnose of mango, etc. are the plant diseases caused by plant pathogenic fungi. Moreover, fungi contribute in the reaction of biodegradation, elucidation of complex life processes; their role is in contaminating the already threatened habitat by their toxins and their prospects in biotechnology and industry in which they have contributed more than any other group of organisms (Dube, 1990).

Taking these facts into consideration, in the present work, seven plant pathogenic fungi, isolated from the economically important plants of Gujarat and identified in the Gujarat Agriculture University, Junagadh, Gujarat, were studied. These fungi are listed in the table given below:

FUNGI	CROP
Aspergillus niger	*Allium cepa* (Onion)
Fusarium solani	*Cuminum cyminum* (Cumin)
Fusarium oxysporum f.sp *cicer*	*Cicer arietinum* (Chick pea)
Rhizoctonia bataticola	*Ricinus communis* (Castor)
Botryodiplodia theobromae	*Mangifera indica* (Mango)
Curvularia sp.	*Pennisetum typhoides* (Bajra)
Alternaria porri	*Allium sativum* (Garlic)

Aspergillus niger which is called a "weed of laboratory" was selected as a major plant pathogenic fungi for the study. The genus is widely distributed from arctic to tropical regions. The air and soil everywhere contains conidia of this organism (Alexopoulos & Mims, 1979). They cause considerable trouble as common contaminants of cultures in bacteriological and mycological laboratories. It is a fungus, which develops on seeds during storage and cause seed rot in the sown crop. It causes crown rot of groundnut seedlings, root rot of groundnut and black mould rot of mango (Mehrotra & Aggarwal, 2004).

Many *Aspergillus sp.* produce aflatoxins on various nuts and grains (peanut, corn and millet) (Alexopoulos & Mims, 2004) and cause spoilage of stored products, food – stuffs, including jams and jellies, salted meat, fish and leather. Some species are weak parasites causing diseases of fruits during storage and transit. The grains stored commercially below 13% of moisture content are attacked by these three species (*A. glaucus, A. halophilicus* and *A. restrictus*) of *Aspergillus* and are thus, called 'storage fungi' (Dube, 1990). It is also used in the manufacture of citric acid, gluconic and itaconic acids and many industrially important enzymes.

Fusarium (vascular pathogen) is a plant pathogen causing wilt and root rot diseases of several economically important plants (Dube, 1990; Alexopoulos & Mims, 2004). It is a facultative parasite occurring commonly in the soil as saprophyte. *Fusarium species* has been described as a causal agent of wilt and dieback of many important crops (Abdalla *et al.*, 2000, Polizzi & Vitale, 2003) and thus is a well known world wide pathogen (Armengol *et al.*, 2005). Many important plant diseases like, "damping-off" of seedlings, "root-rot" of various economically important vascular plants is due to different species of *Fusarium eg. F. udum* causes wilt of *Cajanus cajan*, *F. lini* causes wilt of *Linum usitatissimum*, *F. cubense* causes wilt of *Musa paradisiaca*, *F. vasinfectum* causes wilt of *Gossypium sp.*, *F. lycopersici* causes wilt of *Lycopersicon esculentum* (Pandey & Trivedi, 1977). Varieties of *F. oxysporum* also attack important crops such as sweet potatoes, corns, guava and jute. Mycelium of the fungus invades vascular tissue and along with conidia, physically blocks xylem vessels, prevents the translocation of water and ultimately results in wilting. Most of the species are parasitic causing leaf spot diseases. *Fusarium sp.* cause dry rot of potato, fusarium rot of muskmelon and tomato and also cause seed rot in maize (Mehrotra & Aggarwal, 2004). *F. oxysporum* f. sp. *cubense* is involved in causing the panama disease of banana (Mehrotra & Aggarwal, 2004).

Fusaric acid is produced by several species of *Fusarium* (Bacon *et al.*, 1996) causing wilt disease in tomato, cotton, pea, banana and other plants (Mehrotra & Aggarwal, 2004). It produces pectolytic enzymes which gives good correlation with wilt symptoms. They also produce toxins like zearalenone, which is a mycotoxin, thought to contribute to wilting by affecting the permeability of cell membranes and disrupting cell metabolism (Dube, 1990; Alexopoulos & Mims, 2004). Fumonisins (toxins) are produced on corn by *F. moniliforme* (Alexopoulos & Mims, 2004).

Several *Fusarium species* are widespread pathogens on small grain cereals around the world and can cause root, stem and ear rot and produce mycotoxins in preharvest infected plants and in stored grains, resulting in severe reductions in crop yield (Bottalico & Perrone, 2002). *Fusarium* head blight has received significant attention in recent years (Brennan *et al*., 2003) because of the impact that infection may have on yield (Doohan *et al*., 1999), grain quality (Liggitt *et al*., 1997), mycotoxin contamination of grain (Visconti *et al*., 2000) and the lack of preharvest fungicides for disease control (Doohan *et al*., 1999; Simpson *et al*., 2001).

Rhizoctonia species are commonly found in soil causing root-rot and "damping-off" disease of several economically important plants. It grows as vigorous saprobe in soil (Dube, 1990). *Rhizoctonia bataticola* is a soil borne pathogen, causative agent of soft rot of many plant species and also cause wilt of jute and linseed. It cause root rot of cotton in Gujarat (Mehrotra & Aggarwal, 2004).

It can live saprophytically in the soil on organic matter and can infect several other crops, such as groundnut, cowpea and several leguminous plants, oil seed and vegetable crops on which they are also pathogenic. *Rhizoctonia solani* is capable of attacking a tremendous range of host plants throughout the world, causing a variety of diseases including root rots, cankers, damping off, fruit decay and even foliage disease and produces no spores (Alexopoulos & Mims, 1979).

Botryodiplodia species cause fungal leaf spots (Blight). *Botryodiplodia theobromae* is responsible for causing stem end rot of mango, botryodiplodia rot of guava and also cause seed rot in pearl millet (Mehrotra & Aggarwal, 2004). It is the most common species, which causes fruit rot of mango, cacao, etc. (Dube, 1990).

Curvularia species are common leaf spot fungi and also occur in soil, degrade cellulose and damage textiles (Dube, 1990). *Curvularia* are facultative pathogens of soil, plants and cereals in tropical or subtopical areas, while the remaining few are found in temperate zones and also cause seed rot (Mehrotra & Aggarwal, 2004).

Alternaria species are parasitic causing leaf spot diseases. *Alternaria solani* causes "early-blight" of potato and other members of solanaceae. The characteristic "target-board" like leaf spots, show distinct concentric bands of light and dark colors. Several form species are found as saprobes on dead and dying plant parts in the soil as well as they are also parasitic on plants (Alexopoulos & Mims, 1979). *Alternaria species* causes alternaria blight of mustard, alternaria rot of grapes and muskmelons, brown rot of cauliflower and also causes fungal leaf spots (Blight). *Alternaria solani* causes early blight rot of tomato (Mehrotra & Aggarwal, 2004). Some species of *Alternaria* produce a variety of mycotoxins, including *A. alternata,* which produces 'Black spot' as discoloration of cereal grains (Alexopoulos & Mims, 2004). Some of the species are parasitic on higher plants. "Early blight of potato" is the quite common disease of potato in India, caused by *A. solani*. *A. tenius*, causes "black point disease" of wheat grains. *A. brassicae* is responsible for "leaf spot of crucifers" such as mustard, cabbage, cauliflower, etc. Saprophytic species of *Alternaria*, are of common occurrence on dead and decaying plant parts and in soil (Pandey & Trivedi, 1977).

The history of crop protection has shown two shifts of focus. From the 1st half to 2nd half of the century the attention shifted from pathogen to pathosystem, and at present we are witnessing a further shift to a focus on the whole production system (Miflin, 2000). The world population is expected to reach 7 billion within 25 years, over 10 billion in the year 2050, while agricultural production is growing at the slower rate of about 1.8% annually.

The overwhelming economic losses as well as losses of human lives as a result of plant diseases made the applied branch of agriculture all the way more important (Mayee & Chakrabarty, 2005). So, crop protection is now seen as just one activity among many in agricultural production systems and improvement of crop protection is no longer seen as separate from goals such as maximizing yields and minimizing inputs (Rabbinge & Oijen van, 1997).

Great progress is made in protecting many important plants from attack of fungi by using different cultural practices, disease-resistant varieties and chemical treatment as well as biocontrol methods. Modern agriculture being cost oriented requires greater vigilance to ensure stable and good quality yields and for reducing expenditure on all types of inputs including chemicals for disease management (Mehrotra & Aggarwal, 2004).

Fungicides, defined as a chemical substance which has ability to prevent damage caused by fungi to plants and their products, are used primarily on susceptible crops of high value where losses due to diseases are likely to exceed costs of application. Fungicides alone account for about 18% (approx 2.8×10^9 U.S. dollars) of the total world market of the pesticides (Chaube & Singh, 2000).

Due to the use of such chemical fungicides, the fungal strains are developing resistance. Development of resistant strains of target fungi is now one of the major problems with selective fungicides. It causes unexpected crop losses for the growers and may put him in difficult position if no adequate substitutes are available. It may reduce the profile of manufacturer, which has developed the fungicide at high cost. Apart from this, the use of chemical agents has caused serious environmental hazards and hence its use is dramatically declining (Alexopoulos & Mims, 2004).

One of the areas for concern in plant pathology is to find innovative ways of controlling fungal diseases without using chemicals. One of the challenges in plant biotechnology improvement is the intensified integration of biological control via the use of selected and engineered microorganisms with a biocontrol potential. One of the criteria for the biotechnological development of relationships between plants and microorganisms include biological control of pests, biofertilization and plant growth stimulation and bio and phytoremediation (Altman, 1999). There is a continued need to protect crops from pests and diseases of a diverse and unpredictably changing nature. Fortunately, biological research is at a stage where there are tremendous advances being made in our understanding of organisms and these provide opportunities for enhancing the technologies available for crop improvement.

Biological control of plant pathogens accomplished through host resistance and cultural practices has been worked out since decades and continues to be a predominant disease control strategy. It can be achieved by reducing the inoculum density, displacing pathogen from the host residues, suppression of germination and growth of pathogen and stimulating resistance response of the host (Chaube & Singh, 2000; Mehrotra & Aggarwal, 2004).

Research related to biological control is published in many different scientific journals, particularly those related to plant pathology and entomology (Pal & Gardener, 2006). The biological control of plant pathogens is of paramount importance nowadays, since the conventional use of chemical pesticides has been seriously questioned because of environmental and human health hazard (Spurrier, 1990; Mendgen *et al.*, 1992). By the sub-lethal doses of a number of fungicides, mycotoxin production from *Fusarium* phytopathogens have been increased (Felix D'Mello *et al.*, 1998).

Effective control of silver scurf (*Helminthosporium solani*) of potato tuber through the use of bioagents has been reported (Mehrotra & Aggarwal, 2004). The use of microorganisms for biological purposes has become an effective alternative to control plant pathogens. There are many examples of formulations using bacterial or fungal strains with biocontrol applications (Bernal *et al*., 2002). Much of the research has been carried out for the plant based antimicrobial agents where different parts of the plants are used for the purpose (Ali *et al*., 1999; Bhosale *et al*., 1999; Binns *et al*., 2000; Ranganathan & Balajee, 2000; Kariba & Houghton, 2001; Srinivasan *et al*., 2001). Plants also produce volatile compounds, responsible for their essence or odors, which are called essential oils (Nakajima *et al*., 2005).

Essential oils (EOs) are the subtle, volatile liquids or resins from plants, shrubs, flowers, trees, roots, bushes and seeds. They are antibacterial, anti-inflammatory, antiviral, antifungal and are powerful stimulants in cellular processes. The plants create these oils to help protect them from fungus and bacteria, attract pollinators and help to heal wounds in their bark. EOs are made up of many different volatile compounds and the make up of the oil quite often varies between species. It seems that the anti-fungal and anti-microbial effects are the result of many compounds acting synergistically. This means that the individual components by themselves are not as effective.

These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure, i.e. terpenes. Terpenes or terpenoids are active against fungi (Rana *et al*., 1997). EOs are modified fatty acids, aldehydes, hydrocarbons, esters, volatile alcohols, alkaloids and other shikimic acid derivatives (Nakajima *et al*., 2005).

Many EOs have been evaluated for antifungal activities from Palmarosa, Citronella and Lemongrass. The oils were found to be more potent as compared to synthetic fungicides. Antibacterial and antifungal effects of some essential oils have been previously reported (Njenga *et al*., 2005). Quite a lot of preliminary work has been done to demonstrate the potential of essential oils for use against postharvest pathogens. One group of researchers tested the antifungal activity of a range of essential oils against *Botrytis cinerea. Botrytis* is the main postharvest pathogen of fresh grapes. Their work showed that essential oils from red Thyme (*Thymus vulgaris*), Clove buds (*Eugenia caryophyllata*) and Cinnamon leaf (Cinnamomum *zeylanicum*) prevented the growth of *Botrytis cinerea*. Other researchers have shown that the essential oil of *Monarda citrodora* and *Melaleuca alternifolia* also exhibit antifungal activity against a wide range of common postharvest pathogens.

Fungistatis have been attributed to the involvement of chemical substances of non-volatile and volatile nature of either biotic or abiotic origin. The sclerotia of *Sclerotium cepivorum* were freed from fungistatis by volatiles (allyl cystein sulphoxides) associated with the roots of *Allium species*. Similarly, the volatile compounds like alcohols and aldehydes with low molecular weights released from plant residues were also found to be stimulatory to soil fungi (Chaube & Singh, 2000). Though many EOs have been extensively investigated for their activity against a number of storage fungi, plant pathogens, bacteria and other harmful microorganisms, several EOs against various phytopathogenic fungi remains to be investigated.

The aim is to control the plant disease in order to prevent the economic loss and to increase the economic value of the crop. Control of plant diseases covers a broad, highly technical and rapidly developing field of study. Control measures can be directed against the inoculum of the pathogen or it can be achieved by improving the power of resistance of the plants to infection of pathogenic organisms (Pandey & Trivedi, 1977).

By exploiting antibody engineering and phage display (Winter & Milstein, 1991; Winter *et al*., 1994), it is possible to isolate antibodies that being with high specificity to crucial proteins for pathogenesis resulting in a level of immunity or resistance to the pathogen. The basis of antibody-based resistance is the neutralization of invading pathogens through interactions between high-affinity antibodies and critical pathogen proteins, thus preventing pathogen entry, replication and systemic spread (Nölke *et al*., 2004).

The first plant-derived antibody, a full-length IgG-recognizing phosphonate ester, was produced in transgenic tobacco plant about 15 years ago (Hiatt *et al*., 1989; Düring *et al*., 1990). Antibodies have been used to detect various plant pathogens. Five monoclonal Abs were produced against the *Streptococcus pneumococcal* surface adhesion A (PsaA) 37-kDa common cell wall protein (Crook *et al*., 1998).

Fungal pathogens are the most challenging target for antibody-based resistance because they affect crops in two ways: by destroying plants and seeds in the field and by contaminating the harvested crop with fungal toxins (Nölke *et al*., 2004). During infection, invasive mycelia spread throughout the host plant, secreting enzymes and toxins that are essential for pathogenesis and parasitization. These proteins and toxins are suitable targets for recombinant antibodies and if effective antibody-based strategies could be developed then environmental pollution caused by the extensive use of fungicides could be avoided. The flexibility and specificity of antibodies is a well-known aspect of the vertebrate immune system and the use of antibodies to generate disease resistant plants is therefore an attractive approach (Nölke *et al*., 2004).

The modern trend of research tends to disclose, the inhibition of plant pathogens, the mechanism of fungal toxicity and the importance of extracellular enzymes in the etiology of the disease (Mehrotra & Aggarwal, 2004). Fungi are widely used by man in various kinds of industries. Fungus products, produced commercially, include alcoholic beverages, cheese, organic acids, enzymes, hormones, pigments, vitamins and alkaloids (Pandey & Trivedi, 1977). Fungi have been used commercially to produce variety of chemical compounds including ergosterol, cortisone, various enzymes like α-amylase, rennin, cellulase, catalase, lactase and lipase, acids like fumaric acid, lactic acid, citric acid, succinic acid, oxalic acid, vitamin B and plant growth regulators. For the mass production of a particular enzyme at industrial scale requires high enzyme producing strain, media development, process development and characterization. Carbon sources play an important role in the production of enzymes in higher or lower quantity. So to study different carbon sources in a media different from normal fungal media, changes in pH and its influence on fungal growth and development is necessary.

The growing plant cell wall is a polymeric structure, consisting of crystalline cellulose microfibrils coated with hemicellulose chains which are embedded in a highly hydrated pectin matrix with small amounts of protein (Nicol & Höfte, 1998). One of the mechanisms by which pathogen penetrates into the host is secretion of enzymes which play an important role in weakening the cuticle and in digesting pectin and cellulose (Chaube & Singh, 2000). The importance of cell wall degrading enzymes (CWDE) to the pathogenicity (the ability to cause disease) or virulence (the level of disease induced) of phytopathogenic fungi is not clear. Studies attempting to correlate CWDE production to virulence or pathogenicity in phytopathogenic fungi have been complicated by the multiple isozymes of each CWDE produced by most fungi.

Microbial pathogens of plants secrete enzymes capable of degrading the polysaccharides of plant cell walls. Several fungal pathogens secrete degradative enzymes in a temporal sequence with regard to culture age (Albersheim & Valent, 1974). The study of these enzymes may help in increasing knowledge of mechanism which facilitates the fungi to enter and infect the host.

Moreover, one of the important tasks in plant pathology is the early detection of infectious stages in plants so that control measures can be applied readily. Some frontline areas of research in plant pathology are the molecular biology of disease resistance, plant disease epidemic modeling, forecasting and modern methods of disease control including biocontrol of plant pathogens (Mehrotra & Aggarwal, 2004). Thus detection of plant diseases is important due to large-scale adoption of new agricultural strategies for increasing food production.

Control of plant diseases covers a broad, highly technical and rapidly developing field of study. So the present work is intended to control the plant disease causing fungi, *in vitro*, by using certain biocontrol agents like essential oils and antibodies. The other important aspect considered for the study is to evaluate enzymatic potential of one of the plant pathogenic fungi and its role in pathogenecity. Early detection of pathogen infection is also aimed as a part of this work.

OBJECTIVES

Considering the aforesaid following objectives were designed and worked out for this thesis:

(I)   To collect the fungi infecting major agricultural crops

(II)   To maintain these cultures on Potato Dextrose Agar media at 25 - 28 ^{o}C and study its growth on a defined media.

(III)   To evaluate the *in vitro* efficacy of essential oils on the selected plant pathogenic fungi

    a. Study of 75 EOs against three phytopathogenic fungi

    b. Study of 10 most potent EOs against all the test fungi

    c. GC-MS analysis of EOs

(IV)   Enzymatic study

    a. Studies on optimization of fungal growth

    b. Standardization of extracellular, cytoplasmic and wall bound enzymes in culture media with different sucrose concentrations during entire period of *A. niger* growth and development.

    c. Changes in sugar and protein content during the entire growth period of *A. niger*

(V)   Immunological studies

    a. Raising of antibodies against fungi

        • Preparation of antigen

        • Immunization

        • Collection of antiserum

        • Separation and purification of IgG

    b. Standardization of media for liquid assay and solid assay

(VI)   To study a non-destructive method, GDV (Gas Discharge Visualization) technique, for detecting fungal infection

CHAPTER 2

SCREENING OF ANTIFUNGAL ESSENTIAL OILS

INTRODUCTION TO ESSENTIAL OILS

India is one of the few countries in the world having varied agroclimatic zones suitable for the cultivation of a host of Essential Oils (EOs) bearing plants. The growing awareness of environmental hazards from commercial pesticides and associated problems of pest resistance, detrimental effects on non-target organisms, dictate the need for safe, effective and economical pesticide. Looking into the deleterious effects of synthetic pesticides on the life supporting systems, there is an urgent need to use alternative agents for pest control, using the sources that are harmless. Plants have been an essential part of human society since the dawn of civilization. It is a well-known fact that plant products are less toxic, more systematic, host metabolism stimulatory, easily biodegradable, ecologically safe and non-pollutive (Singh, 1997).

During the recent years, EOs obtained from higher plants has proved usefulness in controlling post harvest fungal diseases and these are considered to have a bright future (Singh, 1997). Higher plants have been used for centuries to cure human diseases. The essence and perfumery produced by aromatic plants have significant aesthetic value in day-to-day life. So, the scientists have been encouraged to screen these plants for various biological activities. EOs and their constituents extracted from aromatic and medicinal plants have also been found to possess medicinal and pharmacological values (Singh, 1997). Recently various workers have successfully tested volatiles of some higher plants obtained in the form of essential oils against a number of storage fungi and insects. Further, it is also heartening that some of these oils have been found more efficacious than some of the prevalent synthetic pesticides (Singh, 1997). This growing interest in plant-originated substances over the last few decades has brought essential oils to the forefront of the international trade. Essential oils are created and stored in specialized plant structures such as secretory cells, glands, glandular hairs and oil or resin ducts, and they are always segregated from other plant tissues within these areas (Keville & Green, 1995).

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The secretory cells that produce the EOs trap the photoelectromagnetic energy of the sun and, with the help of glucose, convert it into biochemical energy in the form of aromatic molecules. In a process similar to photosynthesis, plants create EOs by trapping and transmitting light and energy (Keville & Green, 1995). Essential oils are produced in various parts of plants. For instance, they are found in fruit peels (all Citrus), gums and resins (Frankincense and Myrrh), flowers (Rose and Lavender), leaves (Sage, Lemon balm, Geranium and Peppermint), barks (Cinnamon and Sassafras), roots (Vetiver and Valerian), grasses (Lemongrass and Palmarosa), rhizomes (Ginger) and seeds (Fennel, Anise, Cumin, Celery, Dill and Coriander). The Citrus *species Citrus bigaradia*, commonly known as bitter orange, provides three different essential oils: petitgrain from the leaf, neroli from the blossom, and bitter orange from the fruit peel (Keville & Green, 1995). The latter is popularly applied because EOs represent the essence or odoriferous constituents of the plant. Depending on the plant family, EOs may occur in specialized secretory structure such as glandular hairs (Labiateae) modified parenchyma cells (Piperaceae) oils tubes called vittae (Umbelliferae) or in lysigenous or syzoginous passages (Pinaceae and Rutaceae). They may be formed directly by the protoplasm by decomposition of the resinogenous layer of the cell wall or by the hydrolysis of certain glycosides.

Essential oils are the odorous principles found in various plant parts and because they are evaporated when exposed to the air at ordinary temperature they are called volatile oils, etheral oils or essential oils. This means that a given essential oil is not composed of just one type of molecule. They are pure, natural and extremely concentrated substances. Containing, ozone and negative ions, disease cannot survive in the presence of these substances and hence are believed to be the "life force" which plants use for their own antibiotic or immune stimulants. They are unlike familiar vegetable oils in that they are not greasy and evaporate quickly (Tyler et al., 1981).

There are now 30,000 known aromatic molecules that make up various essential oils. The three primary elements of all essential oils are hydrogen, carbon and oxygen. Its composition is a complex array of components that gives each plant its characteristic odor and flavour. These chemical constituents and the subcomponents they are made up of are what give the essential oils many of their most amazing healing properties (Schnaubelt, 1995). Chemical constituents of volatile oils may be divided into 2 broad classes, (1) terpene derivatives and (2) aromatic compounds. Several components of EOs have been identified with short term (eg. linalool, menthol, pulegone) and long term (eg. Benzaldehyde, carvacrol, carvone, cinnamaldehyde) with fungistatic effects. Most essential oils are complex mixtures involving a few to several hundred compounds (Charlwood & Charlwood, 1991; Bertoli *et al*., 2004; Nakajima *et al*., 2005).

EOs are produced using several techniques. Distillation uses water and steam to remove the oils from dried or fresh plants, and the expression method uses machines to squeeze the oil out of plants. Other techniques may use alcohol or solvents to remove essential oils from plants materials. A number of EOs from Palmarosa, Citronella, Gingergrass, Basil, Mint, Lemongrass, Eucalyptus, Cederwood, Lavender, Davana, Celery seed, Fennel and other oils have been widely used in a variety of products in India (Bhan *et al*., 1997). Out of these the EOs currently being produced in India are the oils of citronella, lemongrass, basil, mint, sandalwood, palmarosa, eucalyptus, cedrus wood, vetiver and geranium (Bhan *et al*., 1997). Rose, lavender, davana, khas and ginger grass oils are produced in small quantities.

In the present study, 75 different EOs are studied. The known chemical constituents of these oils obtained by literature survey are presented in Table 1.

TABLE 1

Literature survey of the tested essential oils for their known chemical constituents

(Ref: Rastogi & Mehrotra, 1993, 1995; Keville & Green, 1995; Schnaubelt, 1995; Nair & Mohanan, 1998, Gogte, 2000; Rajpal, 2002; SBP, 2004)

No.	Botanical name	Common name	Known Chemical Constituents*
1	*Acorus calamus* L. (Araceae)	Calamus	α, β and γ–asarone, acoradin, calamusenone, asaronaldehyde, acorenine, calamenone, calamenene, shyobunone, methyl eugenol, geranyl acetate
2	*Anethum graveolens* L. (Apiaceae)	Dill	Terpenes (phellandrene), carvone, α -phellandrene, piperine, β-sitosterol
3	*Angelica archangelica* L. (Apiaceae)	Angelica	Archengelin, oxypeucedanin, furocomarin, prangolarin, archangelicain, ostruthol, archengelenine, umbelliprenin, isoimperatorin, bergapten, prangolarin, β- sitosterol
4	*Apium graveolens* L. (Apiaceae)	Celery	Carvone, piperitone, eugenol, α-pinene, terpinolene, car-3-ene, myrcene, menthone, β-selinene, limonene, butyl–4,5–dihydrophthalide, caryophyllene, tritylphthalide

5	*Aniba rosaeodora* Ducke (Lauraceae)	Rose wood	Monoterpene alcohol, geraniol, menthol, α-terpineol, terpineol-4, sabinol, linalool, thujanol, cinalol, nerol, cuminol, carveol, borneol, pinocarveol
6	*Azadirachta indica* A. Juss (Meliaceae)	Neem	Azadirachtanin, salannin, 6-O-acetylnimbandiol, 3-desacetylsalannin, nimocin
7	*Barringtonia acutangula* (Linn.) Gaertn. (Barringtoniaceae)	Myrht	Triterpene acids- anhydrobartogenic acid, 19-epibartogenic acid, bartogenic acid
8	*Calendula officinalis* L. (Asteraceae)	Calendula	Flavonoids- calendoflaside, calendoflavoside, calendoflavo-bioside, narcissin, quercetin, isoquercetin, isorhamnetin, rhamnoside
9	*Cananga odorata* (Lam.) Hook.f. and Thoms. (Annonaceae)	Ylang Ylang	Sesquiterpenes, esters, terpenes, germacrene δ, α-humulene, γ and ε-cadinenes, γ-muurolene, α-cedrene, α-amorphene, γ-bisabolene, α-, γ-and δ-cadinols, farnesyl acetate
10	*Carum carvi* L. (Apiaceae)	Caraway	Carvone, terpene, limonene, carvacrol, p-cymene, cuminal-dehyde, β-elemene

11	*Cedrus deodara* (Roxb. Ex D.Don) G.Don (Pinaceae)	Cedar	Longiborneol, α-pinene, β-sitosterol
	Centella asiatica (Linn.) Urban (Apiaceae)	Brahmi	Brahmoside, thankuniside, quercetin, brahminoside, isothankuniside, betulic acid, asiaticoside, asiatic acid, hydro-cotyline, madecassoside, isobrahmic acid, polyacetylene
13	*Cinnamomum camphora* (Linn.) Presl. (Lauraceae)	Camphor	Sesquiterpene hydrocarbons, α-ylangene, β-santalene, δ-guainene, δ-cadinene, calamenene, calacorene, γ-patchoulene, 1,6–dimethy-4-isopropyl–7,8– dihydronaphtha-lene, camphorenone, camphorenol
14	*Cinnamomum cassia* Blume (Lauraceae)	Cassia	Cinncassiol C_1-C_3, Cinncassiol D_1-D_3
15	*Cinnamomum zeylanicum* Blume (Lauraceae)	Cinnamon (bark)	Cinnamaldehyde, eugenol, benzaldehyde, diterpenes, proan-thocyanidin I-III, α-glucan
16	*Cinnamomum zeylanicum* Blume (Lauraceae)	Cinnamon (leaf)	Eugenol, cinnamaldehyde, benzaldehyde, sesquiterpenoids, humulene, selinene, elemene, caryophyllene, δ-nerolidol, cinn-zeylanol, cinnzeylanin, α-pinene, α- phellandrene, p-cymene, linalool, β-caryophyllene, α-terpineol, benzyl acetate, cinnamyl acetate, eugenyl acetate, benzyl benzoate

17	*Citrus aurantium* L. (Rutaceae)	Orange	Flavonoids, α- β-pinenes, limonene, α-phellandrene, decanal, octanol, citronellal, α-terpineol, geranyl acetate, geraniol, citral, auranetin, hesperidin, 5-hydroxyauranetin
18	*Citrus bergamia* Risso and Poit (Rutaceae)	Bergamot	Bergapten, limonene, linalool, linalyl acetate
19	*Citrus limon* (Linn.) Burm.f. (Rutaceae)	Lemon	Limonene, sesquiterpenes, aldehydes, coumarins, furocoumarins, flavonoids, carotenoids, steroids, glycosides- citrusins, coniferin, syringin, dehydrodiconiferyl alcohol-4 β-glucoside, terpene hydrocarbons
20	*Citrus bigaradia* Hook.f. (Rutaceae)	Petitgrain	Terpene alcohols, linalyl acetate, α-methyl-4-hydroxyphenyl-methanol, 7-hydroxy-5,6,3',4'-tetramethoxyflavone, 3'-hydroxy-5,6,7,8,4'-pentamethoxyflavone, limonene
21	*Coriandrum sativum* L. (Apiaceae)	Coriander	Linalool, coriandrol, terpene alcohol (linalool), gnaphaloside α, gnaphaloside β, quercetin, isorhammetin, rutin, luteolin, coriandrin, dihydrocoriandrian, coriandrol, jirenyol, vebriniol
22	*Crocus sativus* L. (Lamiaceae)	Saffron	Picrocrocin, carotenoid

23	*Cuminum cyminum* L. (Apiaceae)	Cumin	Cuminaldehyde, apigenin-7-O-glucoside, luteolin-7-O-glucoside, α-pinene, α-phellandrene, α-terpinene, limonene, p-cymene, thymene
24	*Cupressus sempervirens* L. (Cupressaceae)	Cypress	Terpene hydrocarbons, quercetin, 3-O-α-L-rhamnoside, hinokiflavone, isocryptomerin
25	*Curcuma longa* L. (Zingiberaceae)	Turmeric	Curcumin, 4-hydroxycinnamoyl (feruloyl) bis - (4-hydroxycinnamoyl) methane, anthraquinone, sesquiterpenes, β-turmerone, demethoxycurcumin, bis-demethoxycurcumin, turmerones (sesquiterpene ketones), tertiary alcohols
26	*Cymbopogon flexuosus* (Nees ex Steud.) Wats. (Poaceae)	Lemongrass	Citral, myrcene, α-pinene, α-terpinene, ocimene, p-cymene, limonene, geraniol, citronellol, linalool, cymbopogonol, cymbopogone
27	*Cymbopogon martini* (Roxb.) Wats. (Poaceae)	Palmarosa	Terpene esters, limonene, α-terpinene, myrcene, β-caryophyllene, α-humulene, β- γ-selinenes, geraniol, geranyl butyrate, geranyl isovalerate, geranyl acetate, linalool, methylisoeugenol, farnesyl acetate, farnesols, estragole, neryl acetate, cymbodiacetal

28	*Daucus carota* L. (Apiaceae)	Carrot	Sesquiterpenes hydrocarbons, sesquiterpene alcohol carotol, β-carotene, β-sitosterol, bisabolene, asarone aldehyde, cis-asarone, eugenol, methyl eugenol, car-3-ene, carota-1,4 β-oxide
29	*Elettaria cardamomum* (L.) Maton (Zingiberaceae)	Cardamom	α-terpineol, myrcene, 1,8-cineole, heptane, limonene, menthone, β-phellandrene, α and β-pinenes, sabinene, nerolidol, α-terpinyl acetate
30	*Foeniculum vulgare* Miller (Apiaceae)	Fennel	Trans-anethole, limonene, estragole, fenchone, γ-terpinene, α-pinene, linalool, anethole, fenchone
31	*Geranium macrorhizum* L. (Geraniaceae)	Geranium	Terpene alcohols, esters
32	*Gaultheria frangrantissima* Wall. (Ericaceae)	Wintergreen	Menthyl salicylate and gaultherilene.
33	*Hamamelis virgianiana* L. (Hamamelidaceae)	Witch hazzel	Carvacol, eugenol, hexaenol
34	*Hedychium spicatum* (Buch.) Ham (Zingiberaceae)	Kapura kachari	Terpinen-4-ol, sabinene, p-cymene, limonene, γ-pinene, α-terpinene and α-terpineol.

35	*Hydnocarpus laurifolia* (Dennst.) Sleummer (Flacourtiaceae)	Mogra	Cyclopentenoid cyanohydrin glycosides-(1S,4R) and (1R,4S) 1-[6-O(α-L-rhamnopyranosyl)-β-D-glucopyranosylo-xy]-4-hydroxy-2-cyclopentene-1-carbonitrile, ethyl esters, hydrocarpic acids
36	*Jasminum grandiflorum* L. (Oleaceae)	Jasmine	Methyl jasmonate, salicylic acid, jasminine alkaloid, cis-jasmone
37	*Juniperus communis* L. (Cupressaceae)	Juniper	Manool, 4B-hydroxygermacra 1(10), 5-diene, β-oplopenone, oplopanone, 8-acetoxyelemol, junionins, diterpene acids
38	*Laurus nobilis* L. (Lauraceae)	Bay	1,8-cineole, methyl eugenol, α-pinene, β-pinene, linalool, α-terpineol, limonene, α-terpinvyl acetate, sabinene, eugenol, cinnamic acid, cineole, esters, terpene, alcohol
39	*Lavandula officinalis* Caix (Lamiaceae)	Lavender	Linalool, linalyl acetate, amylalcohol, amylvinylcarbynyl acetate, curcumene, bergamotene, bornyl acetate, δ-cadinene, camphene, car-3-ene, caryophyllene, cineole, p-cymol, dipentene, farnesene, geraniol, isocmyl alcohol, lavandulol, lavandulyl acetate, linalool, linalyl acetate, linalyl isobutyrate, β-myrcene, nerol, neryl acetate, α, β-pinenes, α-santalone, γ-terpinene, terpine-4-ol, terpinyl acetate, α-terpineol, borneol

40	*Marjorana hortensis* Moench./ *Origanum marjorana* L. (Lamiaceae)	Marjoram	carvacrol, thymol, α-terpinene, linalool, geraniol, p-cymene, α-terpineol, terpin-1-en-4-ol, cis-sabinene hydrate, trans-sabinene hydrate, α-terpineol, β-terpineol, terpene alcohol, terpene esters
41	*Matricaria chamomilla* L. (Asteraceae)	Chamomile	Bisabalol, chamazulene, farnesene, spathulenol, bisabolol oxide, borneol, caryophyllene, 1,8-cineole, linalool, myrcene, pulegone, α-terpineol, sesquiterpenes
42	*Melaleuca alternifolia* L. (Myrtaceae)	Tea	Terpinene-4-ol, cymene, pinene, terpinene, cineole.
43	*Melaleuca leucadendron* L. (Myrtaceae)	Cajuput	Terpene alcohols, 1,8- α-cineole, p-cymene, α-terpineol, limonene, α-pinene, linalool, β-pinene, terpinen-4-ol, phellandrene, isoprophylanisole, carveol, α-copaene, β-elemene, humulene, γ-cadinene
44	*Mentha piperita* L. (Lamiaceae)	Peppermint	Menthol, menthone, neomenthone, cineole, menthyl acetate, neomenthol, isomenthol, limonene, pinene, pulegone, α-pinene, phellandrene, terpinene, cardiene, amyl alcohol, acetaldehyde, isovaleric aldehyde, lactone
45	*Michelia champaca* L. (Magnoliaceae)	Champa	Costunolide, parthenolide, dihydroparthenolide, micheliolide

46	*Moringa oleifera* Lamk. (Moringaceae)	Drumstick	Glycoside- moringyne, indole acetic acid, indole acetonitrile
47	*Myristica fragrans* Houtt. (Myristicaceae)	Nutmeg	Myristicin, pinene, myristin, myristic acid, camphene, eugenol, isoeugenol, elemicin, limonene, α and β-pinenes, safrole, α-terpineol, terpine-4-ol
48	*Myristica fragrans* Houtt. (Myristicaceae)	Mace	Myristicin, pinene, myristin, myristic acid, camphene, eugenol, isoeugenol, elemicin, limonene, α and β-pinenes, safole, α-terpineol, terpine-4-ol
49	*Nardostachys jatamansi* (D.Don) DC. (Valerianaceae)	Spikenard	Jatamansin, Nardostachnol, 9-dehydroaristolene, 1(10)-dehydroaristolene, 2β-maaleine, 1,2,9,10- tetrahydroaristolene
50	*Ocimum basilicum* L. (Lamiaceae)	Basil	Methyl chavicol, linalool, citral, methyl cinnamate, eugenol, 1,8-cineole, phenyl propane, terpene alcohols, ocimene, borneol, sambulene, safrole, sesquiterpene hydrocarbon, estragole, eucalyptol, eucalyptol, linalool acetate, menthol, menthone, cyclohexanol, cyclohexanone, myrcenol, juvocimene I, juvocimene II, thymol, xanthomicrol, butyl caffeate, methyl eugenol

51	*Olea europaea* L. (Oleaceae)	Olive	Oleanolic acid, maslinic acid
52	*Pelargonium graveolens* L. (Geraniaceae)	Geranium rose	Geraniol, geranic, citronellol, citronellyl formate, linalol (linalool), eugenol, myrtenol, terpineol, citral, methone and sabinene
53	*Persea americana* Mill. (Lauraceae)	Avocado	Nerolidol, 2,4-decadienal, α-farnesene, β-caryophyllene, caryophyllene oxide and α-copaene.
54	*Pimpinella anisum* L. (Apiaceae)	Anise	Anethole, methyl chavicol, β-methoxy phenylacetone, terpenes, sulphur, α-pinene, camphene, phellandrene, limonene, aniseketone, carvone, cinnamaldehyde, β-elemene, cinnamyl alcohol
55	*Piper cubeba* L. (Piperaceae)	Cubeb	Clusin, cubebin, dihydrocubebin, hinokinin, 2-3-1,4-butanediol, α-ethoxycubebin, β-ethoxycubebin, dihydrocubebin monoacetate, heterotropan, magnosalin, 5''-methoxyhinokinin, 2,4,5-trimethoxybenzaldehyde, terpenes, phellandrene, caryophyllene, dipentene
56	*Piper nigrum* L. (Piperaceae)	Black pepper	Piperine, cubelin, 3,4-dimethoxy-3,4-desnethylenedioxy-cubelin, 3',4'-dimethoxy-3',4'-desmethylenedioxycubelin, terpenes, phellandrene, caryophyllene, dipentene

57	*Polianthus tuberosa* L. (Agavaceae)	Tuberose	Menthyl benzoate, menthyl anthranilate, benzyl alcohol, butyric acid, eugenol, nerol, farnesol and geraniol.
58	*Pogostemon cablin* (Blanco) Benth. (Lamiaceae)	Patchouli	Sesquiterpenes, sesquiterpene alcohols, cycloseychellene, patchouli alcohol, α-bulnesene, α-patchoulene
59	*Primula rosea* Royle (Primulaceae)	Evening primrose	Sapogenin, 14B,26- Epoxy-C-homo-pentacyclic triterpenes (I, II & III)
60	*Prunus amygdalus* Batsch. (Rosaceae)	Sweet almond	Daucosterin, sitosterol, amygdalin
61	*Prunus armeniaca* L. (Rosaceae)	Apricot	Avenasterol-5-ene, β-sitosterol, stigmasterol, 24-dehydrocholesterol, cholesterol, estrone, α-estradiol
62	*Rosa damascena* Mill. (Rosaceae)	Rose (absolute)	Terpene alcohols (citronellal), citronellol, nerol, geraniol, phenylethanol, cyaniding-3,5-diglucoside, α-copaene, n-nonacosanol, farnesol, geranyl acetone, nerol oxide, caryo-phyllene epoxide, neryl laurate, methyl geranate
63	*Rosmarinus officinalis* L. (Lamiaceae)	Rosemary	Cineole, terpene, borneol, bornyl acetate, camphene, camphor, car-3-ene, 1,8-cineole, p-cymene, limonene, linalool, linalyl acetate, ocimene, α-β-pinenes, α-terpinene, terpineol, piperitone, thujone, β-caryophyllene, verbenone

64	*Salvia officinalis* L. (Lamiaceae)	Sage	Thujone, cineole, camphor, pinene, linalyl acetate, borneol, bornyl acetate, farnesol
65	*Salvia sclarea* L. (Lamiaceae)	Clary sage	Borneol, camphor, thujone, phellandrene, salviol and cineole.
66	*Saussurea lappa* Decne (Asteraceae)	Costus	Guaianolides-isodehydrocostuslactone , isozaluzanin C, costunolide, 12-methoxy-dihydrodehydrocostuslactone
67	*Sesamum indicum* L. (Pedaliaceae)	Sesame	Sesamolinol, sesamol, γ -tocopherol
68	*Syzygium aromaticum* (L.) Merrill & Perry (Myrtaceae)	Clove	Eugenol, eugenyl acetate, β-caryophyllene, α-β-lumulene, benzaldehyde, chavicol, naphthalene, eugeniin, acetophenone, benzyl salicylate, α-cadinol, γ-decalactone, fenchone, hexanal, 2-hexanone, methyl palmitate, γ-muurolene, palustrol, propyl benzoate, β selinene, α-thujene
69	*Tagates erecta* L. (Asteraceae)	Tagetes	β-caryophyllene, limonene, myrcene, piperitone, tagetone, valeric acid and ocimene
70	*Thymus vulgaris* L. (Lamiaceae)	Thyme	Thymol, luteolin, apigenin, p-cymene, borneol, carvacrol, p-cymol, α-pinene, β-pinene, camphene, limonene, fenchone, citronellal, terpineol, borneol, citral, anethole, ethyl eugenol, tymonin, cirsilineol

71	*Valeriana jatamansi* Jones (Valerianaceae)	Valerian	Iridoids-valtrate, acevaltrate, α-pinene, limonene, 1,8-cineole, p-cymene, borneol acetate, borneol, nerolidol, maaliol
72	*Vetiveria zizanioides* (L.) Nash (Poaceae)	Vetiver	Sesquiterpene-hydrocarbons, alcohols, ketones, esters, epik-husinol, norkhusinol oxide, khusitoneol, vetiverol, vetivone, vetivene and furfurol.
73	*Vitis vinifera* L. (Vitaceae)	Grapes	Betulinic acid, lupeol, 30-norlupan-3 β -ol-20-one, heptaco-sanol, triacontanol, triacontanyl tridecanoate, stigmasterol, jasmonic acid
74	*Zingiber officinale* Rosc (Zingiberaceae)	Ginger	β-sesquiphellandrene, β-bisabolene, curcumene, α-zingiberene, gingerols, shogaols, diarylheptenenes, 6-gingesulphonic acid, gingerone, gingerol, gingerine
75	*Viola odorata* L. (Violaceae)	Violet	Glucoside: Viola-quercitin

CHAPTER 2A

IN VITRO EFFICACY OF
75 ESSENTIAL OILS AGAINST
ASPERGILLUS NIGER

ABSTRACT

Aspergillus niger is a plant pathogen causing postharvest diseases of stored crops. A study was conducted with 75 different essential oils for the inhibition of hyphae growth and spore formation in *A. niger*. *Cinnamomum zeylanicum* (Bark), *Cinnamomum zeylanicum* (leaf), *Cinnamomum cassia*, *Syzygium aromaticum* and *Cymbopogon citratus* were the top five essential oils which demonstrated marked inhibitory effect against hyphal growth and spore formation of *A. niger*. The chemical composition of ten most active essential oils was investigated by Gas Chromatography-Mass Spectral (GC-MS) analysis. Most of the other essential oils were found challenging to combat *A. niger,* suggesting their use as a safe environmental product for postharvest technology.

Keywords: Aspergillus niger, essential oils, hyphae growth, spore inhibition

INTRODUCTION

Aspergillus niger is a most common plant pathogen. It is an opportunistic human pathogen and frequently colonizes lower respiratory tracts and lungs with localized underlying conditions (healed tuberculous cavity, cystic fibrosis, etc.) even in subjects without systemic predisposing factors (Latge, 1999; Reeves *et al*., 2004). The air and indoor environment is colonized by propagules of the various microorganisms, out of which the relative frequency of *A. niger* alone is 96.8-100% (Motiejūnaitė, 2004).

Black mould in plants is known to cause by *A. niger*. Although the disease can occasionally be seen in the field at harvest, black mould is primarily a postharvest disorder and can cause extensive losses in storage under tropical conditions (Thamizharasi & Narasimham, 1992). Losses caused by postharvest diseases are greater than generally realized because the value of fresh fruits and vegetables increases several fold while passing from the field to the consumer (Eckert & Sommer, 1967). Postharvest losses are estimated to range from 10 to 30% per year despite the use of modern storage facilities and techniques (Harvey, 1978).

Postharvest diseases affect a wide variety of crops particularly in developing countries, which lack sophisticated postharvest storage facilities (Jeffries & Jeger, 1990). Infection by fungi and bacteria may occur during the growing season, at harvest time, during handling, storage, transport and marketing, or even after purchase by the consumer (Dennis, 1983). Since essential oils are less toxic, more systematic, host metabolism stimulatory, easily degradable, ecologically safe and non-pollutive, are widely used to search for an alternative pesticide in the agronomy (Sbragia, 1975; Horn, 1988).

Amongst the pesticides needed to protect crops, fungicides pose more of a carcinogenic risk than insecticides and herbicides together (Wilson *et al.*, 1997). Therefore, synthetic fungicides are suspect in our food chain, and pressure is increasing to find safer alternatives. Essential oils are chemically very diverse in their effect and cause different actions, unlike synthetic chemicals, which basically have one action. Additionally, resistance by pathogens to fungicides has rendered certain fungicides ineffective creating a need for new ones with alternative modes of action. Present activities to find both natural and synthetic fungicides focus on finding compounds that are safe to humans and the environment.

Essential oils show antifungal activity against a wide range of fungi (Kurita *et al.*, 1981; Janssen *et al.*, 1987; Singh, 1997). Essential oils are volatile parts of plants that lend them their characteristic scents (Schnaubelt, 1995). The plants create these oils to help protect them from fungus and bacteria, attract pollinators and help to heal wounds in their bark. Essential oils are made up of many different volatile compounds and the make up of the oil quite often varies between species (Jobling, 2000). The essence and perfumery produced by aromatic plant have significant aesthetic value in day to day life, and found to possess medicinal and pharmacological values (Singh, 1997).

Essential oil from red Thyme (*Thymus vulgaris*), Clove buds (*Syzigium aromaticum*) and Cinnamon leaf (*Cinnamomum zeylanicum*) prevented the growth of *Botrytis cinerea* (Reddy *et al.*, 1997; Wilson *et al.*, 1997). Additionally, essential oils of *Melaleuca alternifolia* also exhibit antifungal activity against a wide range of common postharvest pathogens (Bishop & Reagan, 1998). Consequently, plant-derived antimycotics are attracting the attention of botanists and mycologists because they are natural, cheaper, safer and eco-friendly.

Thus considering the importance of essential oils, an attempt was made to test 75 different essential oils against *A. niger* out of which some have been previously tested (Guynot *et al*., 2003; Jantan *et al*., 2003; Kalemba & Kunika, 2003). The present investigation is focused on the screening of the anti *A. niger* activity of 75 oils altogether and to compare them with previous data by finding details of inhibition of hyphae growth and spore formation in *A. niger*.

MATERIALS AND METHODS

Essential oils

Seventy-five essential oils used in this experiment were obtained from Vimal Research Society for Agrobiotech and Cosmic Powers (VIRSACO), Rajkot, Gujarat, India.

Gas chromatography – mass-spectral analysis

The essential oils of *Cinnamomum zeylanicum (bark), Cinnamomum cassia, Syzygium aromaticum, Cinnamomum zeylanicum (leaf), Cymbopogon citratus, Primula rosea, Ocimum basilicum, Rosa damascena, Citrus bergamia* and *Cuminum cyminum* were subjected to gas chromatography-mass spectra (GC-MS) analysis on Perkin Elmer Auto System XL Gas chromatograph equipped with Perkin Elmer Turbo Mass, mass spectrometer as a detector and National Institute of Standards and Technology (NIST) database. The capillary column was DB-1 (30 m x 0.25 mm, 0.25 µm film thickness) while helium was used as a carrier gas. Flow rate was 1 ml/min and temperature of injector and detector was 250 and 280 ^{o}C, respectively. The oven temperature was programmed from 60 to 250 ^{o}C at 5° /min. The amount of oil injected into the GC-MS was 0.4 µl. The injector operated at split mode (1:40) was held at 250 ^{o}C. The sample transfer line was heated to 280 ^{o}C. The identification of oil components was based on their retention time, and by comparison of their mass spectral fragmentation pattern with NIST database. The components from GC-MS analysis are listed in Table 2 (only those components in order with maximum percent area in oil were subjected to mass spectral analysis). The GC-MS analysis was accomplished in SIKART, Vallabh Vidyanagar, Gujarat, India.

Test Fungus

The test fungus was *A. niger* (VP-001). It was isolated from *Allium cepa* L., cultured on Potato Dextrose Agar (PDA) media and identified in the Gujarat agricultural University, Junagadh, Gujarat.

Preparation of inoculum

The fungus was cultured on PDA media and after 7 days of inoculation disc of 1.1 cm was cut from periphery with the help of cup borer and suspended in 10 ml of sterile distilled water. It was shaked vigorously to dislodge the spores in water and then the agar debris was removed by centrifugation. The supernatant was used as a culture suspension for inoculation. The colony forming units per milliliter (CFU/ml) were calculated by Neubauer's chamber (1×10^5 approximately).

Studied activity

Antifungal activity was studied. Potato Dextrose Agar media was used for the culture maintenance and the bioassays. 0.1 ml of the prepared inoculum was spread on the PDA plates. Antifungal activity testing was done using standard disc diffusion assays (Bauer *et al*., 1966). For preparing the test discs, 5 µl of the essential oil was pipetted onto 5-mm filter paper discs made from Whatman filter paper no.1, which were carefully transferred onto the surface of seeded agar plate. Hexaconazole was used as a positive control. Stock was prepared by mixing 0.1 ml Hexaconazole with 0.9 ml distilled water. From this stock, 0.5 µl was pipetted on the disc (i.e. 0.05 µl Hexaconazole/disc). The plates were incubated at 28 ^{o}C for 48 h and the diameters of the zone of hyphae inhibition (both, hyphae and spores absent) and zone of spore inhibition (zone of hyphae inhibition + zone where only hyphae is present but spores are absent) was recorded for all the 75 oils in millimeters after the incubation period. The hyphae diameter inhibition was determined for six essential oils with the help of Carl Zeiss Axioskop 2 plus microscope with CCD camera at 45X objective lens. The anti *A. niger* activity of the oils is summarized in Table 3.

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Spore count

A borer with 5 mm diameter was used to cut a piece of agar with complete sporulation (i.e. after the period of incubation) from control plate. This was mixed with 1 ml distilled water, shaked vigorously and as stated above, the supernatant was used for spore count, which was carried out with Neubauer's chamber (Pfeller *et al*., 1988). This total number of spores was present on 5 mm diameter agar disc area (3.14 x r^2). Thus the total number of spores in control plate with diameter 100 mm was calculated. A spore count for 100 mm diameter area was obtained (50 x 10^6) with the help of which the possible number of spores in inhibited zone diameter was calculated accordingly.

RESULTS

The list of chemical compounds in order with their maximum percent area in oil is presented in Table 2 and the typical chromatograms of these oils are shown in Plate 1. Hyphal inhibition was studied as inhibition in hyphae diameter (Fig. 1) and zone of its inhibition whereas, for spore, zone and its number inhibited was calculated for each essential oil (Table 3). It was observed that *A. niger* was highly sensitive to *Cinnamomum zeylanicum (bark), Cinnamomum cassia, Syzygium aromaticum, Cinnamomum zeylanicum (leaf) and Cymbopogon citratus* essential oils. The oils of *Primula rosea, Ocimum basilicum, Rosa damascena, Citrus bergamia* and *Cuminum cyminum* also exhibited distinct antifungal activity. Highest activity was found with Cinnamon bark oil (Plate 2).

Zone of hyphal inhibition was 43 mm and spore inhibition was up to 50 mm. Cassia oil was also found to be highly active showing a zone of spore inhibition up to 45 mm. The essential oil of Clove and Cinnamon leaf exhibited the zone of spore inhibition 36 mm and 35 mm, respectively (Table 3). Zone of spore inhibition between 20 and 32 mm was exhibited by the essential oils of Bergamot, Cumin, Bay, Basil, Geranium rose, Evening primrose and Rose.

In Lemongrass and Cumin oil complete zone of hyphae and spore inhibition was observed up to 48 h while after 72 h the spore formation was observed. In this study the later data is included (Table 3). Rose and Bergamot oils also showed very high antifungal activity ranging between 18 and 27 mm (hyphae and spore both). Most of the oils showed activity between 6 and 15 mm and many other oils had no effect on *A. niger* growth (Table 3).

As an influence of essential oils on *A. niger* growth, three different zones were observed i.e. (i) zone of complete inhibition (spore + hyphae) (ii) zone of spore inhibition and (iii) growth zone. In second zone, six selected different inhibitor essential oils were studied for the measurement of hyphae diameter (Fig. 1). Although, in Cinnamon bark maximum zone of inhibition was observed, inhibition in hyphae diameter was nearly 25% as compared to 75% in Cinnamon leaf. In other essential oils inhibition was observed between 75 and 50%. In third zone hyphae diameter was observed near to control (data not presented).

DISCUSSION

The physical nature of essential oils, i.e. low molecular weight combined with pronounced lipophilic tendencies, allow them to penetrate cell membrane more quickly than other substances. The amazing ability of essential oils to penetrate tissue has been proven repeatedly in scientific experiments. Essential oils penetrate tissue roughly 100 times faster than water and 10,000 times faster than salts (Römmelt *et al*., 1974). Essential oils are made up of many different volatile compounds and make up of the oil quite often varies between species (Table 2, Plate 1). It seems that the antifungal and antimicrobial effects are the result of many compounds acting synergistically. This means that the individual components by themselves are not as effective (Jobling, 2000).

In the present study, anti *A. niger* activity was quantitatively assessed by measuring the diameter of zone of inhibition (both hyphae and spore) around the disc, which was ultimately expressed in millimeters (Table 3, Plate 2). It was observed that all the tested essential oils demonstrated varying degrees of anti *A. niger* activity. Highest inhibitory effect was exhibited by essential oils of *Cinnamomum zeylanicum* (bark), *Cinnamomum cassia, Syzygium aromaticum, Cinnamomum zeylanicum* (leaf) *and Cymbopogon citratus.* The chemical composition of these oils along with other five EOs, found to be potent inhibitors next to them, were investigated in an effort to correlate the constituents of the oils and their anti *A. niger* activities (Table 3)

In this study, highest percentage of (E)-cinnamaldehyde was observed in Cinnamon bark (64.13%), Cassia (66.36%) and Cinnamon leaf (58.49%; Table 2). A recent study suggested that the antifungal activity of Cinnamon and Thyme oils were probably due to their major components, cinnamaldehyde and carvacrol, respectively (Ferhout *et al*., 1999).

In other study, the main component was trans-cinnamaldehyde in the oil of Cinnamon, which showed the strongest antifungal activity (Simi *et al*., 2004). Considering these reports, it can be concluded that in the present study cinnamaldehyde may have a role in inhibiting *A. niger* growth. Besides (E)-cinnamaldehyde, benzyl benzoate, linalool, eugenol and cinnamyl acetate were also found abundant in Cinnamon bark and Cassia oil. Previous studies have indicated that the essential oils showing high levels of benzyl benzoate show high toxicity values against insects (Jantan *et al*., 2005). Linalool was found to be responsible for the antifungal activity performed previously (D'Auria *et al*., 2005).

The essential oils of Clove and Lemongrass also revealed elevated inhibitory effect against *A. niger*. The content of eugenol (47.64%) was highest followed by benzyl alcohol (34.10%) in clove whereas citral (geranial) (29.40%) and β-citral (neral) (21.39%) occupied highest area in the oil of Lemongrass along with linalool (3.20%), geraniol (3.25), nerol acetate (10.81) and methyl heptenone (3.21%). There are reports where citral a and b are indicated as fungicidal constituents in Lemongrass oil (Abdullah *et al*., 1975; Paranagama *et al*., 2003). Previous work on antimicrobial activities of several essential oil components indicated that cineole, citral, geraniol, linalool and menthol were active against several yeast-like and filamentous fungi (Pattnaik *et al*., 1997). Thus the presence of citronellal, linalool and geraniol in Lemongrass oil might have played role in inhibiting spore formation for a longer period of time, in the present study. The fungitoxic properties of the oil of Lemongrass have been found to be thermostable and unaltered for 7 months (Mishra & Dubey, 1994). The effect of clove oleoresin was found to be satisfactory for *A. niger* in previous studies (Núñez *et al*., 2001). The phenolic major components of essential oils have been suggested to have a potent antifungal activity (Viollon & Chaumont, 1994). Carvacrol is proved to be the most important fungitoxic compound among the Thyme essential oil constituents (Arras & Usai, 2001).

Cumin showed inhibition after 72 h, which may be attributed to its chemical constituents like cuminol and p-cumic aldehyde. β-citronellol, cis-geraniol, iso-eugenol, caryophyllene, β-linalool, cinnamaldehyde, etc., are the other most important constituents of Rose, Basil, Evening primrose and Bergamot that may be responsible for such a high inhibitory activity. Citronellal, geraniol and citral are previously tested individually for their antifungal activity (Saikia *et al*., 2001). In earlier studies citronellol, geraniol, linalool, limonene and citronellal are found in Rose, Cumin and Bergamot oils and are ascribed to have a role in exhibiting good antifungal activity (Carson & Riley, 1995; Singh, 1997). Remarkable number of spores found to be inhibited by 5 µl of essential oils (Table 3), in the present study, supports the importance of these oils in anti *A. niger* activity. In nature this concentration of spores is unpredictable and therefore essential oils as a fungicide can be safe and economic approach.

Various workers have reported anti activity against a number of storage fungi and insects (Caccioni & Guizzardi, 1994; Mishra & Dubey, 1994; Caccioni *et al*., 1998; Hammer, 1999; Bankole & Joda, 2004). Essential oil from red Thyme (*Thymus vulgaris*), Clove buds (*Syzigium aromaticum*) and Cinnamon leaf (*Cinnamomum zeylanicum*) prevented the growth of *Botrytis cinerea* (Reddy *et al*., 1997; Wilson *et al*., 1997). Additionally, essential oils of *Melaleuca alternifolia* also exhibit antifungal activity against a wide range of common postharvest pathogens (Bishop & Reagan, 1998).

Huge losses are incurred on account of different types of postharvest diseases. Economic losses caused by postharvest diseases are considerably more than realized because fruits and vegetables increase manifold in unit value while passing from the field at harvest to the consumer (Mehrotra & Aggarwal, 2004).

Species of *Aspergilli* are very often responsible for the deterioration of stored grains. *A. flavus* produces a fungal toxin known as aflatoxin in groundnut kernels as well as several other fungi, for eg. *Alternaria* and *Helminthosporium* brings about the deterioration of grains in storage (Mehrotra & Aggarwal, 2004). Different control strategies have been utilized to treat postharvest diseases, like various cultural practices, chemical treatment as well as biocontrol methods.

Although there are certain antibacterial and antifungal agents available, they usually are very caustic and potentially toxic. *A. niger* is the most common postharvest pathogen for the stored crops like onion, garlic and causing Aspergillus rot in fruits like grapes (Kelman, 1989). The important goal to use essential oils is to prevent such postharvested crops which are stored in a closed chamber and where essential oils can be used economically.

TABLE 2

Chemical composition of essential oils as determined by GC-MS analysis

Essential oil	Compound	Retention time	% Area
Cinnamomum zeylanicum (bark)	β-linalool	11.647	10.25
	(E)-Cinnamaldehyde	15.277	64.13
	Eugenol	15.919	9.04
	Cinnamyl acetate	17.093	4.47
	Eugenyl acetate	17.844	0.75
	Benzyl benzoate	21.163	9.26
	Others		2.1
Cinnamomum zeylanicum (leaf)	Benzaldehyde	9.135	11.18
	Benzene acetaldehyde	10.749	2.40
	Acetophenone	11.152	1.27
	Benzoic acid	14.379	3.64
	(E)-Cinnamaldehyde	15.259	58.49
	Cinnamic acid	17.697	16.57
	Diethyl pthalate	18.834	1.42
	Others		5.03

Cinnamomum cassia	β -linalool	11.666	9.16
	(E)-Cinnamaldehyde	15.369	66.36
	Eugenol	15.956	7.74
	Cinnamyl acetate	17.111	3.97
	Eugenyl acetate	17.862	0.52
	Benzyl benzoate	21.181	10.24
	Benzaldehyde	8.916	0.15
	Benzyl alcohol	10.474	0.23
	Others		1.63
Syzygium aromaticum	Benzyl alcohol	11.104	34.10
	Eugenol	16.182	47.64
	Vanillin	16.604	0.91
	Caryophyllene	16.732	1.00
	3allyl-6-Methoxyphenol	17.961	4.98
	Caryophyllene oxide	18.896	1.35
	Others		10.02
Primula rosea	β- citronellol	14.092	28.52
	cis - geraniol	14.77	14.49
	Diethyl phthalate	19.024	11.33
	Phenyl ethyl alcohol	12.241	10.86
	Musk xylene	21.903	5.82
	Citronellal	14.991	5.73
	Others		23.25

Ocimum basilicum	Iso-eugenol	16.256	47.19
	β - elemene	16.402	9.63
	Caryophyllene	16.934	21.63
	α - Caryophyllene	17.264	2.27
	α - Guaiene	17.704	3.14
	Caryophyllene oxide	18.951	5.00
	Others		11.14
Cymbopogon citratus	Methyl heptenone	9.325	3.21
	β -Linalool	11.599	3.20
	β -Citral	14.166	21.39
	Geraniol	14.386	3.25
	Citral	14.716	29.40
	Nerol acetate	15.962	10.81
	Others		28.74
Rosa damascena	β -Linalool	11.654	4.52
	Phenyl ethyl alcohol	12.754	47.04
	Formic acid, 2-phenyl Ethyl ester	13.212	2.53
	β - Citronellol	14.037	16.73
	Cis- geraniol	14.422	10.74
	α – Santalol	19.867	1.93
	Others		16.51

Citrus bergamia	Limonene	10.224	2.67
	β -Linalool	11.599	3.11
	(E)-3(10)-Caren-2-ol	13.744	2.77
	Cinnamaldehyde	14.734	2.60
	Limonene oxide	15.761	3.30
	Diethyl phthalate	19.354	70.47
	Others		15.08
Cuminum cyminum	o - Cymene	10.205	6.69
	p – Cumic aldehyde	14.569	43.05
	Cuminol	15.119	15.43
	Benzoic acid	16.256	3.60
	Acoradien	17.557	14.85
	α – terpineol	13.377	2.75
	Others		13.63

TABLE 3

Anti *A. niger* activity of 75 different essential oils

No.	Botanical name	Common name	Zone of hyphae inhibition (mm)	Zone of spore inhibition (mm)
1	*Acorus calamus* L. (Araceae)	Calamus	7	12 (72×10^4)[1]
2	*Anethum graveolens* L. (Apiaceae)	Dill	9	13 (84.5×10^4)
3	*Angelica archangelica* L. (Apiaceae)	Angelica	-	-
4	*Apium graveolens* L. (Apiaceae)	Celery	9	11 (60.5×10^4)
5	*Aniba rosaeodora* Ducke (Lauraceae)	Rose wood	8	10 (50×10^4)
6	*Azadirachta indica* A.Juss (Meliaceae)	Neem	-	-
7	*Barringtonia acutangula* (Linn.) Gaertn. (Barringtoniaceae)	Myrht	10	16 (128×10^4)
8	*Calendula officinalis* L. (Asteraceae)	Calendula	-	-
9	*Cananga odorata* (Lam.) Hook.f. and Thoms. (Annonaceae)	Ylang Ylang	11	17 (144.5×10^4)
10	*Carum carvi* L. (Apiaceae)	Caraway	7	7 (24.5×10^4)

11	*Cedrus deodara* (Roxb. Ex D.Don) G.Don (Pinaceae)	Cedar	10	15 (112.5×10^4)
12	*Centella asiatica* (Linn.) Urban (Apiaceae)	Brahmi	-	-
13	*Cinnamomum camphora* (Linn.) Presl. (Lauraceae)	Camphor	-	-
14	*Cinnamomum cassia* Blume (Lauraceae)	Cassia	40	45 (1012.5×10^4)
15	*Cinnamomum zeylanicum* Blume (Lauraceae)	Cinnamon (bark)	43	50 (1250×10^4)
16	*Cinnamomum zeylanicum* Blume (Lauraceae)	Cinnamon (leaf)	30	35 (612.5×10^4)
17	*Citrus aurantium* L. (Rutaceae)	Orange	6	6 (18×10^4)
18	*Citrus bergamia* Risso and Poit (Rutaceae)	Bergamot	18	25 (312.5×10^4)
19	*Citrus limon* (Linn.) Burm.f. (Rutaceae)	Lemon	7	7 (24.5×10^4)
20	*Citrus bigaradia* Hook.f. (Rutaceae)	Petitgrain	-	-
21	*Coriandrum sativum* L. (Apiaceae)	Coriander	-	-
22	*Crocus sativus* L. (Lamiaceae)	Saffron	-	-
23	*Cuminum cyminum* L. (Apiaceae)	Cumin	18	28 [2] (392×10^4)

24	*Cupressus sempervirens* L. (Cupressaceae)	Cypress	-	-
25	*Curcuma longa* L. (Zingiberaceae)	Turmeric	-	-
26	*Cymbopogon citratus* (DC.) Stapf (Poaceae)	Lemon grass	21	33 [2] (544.5 x 10⁴)
27	*Cymbopogon martini* (Roxb.) Wats. (Poaceae)	Palmarosa	8	12 (72 x 10⁴)
28	*Daucus carota* L. (Apiaceae)	Carrot	6	6 (18 x 10⁴)
29	*Elettaria cardamomum* (L.) Maton (Zingiberaceae)	Cardamom	8	13 (84.5 x 10⁴)
30	*Foeniculum vulgare* Miller (Apiaceae)	Fennel	7	7 (24.5 x 10⁴)
31	*Geranium macrorhizum* L. (Geraniaceae)	Geranium	-	-
32	*Gaultheria frangrantissima* Wall. (Ericaceae)	Wintergreen	9	12 (72 x 10⁴)
33	*Hamamelis virgianiana* L. (Hamamelidaceae)	Witch hazzel	-	-
34	*Hedychium spicatum* (Buch.) Ham (Zingiberaceae)	Kapura kachari	8	8 (32 x 10⁴)
35	*Hydnocarpus laurifolia* (Dennst.) Sleummer (Flacourtiaceae)	Mogra	-	-

36	*Jasminum grandiflorum* L. (Oleaceae)	Jasmine	6	6 (18×10^4)
37	*Juniperus communis* L. (Cupressaceae)	Juniper	5	5 (12.5×10^4)
38	*Laurus nobilis* L. (Lauraceae)	Bay	25	32 (512×10^4)
39	*Lavandula officinalis* Caix (Lamiaceae)	Lavender	10	15 (112.5×10^4)
40	*Marjorana hortensis* Moench./ Origanum marjorana L. (Lamiaceae)	Marjoram	10	10 (50×10^4)
41	*Matricaria chamomilla* L. (Asteraceae)	Chamomile	-	-
42	*Melaleuca alternifolia* L. (Myrtaceae)	Tea	7	13 (84.5×10^4)
43	*Melaleuca leucadendron* L. (Myrtaceae)	Cajuput	7	13 (84.5×10^4)
44	*Mentha piperita* L. (Lamiaceae)	Peppermint	-	-
45	*Michelia champaca* L. (Magnoliaceae)	Champa	10	15 (112.5×10^4)
46	*Moringa oleifera* Lamk. (Moringaceae)	Drumstick	-	-
47	*Myristica fragrans* Houtt. (Myristicaceae)	Nutmeg	-	-
48	*Myristica fragrans* Houtt. (Myristicaceae)	Mace	5	5 (12.5×10^4)
49	*Nardostachys jatamansi* (D.Don) DC. (Valerianaceae)	Spikenard	-	-

50	*Ocimum basilicum* L. (Lamiaceae)	Basil	23	31 (480.5 x 10^4)
51	*Olea europaea* L. (Oleaceae)	Olive	-	-
52	*Pelargonium graveolens* L. (Geraniaceae)	Geranium rose	11	25 (312.5 x 10^4)
53	*Persea americana* Mill. (Lauraceae)	Avocado	-	-
54	*Pimpinella anisum* L. (Apiaceae)	Anise	-	-
55	*Piper cubeba* L. (Piperaceae)	Cubeb	-	-
56	*Piper nigrum* L. (Piperaceae)	Black pepper	7	7 (24.5 x 10^4)
57	*Polianthus tuberosa* L. (Agavaceae)	Tuberose	10	12 (72 x 10^4)
58	*Pogostemon cablin* (Blanco) Benth. (Lamiaceae)	Patchouli	-	-
59	*Primula rosea* Royle (Primulaceae)	Evening primrose	15	23 (264.5 x 10^4)
60	*Prunus amygdalus* Batsch. (Rosaceae)	Sweet almond	-	-
61	*Prunus armeniaca* L. (Rosaceae)	Apricot	-	-
62	*Rosa damascena* Mill. (Rosaceae)	Rose (absolute)	19	27 (364.5 x 10^4)
63	*Rosmarinus officinalis* L. (Lamiaceae)	Rosemary	7	10 (50 x 10^4)
64	*Salvia officinalis* L. (Lamiaceae)	Sage	10	10 (50 x 10^4)

65	*Salvia sclarea* L. (Lamiaceae)	Clary sage	-	-
66	*Saussurea lappa* Decne (Asteraceae)	Costus	-	-
67	*Sesamum indicum* L. (Pedaliaceae)	Sesame	-	-
68	*Syzygium aromaticum* (L.) Merrill & Perry (Myrtaceae)	Clove	28	36 (648×10^4)
69	*Tagates erecta* L. (Asteraceae)	Tagetes	-	-
70	*Thymus vulgaris* L. (Lamiaceae)	Thyme	12	17 (144.5×10^4)
71	*Valeriana jatamansi* Jones (Valerianaceae)	Valerian	-	-
72	*Vetiveria zizanioides* (L.) Nash (Poaceae)	Vetiver	-	-
73	*Vitis vinifera* L. (Vitaceae)	Grapes	-	-
74	*Zingiber officinale* Rosc (Zingiberaceae)	Ginger	-	-
75	Viola odorata L. (Violaceae)	Violet	7	11 (60.5×10^4)
76	Hexaconazole		15	30 (450×10^4)

[1] Values in paranthesis indicates number of the spores inhibited
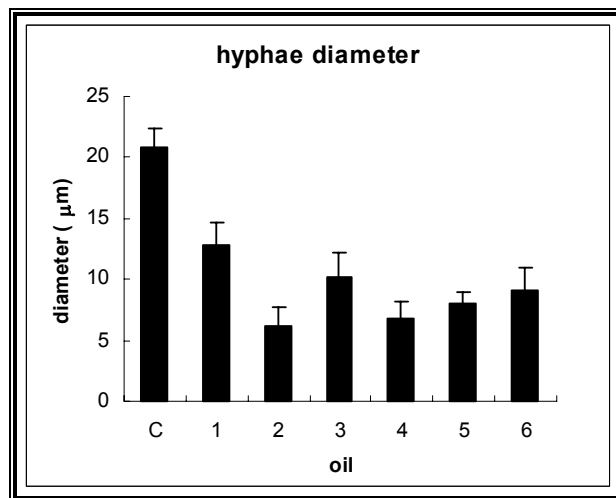
[2] After 72 h

Figure 1

Inhibition of hyphae diameter

C - Control; 1 - Cinnamon bark; 2 - Cinnamon leaf; 3 - Cassia;
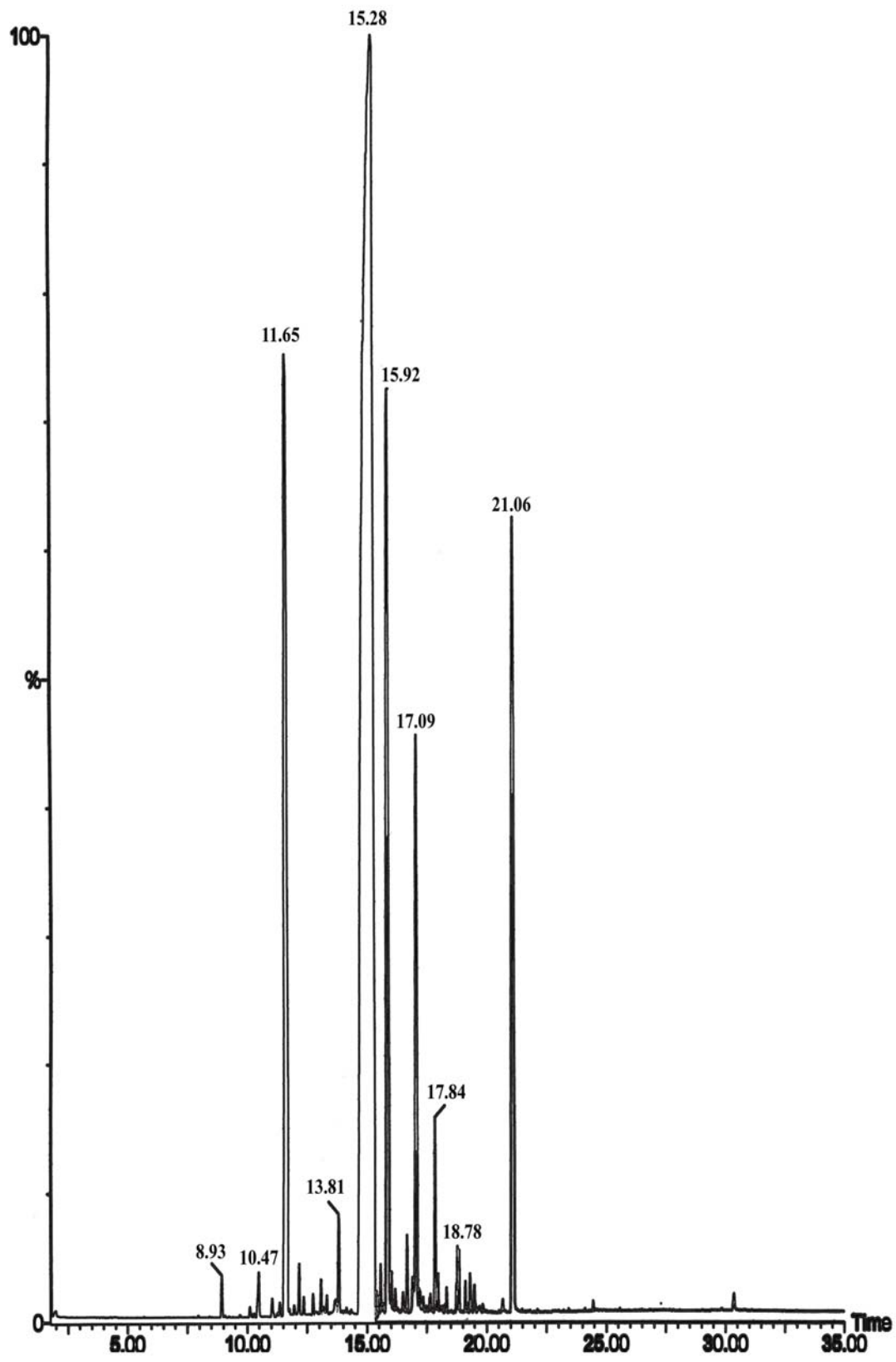
4 - Clove; 5- Thyme; 6- Cumin

Plate 1a
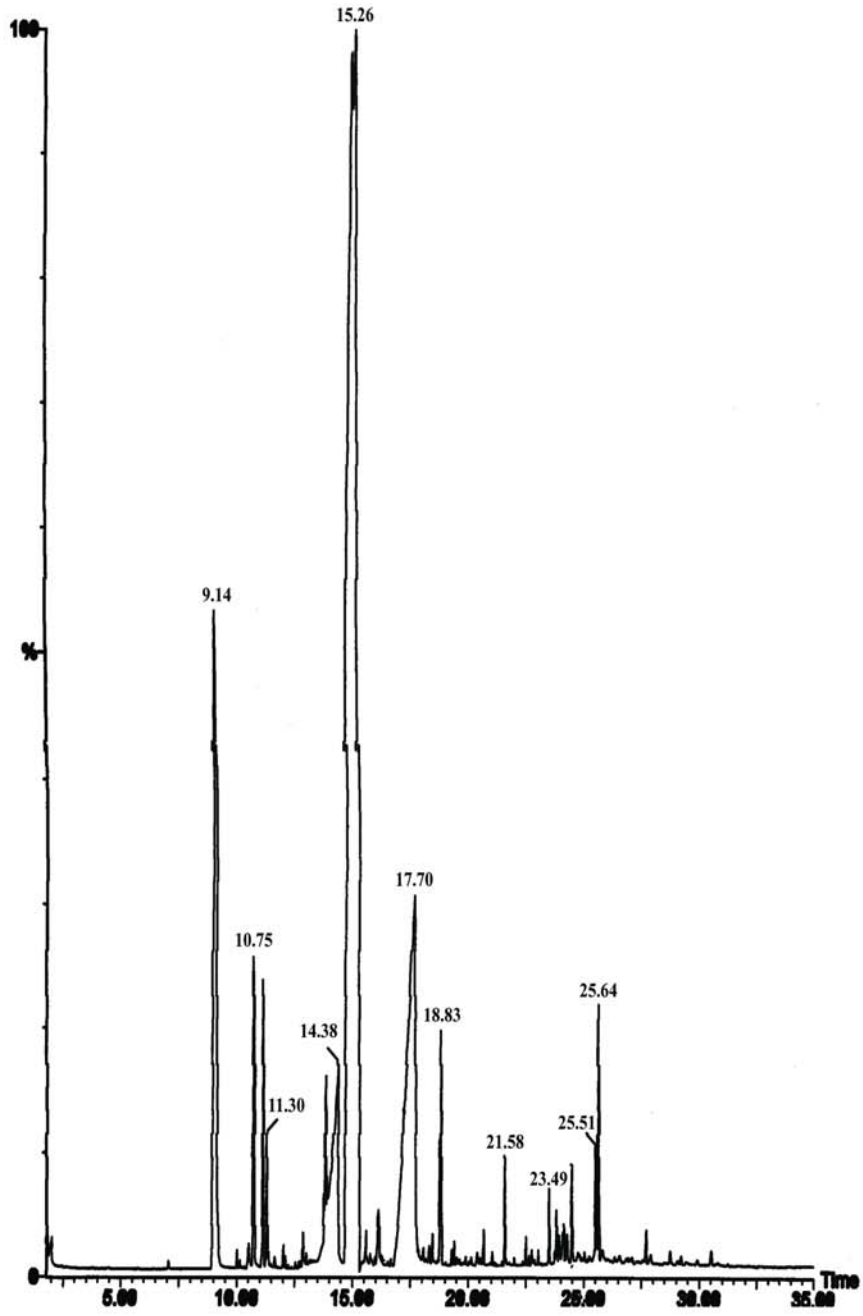**Chromatogram of Cinnamomum zeylanicum (bark) EO**

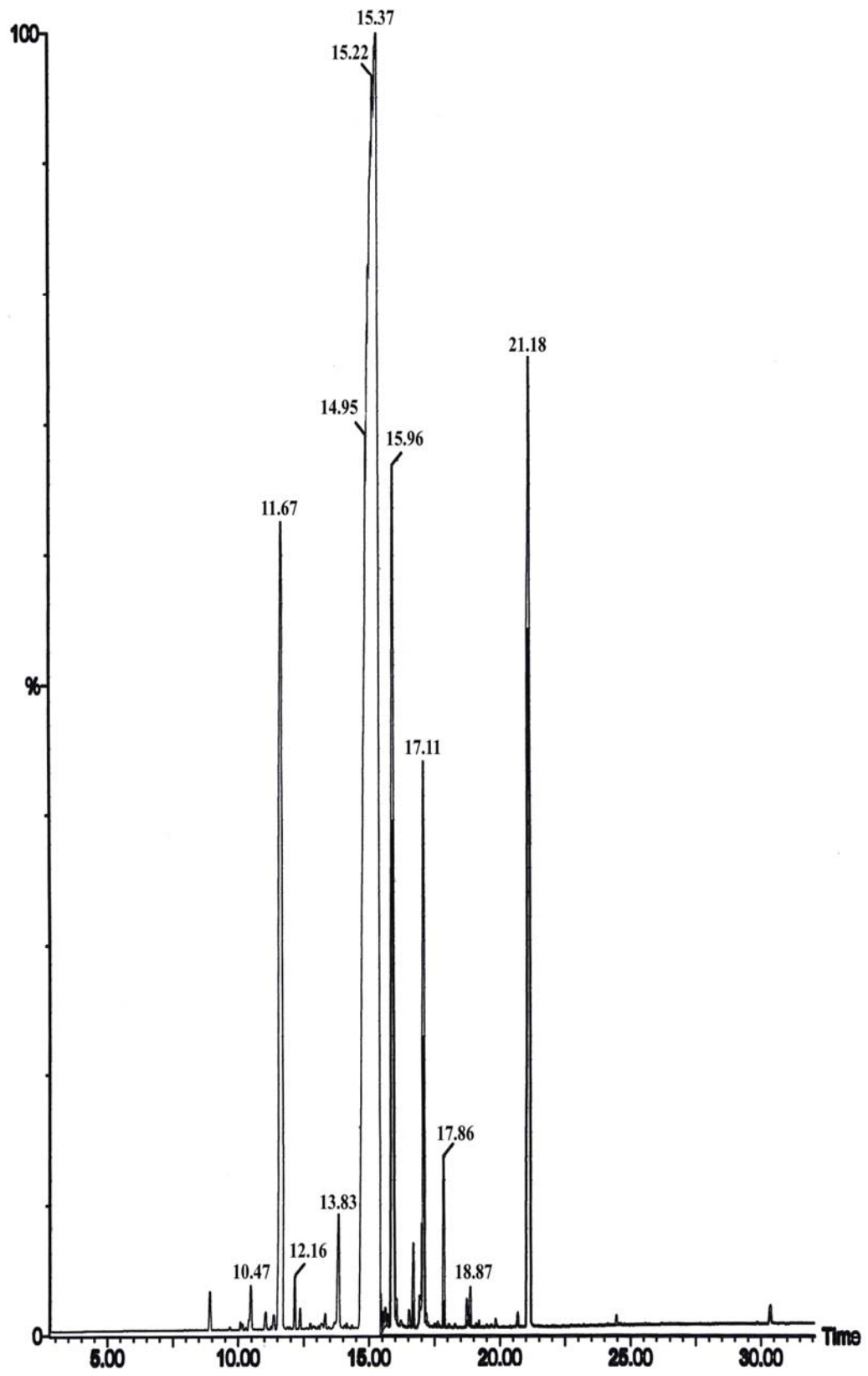**Plate 1b**
Chromatogram of Cinnamomum zeylanicum (leaf) EO
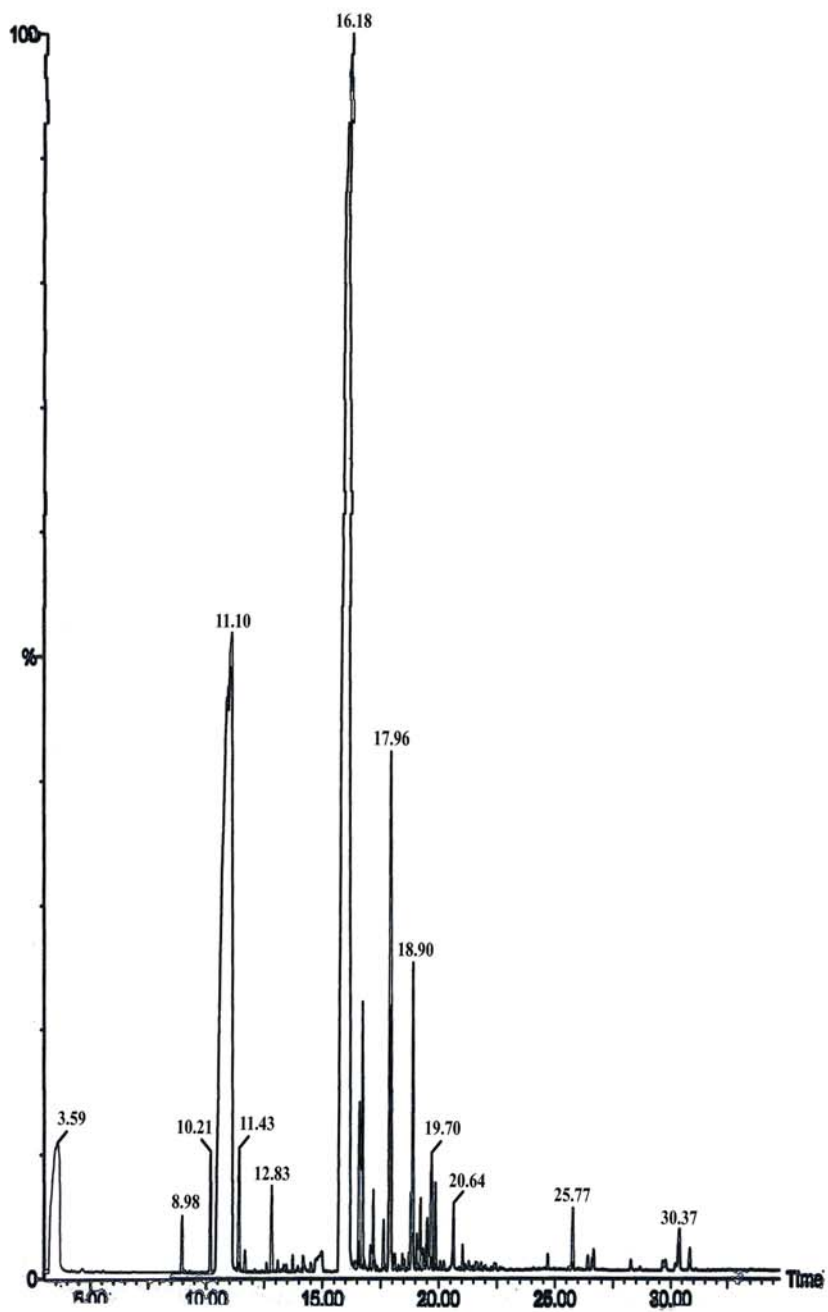
Plate 1c
Chromatogram of Cinnamomum cassia EO
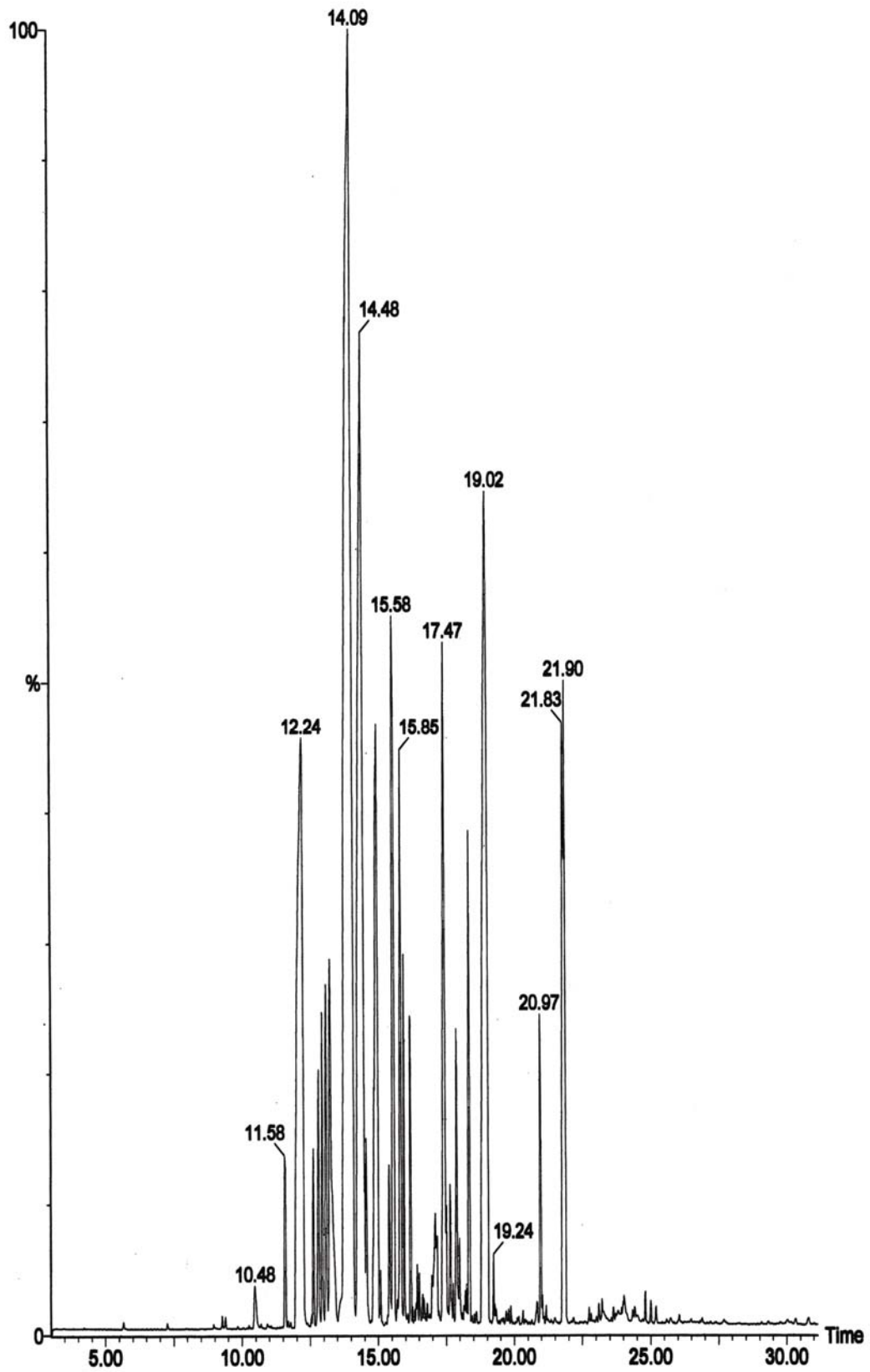
Plate 1d
Chromatogram of Syzygium aromaticum EO

Plate 1e
Chromatogram of Primula rosea EO
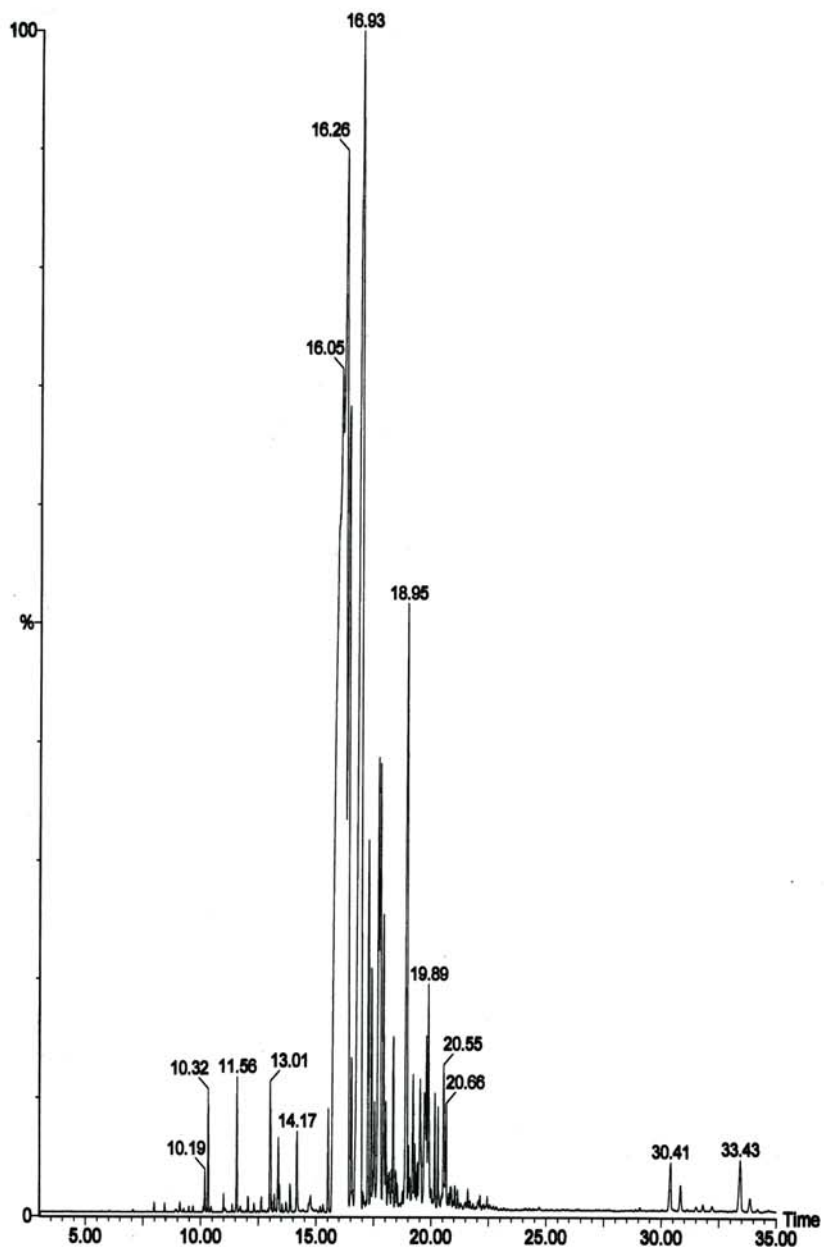
Plate 1f
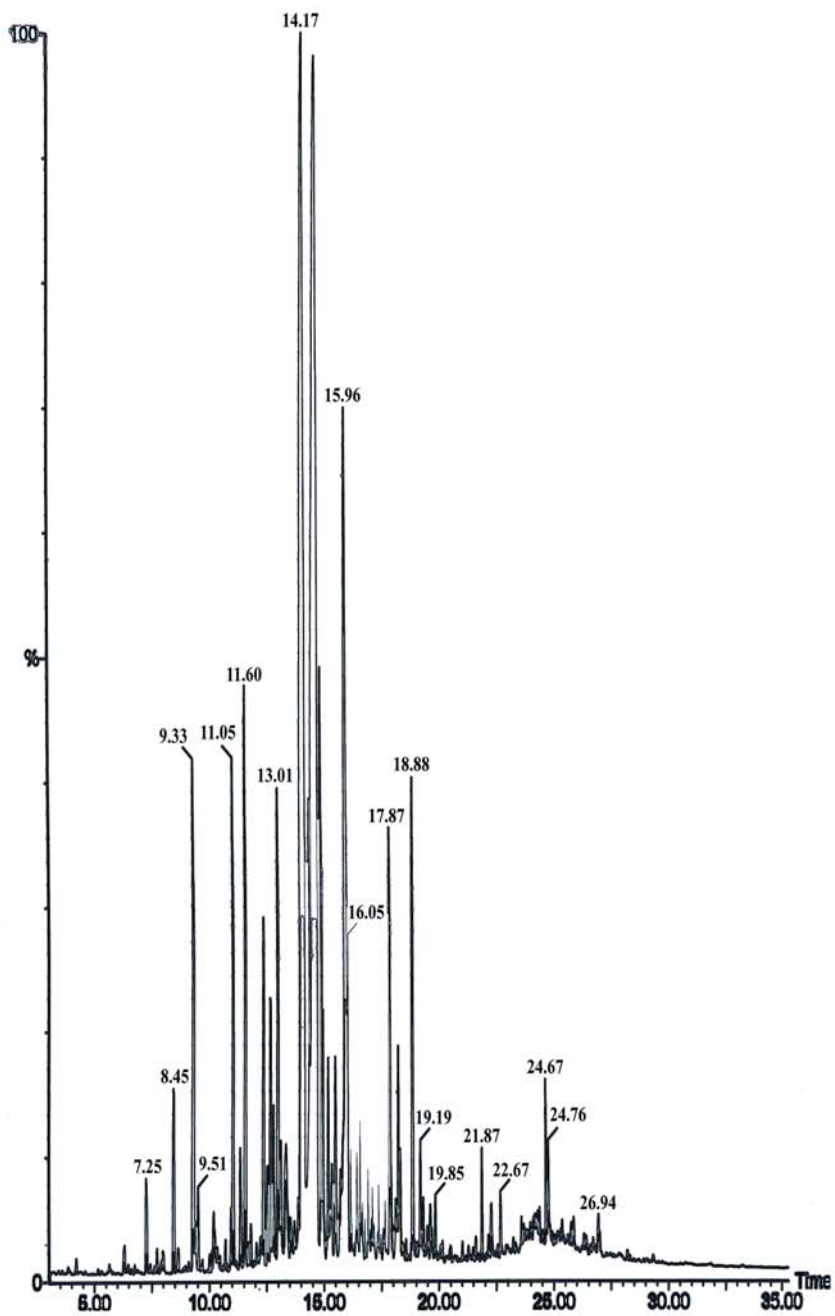**Chromatogram of Ocimum basilicum EO**

Plate 1g
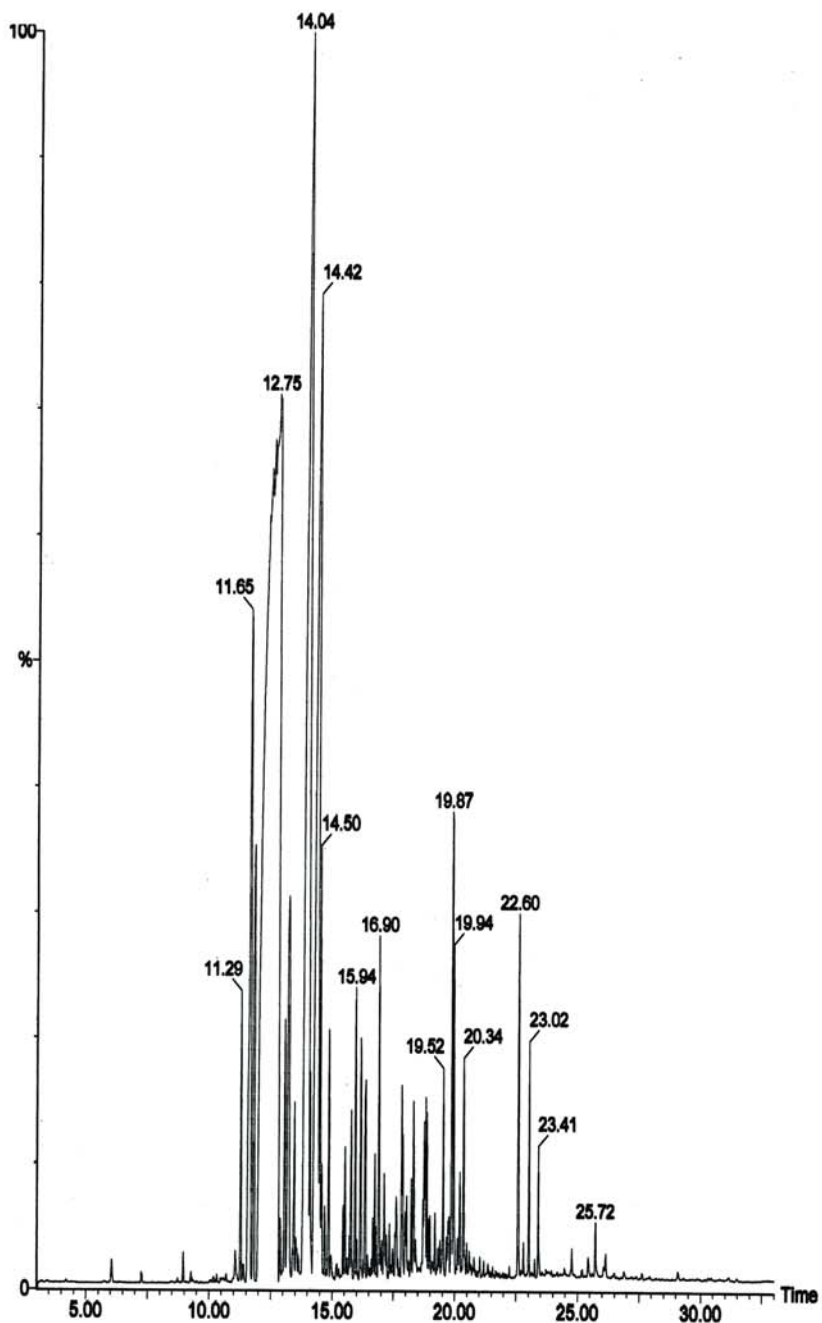**Chromatogram of Cymbopogon citratus EO**

Plate 1h
Chromatogram of Rosa damascena EO
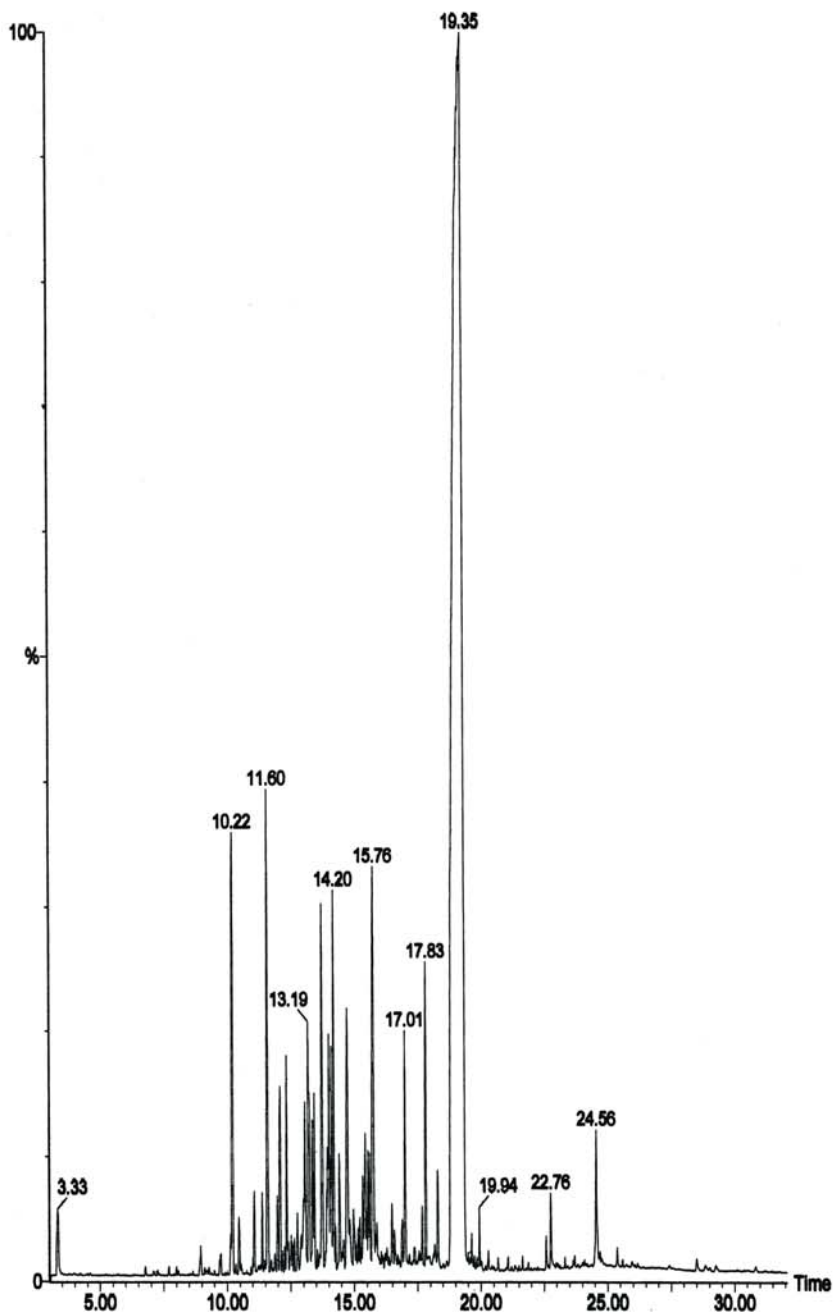
Plate 1i
Chromatogram of Citrus bergamia EO
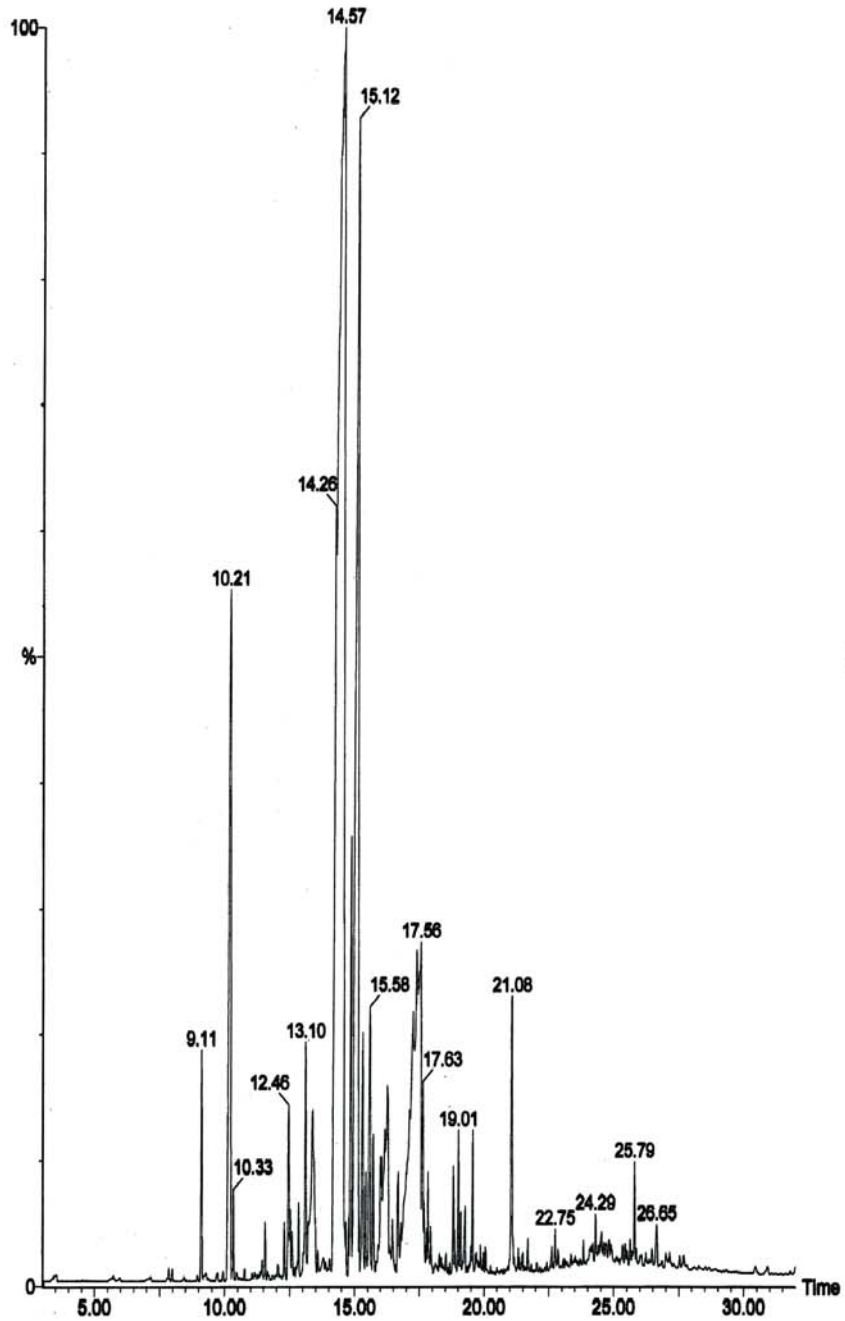
Plate 1j
Chromatogram of Cuminum cyminum EO

LEGEND TO PLATE 2

Zone of complete inhibition and spore inhibition of *A. niger* by different essential oils

a   Cinnamon bark

b   Cassia

c   Cinnamon leaf

d   Clove

e   Bay

f   Basil

g   Rose

h   Bergamot

i   Evening primrose

j   Thyme

k   Geranium rose

l   Ylang Ylang

Plate 2

CHAPTER 2B

EVALUATION ANTI-*FUSARIUM OXYSPORUM* F.SP.*CICER* AND ANTI-*ALTERNARIA PORRI* EFFECT OF SOME ESSENTIAL OILS

ABSTRACT

Anti *Fusarium oxysporum* f.sp *cicer* (*FOC*) and anti *Alternaria porri* (*A. porri*) effect was evaluated for 75 different essential oils. Most active essential oils found were those of Lemongrass, Clove,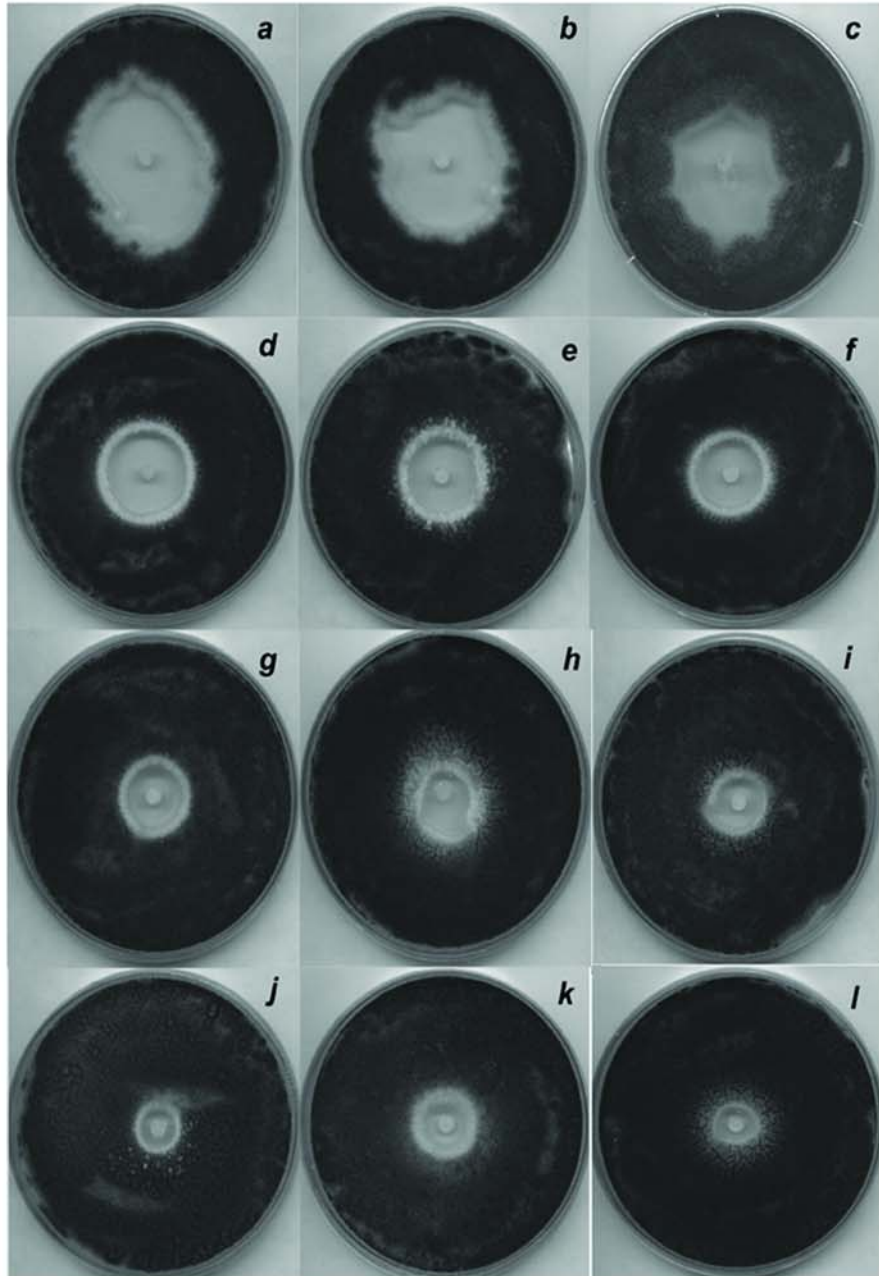 Cinnamon bark, Cinnamon leaf, Cassia, Fennel, Basil, Evening primrose and Cumin. However, the effectiveness of these essential oils with both the tested fungi showed different response. The level of inhibition was compared with Hexaconazole. GC-MS analysis for ten oils amongst the seventy-five tested essential oils was performed. The potential of these essential oils in ecofriendly and economic approach as a fungicide for *FOC* and *A. porri* is discussed.

Keywords: *Alternaria porri (A. porri)*, essential oils, *Fusarium oxysporum* f.sp *cicer (FOC),* GC-MS

INTRODUCTION

Fusarium is a plant pathogen causing wilt diseases of several economically important plants and is also known to produce toxins thought to contribute to wilting by affecting the permeability of cell membranes and disrupting cell metabolism (Alexopoulos & Mims, 1979). Several *Fusarium species* are widespread pathogens on small-grain cereals around the world and can cause root, stem and ear rot, resulting in severe reductions in crop yield, often estimated at between 10% and 40% (Antonio & Giancarlo, 2002). The wilt of castor caused by *F. oxysporum* f.sp *ricini*, is the most serious disease of castor in India (Desai & Dange, 2003). Fusarium wilt in tomato plants (*Lycopersicon esculentum Mill.*) caused by *F. oxysporum* f.sp *lycopersici* have an economic impact. As with other vascular plant diseases, chemical control is not effective and sanitation measures are difficult to apply (Brayford, 1992).

Most of the *Alternaria sp.* are parasitic causing leaf spot diseases. *A. solani* causes "early-blight" of potato and other members of solanaceae. Several form species are found as saprobes on dead and dying plant parts in the soil from which the conidia are picked up by the wind, and they invade laboratories where they are troublesome as contaminants of cultures (Alexopoulos & Mims, 1979). Alternaria black spot disease of rapeseed-mustard caused by *A. brassicae* (Berk.) Sacc, in India causes an average yield loss of 35-40% (Kolte *et al.,* 1987). *A. alternata* Japanese Pear pathotype causing black spot disease of the Japanese pear is a pathogen associated with severe economic losses (Evelyn *et al.,* 2004). Alternaria blight (*A. helianthi*) and Brown spot of citrus (*A. alternata* pv. *citri*) are serious diseases of sunflower (Theertha, 2003) and citrus fruits (Vicent *et al.,* 2004), respectively in India as well as in many parts of the world. *Alternaria* infected fruits are unmarketable due to lesions, resulting in important economic losses (Vicent *et al.,* 2000). *A. porri* infection may provide entrance to bacteria that cause bulb decay in storage.

Volatile compounds from plants, especially essential oils, have antimicrobial, fungicidal and insecticidal activity (Wilson *et al.,* 1997). Effectiveness of plant essential oils as soil fumigants to manage bacterial wilt (caused by *Ralstonia solanacearum*) in tomato has been studied (Pradhanang *et al.,* 2003). Preliminary *in vitro* and greenhouse experiments conducted with several plant essential oils and their components showed that some essential oils have significant efficacy against *R. solanacearum* (Momol *et al.,* 1999) and against several soil borne fungi of tomato (Momol *et al.,* 2000). Formulations of Clove oil, Neem oil, Pepper extract and Mustard oil, Cassia extract, synthetic Cinnamon oil were tested against *Phytophthora nicotianae* (Browers & Locke, 2004). Essential oils from Pepper, Mustard, Cassia tree and Clove suppressed disease development caused by *F. oxysporum* f.sp *melonis* on Muskmelon and reduced population density of *F.oxysporum* f.sp *chrysanthemi* in greenhouse experiments (Browers & Locke, 2000).

Public demands to reduce pesticide use, stimulated by greater awareness of environment and health issues, as well as development of resistance of some of the pathogens to fungicides, limits the postharvest application of chemicals to agricultural products (Karabulut & Baykal, 2003). Only a few essential oils have been evaluated for their anti *Fusarium* activity (Browers & Locke, 2000; Rai *et al.,* 1999) and up to our knowledge no reports are there for evaluation of essential oils against *A. porri.* In the present study, 75 essential oils were evaluated for their antifungal effects on *FOC* and *A. porri.*

MATERIALS AND METHODS

Test Fungus

The test fungi *FOC* (VP-003) and *A. porri* (VP-010) were isolated from Chick Pea and Garlic plants, respectively and identified in the Gujarat Agricultural University, Junagadh, Gujarat. The cultures of these fungi were maintained on Potato Dextrose Agar media at 4 ^{o}C.

Culture suspension

A disc (1.1 cm) from one week old culture of both the fungi was cut from periphery and suspended in 10 ml of sterile distilled water. It was shaked vigorously and centrifuged to remove the agar particles. The supernatant was used as a culture suspension for spreading.

Essential oils

Same as presented in Chapter 2A

Gas chromatography – mass-spectral analysis

Same as presented in Chapter 2A

RESULTS

In both of the fungal species, different oils exhibited different antifungal activities. Maximum antifungal activity for *FOC* was exhibited by the oils of Lemongrass (77 mm), Cumin (68.5 mm), Fennel (49.8 mm), Cinnamon bark (40 mm), Cassia (37 mm) and Cinnamon leaf (35 mm), respectively (Plate 3). Other oils like Bergamot (18.6 mm), Bay (15.3 mm) and Basil (17.25 mm) exhibited activity between 15 mm to 20 mm. However most of the oils showed activity between 7-15 mm and the rest were ineffective (Table 4).

For *A. porri* the oils of Cinnamon bark (50 mm), Cinnamon leaf (50 mm), Cassia (45 mm) and Clove (40.5 mm) exhibited highest activity i.e. between 40-50 mm (Plate 4). The inhibitory zones of *A. porri* by the essential oils of Champa, Anise and Evening primrose were 31.25 mm, 35 mm and 37.3 mm, respectively. Most of the other oils like Cumin (28.5 mm), Basil (26.9 mm), Thyme (26.6 mm), Wintergreen (24.6 mm), Ylang Ylang (24 mm) and Rosemary (21 mm) exhibited activity between 20-30 mm. Thirty seven other essential oils were moderately effective showing the zone diameter between 5 to 20 mm and rest of the oils were not effective at all (Table 4).

The GC-MS analysis of ten oils amongst the seventy five essential oils tested was performed (Table 2) and the typical chromatograms of these oils are shown in Plate 1. The highest % area of citral (29.4) and β-citral (21.39) was found in Lemongrass oil. Cinnamaldehyde occupied the maximum portion of Cinnamon bark (64.13), Cassia (66.36) and Cinnamon leaf (58.49). The highest amount of eugenol (47.64) was detected in Clove (Table 2). β-linalool, (E)-cinnamaldehyde, eugenol, cinnamyl acetate, eugenyl acetate and benzyl benzoate were the common components found in the essential oils of Cinnamon bark and Cassia.

Benzyl alcohol and eugenol were detected in Cassia and Clove oils but the percentage of these components in Clove oil was more than that found in Cassia oil (Table 2). On the other hand, cuminol, p-cumic aldehyde, β-citronellol, cis-geraniol, iso-eugenol, caryophyllene, β-linalool, cinnamaldehyde, etc., are the other most important constituents of Cumin, Rose, Basil, Evening primrose and Bergamot oils.

Cinnamon bark, Cinnamon leaf and Cassia oils studied for their antifungal activity were found to be highly inhibitory against both the phytopathogenic fungi. Amongst both the fungi, *A. porri* was found to be more susceptible towards the oils of Clove, Ylang Ylang, Wintergreen, Champa, Basil, Anise, Evening primrose, Rosemary and Thyme, whereas *FOC* was more prone towards the inhibitory action of Lemongrass, Cumin and Fennel as compared to *A. porri.*

DISCUSSION

Several studies are going on to explore the potential of essential oils as antifungal agents (Ko *et al.,* 2003; Oxenham *et al.,* 2005). In this study, 75 essential oils all together have been studied against *FOC* and *A. porri.* Both of these fungi are plant pathogenic and are responsible for disease at both, storage and field levels. So considering the importance of essential oils as ecofriendly agents, they were studied against these fungi.

It is interesting to note that Cinnamon bark, Cinnamon leaf and Cassia oils exhibited high antifungal activity against both the tested fungi. Lemongrass and Cumin oil, exhibited highest antifungal activity against *FOC* but it was not found equally effective against *A. porri.* On the other hand, Clove, Evening primrose and Basil oils were found to be highly active against *A. porri* but not for *FOC.* Considering this fact, these oils were subjected to GC-MS analysis to investigate the components present in them and detect the reason behind this opposite activity.

Lemongrass oil consisted highest percentage of citral and β-citral (Table 2). There are reports where citral a and b are indicated as fungicidal constituents in Lemongrass oil (Paranagama *et al*., 2003). Previous work on antimicrobial activities of several essential oil components indicated that cineole, citral, geraniol, linalool and menthol were active against several yeast-like and filamentous fungi (Pattnaik *et al*., 1997). Lemongrass oil has also exhibited antifungal activity against *Botrytis cinerea* (Wilson *et al.,* 1997).

Moreover the highest % area in Cumin oil was covered by p-cumic aldehyde which may be highly inhibitory against *FOC*. Thus it may be concluded that citral and p-cumic aldehyde are acting against *FOC* but they are not efficient in inhibiting *A. porri.*

Clove and Basil oils were dominated by eugenol and iso-eugenol, respectively. There are reports stating eugenol and clove oil as an antifungal agents (Núñez *et al.,* 2001; Chami *et al.,* 2004). But in this study, the inhibitory effect of Clove oil on *FOC* is low compared to *A. porri.* On the other hand, oil of Evening primrose, in which β-citronellol occupied the maximum area, was also found to be less effective for *FOC* compared to *A. porri.* Thus eugenol and β-citronellol may not have the potential to inhibit *FOC.*

Cinnamaldehyde occupied the highest % area in Cinnamon bark, Cinnamon leaf and Cassia oils and above all, these oils are inhibitory to both the tested fungi. Recent studies suggested that the antifungal activity of Cinnamon oil was probably due to the major component, cinnamaldehyde (Simi *et al.,* 2004). Considering these reports, it may be concluded that, cinnamaldehyde may also have a probable role in the inhibition of both the fungi tested. It was observed that 0.5 µl of Hexaconazole per disc could not have much influence on *FOC*, whereas, *A. porri* was more susceptible to its poisoning effect (Table 4).

The inhibitory effects of essential oils of Eucalyptus, *Ocimum basilicum*, *Prosopis cinerea, Derris indica* (Rai *et al.,* 1999) and *Chukrasia tabularis, Melia dubia* and *Hyssopus officinalis* (Nagalakshmi *et al.,* 2003) were evaluated against *F. oxysporum*. Antifungal properties of essential oils of three *Pistacia species* have also been studied for *F. sambucinum* (Duru *et al.,* 2003). The essential oil from *Melaleuca alternifolia* has also shown potential to reduce disease incidence and severity from foliar *A. solani* on potato and *Cercospora beticola* on sugar beets (Caolotanski *et al.,* 2002). Lack of effectiveness of tea tree oil is proved against *R. solanacearum* even though this essential oil in high concentrations is effective *in vitro* against *B. cinerea* (Wilson *et al.,* 1997).

In the present investigation *Melaleuca alternifolia* has also revealed good antifungal activity. The antifungal activity of Thyme oils is well established against fungi such as *B. cinerea* (Wilson *et al.,* 1997), *Rhizopus stoloniger* (Reddy *et al.,* 1997), *Aspergillus sp.* (Paster *et al.,* 1995), *Rhizoctonia solani, Pythium ultimum, Fusarium solani* and *Colletotrichum lindemuthianum* (Zambonelli *et al.,* 1996), and thymol appears to be one of the predominant active components of these oils (Reddy *et al.,* 1997; Zambonelli *et al.,* 1996).

Recently however, the use of fungicidal chemicals has fallen into disfavor because of their detrimental effects on nontarget organisms and environment pollution (Evelyn *et al.,* 2004). If natural plant products can reduce populations of soil borne plant pathogens and control disease development, then these plant extracts have potential as environmentally safe alternatives and as components in integrated pest management programmes (Browers & Locke, 2004). Moreover, the essential oils as such may be exploited for their fungitoxic potency because of the synergistic activity of their different compounds (Tripathi *et al.,* 2004; Sharma & Tripathi, 2005). Application of essential oils or their main components as a biorational alternative to conventional fumigants in field conditions will require further experimentation (Pradhanang *et al.,* 2003).

In the present study, out of the 75 oils tested, ten oils were selected for GC-MS analysis in which, three oils had antifungal activity against both the fungi while rest of the oils showed activity against either of the fungi. Thus cinnamaldehyde, citral and p-cumic aldehyde probably may be the active constituents against *FOC* and on the contrary, cinnamldehyde, eugenol and β-citronellol may be the active inhibitors of *A. porri*. It would be interesting to workout the real cause for the anti fungal activity and identify the target sites of these components.

However attempts have been made to explore the mode of action of essential oils against few fungi, where hyphae wall synthesis was found to be affected by the oils (Zambonelli *et al.,* 1996; Sharma & Tripathi, 2005). Thus these essential oils are potent antifungal agents with broad spectrum activity with possible potential for the control of fungal infections in plants as well as that of postharvest spoilage of many crops and crop products. Further studies need to be done to evaluate the efficacy of these oils against fungal infections so that its importance as an alternative to synthetic pesticides can be properly assessed. The sources of these oils are available easily in the local market and if they are used synergistically in the field conditions, they may prove to be more economic and environmentally safe.

TABLE 4

Activity of 75 different essential oils against *FOC* [a] and *A. porri* [a]

No.	Botanical name	Common name	*FOC* [b] Zone of inhibition (mm)	*A. porri* [b] Zone of inhibition (mm)
1	*Acorus calamus* L. (Araceae)	Calamus	6.6 ± 2	15.1 ± 0.15
2	*Anethum graveolens* L. (Apiaceae)	Dill	11.3 ± 1.7	9 ± 1
3	*Angelica archangelica* L. (Apiaceae)	Angelica	-	-
4	*Apium graveolens* L. (Apiaceae)	Celery	14 ± 1.6	13.3 ± 1.5
5	*Aniba rosaeodora* Ducke (Lauraceae)	Rose wood	12.5 ± 2.5	-
6	*Azadirachta indica* A.Juss (Meliaceae)	Neem	-	-
7	*Barringtonia acutangula* (Linn.) Gaertn. (Barringtoniaceae)	Myrht	13 ± 1.58	19.3 ± 1.15
8	*Calendula officinalis* L. (Asteraceae)	Calendula	-	-
9	*Cananga odorata* (Lam.) Hook.f. and Thoms. (Annonaceae)	Ylang Ylang	10 ± 1.41	24 ± 1
10	*Carum carvi* L. (Apiaceae)	Caraway	11 ± 2.8	7.4 ± 0.3
11	*Cedrus deodara* (Roxb. Ex D.Don) G.Don (Pinaceae)	Cedar	8.5 ± 1.29	11 ± 1

12	*Centella asiatica* (Linn.) Urban (Apiaceae)	Brahmi	8.8 ± 1.7	-
13	*Cinnamomum camphora* (Linn.) Presl. (Lauraceae)	Camphor	10 ± 1.41	13 ± 1
14	*Cinnamomum cassia* Blume (Lauraceae)	Cassia	37 ± 2	45 ± 0.9
15	*Cinnamomum zeylanicum* Blume (Lauraceae)	Cinnamon (bark)	40 ± 1.7	50 ± 0.8
16	*Cinnamomum zeylanicum* Blume (Lauraceae)	Cinnamon (leaf)	35±1.5	50±2
17	*Citrus aurantium* L. (Rutaceae)	Orange	10 ± 1.4	10 ± 1
18	*Citrus bergamia* Risso and Poit (Rutaceae)	Bergamot	18.6 ± 2.3	15.5 ± 1.3
19	*Citrus limon* (Linn.) Burm.f. (Rutaceae)	Lemon	13.2 ± 1.5	7.3 ± 0.6
20	*Citrus bigaradia* Hook.f. (Rutaceae)	Petitgrain	10.5 ± 2.12	8 ± 1
21	*Coriandrum sativum* L. (Apiaceae)	Coriander	11.5 ± 2.12	12 ± 1
22	*Crocus sativus* L. (Lamiaceae)	Saffron	13.3 ± 1.5	12.3 ± 0.6
23	*Cuminum cyminum* L. (Apiaceae)	Cumin	68.5 ± 2.6	28.5 ± 0.5
24	*Cupressus sempervirens* L. (Cupressaceae)	Cypress	-	-

25	*Curcuma longa* L. (Zingiberaceae)	Turmeric	-	9.6 ± 0.57
26	*Cymbopogon citratus* (DC.) Stapf. (Poaceae)	Lemon grass	77 ± 2.4	13.3 ± 0.6
27	*Cymbopogon martini* (Roxb.) Wats. (Poaceae)	Palmarosa	13.3 ± 1.5	9.6 ± 0.5
28	*Daucus carota* L. (Apiaceae)	Carrot	-	7.1 ± 0.1
29	*Elettaria cardamomum* (L.) Maton (Zingiberaceae)	Cardamom	12 ± 2	14.5 ± 0.5
30	*Foeniculum vulgare* Miller (Apiaceae)	Fennel	49.8 ± 1.86	9 ± 1
31	*Geranium macrorhizum* L. (Geraniaceae)	Geranium	9.5 ± 1.29	-
32	*Gaultheria frangrantissima* Wall. (Ericaceae)	Wintergreen	10 ± 2.8	24.6 ± 0.57
33	*Hamamelis virgianiana* L. (Hamamelidaceae)	Witch hazzel	11.3 ± 1.52	-
34	*Hedychium spicatum* (Buch.) Ham (Zingiberaceae)	Kapura kachari	-	-
35	*Hydnocarpus laurifolia* (Dennst.) Sleummer (Flacourtiaceae)	Mogra	8.5 ± 1.2	-
36	*Jasminum grandiflorum* L. (Oleaceae)	Jasmine	10.6 ± 1.52	9.66 ± 0.57
37	*Juniperus communis* L.	Juniper	7.5 ± 1.3	11 ± 1

	(Cupressaceae)			
38	*Laurus nobilis* L. (Lauraceae)	Bay	15.3 ± 1.53	-
39	*Lavandula officinalis* Caix (Lamiaceae)	Lavender	9.8 ± 1.7	13.3 ± 0.57
40	*Marjorana hortensis* Moench./ Origanum marjorana L. (Lamiaceae)	Marjoram	9.5 ± 1.29	7.6 ± 0.57
41	*Matricaria chamomilla* L. (Asteraceae)	Chamomile	9 ± 1	9.7 ± 0.5
42	*Melaleuca alternifolia* L. (Myrtaceae)	Tea	10.5 ± 0.7	15.3 ± 1.52
43	*Melaleuca leucadendron* L. (Myrtaceae)	Cajuput	13.5 ± 2.12	8.63 ± 0.55
44	*Mentha piperita* L. (Lamiaceae)	Peppermint	10 ± 1.8	10.6 ± 1.15
45	*Michelia champaca* L. (Magnoliaceae)	Champa	13 ± 1.58	31.25 ± 1.25
46	*Moringa oleifera* Lamk. (Moringaceae)	Drumstick	-	-
47	*Myristica fragrans* Houtt. (Myristicaceae)	Nutmeg	-	8.3 ± 0.57
48	*Myristica fragrans* Houtt. (Myristicaceae)	Mace	-	8.6 ± 0.57
49	*Nardostachys jatamansi* (D.Don) DC. (Valerianaceae)	Spikenard	-	-
50	*Ocimum basilicum* L. (Lamiaceae)	Basil	17.25 ± 1.7	26.9 ± 1.3

51	*Olea europaea* L. (Oleaceae)	Olive	9.5 ± 1.29	-
52	*Pelargonium graveolens* L. (Geraniaceae)	Geranium rose	9.5 ± 2.12	-
53	*Persea americana* Mill. (Lauraceae)	Avocado	-	-
54	*Pimpinella anisum* L. (Apiaceae)	Anise	15 ± 2.5	35 ± 2.2
55	*Piper cubeba* L. (Piperaceae)	Cubeb	7 ± 1	7.5 ± 1.29
56	*Piper nigrum* L. (Piperaceae)	Black pepper	9.5 ± 1.29	8.5 ± 0.7
57	*Polianthus tuberosa* L. (Agavaceae)	Tuberose	9 ± 1	11.3 ± 0.57
58	*Pogostemon cablin* (Blanco) Benth. (Lamiaceae)	Patchouli	-	-
59	*Primula rosea* Royle (Primulaceae)	Evening primrose	14.5 ± 1.29	37.3 ± 2.5
60	*Prunus amygdalus* Batsch. (Rosaceae)	Sweet almond	8 ± 1	-
61	*Prunus armeniaca* L. (Rosaceae)	Apricot	-	-
62	*Rosa damascena* Mill. (Rosaceae)	Rose (absolute)	13 ± 2.8	10 ± 1
63	*Rosmarinus officinalis* L. (Lamiaceae)	Rosemary	11 ± 2.8	21 ± 1
64	*Salvia officinalis* L. (Lamiaceae)	Sage	11 ± 1	7.83 ± 0.76
65	*Salvia sclarea* L. (Lamiaceae)	Clary sage	9 ± 1.4	-

66	*Saussurea lappa* Decne (Asteraceae)	Costus	-	10.7 ± 0.68
67	*Sesamum indicum* L. (Pedaliaceae)	Sesame	-	-
68	*Syzygium aromaticum* (L.) Merrill & Perry (Myrtaceae)	Clove	12 ± 1.4	40.5 ± 0.5
69	*Tagates erecta* L. (Asteraceae)	Tagetes	12 ± 1	-
70	*Thymus vulgaris* L. (Lamiaceae)	Thyme	10.5 ± 2.1	26.6 ± 2.8
71	*Valeriana jatamansi* Jones (Valerianaceae)	Valerian	-	-
72	*Vetiveria zizanioides* (L.) Nash (Poaceae)	Vetiver	9.5 ± 0.7	9 ± 1
73	*Vitis vinifera* L. (Vitaceae)	Grapes	-	-
74	*Zingiber officinale* Rosc (Zingiberaceae)	Ginger	-	12.6 ± 2.5
75	Viola odorata L. (Violaceae)	Violet	12.6 ± 2.5	-
76	Hexaconazole		15.3 ± 0.57	57.5 ± 1.29

[a] Mean growth measurements calculated from three replicates of both the fungi

[b] after three days of inoculation

LEGEND TO PLATE 3

Zone of inhibition of *FOC* by different essential oils

        a   Cinnamon leaf

        b   Cassia

        c   Cedar

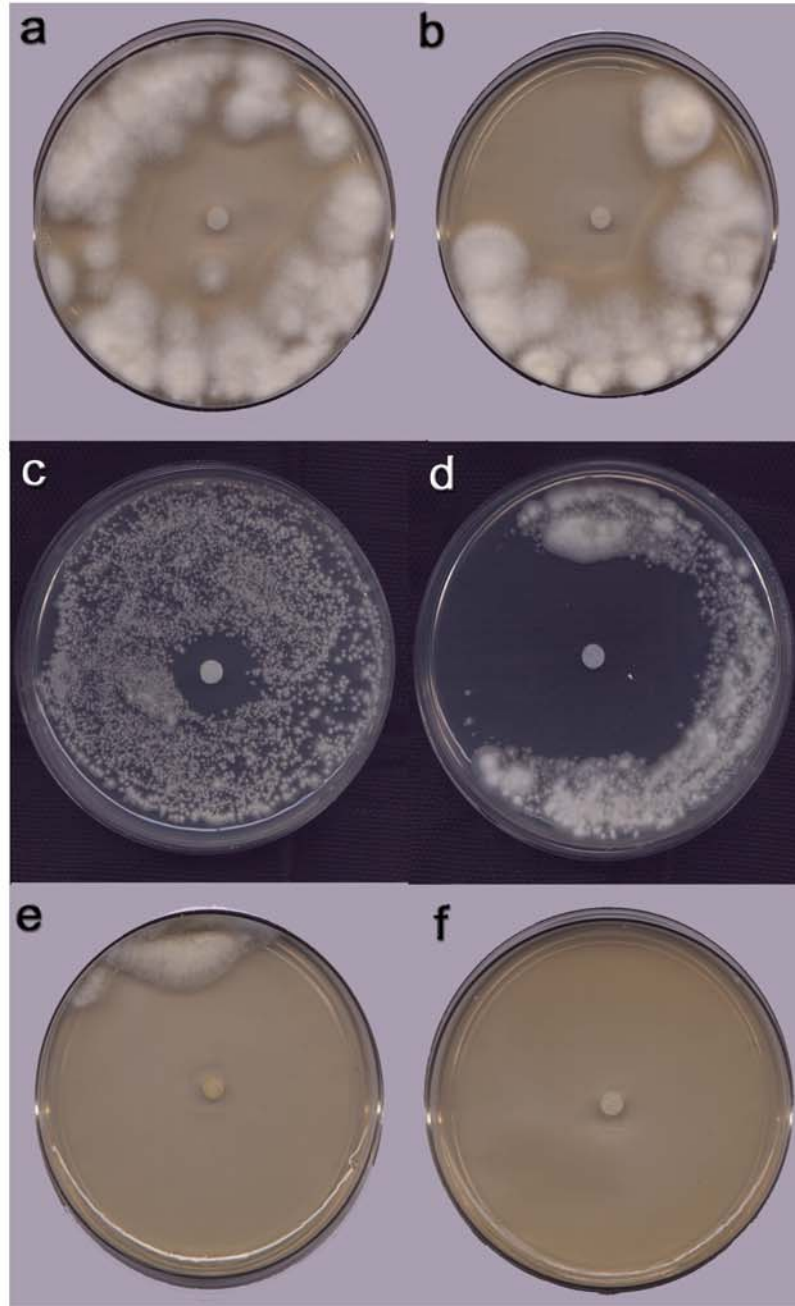   **d**   Cinnamon bark

        e   Cumin

        f   Lemongrass

Plate 3

LEGEND TO PLATE 4

Zone of inhibition of *A. porri* by different essential oils

a Cassia

b Clove

c Calamus

d Champa

e Costus

f Ginger

g Evening primrose

h Rose
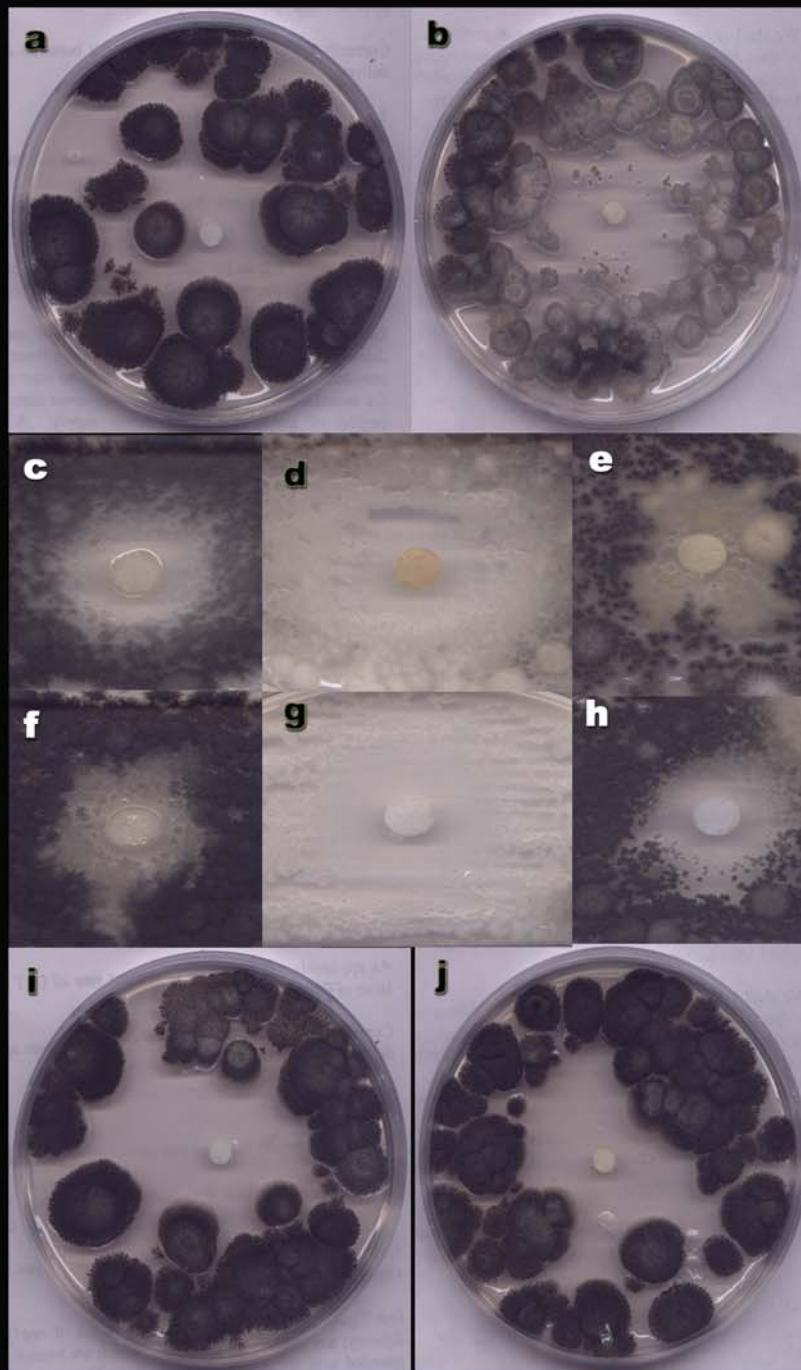
i Cinnamon bark

j Cinnamon leaf

Plate 4

CHAPTER 2C

ANTIFUNGAL ACTIVITY OF 10 ESSENTIAL OILS AGAINST FEW PHYTOPATHOGENIC FUNGI

ABSTRACT

Ten different essential oils were evaluated against four plant pathogenic fungi, *Fusarium solani, Rhizoctonia bataticola, Botryodiplodia theobromae* and *Curvularia sp.* Gas Chromatography-Mass Spectrometry (GC-MS) analysis of these essential oils was performed to detect their chemical composition. The level of inhibition was compared with Hexaconazole. The essential oil of *Cuminum cyminum* was found to be the most potent against *F. solani* and *Curvularia sp.* The essential oil of *Cinnamomum zeylanicum* (leaf), *Primula rosea* and *Cuminum cyminum* demonstrated a broad spectrum of antimicrobial activity.

Keywords: Antifungal activity, *Botryodiplodia theobromae, Curvularia sp.*, essential oils, *Fusarium solani, Rhizoctonia bataticola*

MATERIALS AND METHODS

Essential Oils

The samples of essential oils used in this experiment were obtained from Vimal Research Society for Agrobiotech and Cosmic Powers (VIRSACO), Rajkot, Gujarat, India.

Ten different essential oils of *Cinnamomum zeylanicum (*leaf), *Cinnamomum zeylanicum (*bark), *Cinnamomum cassia*, *Syzygium aromaticum, Primula rosea, Oscimum basilicum, Cymbopogon citratus, Rosa damascena, Citrus bergamia and Cuminum cyminum were used*.

Gas chromatography – mass-spectral analysis

Same as described in Chapter 2A

Used Fungi

Five plant pathogenic test fungi *Fusarium solani* (VP-002), *Rhizoctonia bataticola* (VP-004), *Botryodiplodia theobromae* (VP-007) *and Curvularia sp.* (VP-008), were isolated from *Cuminum cyminum, Ricinus communis*, *Mangifera indica and Pennisetum typhoides,* respectively. They were cultured on Potato Dextrose Agar (PDA) media and maintained at 4 ^{o}C.

Antifungal activity

Same as described in Chapter 2A

RESULTS

Chemical composition of essential oils obtained by GC-MS analysis is reported in Table 2.

The results of antifungal activity are given in Table 5.

CONCLUSIONS

Tested oils showed different activities against all the four plant pathogenic fungi. Their chemical composition contained different compounds with β-linalool found in 5 oils and cinnamaldehyde present in 4 oils amongst the tested 10 essential oils. *C. cyminum* oil exhibited the pronounced antifungal activity against *F. solani* (10 cm) and *Curvularia sp.* (10 cm). This oil was prominent with cumic aldehyde and cuminol. The essential oils of *C. zeylanicum* (leaf), *P. rosea* and *C. cyminum* were effective against all the studied fungi but their inhibition degree varied with the fungal species. Cinnamaldehyde occupied the major portion of *C. zeylanicum* oil whereas β-citronellol content was highest in the oil of *P. rosea*. *R. bataticola* and *B. theobromae* were found to be resistant against 6 and 4 oils, respectively, amongst the ten essential oils tested. Thus the difference in the fungitoxic activity depends upon the chemical constituents of the oils tested and toxicity for the particular fungi.

TABLE 5

Antifungal activities of essential oils

	Zone of inhibition (cm) [#]			
Essential oils	*Fusarium solani* [*]	*Rhizoctonia bataticola* [*]	*Botryodiplodia theobromae* [*]	*Curvularia sp.* [*]
***Cinnamomum zeylanicum* (bark)**	5.46 ± 0.15	NZ	4.16 ± 0.28	4.16 ± 0.15
***Cinnamomum zeylanicum* (leaf)**	5.43 ± 0.21	2.36 ± 0.21	2.73 ± 0.25	6.4 ± 0.28
Cinnamomum cassia	4.9 ± 0.3	NZ	3.7 ± 0.17	4.3 ± 0.7
Syzygium aromaticum	3.2 ± 0.26	2.4 ± 0.1	NZ	4.26 ± 0.25
Primula rosea	2.46 ± 0.057	1.2 ± 0.2	3.13 ± 0.30	1.26 ± 0.05
Oscimum basilicum	3.23 ± 0.25	NZ	NZ	4.7 ± 0.98
Cymbopogon citratus	3.46 ± 0.45	NZ	NZ	5.2 ± 0.72
Rosa damascena	3.06 ± 0.11	NZ	NZ	3.7 ± 0.86
Citrus bergamia	2.13 ± 0.11	NZ	1.16 ± 0.15	1.16 ± 0.28
Cuminum cyminum	10	2.13 ± 0.23	2.4 ± 0.17	10
Hexaconazole	2.76 ± 0.25	2.06 ± 0.11	1.1 ± 0.14	4.56 ± 0.49

\# after 72 h of inoculation and done in 100 mm petri plate

* mean growth measurements in cm calculated from three replicates ± standard deviation

Hexaconazole (0.5 μl/disc); diameter of disc - 5 mm

EO's were analysed at 5 μl/disc concentration; NZ - no zone of inhibition

CHAPTER 3

STUDIES ON INHIBITORY ACTIVITIES OF ANTIBODIES AGAINST PHYTOPATHOGENIC FUNGI: AN IMMUNOLOGICAL APPROACH

ABSTRACT

The major problem in agriculture is that of the fungal pathogens. In this era, where biological control is at focus, and is the centre of crop protection as well as environmental protection, synthesis of new bio bodies is the utmost need. Fungicides available in the market, though potential, are pathogen specific and highly pollutive. An attempt was made to raise polyclonal antibodies against *Aspergillus niger*. Following the particular standardized immunization schedule, regular injections and periodic tapping was carried out. The IgG purified was used to check cross reactivity with different crop fungi like *Fusarium solani, Fusarium oxysporum* f.sp *cicer (FOC), Rhizoctonia bataticola, Botryodiplodia theobromae, Curvularia sp., Alternaria porri* and also with *Aspergillus niger* by two different methods. Liquid test media and radial growth inhibition test performed in solid media was used to check the inhibition of fungi and cross reactivity. The results were subjected to analysis of variance (ANOVA) by using Tukey test at the significance level of $p < 0.05$. The antibodies were active against all the fungi for more than fifteen days except for *A. niger* in which from the seventh day onwards spore germination was observed. The probable role of antibodies to detect the common antigenic molecule that may be present in all the tested fungi and their role in inhibiting these fungi is discussed.

Keywords: Alternaria porri, Antibody, *Aspergillus niger*; *Botryodiplodia theobromae,* cross reactivity, *Curvularia sp.*, *Fusarium oxysporum* f.sp *cicer (FOC)*, *Fusarium solani*, *Rhizoctonia bataticola*

INTRODUCTION

All human beings depend on agriculture that produces food of the appropriate quality at the required quantities. The greater part of the twentieth century, saw the increased reliance on chemical means to control pests and diseases (Rabbinge & Oijen van, 1997). In today's century there are a number of challenges to be faced to maintain the necessary food production (Miflin, 2000) and one of the challenges in plant biotechnology improvement is the intensified integration of biological control (Altman, 1999). The 20th century has seen a tremendous increase in crop yields (Rasmussen *et al*., 1998), one of the reasons for which is the intensified use of chemical fungicides. However, crop protection has become a matter of searching for the optimal combination of different control measures rather than reliance on one-chemical measure alone (Rabbinge & Oijen van, 1997).

Fungi are one of the agents causing diseases in plants and are the major problem in agricultural crops along with other pests. But most of the fungicides are chemical based and each fungus requires a specific fungicide. Biological control can be achieved by suppression of germination of fungal spores and suppressing or inhibiting growth of the pathogen (Chaube & Singh, 2000). There is a dire need for new fungicide treatments that could in part be alleviated by using biological control agents (Utkhede & Sholberg, 1993; Wisniewski & Wilson, 1992). Recently different biocontrol agents like fungi (Nel *et al*., 2006) and bacteria (Omar *et al*., 2006) have been used against fungi.

Antibodies are bioactive molecules that, owing to their individual and specific binding properties, allow a large diversity of potential applications like medical diagnosis and therapy, the sensitive detection and removal of environmental contaminants, control of pathogens and industrial purification processes (Stoger *et al*., 2002).

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Antibodies can react specifically with the pathogen or its metabolites and may be used for plant disease diagnosis. Fungal proteins and polysaccharides elicit an immune response when introduced into a higher animal, typically a rabbit, resulting in antibody production (Clausen & Green III, 1997). The capacity of the mammalian immune system to produce serum glycoproteins (antibodies) in direct response to immunization with foreign proteins or polysaccharides (antigens) has been exploited in many creative ways in plant pathology (Clausen & Green III, 1997). Once harvested, these antibodies possess the capacity to recognize, bind to, and neutralize the immunizing agent (antigen) (Clausen & Green III, 1997).

A large number of fungal products have been used successfully as direct fungal material, eg. mycelium fragments (pellets of homogenized mycelium), extracts of freeze-dried mycelium, spore suspension, and mixtures of spores and mycelia; and indirect fungal material, i.e. surface washes from the growth medium (extracellular polysaccharide) obtained directly from the growth medium or after column chromatography (Shapira *et al.*, 1997).

Many different techniques for the detection and identification of decay fungi (Jellison & Jasalavich, 2000) in plants as well as in human systems have been developed, like RIA and ELISA (Menning *et al.*, 2004), Immunohistochemistry or molecular techniques, such as PCR based diagnosis (Hamilton & Gómez, 1998), amplification and detection of specific fungal DNA sequences and the detection and quantification of specific fungal metabolic products (Yeo & Wong, 2002) as well as some cantilever array methods (Nugaeva *et al.*, 2005) have been developed.

In the present work polyclonal antibodies are produced against *A. niger*. Two batches of antibodies i.e. Ab1 with low titer and Ab2 with high titer are studied against seven crop fungi including *A. niger.* Inhibitiory activity of these antibodies with the tested fungi is checked by using two test methods i.e. solid and liquid.

Antibodies can also be used as a form of biological control and monoclonal antibodies have been identified that inhibit fungal growth (Hiatt *et al*., 2001). Antibodies have also been used to examine the degree of homology among fungal cellobiohydrolases (Ishihara *et al*., 1993) and biochelators (Jellison *et al*., 1991). To screen any biological agent one of the important attributes to understand is the infection process (Khamis *et al*., 2005). As antibodies may prove to be good biological control agents and excellent research tools, the ultimate goal of this work is to develop an understanding regarding the common antigen or metabolite/s which may be responsible for fungal growth in the host. In general, to screen out such common metabolite/s an immunological approach may prove to be easier, sensitive, economic and less laborious than other techniques.

MATERIALS AND METHODS

Test Fungi

In this study seven different fungi were isolated from different plants, like *Aspergillus niger (Allium cepa)*, *Fusarium solani (Cuminum cyminum)*, *Fusarium oxysporum* f.sp. *cicer (Cicer arietinum)(FOC)*, *Rhizoctonia bataticola (Ricinus communis)*, *Botryodiplodia theobromae (Mangifera indica)*, *Curvularia sp. (Pennisetum typhoides)*, *Alternaria porri (Allium sativum)* and were identified in Gujarat Agriculture University, Junagadh. The fungi were cultured and subcultured, thus maintained on Potato Dextrose Agar (PDA) media.

Preparation of Antigen

Fungal antigen was extracted from one week old *A. niger* growth. Whole cells were used as an antigen and immunized into two different rabbits (both the rabbits reacted equally but here the serum of only one rabbit was used).

Immunization

Each rabbit received intramuscular injections of antigen (1 mg/0.5 ml) emulsified with an equal volume of Freund's complete adjuvant (total 1 ml was administered) at 7 days interval. Blood was collected by ear vein puncture. The titer of raised antibodies was checked and it was found to be 1:1000 (Ab 1) after 3 months and 1:10000 (Ab 2) after 7 months of regular immunization.

Separation of Antibodies

Serum was clarified from blood clot by centrifugation. The IgG molecules were separated from serum by Ion Exchange Chromatography using DiEthyl Amino Ethyl (DEAE) cellulose column. These IgG molecules (serum) were collected in sterile eppendorfs and were further used in the experimental purpose.

Preparation of Inoculum

The fungi were cultured on PDA and after seven days of inoculation, discs (to maintain the same physiological age) of 1 cm were cut from the periphery with the help of cup borer and suspended in 10 ml of sterile distilled water. It was shaked vigorously and left as such for 2 days and then centrifuged to remove the agar particles and other debris. The supernatant was used as a culture suspension for inoculation.

Preparation of liquid test media

Murashige and Skoog (MS) media was used for testing the inhibitory activity of antibodies (Murashige & Skoog, 1962). MS media contains all the salts in the form of macro and micro nutrients in known concentrations and hence it is a defined media. Almost all the test fungi grew well in MS media (tested before by culturing the fungi in MS media) with 2% of sucrose concentration. Tubes were filled with 10 ml of MS media (without agar). The antibodies were added in the concentration of 2 µl/ml (86 µg protein/2 µl) MS media. The inoculum (0.1 ml) was added in the test tubes along with the controls. Results were noted daily in the form of positive signs (Table 6) and microscopic slides prepared were observed (Fig. 2). The hyphal diameter was observed with the help of Carl Zeiss microscope at 45X objective lens. The test was done in static conditions in the tubes. After inoculation, the tubes were shaked well and left static at 22 ^{o}C – 25 ^{o}C temperature.

Antifungal Bioassay (Radial growth assay in solid media)

The inhibitory activity of the antibodies (Ab 2) against all the seven fungi was evaluated using the Poisoned Food Technique (Grover & Moore, 1962; Janssen *et al*., 1987). Ten microlitres of antibodies was added to molten PDA media (10 ml) to give a concentration of 1 µl/ml. Similarly higher concentrations of 2, 3, 4, 5 and 6 µl/ml were prepared. The medium with antibodies was gently mixed between palms to avoid foaming. It was poured in 60 mm score top petri plates.

The centre of each PDA plate was inoculated with one fungal disc (7 mm diameter and seven days old as mentioned earlier) of the respective fungus. The plates were incubated at 27 ^{o}C for seven days. The antifungal activity of the antibodies was evaluated every 24 h by measuring the diameter of test and control colonies in millimeter. The fungitoxity of antibodies was recorded in terms of % mycelial inhibition (Khamis *et al*., 2005) against phytopathogenic fungi (Fig. 3).

Statistical analysis

The percent mycelial inhibition of radial growth was statistically evaluated with Analysis Of Variance (two way ANOVA) using Tukey test at $p < 0.05$ significant level to detect the significant differences between different test dosages and test period.

RESULTS

In the liquid test media, Ab 2 inhibited *F. solani* and *Curvularia sp.* for 15 days which was the test period, *R. bataicola* was inhibited up to 8 days whereas *FOC, B. theobromae* and *A. porri were* inhibited up to 5 days (Table 6). But *A. niger* was inhibited completely only for 4 days after that it started growing (Table 6). A clear difference was observed amongst the inhibitory action of Ab 1 and Ab 2 but both the antibodies inhibited *Curvularia sp.* during the complete test period. Thus *Curvularia sp.* was inhibited even at the low antibody titer. The results are expressed by the positive signs, each additional sign presents the increasing amount of growth and it is compared with that of control (Table 6).

In all the seven fungi the diameter of hyphae wall and complete hyphae was measured continuously for 5 days for Ab 1 only, because in Ab 2 there was no growth in most of the tested fungi at 2 µl/ml concentration for first 5 days of test period (Fig. 2). The diameter of hyphae (wall and complete hyphae) was observed to be less than that of control.

The radial growth assay was performed using Ab 2 which showed that, at the highest concentration tested (6 µl/ml); *F. solani* was inhibited for all the 7 days (Fig. 3b; Plate 5). *FOC, R. bataicola* and *A. porri* were inhibited up to 4 days, after that the degree of inhibition gradually decreased (Figs. 3c, 3d, 3g; Plate 5, 6, 7). In *B. theobromae* and *Curvularia sp.* up to 2 days the inhibition was observed but from 3rd day onwards gradual decrease in % mycelial inhibition was observed (Figs. 3e, 3f; Plate 6, 7). In *A. niger* for 3 days the % mycelial inhibition was observed but it was zero from the 4^{rth} day (Fig. 3a) onwards. At lower concentrations, as the incubation time increased, % mycelial inhibition decreased. The statistical analysis showed significant differences between and amongst test dosages of Abs for all the fungi (Table 7).

DISCUSSION

Antibodies provide an invaluable tool in fundamental research, because of their ability to interfere with metabolic processes within an organism (Stoger *et al*., 2002). In the present study polyclonal antibodies are produced against an antigen of *A. niger.* Generally polyclonal antibodies recognize multiple chemical sites of the targeted molecule, a fungal glycoprotein while monoclonal antibodies recognize one specific protein sequence on the targeted molecule. Reviews on the use of immunological techniques in mycology have been presented by Preece (1971) and Pepys and Longbottom (1978).

In the radial growth inhibition assay, there was a highly significant difference observed (two way ANOVA and Tukey test) in % mycelial inhibition for the consecutive days and increasing concentration of test dosages for the tested fungi except *A. niger* (Table 7). Maximum antifungal activity was found against *F. solani*, followed by *FOC*, A*. porri* and *R. bataticola.* The same results were obtained for *F. solani* in both the assay methods. But for all other fungi, up to 7 days, at 2 µl/ml concentration, the assay done in liquid media showed higher activity compared to the assay performed in solid media.

Here a comparison of two simple test methods for inhibitory activity of antibodies has been done. Many serological methods have been tested previously which showed cross reactivity with different fungi (Yeo & Wong, 2002). In general, antigen tests that use polyclonal antibodies raised against crude fungal antigens have significant cross reactivities with several pathogenic fungi (de Repentigny, 1992; Hamilton, 1998). Polyclonal and monoclonal antibodies have been produced and the mechanism of fungal infection of living cells and the role of enzymes has been previously investigated (Call & Mücke, 1996; Goodell *et al*., 1998).

However others have explained the role of low molecular weight chelators in degradation of white rot fungi (Goodell *et al*., 1997). It has been established that non-enzymatic oxidative degradation processes are involved in the brown rot attack of lignocellulose (Goodell *et al*., 1997; Kerem *et al*., 1999; Paszczynski *et al*., 1999). Antibodies against fungal melanin (Nosanchuk *et al*., 1998), six common brown rot fungi (Clausen *et al*., 1991) and against two white rot fungi *Armillaria mellea* and *Heterobasidium annosum* have also been produced (Zollfrank *et al*., 1987). Polyvalent antibodies are produced against bacteria (Leversee & Glatz, 2001) and extracellular proteins of anaerobic fungi (Xin-Liang *et al*., 1997) and monoclonal antibodies are also produced against *Plasmapare halstedii* fungi causing downy mildew (Bouterige *et al*., 2000). Moreover polyclonal and monoclonal antibodies have been prepared against xylanase from *Postia placenta* (Clausen *et al*., 1993).

But most of the research work has been targeted for detecting fungi in small sample size where cross reactivity has been a problem so monoclonal Ab were produced to detect a specific fungus. The present work also shows cross reactivity with fungi which has been tested through ELISA (data not presented) and is also depicted from the results presented here but due to this inhibition is resulted. Thus these antibodies which are active against the tested 7 fungi suggest that they might have some common antigenic protein. However, the important thing is that, the fungus which was used as an antigen to produce the polyclonal Abs, is resistant compared to all the other fungi which were found to be highly susceptible. The reason might be that, *A. niger* have developed a counteracting mechanism to allow its growth either by producing certain enzymic isoforms or changing the metabolic cycle which might not be affected by the tested Abs. Whereas other fungi might be poor in such changes, and hence are more susceptible. This gives us an idea that Ab works at molecular level and their cross reaction may help in exploring the mechanism of fungal infection as well as in finding the key metabolites

essential for their growth and development. In liquid media, though the concentration of Abs tested was 2 µl/ml, but as it was in dissolved form, the fungi would be in direct contact with each molecule of Abs hence were inhibited for several hours. However in radial growth assay, the Abs molecules were fixed in solid media, so due to poor interaction or diffusion of Abs molecules the fungi might have grown. Perhaps, a distinct physiological lag was observed at higher concentrations leading to the conclusion that by increasing the Abs concentration, the interaction of fungal metabolites with Abs would be more and hence drastic inhibition was observed. Diameter of the hyphal wall and complete hyphae was found to be less than that of control which gives an idea about the effect of Abs at physiological as well as molecular level. Accessibility of antibody to the cell wall structure suggests that antibody responses to cell wall antigen during the course of infection may be biologically relevant (Nosanchuk *et al*., 1998).

In the present work a distinct lag phase was observed which led to a conclusion that the interaction of antibody with fungi might have been restricted due to poor mobility of the antibody. However, antibody with a higher titer increased the lag phase of fungi for more than fifteen days which was observed in the liquid assay. It is concluded that all the tested fungi might possess a common antigen which can be traced out by the help of antibodies produced against them. It may also be possible that the molecule against which the Abs are produced may be important for growth and development of all the tested fungi. Further testing the enzyme patterns, of all of these fungi, is needed which would be highly useful in the area of plant pathology. This immunological approach may prove to be better in elucidating the pathogenic molecule and its extent, giving us the knowledge of its control measures to be implemented. These antibodies could help to identify the antigenic protein and thus may lead to its further characterization. This knowledge may also prove to be helpful in the production of plantibodies.

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TABLE 6

Comparison of growth between control and test, of the tested fungi in MS liquid media incorporated with antibodies (Ab 1)

Fungus	Day	Control	Ab 1	Ab 2
Aspergillus niger	1	+	-	-
	2	++	-	-
	3	+++	++	-
	4	+++	++	-
	5	++++	+++	+
	7	++++	++++	+
	8	++++	++++	++
	9	++++	++++	+++
	12	++++	++++	+++
	15	++++	++++	++++
Fusarium solani	1	++	-	-
	2	+++	-	-
	3	+++	+	-
	4	+++	+	-
	5	++++	++	-
	7	++++	+++	-
	8	++++	+++	-
	9	++++	+++	-
	12	++++	+++	-
	15	++++	++++	-

***Fusarium oxysporum* f.sp. cicer (FOC)**	1	+	-	-
	2	++	-	-
	3	+++	-	-
	4	+++	-	-
	5	++++	+	-
	7	++++	++	+
	8	++++	+++	+
	9	++++	+++	++
	12	++++	+++	++
	15	++++	++++	++++
Rhizoctonia bataticola	1	-	-	-
	2	-	-	-
	3	++	-	-
	4	+++	-	-
	5	++++	-	-
	7	++++	+	-
	8	++++	+	-
	9	++++	++	+
	12	++++	++	++
	15	++++	+++	+++
Botryodiplodia theobromae	1	-	-	-
	2	+	-	-
	3	++	-	-
	4	+++	-	-
	5	++++	+	-
	7	++++	++	+
	8	++++	++	+
	9	++++	+++	+
	12	++++	+++	++
	15	++++	++++	++

Curvularia sp.	1	-	-	-
	2	-	-	-
	3	+	-	-
	4	++	-	-
	5	+++	-	-
	7	++++	-	-
	8	++++	-	-
	9	++++	-	-
	12	++++	-	-
	15	++++	-	-
Alternaria porri	1	++	-	-
	2	++	-	-
	3	+++	-	-
	4	+++	-	-
	5	+++	+	-
	7	++++	++	+
	8	++++	+++	+
	9	++++	+++	+
	12	++++	+++	+
	15	++++	+++	+

TABLE 7

P – value of all the tested fungi between days and amongst the different test dosages of antibodies at p < 0.05 significance level, degrees of freedom is 6 and 5 respectively

Test Fungi	Source of variation	
	Between days	**Amongst test dosages**
Aspergillus niger	7.54E-08	0.057408
Fusarium solani	1.8E-05	1.5E-15
Fusarium oxysporum **f.sp.** *cicer* (FOC)	3.33E-08	4.44E-07
Rhizoctonia bataticola	1.93E-09	3.11E-05
Botryodiplodia theobromae	1.63E-17	7.13E-12
Curvularia sp.	6.86E-10	3.99E-06
Alternaria porri	9.52E-06	5.79E-08

a. *Aspergillus niger*
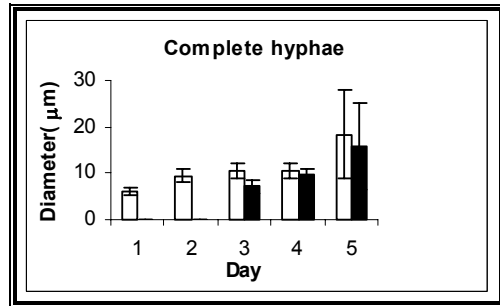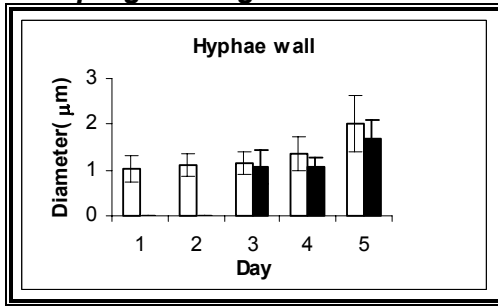
b. *Fusarium solani*
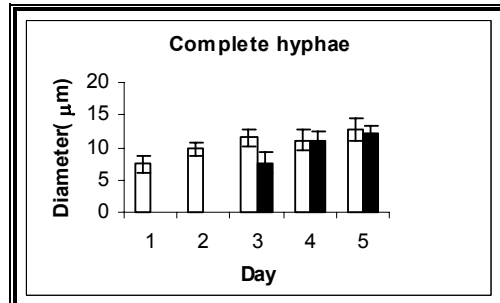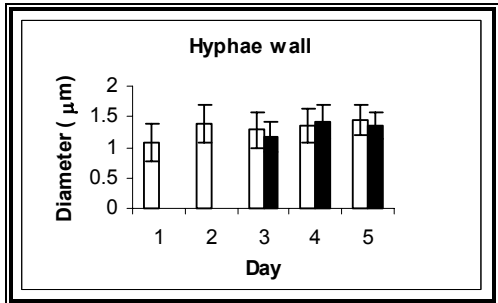
c. *Fusarium oxysporum* **f.sp.** *cicer*
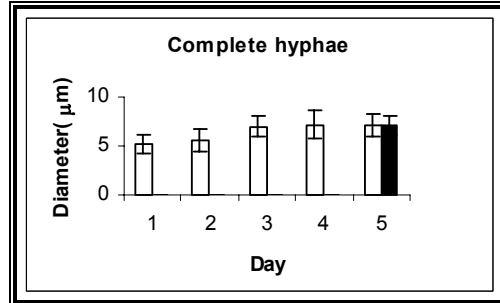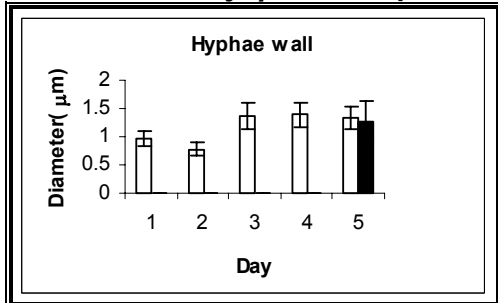
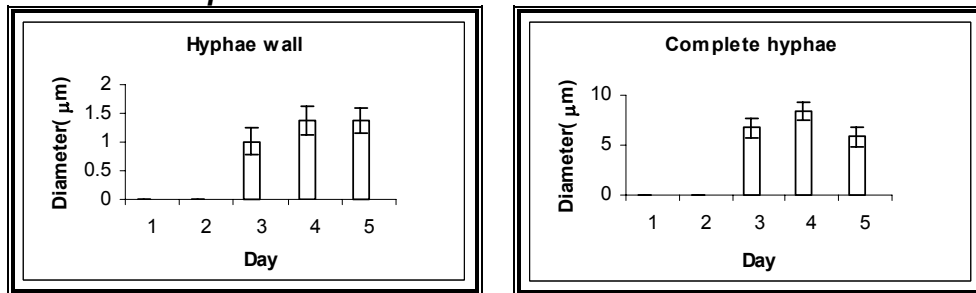**d.** *Rhizoctonia bataticola*
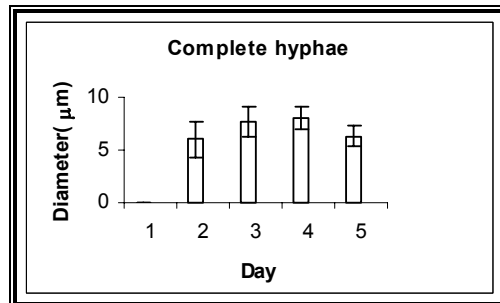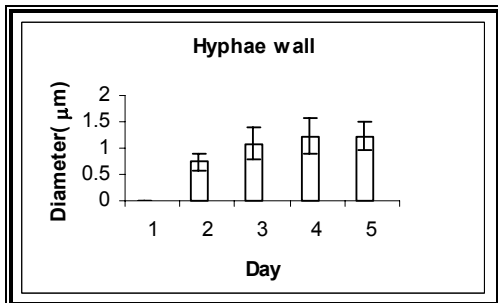
Figure 2 …..

Diameter of hyphae: A comparison of control and test grown fungal hyphae (■ – test; □ - control)
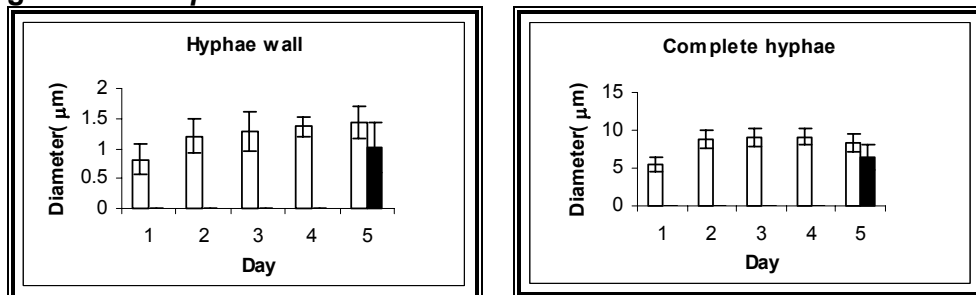
e. *Botryodiplodia theobromae*
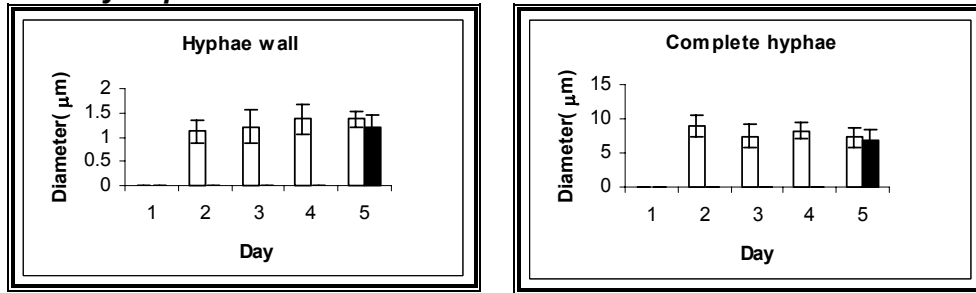
f. *Curvularia sp.*

g. *Alternaria porri*

….. Figure 2

Diameter of hyphae: A comparison of control and test grown fungal hyphae   (■ – test;   □ - control)

Figure 3 …..

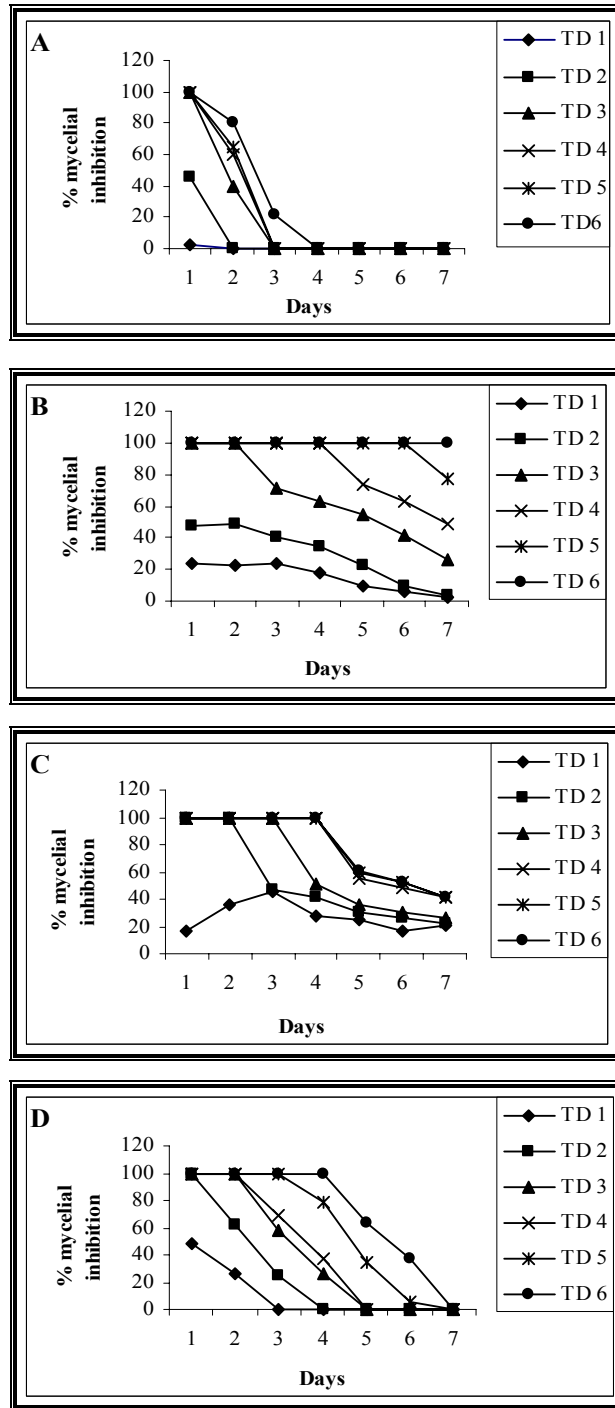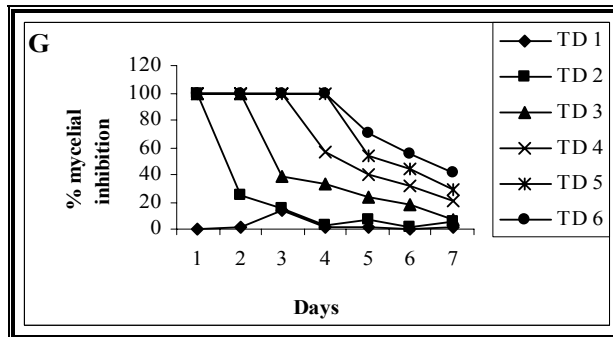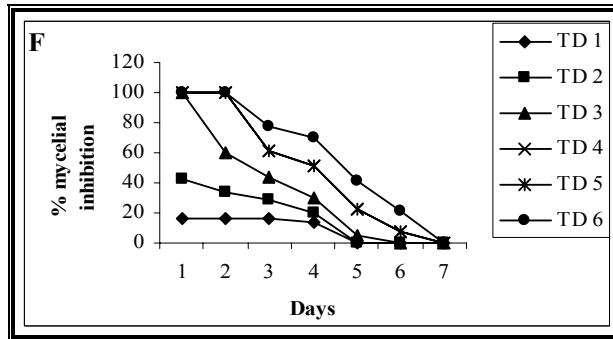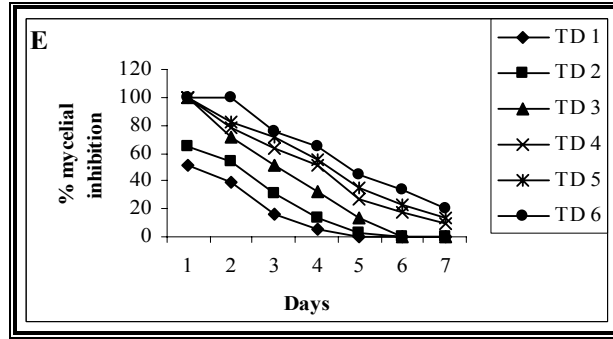**Changes in % Mycelial inhibition in response to antibody concentrations against time (A)** *A. niger* **(B)** *F. solani* **(C)** *F. oxysporum f.sp. cicer* **(D)** *R. bataticola*

TD(Test dosage)1 – 1µl ab/ml, TD2 - 2µl ab/ml, TD3 - 3µl ab/ml, TD4 - 4µl ab/ml, TD5 - 5µl ab/ml, TD6 - 6µl ab/ml
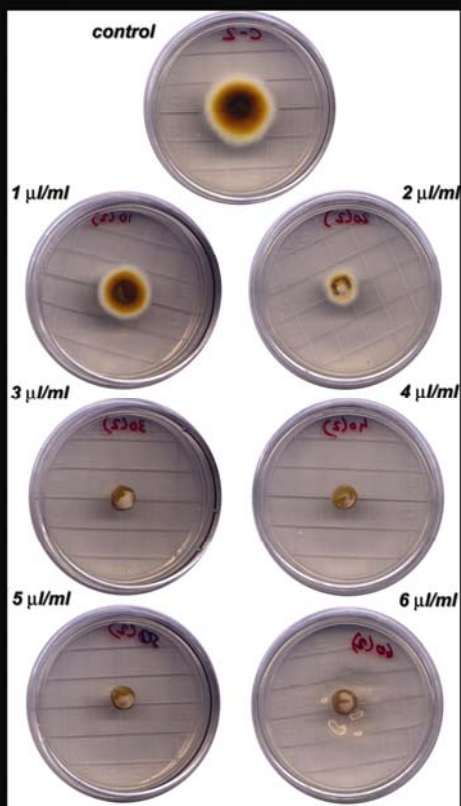
….. **Figure 3**

Changes in % Mycelial inhibition in response to antibody concentrations

against time (E) *B. theobromae* **(F)** *Curvularia sp.* **(G)** *A. porri*

TD(Test dosage)1 – 1µl ab/ml, TD2 - 2µl ab/ml, TD3 - 3µl ab/ml, TD4 - 4µl ab/ml,

TD5 - 5µl ab/ml, TD6 - 6µl ab/ml

Fusarium solani

control

1 μl/ml 2 μl/ml

3 μl/ml 4 μl/ml

5 μl/ml 6 μl/ml

Fusarium oxysporum f.sp.cicer
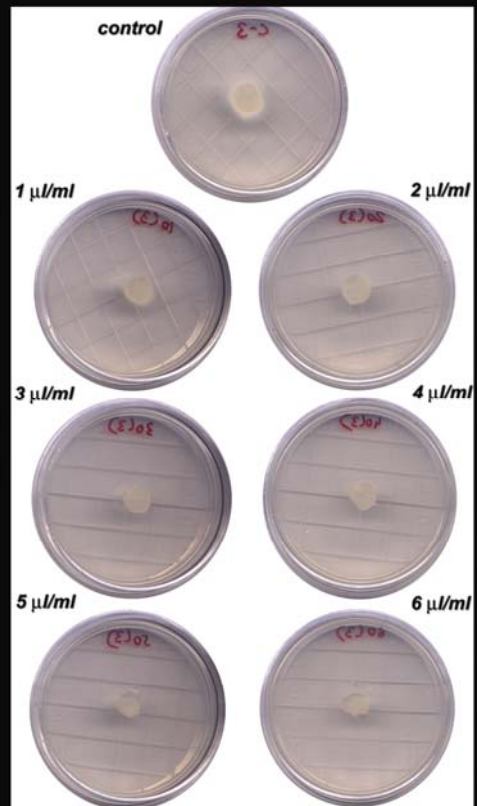
control

1 μl/ml 2 μl/ml

3 μl/ml 4 μl/ml

5 μl/ml 6 μl/ml

Plate 5. Inhibition of Fungi in radial growth assay (48 h)

Rhizoctonia bataticola

control

1 μl/ml   2 μl/ml

3 μl/ml   4 μl/ml

5 μl/ml   6 μl/ml

Botryodiplodia theobromae
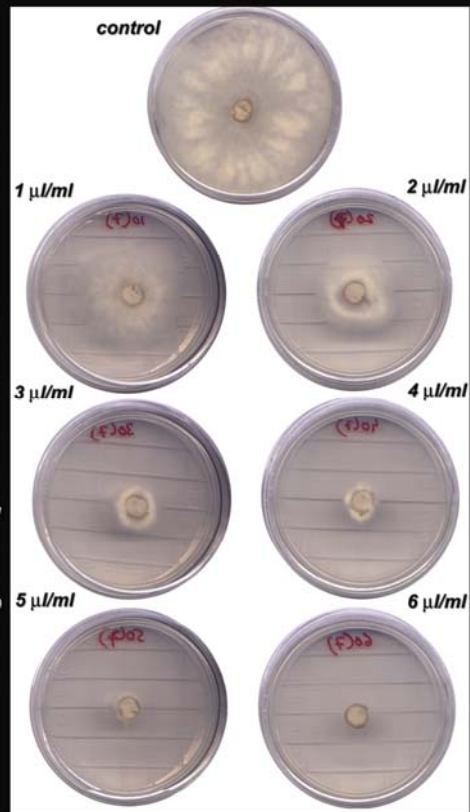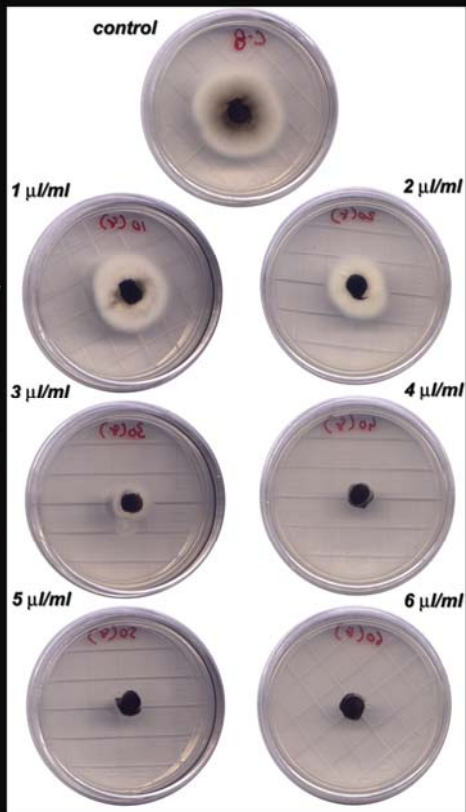
control

1 μl/ml   2 μl/ml

3 μl/ml   4 μl/ml

5 μl/ml   6 μl/ml
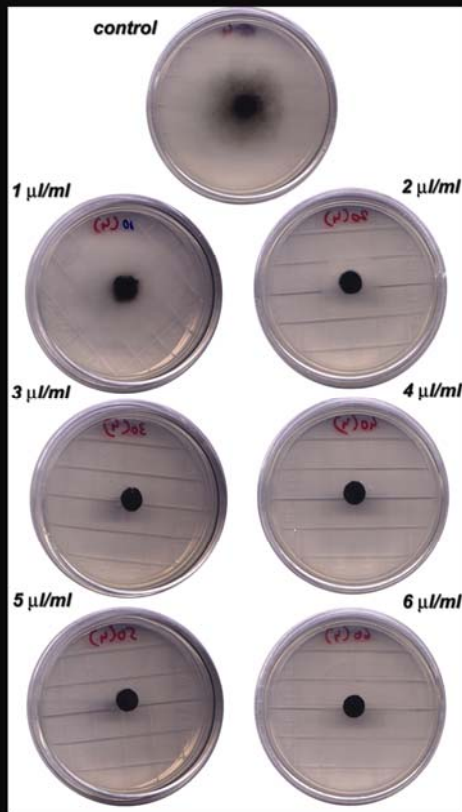
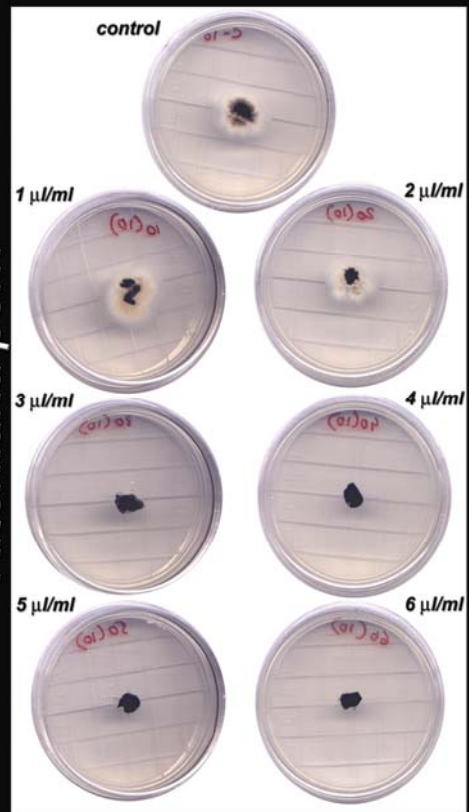Plate 6. Inhibition of Fungi in radial growth assay (48 h)

Plate 7. Inhibition of Fungi in radial growth assay (48 h)

CHAPTER 4

ENZYMATIC STUDIES
OF *ASPERGILLUS NIGER*

CHAPTER 4A

STUDY OF
ACID PHOSPHASE AND
INVERTASE ACTIVITY

ABSTRACT

Aspergillus niger is widely used as a source of enzymes in industries. Considering its enzymic potential, it was studied for its acid phosphatase *(EC 3.1.3.2, orthophosphoric monoester phosphohydrolase)* and invertase *(EC 3.2.1.26, β-fructofuranoside fructohydrolase)* in a defined media supplemented with 1%, 3% and 5% sucrose concentrations. Both of these enzymes play a key role in phosphate and carbon metabolism in plants, animals and microorganisms and hence are interesting from the standpoint of biotechnological applications. Ontogenic changes in extracellular, cytoplasmic and wall bound enzyme activities of *A. niger* are studied. The growth in terms of fresh weight showed inverse correlation with pH. At higher pH values, both enzyme activities were higher in the medium supplemented with low sucrose concentration. It was observed that with the decrease in fresh weight of fungi the enzyme activities enhanced suggesting that these enzymes may participate in autolysis of fungi and on the other hand, could prove to be a potential source of industrial application and exploitation.

Keywords: acid phosphatase, *Aspergillus niger*, invertase

INTRODUCTION

The filamentous fungi are a heterogeneous group of microorganisms which can use wide variety of carbon and nitrogen sources for growth; exhibit a large metabolic diversity; and can produce a wide range of secondary metabolites and hydrolytic enzymes which are widely used in the food, beverages and pharmaceutical industries (Metwally, 1998). They produce extracellular enzymes to degrade insoluble substrate into smaller fragments and finally into soluble units which are then absorbed by hyphae (Chaube & Singh, 2000). The property of microorganisms to produce enzymes is exploited vastly for industrial purposes.

A. niger, cosmopolitan in distribution, important industrial source for citric acid and enzymes, such as amylase, enjoy a GRAS ("generally regarded as safe") status (Martens-Uzunova *et al*., 2006). The starch degrading enzyme amyloglucosidase and extracellular inducible enzyme from filamentous fungi (*Aspergilli*) is the second most widely produced enzyme worldwide after *Bacillus* protease (Metwally, 1998). Thus study of the stability of enzymes is an important aspect to consider in biotechnological processes, as this can provide information on the structure of the enzymes and facilitate an economical design of continuous processes in bioreactors (Jurado *et al*., 2004).

Acid phosphatases (*EC 3.1.3.2, orthophosphoric monoester phosphohydrolase*) are a group of enzymes that catalyze the hydrolysis of external phosphate esters (Joh *et al*., 1996). They play an important role in the mineralization of organic carbon, organic phosphate and low levels of free inorganic ions (Pi) (Straker & Mitchell, 1986) and transport of the free phosphatases into the cells. Orthophosphate anion ("inorganic phosphate" Pi) plays a vital functional role in energy transfer and metabolic regulation and is also an important structural constituent of many biomolecules (Ehsanpour & Amini, 2003).

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Acid phosphatases of *Saccaromyces cereviseae* have been extensively studied (Schweingruber *et al*., 1992). They have also been studied in *A. nidulans* (Arst *et al*., 1980), ectomycorrhizal fungi (Straker & Mitchell, 1986) and molds (Haas *et al*., 1992). It is reported to be located in the cell wall and septa of hyphae as determined by cytochemical studies by TEM (Gutiérrez-Miceli *et al*., 2005).

Invertases *(EC 3.2.1.26, β-fructofuranoside fructohydrolase)*, a group of ubiquitous enzymes with different pH optima and subcellular localization (Verma & Dubey, 2001), hydrolyze sucrose to glucose and fructose (Heil *et al*., 2004). Considerable attention has been paid to the carbohydrate physiology of mycorrhizas, as the carbohydrate supply of the fungus is pivotal for the functioning of this symbiosis (Schaeffer *et al*., 1995).

Non-photosynthetic tissues prefer sucrose as a main carbon source, probably due to its high solubility, low reactivity, and energy storage capacity (Giaquinta, 1980), where Pi plays a decisive role in multiple biosynthetic pathways (Chávez-Bárcenas *et al*., 2000). The intracellular phosphatases play an important role in the metabolism of carbohydrates (Giaquinta, 1980).

Since fungi are opportunistic organisms we have used sucrose with defined media for the study of acid phosphatase and invertase activities for their potential extraction. To the best of our knowledge no such study has conducted in *A. niger*. Thus considering the importance of culture medium for growth and enzyme production, the present study, aims to investigate the extracellular, intracellular and cell wall bound acid phosphatase and invertase activities of *A. niger* with time course and different levels of sucrose as a sole carbon source of the culture medium.

MATERIALS AND METHODS

Fungal strain and preparation of inoculum

A. niger (VP-001) strain was used for this work. The inoculum was prepared as described in Chapter 2A. The spore count was adjusted to 3.2×10^4 spores/ml.

Media preparation and culture conditions

Murashige and Skoog (MS) media (Murashige & Skoog, 1962) (PT0018) procured from Hi media Laboratories, Mumbai, India was used for the study. The carbon sources supplemented were various concentrations of sucrose i.e. 1% (A), 3% (B) and 5% (C). The pH of individual basal medium was adjusted to 5.5, of which 50 ml was taken in each of the 250 ml conical flask. The media was autoclaved for 15 min and allowed to cool to room temperature. The inoculum (0.1 ml) was added to each flask and maintained in a stationary condition at 28 ^{o}C.

Determination of hyphae diameter

It was measured as described in Chapter 2A.

Extracellular, cytoplasmic and wall-bound enzyme extraction

After 24 h and at intervals of 12 h, culture media were collected from individual flasks under aseptic conditions and filtered through a disk of Whatman no.1 filter paper. This filtrate constituted extracellular fraction which was used for further assays. The mycelium remained after removing the filtrate was gently blotted over the layers of filter paper and weighed. The mycelial mass was homogenized in chilled pestle - mortar with acid washed sand in sodium acetate buffer (100 mM, pH 5). This homogenate was centrifuged at 4 ^{o}C at 8000 g for 30 min. The supernatant constituted cytoplasmic fraction and the residue was washed several times with repeated centrifugation by distilled water.

It was then incubated in 1M NaCl at RT (30 ± 2 ^{o}C) overnight to release wall bound fraction. Preliminary studies showed that 1M NaCl was the optimal concentration for the release of wall bound protein (Straker, 1986). Three samples of each culture media were taken each time.

Acid phosphatase assay

The assay medium consisted of 0.3 ml, 100 mM sodium acetate buffer (pH 5), 0.4 ml enzyme solution and 2.3 mM p-nitrophenyl phosphate (pNPP) (in buffer) in a final volume of 1 ml. The mixture was incubated at RT (30 ± 2 ^{o}C) and after 15 min incubation it was terminated by the addition of 2 ml of 1M Na_2CO_3. In control reaction, Na_2CO_3 was added prior to the addition of enzyme. The absorbance of the yellow pNP released was measured at 405 nm using ELISA Reader (µ Quant, Bio-tek, USA). The quantity of pNP released was calculated from the calibration curve prepared using a range of pNP concentration from 0.036 to 0.7 mM prepared in same buffer. The acid phosphatase activity was expressed as mM pNP released/gfwt/min for all the three fractions.

Invertase assay

The reaction mixture contained 0.15 M sucrose and enzyme solution. After 60 min incubation at RT (30 ± 2 ^{o}C), the reducing sugars were estimated with the 3, 5-dinitrosalicylic acid method (Miller, 1959). Control was prepared by addition of Miller's reagent prior to the substrate. Absorbance was measured at 520 nm using ELISA Reader (µ Quant, Bio-tek, USA). The activity was calculated from the standard curve prepared by using glucose. The activity was expressed as mg sugar released/gfwt/min for all the three fractions.

Protein determination

Total protein was determined from extracellular and cytoplasmic fractions, by the method of Lowry *et al*. (1951) using BSA as the standard.

Sugar estimation

The sugar was estimated from extracellular and cytoplasmic fractions. These fractions were mixed with 80% ethanol and incubated overnight. They were heated in a boiling water bath till the alcohol evaporated off completely. The volume (10 ml) was made with distilled water and the extract was used for sugar estimation by the method of Thaker *et al*. (1992).

Statistical analysis

The mean values were statistically analyzed by Analysis Of Variance (one way ANOVA) using Tukey test at $p < 0.05$ significant level to detect the significant differences between media containing different sucrose concentrations A (1%), B (3%) and C (5%).

RESULTS AND DISCUSSION

The media used in the present study is Murashige and Skoog's, which is a defined media. Known concentrations of sucrose were added to study their influence on enzyme production at different growth stages. There are very few reports which have investigated the growth of molds considering the type of media (Bhaskaran & Smith, 1993; Meletiadis *et al*., 2001). Sucrose is the major transport form for photoassimilates in higher plants; it is highly soluble, cheap and is the major carbon source in molasses and attractive feedstock for large-scale fermentation (Skowronek & Fiedurek, 2006). It is the principle carbohydrate substrate for the synthesis of cytoplasmic and cell wall constituents (Thaker *et al*., 1992). It has also been established that cAMP alteration of growth rate of *A. fumigatus* and *A. niger* is carbon source dependent (Oliver *et al*., 2002).

The diameter in cellulose hyperproducing mutant strain of *Penicillium* has been reported (Brown *et al*., 1987). In the present study, measurements of hyphae diameter showed increase with time in a sigmoid pattern, but no statistically significant difference was observed within A, B and C (Fig. 4). With time the diameter increased and in later hours it was stabilized. In this thesis, hyphae diameter measurements were done in the inhibition studies of essential oils (Chapter 2A) where it was less in tests than in control. But no such difference was observed with changes in sucrose concentration in this study, suggesting that it may not have any effect on the thickness of hyphae (Fig. 4).

Extracellular enzyme production in filamentous fungi is tightly coupled to hyphal tip growth and the increase in number of hyphal branches which can be envisaged with increase in fresh weight (Fig. 5). The maximum yield of mycelium in A reached at 60 h (730 mg/flask) and after that it declined. Whereas, the maximum yield of mycelium in B (1650 mg/flask) and C (1650 mg/flask) reached at 96 h (Fig. 5). In the initial phase, up to

60 h, the growth was directly proportional to the sucrose levels. In A the fresh weight was lowest followed by B and C, respectively. After 60 h, the growth of fungi moved parallel in B and C but later on it declined in B and was stabilized in C. There was a statistically significant difference of A with B and C ($p < 0.01$) but it was not found between B and C.

Fungi as a group, tends to be acidophilic with an optimum in the acid range. The pH of culture fluid with 1% sucrose (A) increased abruptly after 36 h till 60 h (7.36) and remained constant thereafter. In B it remained in between 6 to 3 but in C the pH sharply decreased and turned acidic from 72 h onwards (Fig. 6).Thus inverse correlation was observed amongst pH and growth of the fungi. Changes in pH with different levels of glucose have been studied which showed that in low concentration of glucose pH was high and in high concentration of glucose pH was low (Maldonado & Strasser de Saad, 1998). With the change in sucrose concentration the pH and fresh weight of the fungal mycelia do change accordingly. Thus carbon source highly influence the growth, in higher concentration the growth is maximum. It has been revealed that natural autolysis of *A. niger* occurs at pH 6.7 (Perez-Leblic *et al*., 1982). Thus this might be the reason for decreasing fresh weight of the fungus in A because of changing the pH towards alkalinity.

In the acid phosphatase assay pNPP is used as a substrate which is shown to be most efficiently hydrolyzed (Straker & Mitchell, 1986) in the previous studies. It has been reported that the catalytic efficiency of the enzyme given by the Vmax/Km ratio is much higher for the synthetic substrate (pNPP) than for the natural substrates (Gonnety *et al*., 2006). Acid phosphatase have been studied in yeasts and speculated to be associated with cell elongation (Miyata & Miyata, 1978) and involved in morphogenetic and behavioral differentiation. Their role in membrane processes costs an intriguing new light on the fact that these enzymes are periplasmic and extracellular in many microorganisms including *A. nidulans* (Arst *et al*., 1980).

In the present study, acid phosphatase activity was highest at 72 h in extracellular fraction of A (Fig. 7a). In C the activity remained consistently low till the entire time course but in B peak was observed at 24 and 120 h, i.e. in the initial and later phases of growth (Fig. 7a). The enzyme activity in A was significantly higher than B and C. In the cytoplasmic fraction, at 24 h, maximum enzyme activity was observed in A, B and C. Similar to extracellular fraction, activity in cytoplasmic fraction of A was maximum followed by B and C. In later hours, the activity peaked at 72 h in A and at 60 h in B and C thereafter it declined (Fig. 7b). In the wall bound fraction also the activity declined after 48 h but at 72 h it showed a peak in A (Fig. 7c). Thus highest enzyme production was obtained in the initial phase at 24 h in cytoplasmic fraction of A, B and C. The media supplemented with low sucrose concentration exhibited highest enzyme activity.

Extracellular phosphatase in the endophyte of *Erica hispidula* was more active than the cytoplasmic and wall bound enzymes 18 d after inoculation (Straker & Mitchell, 1986). Acid phosphatase have been reported in *Pholiota nameko* (Yazaki *et al*., 1997), several ectomycorrhizal fungi and VAM fungi (Dodd *et al*., 1987), suggesting their potential for degradation of complex phosphorus containing compounds (Dodd *et al*., 1987), and associated with the growth and development of the fungus within the host tissue (Gianinazzi *et al*., 1979). With the bulk of phosphate being organically bound, the phosphatases secreted by microorganisms would be important in the catalytic release of Pi from bound complexes (Straker & Mitchell, 1986).

The increase in extracellular and wall bound invertase activity was detected only during the later period of mycelial growth (Figs. 8a and 8c) but in the extracellular fraction at 24 h, the activity was found to be at the highest levels in A (25.57) followed by C (18.67) and B (10.3). The cytoplasmic invertase activity was also highest at 24 h in A (3.201) followed by B (1.058) and C (1.005), later on it progressively increased at

60 h, thereafter a sharp decrease in the activity occurred (Fig. 8b). Thus the highest enzyme production was obtained in the initial phase at 24 h in cytoplasmic as well as extracellular fraction of A, B and C. The media supplemented with low concentration of sucrose exhibited the highest invertase activity.

The extracellular and cytoplasmic total sugars also peaked at 24 h in A followed by B and C, respectively (Fig. 9a and 9b). In the later phase of growth total sugars were highest in both the fractions of A. On the contrary, they were lowest in B and C during this period which may be attributed to their maximum weight of mycelia during later phase compared to the low weight of mycelia in A. The sugars which are produced may be utilized in increasing the biomass in B and C.

The extracellular reducing sugar levels in A, B and C were also maximum at 24 h, but here the value was highest in C (853.84) followed by B (276) and A (195) respectively, but decreased gradually with an increase in time period in all the three media (Fig. 10a). The influence of different concentrations of sugar is evident here. On the other hand, the cytoplasmic reducing sugar levels, which were also found maximum at 24 h, peaked in A (196.27) followed by C (20.1) and B (16.88), respectively (Fig. 10b). In the later hours it peaked at 60 h in C, 168 h in B and at 120 h in A. Highest reducing sugars at 24 h correlate with maximum invertase activity at the same time. Cytoplasmic reducing sugars were high at 60 h in C with maximum invertase activity. Invertase activity after 72 h gradually declined in all (Figs. 8b and 10b).

In *Saccharomyces cerevisiae,* the utilization of sucrose involves extracellular hydrolysis followed by transport of the liberated hexoses into the cell (Sutton & Lampen, 1962). Thus the total conversion of sucrose to reducing sugars and its decline overtime supports this conclusion (Bhaskaran & Smith, 1993). Extracellular invertase activity was low at 36

and 48 h in A and B, it reached maximum at 120 h. But the reducing sugars were high at initial hours and decreased later on. Thus as the invertase activity is increasing more reducing sugars are accumulated in the cytoplasmic fraction and less sugars are found in the extracellular fractions in the later phase. To produce more reducing sugars in the later phase may be the invertase activity is increasing.

In the previous studies it has been speculated that *Sporisorium relianum* is potentially capable of producing extracellular enzymes for the release of hexoses (glucose and fructose) into the medium which are used for growth in the yeast form (Bhaskaran & Smith, 1993). Thus by using the defined carbohydrates, they showed that invertase is produced by the fungus (Bhaskaran & Smith, 1993). Wall bound invertase activity was also found to be highest at 60 h to 120 h. This proves that maximum uptake of sugars is done during later phase to facilitate the growth of fungi. Invertase activity is also highest in A compared to B and C. Thus it is possible that to strive in the alkaline conditions and to increase growth in the later phase, the fungus with 1% sucrose concentration is more in its activity, and it was less in the medium with 3% and 5% sucrose concentration.

In several host pathogen interaction studies invertase and acid phosphatases have been studied. A reduction in phosphatase activity was shown in tobacco leaves after invasion with *Pholiota tobacina* in the necrotic phase of the development of the disease. This decrease, as it had become clear from the cytochemical investigations, affects the tissues of the host, while the hyphae of the parasite have highly active phosphatase (Edreva & Georgieva, 1977). Alternatively, the acid phosphatases may play a role in recognition and/or infection processes, as has already been suggested for such enzymes in other biological systems.

Reviewing nutrient relations in biotrophic infections emphasized that acid invertase is the most studied relevant enzyme in infected leaves and it is of central interest in view of its probable role in hydrolysis of sucrose prior to sugar uptake by fungi (Schaeffer *et al*., 1995). The acid invertase activity was inversely proportional to the starch content and closely related to the changes in saccharose and glucose contents (Pius *et al*., 1998). The significantly enhanced enzyme activity began with the onset of sporulation and reached a maximum at the stage of maximum basidiospore release, when it was nearly 10 times higher than in the control (Pius *et al*., 1998). Although the localization of invertase is not clear yet (Scholes *et al*., 1994), its increasing activity during sporulation may be due to a fungal enzyme as proposed previously (Xiu *et al*., 1993).

Evaluation of protein in extracellular fraction of A, B and C showed that at the initial phase protein of A was maximum (1439 mg/gfwt) followed by B (911 mg/gfwt) and C (674 mg/gfwt) respectively, but at 72 h the protein value was more in medium with more sucrose concentration (Fig. 11a). The cytoplasmic protein value was more in C (4.98 mg/gfwt) at 24 and 36 h after that it declined gradually. However the protein value in A and B peaked at 48 h and then declined (Fig. 11b).

In the present study, the enzyme activities expressed in 1% sucrose concentration is many folds high compared to 3% and 5%, in the early and later growth phases. As the pH turns towards alkaline condition, the weight of the fungus decreases. Thus with low concentration of sucrose, though the biomass is low, the enzyme activity is enhanced. Also the high enzyme activity could be in part due to the increase in pH. It might be inferred that the alkaline pH might be stressful for growth and development and so to strive in such conditions the fungi might have produced more enzyme, so that its nutritional requirements can be fulfilled.

Another possibility also cannot be ruled out that during autolytic condition at higher pH generally the lytic enzymes which attack the cell wall and the reserve carbohydrates get activated (Gomez *et al*., 1977). Moreover in the culture medium with 3% and 5% sucrose concentration the enzyme activities remained low compared to the culture medium with 1% sucrose concentration. Because of suitable carbon source, optimum concentration and pH, the fungi in 3% and 5% sucrose concentration, grows well with more protein value and with minimum need to increase the acid phosphatase and invertase activities, i.e. the enzymes involved in phosphate and carbohydrate metabolism. Thus the present work showed that concentration of sucrose influences the growth, pH and enzyme production. It can be used to understand the host-pathogen relationship as well as the exploitation of these enzymes can be done industrially.
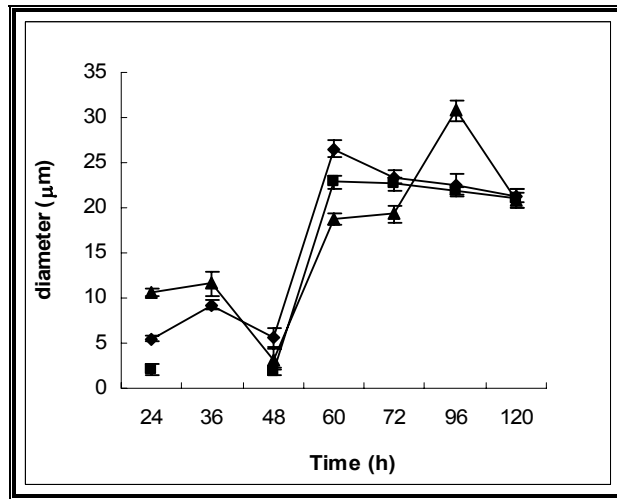
Figure 4

Diameter of *A. niger* measured microscopically at different hours in
media with 1% sucrose level A(♦), 3% sucrose level B(▲) and 5%
sucrose level C(■) of sucrose concentration
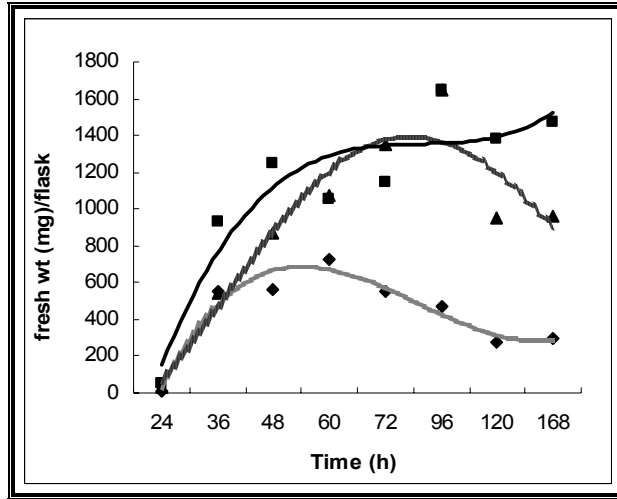The vertical bars represent ± standard deviation

Figure 5

Growth determination of *A. niger* grown in A (♦), B (▲) and C (■) in
terms of fresh weight at different time period. The values are
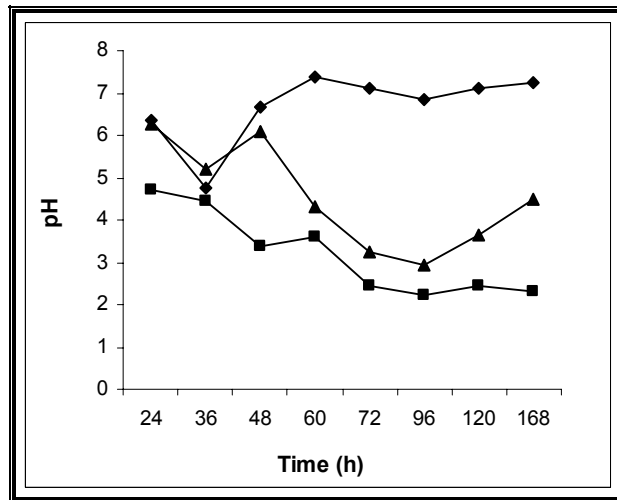statistically significant (at p ≤ 0.05) between A and C

Figure 6

Changes in pH with time in A (♦), B (▲) and C (■). The values are
statistically significant (at p ≤ 0.05) between A with B and C
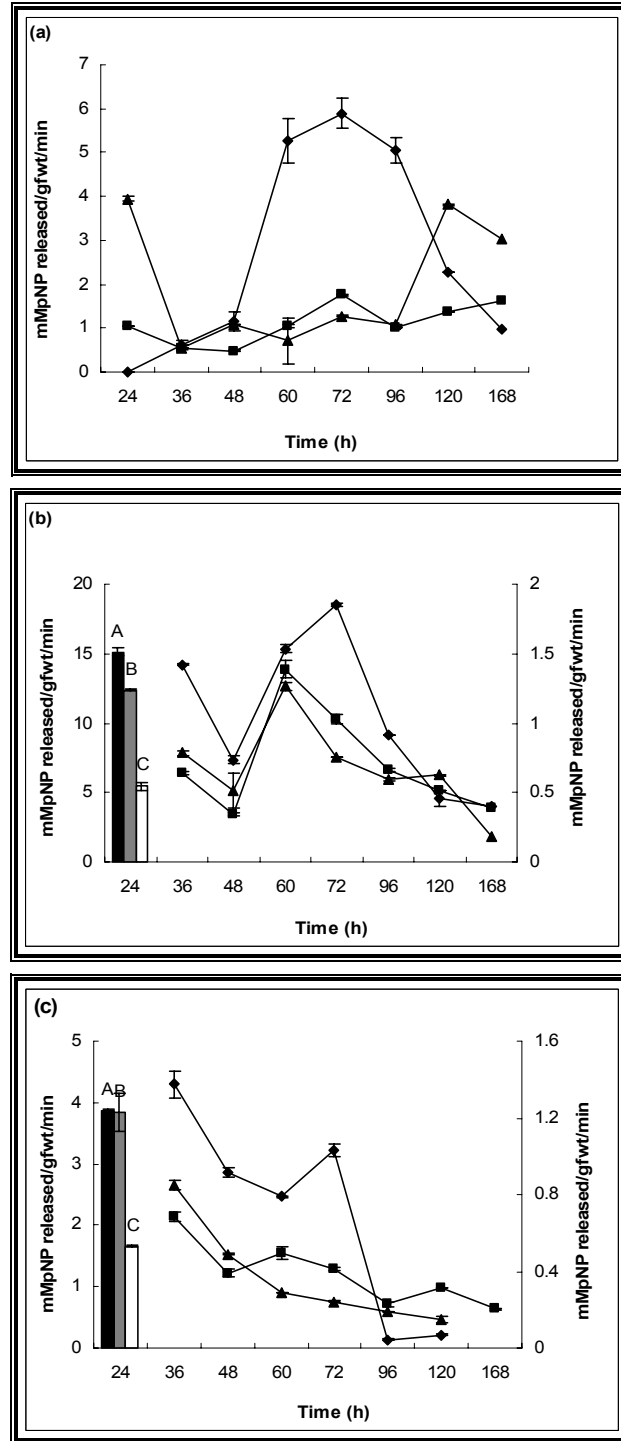
Figure 7

Acid phosphatase activity expressed on fresh weight basis in A (♦), B (▲)

and C (■)   (a) extracellular (b) cytoplasmic and (c) wall bound

The primary axis represents the columns
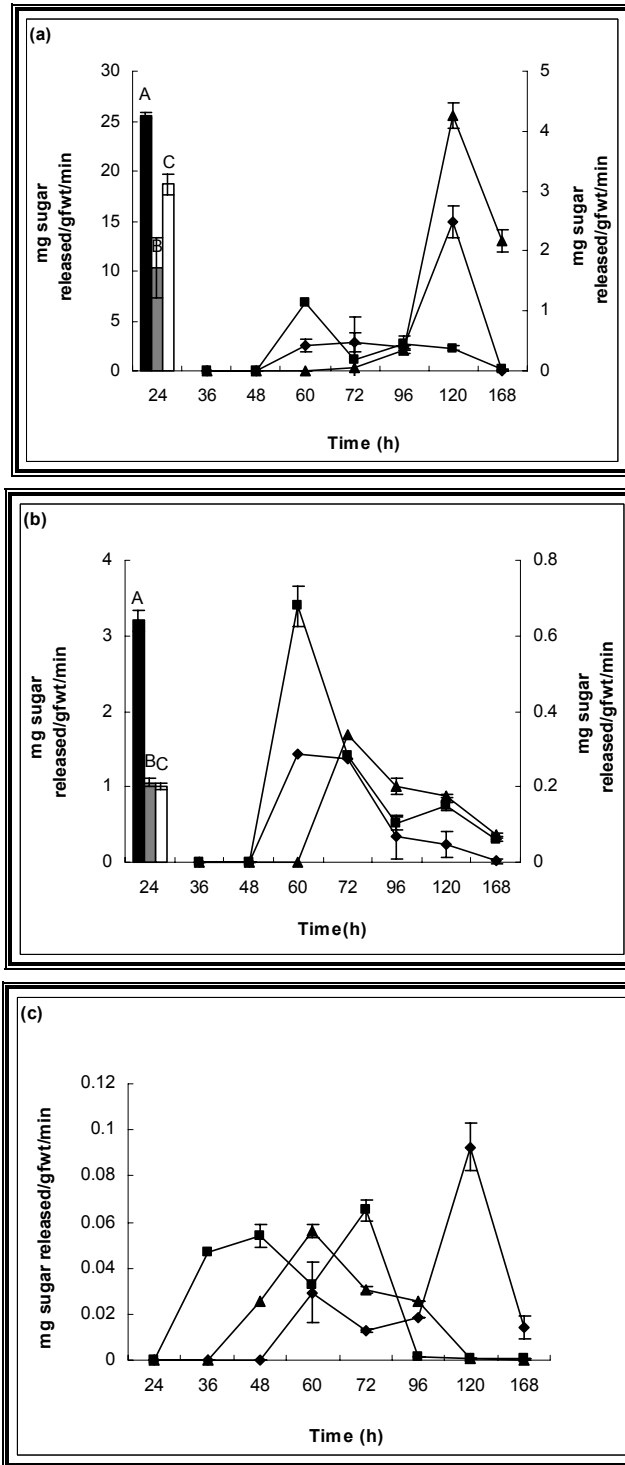
Figure 5

Invertase activity expressed on fresh weight basis in A (♦), B (▲) and C (■)

(a) extracellular (b) cytoplasmic and (c) wall bound

The primary axis represents the columns

Figure 9

Changes in total sugars in A (♦), B (▲) and C (■) with time

(a) extracellular (b) cytoplasmic

The primary axis represents the columns
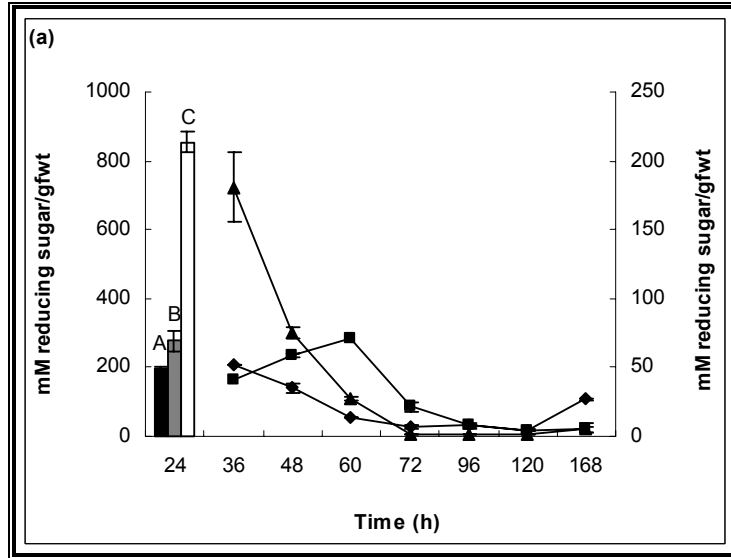
Figure 10

Changes in reducing sugars in A (♦), B (▲) and C (■) with time

(a) extracellular (b) cytoplasmic

The primary axis represents the columns
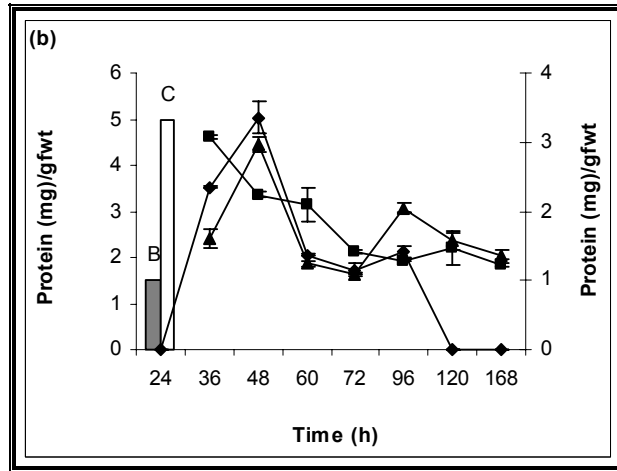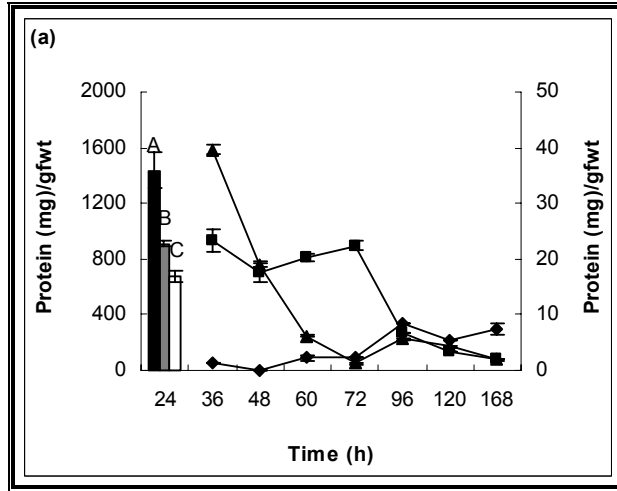
Figure 11

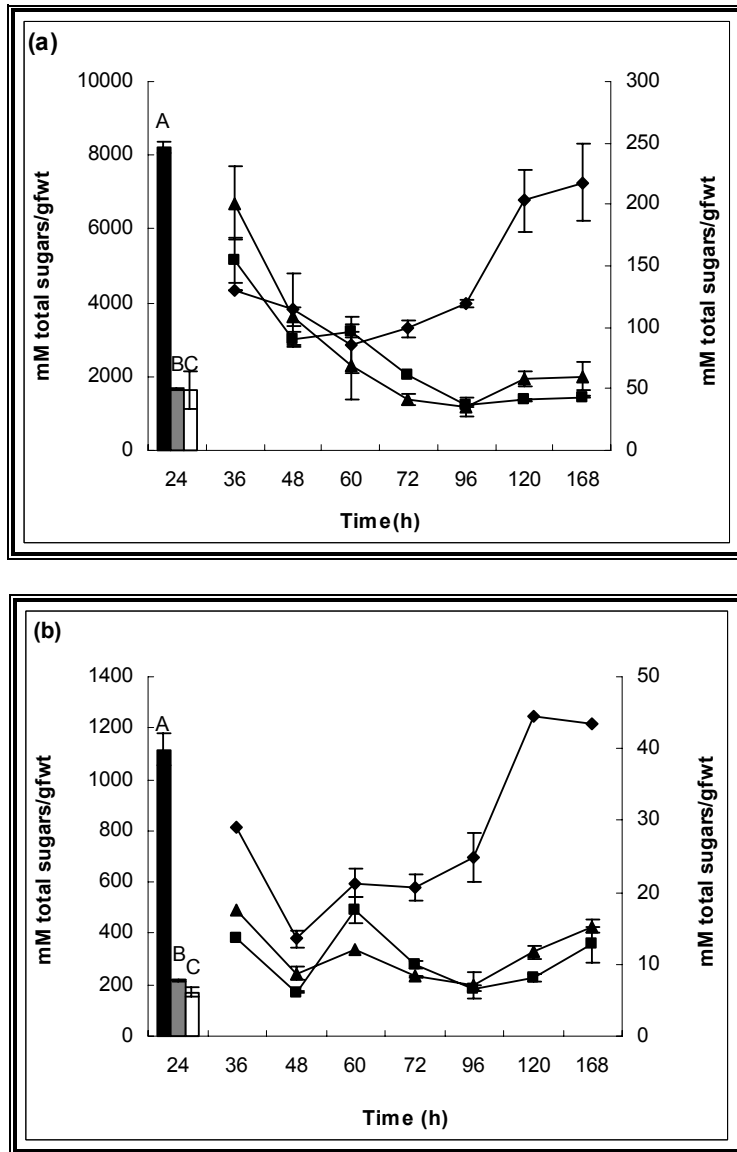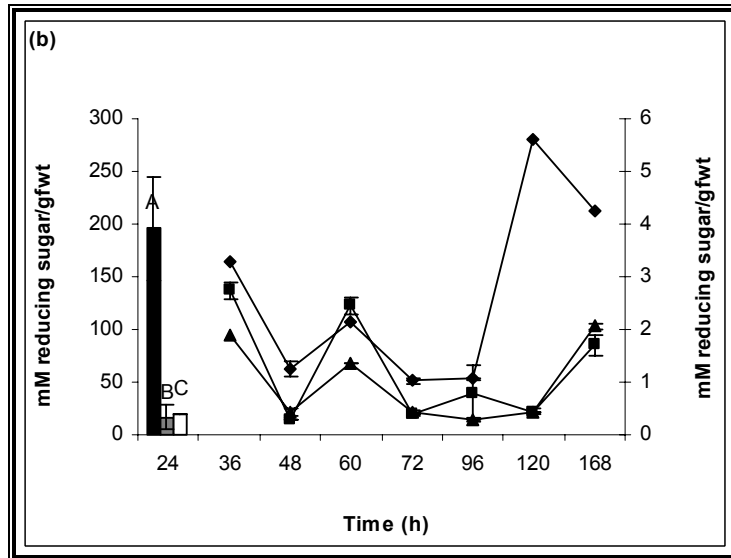Protein content changes in A (♦), B (▲) and C (■) with time

(a) extracellular (b) cytoplasmic

The primary axis represents the columns

CHAPTER 4B

STUDY OF
CELL WALL
DEGRADING ENZYMES

ABSTRACT

Aspergillus niger, the plant pathogenic fungus was studied for the production of cell wall degrading enzymes with the Murashige and Skoog's media and different concentrations of sucrose that was used as a sole carbon source. The extracellular, cytoplasmic and wall bound fractions were evaluated for polygalacturonase, xylanase, α/β-galactosidase and β-glucosidase activities along with the time course. Polygalacturonase activity was found to be the highest amongst the studied enzyme activities and extracellular fractions exhibited highest activity amongst all the three fractions studied. Thus the study supports the view of polygalacturonase being produced the first enzyme during pathogenesis.

Keywords: Aspergillus niger, glycosidases, polygalacturonase, xylanase

INTRODUCTION

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They make up 90% of the plant cell wall and can be divided into three groups: cellulose, hemicellulose, and pectin (McNeill *et al*., 1984). During pathogenesis, cell walls act as the first line of defense that plant pathogenic fungi encounter to colonize the plant tissue and obtain nutritional requirements (Di *et al*., 2006). Plant pathogenic fungi produce many kinds of macerating enzymes that cause the degradation of plant cuticle and cell wall during the infection (Hoshino *et al*., 1997). They produce cell wall degrading enzymes (CWDE) in sequence, with pectic enzymes forming first and hemicellulose and cellulose degrading enzymes produced later (Zhang *et al*., 1997). In general, CWDE produced by plant pathogenic fungi are considered important pathogenecity factors (Wanjiru *et al*., 2002). Plant pathogens can produce a series of plant cell wall degrading enzymes involved in pre and post harvest diseases (Bruton *et al*., 1992).

Aspergillus niger is a plant pathogenic fungi causing black mould in plants. Although the disease can occasionally be seen in the field at harvest, black mould is primarily a post harvest disorder and can cause extensive losses in storage under tropical conditions (Thamizharasi & Narasimham, 1992). It is the group of filamentous fungi with a large number of species. The black *Aspergilli* have a number of characteristics which make them ideal organisms for industrial applications, such as good fermentation capabilities and high levels of protein secretion. They enjoy the GRAS (generally regarded as safe) status, grow rapidly on inexpensive media and have a well-studied genetic background (Rose & van Zyl, 2002). They are used for the production of many industrial enzymes including extracellular enzymes such as amylases, proteases, cellulases, hemicellulases and lipase (Rao *et al*., 2002).

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Aspergillus species are known to be good producers of β-glucosidase and commonly used for glycosyl hydrolases production in the industry (Günata & Vallier, 1999). Previously, it has been shown that *Aspergillus niger* isolated from rotten lemons was able to produce extracellular pectinestarase (PE) and polygalacturonase (PG) (Maldonado *et al*., 1986). Several different species of *Aspergilli* have been reported to produce xylanases (dos Reis *et al*., 2003; Butt *et al*., 2002).

Among the pectic enzymes, polygalacturonase (PG) (E.C.3.2.1.15) has been associated with pathogenesis and disease severity in a variety of host/pathogen interactions (Zhang *et al*., 1997). These are the first-enzymes to be secreted by fungal pathogens when they encounter plant cell walls (Jones *et al*., 1972). They degrade plant cell walls which allow other hydroxylation enzymes to also degrade cell walls more easily (Di *et al*., 2006). Xylan is the main form of hemicelluloses present in plant cell walls and can constitute up to 35% of the dry weight of plants (Rose & van Zyl, 2002). β-1,4 xylanases (1,4 β-D-xylan-xylanohydrolase, E.C. 3.2.1.8) catalyze the hydrolysis of xylan, the major component of hemicellulose of plant cell walls (dos Reis *et al*., 2003). A variety of microorganisms, including bacteria, yeast and filamentous fungi, have been reported to produce xylanase, of which the most potent producers are fungi (Haltrich *et al*., 1996). On an industrial scale, xylanases are produced mainly by *Aspergillus* and *Trichoderma sp*. (Park *et al*., 2002). Glycosidases like β-glucosidases (EC 3.2.1.21) (Aristidou & Penttila, 2000), α, β-galactosidase (Kang *et al*., 1999) are required for the degradation of cellulose to glucose. These released sugar molecules are used as a nutritional source for the fungus during its growth through plant tissue. β-galactosidases (EC 3.2.1.23) are widely distributed in various plant tissues, including developing fruits (Ranwala *et al*., 1992). Among *Aspergillus* species*, A. niger* is the most efficient producer of β-glucosidase (Kang *et al*., 1999).

The present study is aimed to evaluate the extracellular, intracellular and cell wall bound CWDE *in vitro* produced from *A. niger* with time course using different concentrations of sucrose in a well defined medium. As it is a well known fact that sucrose is the common carbon source which is translocated in the plant system and is commonly used in tissue culture media, it was selected as a carbon source to be used in the present study. Here a comparison is done between different concentrations of sucrose and between extra and intracellular enzymes produced.

MATERIALS AND METHODS

Fungal strain and preparation of inoculum

Same as described in Chapter 4A.

Media preparation and culture conditions

Same as described in Chapter 4A.

Extracellular, cytoplasmic and wall-bound enzyme extraction

Same as described in Chapter 4A.

Polygalacturonase and Xylanase assay

The assay medium consisted equal volume of buffered substrate [2 mg/ml polygalacturonic acid or xylan in 100 mM sodium acetate buffer (pH 5)] and enzyme solution. After 60 min incubation at RT (30 ± 2 °C), the reaction was terminated by the addition of 3, 5-dinitrosalicylic acid (Miller, 1959) and reducing sugars were estimated. Absorbance was measured at 520 nm using ELISA Reader (µ Quant, Bio-tek, USA). The activity was calculated form the standard curve prepared by using glucose. The activity was expressed as mg sugar released/gfwt/min for all the three fractions. Control was prepared by addition of Miller's reagent prior to the substrate. The assay was performed in triplicates for each enzyme extract and mean values with ± standard deviation were calculated.

Glycosidases assay

This activity was determined as described by Thaker *et al*. (1987). The assay medium consisted equal volume of enzyme extract and buffered substrate [1 mg/ml p-nitrophenyl α/β-D galactopyranoside or p-nitrophenyl β-D-glucopyranoside in 100 mM sodium acetate buffer (pH 5)]. After 60 min incubation at RT (30 ± 2 °C), the reaction was terminated by the addition of 1M Na_2CO_3 solution in one and a half times the volume of the reaction mixture.

In control reaction, Na_2CO_3 was added prior to the addition of enzyme. The absorbance of the yellow pNP released was measured at 405 nm using ELISA Reader (µ Quant, Bio-tek, USA). The quantity of pNP released was calculated from the calibration curve prepared using a range of pNP concentration from 0.036 to 0.7 mM prepared in same buffer. The activity was expressed as mM pNP released/gfwt/min for all the three fractions. The assay was performed in triplicates for each enzyme extract and mean values with ± standard deviation were calculated.

Statistical analysis

The mean values were statistically analyzed by Analysis Of Variance (one way ANOVA) using Tukey test at $p < 0.05$ significant level to detect the significant differences between media containing different sucrose concentrations A (1%), B (3%) and C (5%).

RESULTS

Growth

The maximum yield of mycelium in A (1% sucrose) reached at 60 h (730 mg/flask) and after that it declined. Whereas, the maximum yield of mycelium in B (1650 mg/flask) and C (1650 mg/flask) reached at 96 h (Fig. 5). In the initial phase, up to 60 h, the growth was directly proportional to the sucrose levels. In C the fresh weight was highest followed by B and A respectively. After 60 h, the growth of fungus moved parallel in B and C but later on it declined in B and was stabilized in C. There was a statistically significant difference of A with B and C ($p < 0.01$) but it was not found between B and C.

Polygalacturonase

The extracellular activity was highest in A compared to B and C at 24 h, 60 h and 168 h (Fig. 12a). Cytoplasmic polygalacturonase activity in A was highest at 24 and 36 h, declined thereafter with a slight peak at 72 h and again it raised at 168 h. Thus at initial and later phases the activity in A was higher. The activity in B and C was less compared to A at 24 and 168 h, but they showed peak at 72 h (Fig. 12b). Wall bound activity was also maximum in A at 24 and 96 h, whereas B and C peaked at 60 h (Fig. 12c).

Xylanase

Extracellular xylanase activity was also highest in A at 24 h followed by B and C respectively. In B the activity peaked at 60 h whereas in C it was at 72 h. However the maximum activity was at 24 h (Fig. 13a). The cytoplasmic xylanase activity was highest at 36 h and declined thereafter with a slight increase at 72 h in A, B and C. Maximum activity was found in A (Fig. 13b). Wall bound activity also peaked at 24 h with the highest in A and declined after 36 h (Fig. 13c).

α-D-Galactosidase

The extracellular activity in A was maximum at 24 h; with a peak again at 72 h but the activity was constantly low in C after 48 h but B peaked at 168 h (Fig. 14a). The cytoplasmic activity was maximum in C at 36 h and declined afterwards. In B the peak was at 48 h and in A it was at 36 h (Fig. 14b). Wall bound activity was maximum in A compared to B and C at 24 h. B peaked at 48 h whereas C peaked at 36 h and then the activity declined (Fig. 14c).

β-D-Galactosidase

The extracellular activity was maximum in A at 120 h, but it remained constantly low with slight peak at 96 h in B and C (Fig. 15a). The cytoplasmic β-galactosidase activity in A was maximum at 36 h while B and C peaked at 96 h (Fig. 15b). Wall bound activity was sharp at 72 h in A , in B at 24 h, 60 h, 120 h and in C at 60 h (Fig. 15b).

β-D-Glucosidase

Extracellular activity was also maximum in A at 120 h, in B at 120 and 160 h but in C it remained constantly low (Fig. 16a). The cytoplasmic activity in A started a rise from 48 h till 96 h with a peak at 72 h (Fig. 16b). While in B the peak was at 120 h and in C at 60 h. Wall bound activity rose from 48 h till 96 h with a peak at 72 h. B and C showed a peak at 120 h (Fig. 16c).

DISCUSSION

Most phytopathogenic microorganisms produce many different enzymes that can degrade cell wall polymers (Di *et al*., 2006). *A. niger* is the omnipresent fungi as well as it is known for its pathogenecity. The objective of the present work was to study the CWDE of this fungus *in vitro* by providing Murashige and Skoog media which is a defined media and known concentrations of sucrose were added to study their influence in enzyme production at different growth stages. There are very few reports which have investigated the growth of molds considering the type of media (Meletiadis *et al*., 2001; Bhaskaran & Smith, 1993). Sucrose is the major transport form for photoassimilates in higher plants. Moreover it is the principle carbohydrate substrate for the synthesis of cytoplasmic and cell wall constituents (Thaker *et al*., 1992) and hence was used here as the sole carbon source. Both saprophytic and plant parasitic fungi produce extracellular enzymes which can degrade the cell wall components of plants (Annis & Goodwin, 1997). Most fungi appear to produce number of enzymes to degrade pectic polymers (Cooper, 1983). They digest plant cell wall to aid in penetrating cells and spreading through plant tissue.

DeBary (1986) was the first to suggest that extracellular enzymes may be involved in the infection process of plant pathogenic fungi. Since then, much research has been focused on trying to determine the role and importance of CWDE to the virulence of plant pathogenic fungi (Annis & Goodwin, 1997). The net effect of these three enzymes is to rapidly decrease the polymer length with a slow increase in reducing group (Coughlan, 1989). In this work, the effect of growth period and concentration of carbon source, which is sucrose, on the production of polygalacturonase, xylanase, α-D-galactosidase, β-D-galactosidase and β-D-glucosidase from *A. niger*, is emphasized.

PG activity was more in the media containing low concentration of sucrose both at initial and later phases in all the three fractions. However, the activity in the extracellular fraction was highest in A, B and C. It may be possible that this low concentration leads the fungus to produce more PG so that it could strive in such nutrient conditions. In order to penetrate the cell wall, PGs are produced by many plant fungal pathogens and PGs are the first cell wall degrading protein to be secreted (Di *et al*., 2006). Degradation and solubilization of pectic polymers accompanied by the dramatic increase in PG activity have often been observed at softening stages of many fruits (Ranwala *et al*., 1992). PG was studied in relation to different carbon sources and various stages of cantoloupe fruit development (Zhang et al., 1997). Its activity has been studied in some plant pathogenic fungi as *Botrytis cinerea* (Tobias *et al*., 1995), *Phomopsis cucurbitae* (Zhang *et al*., 1997), *Pythium ultimum* (Campion *et al*., 1997), etc.

Xylan is the most abundant component of hemicellulases and is a β-1,4 linked polymer of xylose substituted with side-chain of other pentoses, hexoses and uronic acid depending on its botanical origin (Rao *et al*., 2002). Carpita and Gibeaut (1993) have proposed that xyloglucans are interlaced with cellulose microfibrils and act as a network providing tensile strength to the cell wall. Much effort has been directed at increasing xylanase production by *Aspergilli* because of their many potential applications. Xylanase was also found to be the utmost in A at the initial phase but it depleted in the later phases, in present work. Xylanases may have played a role in the growth of the fungus. It was also highest in the extracellular followed by wall bound and cytoplasmic fractions respectively. Production of xylanases has been found predominantly with grass pathogens and synthesis of xylanases is induced by xylan and plant cell walls (Wanjiru *et al*., 2002). This indicates that xylan degrading enzymes are important for the colonization of the host tissue (Wanjiru *et al*., 2002).

The changes in activities of α, β-galactosidases and β-glucosidase were also investigated throughout the development period of *A. niger*. *Aspergillus sp* is known to produce high levels of β-glucosidase activity, but have weak levels of endo glucanase activity (Rose & van Zyl, 2002). α-galactosidase was almost similar in extracellular and wall bound fraction in A but was comparatively low in cytoplasmic fraction where the activity in C was more in the initial phase. β-galactosidase and β-glucosidase activity was less in the initial hours but increased in the later hours suggesting that it may not have the role in the initial growth or degradation but it may become active in the later hours. Also α-galactosidase activity was more in extracellular fraction followed by wall bound and cytoplasmic fraction, respectively whereas β-galactosidase and β-glucosidase activity though more in extracellular fraction, it was followed by cytoplasmic and wall bound fractions, respectively. The possible involvement of β-galactosidases in modification of cell wall components during the ripening of fruits has been investigated (Ranwala *et al*., 1992).

A correlation between growth rate of the tissue and specific activity of the wall-associated β-glucosidase has also been established (Murray & Bandurski, 1975). β-glucosidase plays an important role in the hydrolysis of cellulose by converting cellobiose to glucose. Previously, the changes in activities of soluble β-galactosidase and two forms of wall-bound β-galactosidases extracted with NaCl and EDTA were investigated throughout the development of muskmelon fruits (Ranwala *et al*., 1992). Data from several laboratories suggest that glycosidases play a role in wall plasticization, thus permitting cell elongation during extension growth (Murray & Bandurski, 1975). To investigate whether or not some glycosidases, namely α and β-galactosidases, β-glucosidases, and α-mannosidase, are involved in the softening of melon fruits, the changes in their total activities throughout fruit development were determined (Ranwala *et al*., 1992).

In the earlier reports, the cell-wall degrading enzymic potential was tested *in vitro* (Campion *et al*., 1997). Cell wall degrading enzymes like cellulose was found to be the major factor of virulence and susceptibility of pathogen and host respectively (Lakshmesha *et al*., 2005). They can degrade plant cell wall, cause cell wall collapse, and provide nourishment for fungal pathogens (Di *et al*., 2006). Pectinase and cellulase enzymes were found to be the major pathogenic chemical factors that influence the degree of pathogenecity of *Colletotricum capsici* spoilage of fresh fruits and vegetables (Lakshmesha *et al*., 2005). Enzymes involved in cellulose degradation include endo-1,4-glucanase which cleaves internal bonds, and cellobiose from the end of the polymer (Sheppard *et al*., 1994). Fungal arabinases, arabinogalactanases, xylanases, and glycosidases may act in concert to increase access to the main polymers by degrading their respective substrates (Annis & Goodwin, 1997).

The extracellular CWDE are potentially involved in pathogenesis of *Fusarium graminearum* on wheat (Wanjiru *et al*., 2002). Fungal glycosidases such as β-galactosidase, xylosidase and arabinosidase may release sugar molecules that can be used as a nutritional source for the fungus during its growth through plant tissue (Annis & Goodwin, 1997). Pectinase is produced during the natural ripening process of some fruits, where together with cellulose; they help to soften the cell wall. These enzymes are also secreted by plant pathogens and soft-rot bacterium as part of their strategy to penetrate the host cell wall (Lakshmesha *et al*., 2005).

This study is conducted with the MS medium which is the most common medium for the tissue cultural studies. Also the carbon source used is sucrose which is the main compound used by most plant species to translocate photoassimilates from the leaves to non-photosynthetic tissues (Sheppard *et al*., 1994).

When the pathogen is studied in association with its host plant, extracellular release of the enzyme by the pathogen into the host tissues can be mistaken for an enzyme of plant origin (Bhaskaran & Smith, 1993). Here the PG activity is highest, followed by xylanase and other glycosidases in the extracellular fraction. This experiment clearly depicts that the fungus at its initial phase of growth, secretes high wall degrading enzymes and release more reducing sugars and utilize more energy which might be useful in its pathogenic activities and also supports the view of PG being produced the first enzyme during wall degradation.

Figure 12

Polygalacturonase activity expressed on fresh weight basis in A (♦),

B (▲) and C (■)   (a) extracellular (b) cytoplasmic and (c) wall bound

The primary axis represents the columns

Figure 13

Xylanase activity expressed on fresh weight basis in A (♦), B (▲) and C (■)

(a) extracellular (b) cytoplasmic and (c) wall bound

The primary axis represents the columns

Figure 14

α-D-Galactosidase activity expressed on fresh weight basis in A (♦), B (▲) and C (■)

(a) extracellular (b) cytoplasmic and (c) wall bound

The primary axis represents the columns

Figure 15

β-D-Galactosidase activity expressed on fresh weight basis in A (♦), B (▲) and C (■)

(a) extracellular (b) cytoplasmic and (c) wall bound
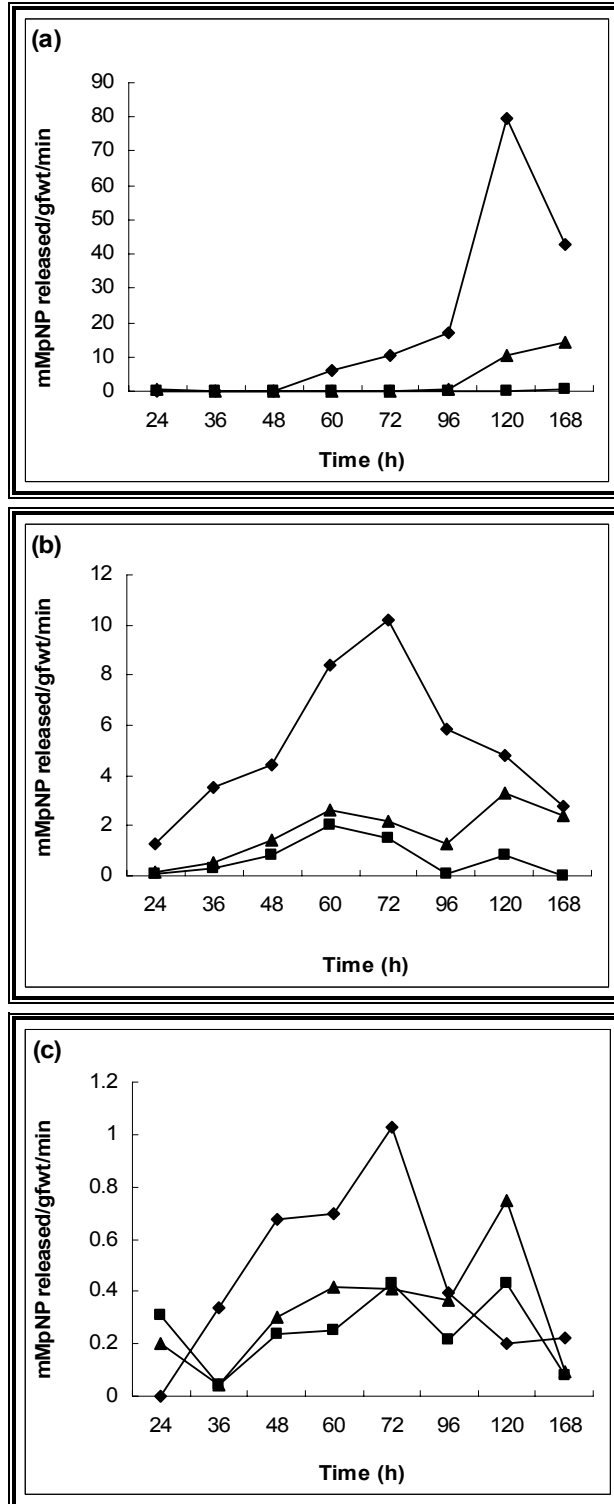
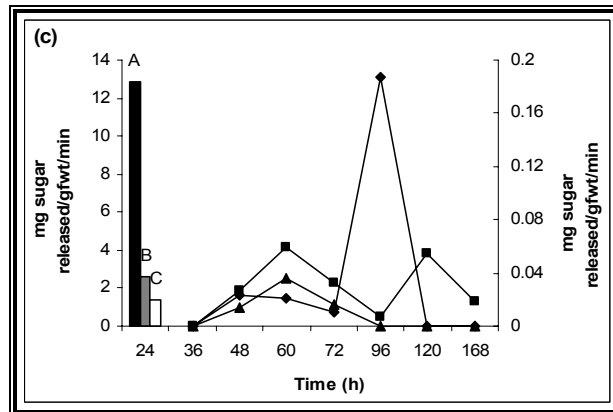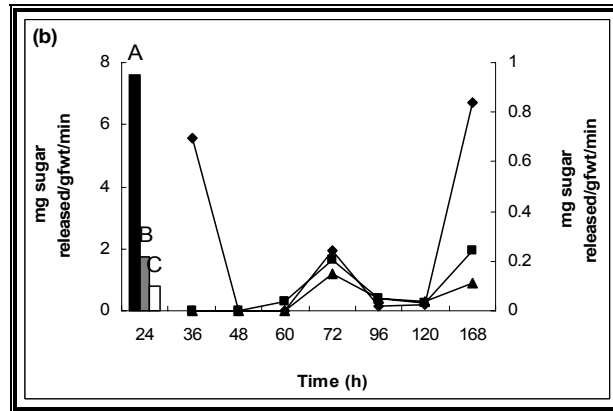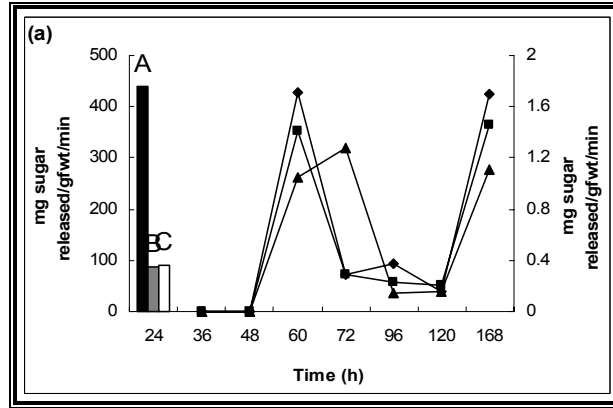**Figure 16**

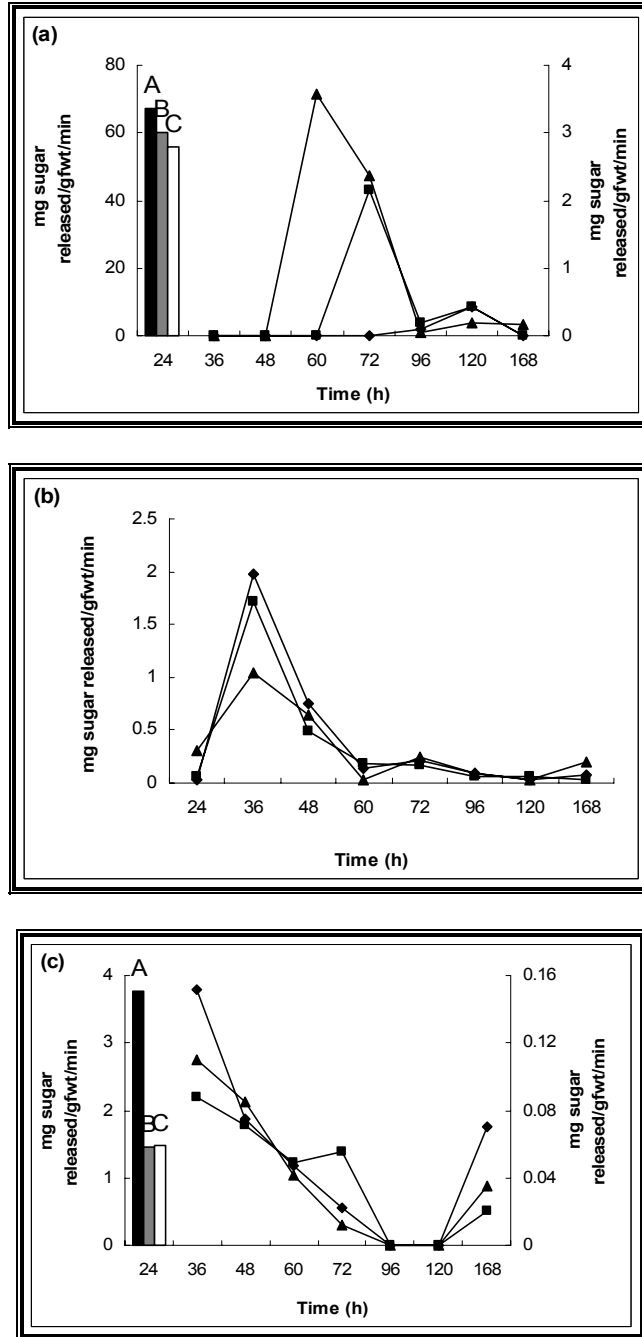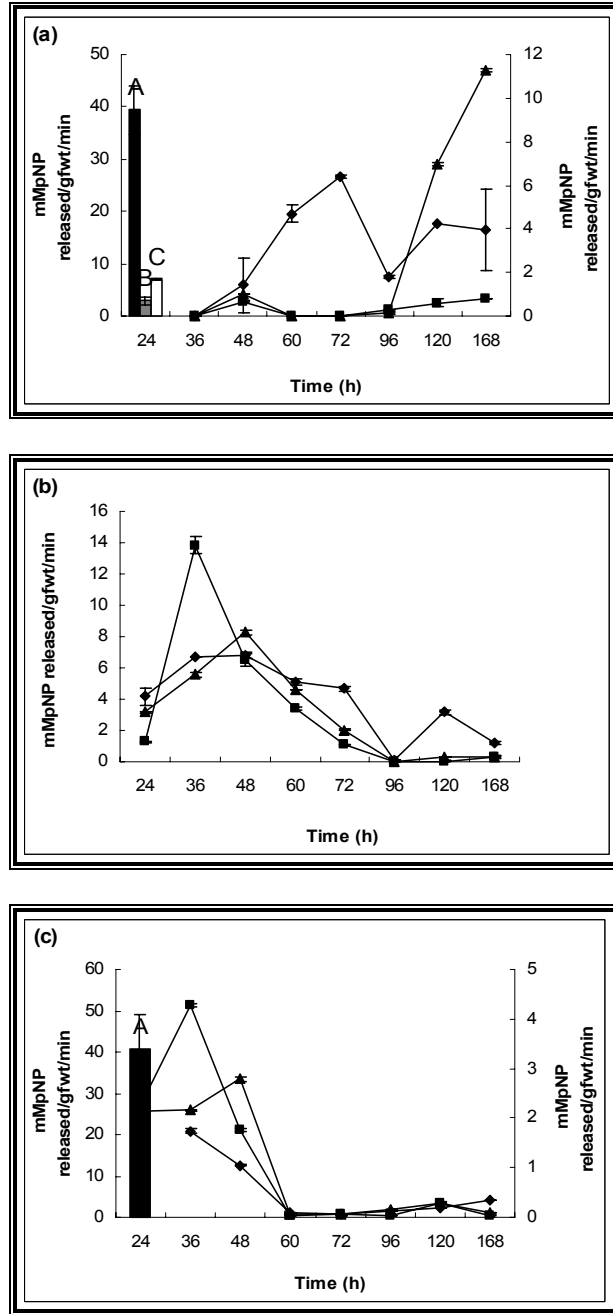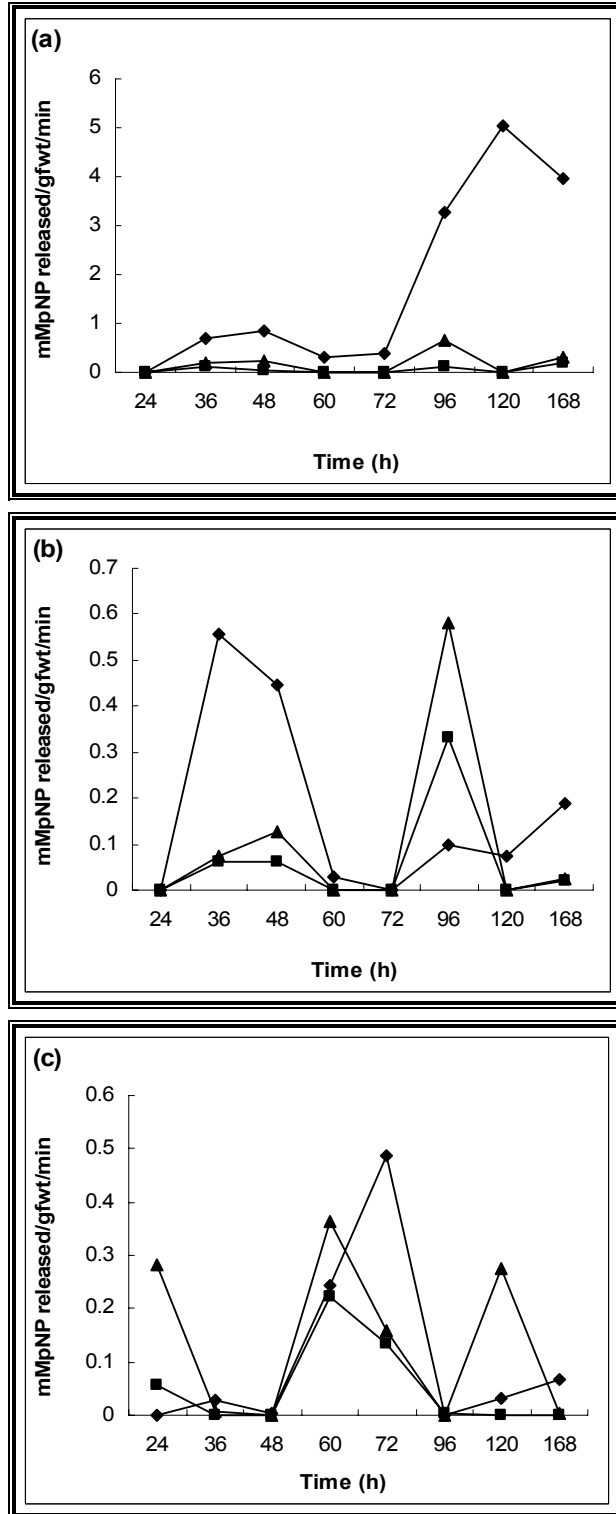β-D-Glucosidase activity expressed on fresh weight basis in A (♦), B (▲) and C (■)

(a) extracellular (b) cytoplasmic and (c) wall bound

CHAPTER 5

GDV ANALYSIS OF DISEASED LEAVES

ABSTRACT

To predict diseased condition in plant, a very important aspect of plant pathology, is rather difficult and time consuming. Disease prediction is challenging and its work is still in a developing condition. In this study, a biophysical approach was used to detect diseased condition in groundnut leaves. Groundnut leaves with increasing order of infection and necrotic lesions were selected. The images of these leaves were captured with GDV (Gas Discharge Visualization) technique and with the GDV analysis software its parameters were calculated from the GDV grams. There were significant differences ($p < 0.05$, Tukey test) observed in these parameters between all the selected leaf categories. ABA, a stress hormone, being very important in host-pathogen interaction was also measured. For this, the antibodies against ABA were raised and IgG was purified. Indirect ELISA technique was followed to estimate the endogenous ABA levels in leaves. ABA was taken as a base to study the machine based learning of pathogen interaction prior to complete infection. This preliminary experiment based on the leaves physiological parameters like weight, area, infected area and ABA measurement during interaction and complete infection till the appearance of visual symptoms, may provide the best study tool for the researchers to predict disease before severe infection. The importance of GDV technique and its use in the field of plant pathology is discussed.

Keywords: ABA, GDV technique, leaf area, non invasive

INTRODUCTION

Plant pathogens exhibit parasitic relationship with the host in which they harm the plant. They typically weaken or destroy cells and tissues of plants, reduce or eliminate their ability to perform normal physiological functions, reduce plant growth and result in disease symptoms or may cause death. Thus there is a chain of events responsible for the causation of any disease in which symptoms and manifestation of injury to the plant is the last link (Chaube & Singh, 2000). Diseases of agricultural crops cause serious economic losses, worldwide due to which crop protection is increasingly seen as an integral part of production system (Rabbinge & Oijen, 1997). Physiology and Biochemistry have been developed as powerful disciplines during the 20th century, but only in a limited number of instances they have led to crop improvement (Miflin, 2000). Protection of crops is depended on the development of the agrochemical industry that has developed sophisticated chemical syntheses and screening technologies (Miflin, 2000).

Arachis hypogaea L. (Groundnut) is an important oil seed crop in Gujarat. It is a crop of global economic significance because of its wide adaptability and use as a source of diverse food products (Anjaiah *et al*., 2006). The leaf spot disease of groundnut caused due to fungus has led much of the crop loss. Most of the fungicides which are chemical in nature are used to eradicate the fungus because until the visual symptoms appear it becomes too late for a biocontrol agent to be used. The usage of fungicides and pesticides are not ecofriendly and hence biocontrol agents need to be emphasized. The use of biocontrol methods would be definitely challenging to eradicate diseases if it is possible to forecast the disease inception at early stages in the chain of events causing disease. Plant pathologists are working upon disease forecasting but it is still in a juvenile form and is developing.

Prediction of disease initiation in plants is difficult and time consuming however it is done on the basis of weather condition, inoculum intensity and host physiology (Mehrotra & Aggarwal, 2004). In the present study, a biophysical approach is used to detect diseased condition in groundnut leaves with the support of some physiological parameters.

Optical methods are used more and more in biological, food and medical research. In comparison with other methods, they have two main advantages: high speed and non-invasiveness. Here an attempt is made to assess the level of pathogenecity in the leaves of groundnut through Gas Discharge Visualization (GDV) Technique. This technique is based on the concept of Kirlian photography. In 1939, Russian technician Semyon D. Kirlian noticed that through the interaction of electric currents and photographic plates, imprints of living organisms developed on film. Later on in 1995, new scientific approach, based on CCD (Charge Coupled Device) video techniques, modern electronics and computer processing of data - the GDV technique (Korotkov, 1998) came into existence. Image formation in gas discharge around objects of a different nature initiated by strong impulsive electromagnetic fields is known for more than two centuries (Korotkov, 1998). So far the main direction of investigation of the effect has been purely practical, it turned out that gas discharge images around biological objects could provide substantial information about the internal state of the object (Korotkov & Korotkin, 2001).

In this apparatus (Korotkov & Korotkin, 2001), by a vacuum photogalvanoplastic process a thin metal grid with 10-micron wires is evaporated on the bottom surface of the glass plate. The sequence of electrical impulses from the highly stabilized generator is applied to this grid generating an electromagnetic field around the subject. Under the influence of this field the subject produces a burst of electron-ion emission and optical radiation light quanta in visual and ultraviolet range.

These particles and photons initiate electron-ion avalanches, giving rise to the sliding gas discharge along dielectric surface. Spatial distribution of discharge channels is registered via glass plate by the optical system with CCD camera and digitized in the computer. Thus the technique is called GDV technique and images after processing are called GDV-grams (Korotkov, 1998). It was demonstrated that Crown-TV Kirlian camera (Plate 8) and GDV technique can provide useful information for some problems in agronomy, i.e. distinguishing healthy from stressed or infected plants or differentiating between distinct varieties of the same family of plants (Skočaj *et al*., 2000; Sadikov & Kononenko, 2002). This technique is also able to point out differences between plants grown using distinct nutrition schemes.

To assess the significance of the results obtained by GDV technique, other parameter i.e. ABA (abscisic acid) is also measured in the leaves of groundnut with the well-established immunological technique, ELISA (Enzyme Linked Immunosorbent Assay). ABA is a sesquiterpenoid plant hormone participating in the control of numerous essential physiological processes such as seed development and germination, and also in plant responses to different stresses (Leung & Giraudat, 1998). The implication of ABA in abiotic stress interactions has been widely studied (Zhu, 2002), but much less reports exist about the influence of this hormone in biotic interactions (Flors *et al*., 2005). An increasing body of information also points to an involvement of ABA in the plants' responses to pathogen attack (Audenaert *et al*., 2002; Anderson *et al*., 2004). The quantification of endogenous ABA levels has been done by HPLC-UV (High-pressure liquid chromatography and UV detection), GC-FID (gas chromatography flame ionization) and GC-MS (gas chromatography-mass spectrometry) methods but they require rigorous clean up procedures to ensure elimination of contaminants (Norman *et al*., 1998) and make routine analysis difficult (Harris & Dugger, 1986).

Several RIA (Radio Immuno Assay) methods for ABA quantization have also been reported (Norman *et al*., 1998). However, immunological methods minimize the need for purification and allow for rapid analysis of many samples using little plant material. The immunological method EIA (Enzyme Immuno Assay) has been proved advantageous for hormone analysis because of its ability to use crude plant extracts without sacrificing sensitivity and selectivity (Sharma & Thaker, 2001). To amplify the reaction, indirect ELISA technique was used previously to quantify endogenous levels of ABA in cotton leaves (Gokani *et al*., 1998).

Though the immunological methods are sensitive and non laborious, the optical method of detecting pathogenecity exhibit few more advantages such as non-invasiveness, rapidity and less material consumption. Improved detection techniques of pathogen in plants may help in improving the crop quality and increasing the agricultural crop yield. Taking this into consideration the machine learning technology i.e. GDV was used to record the Bioelectromagnetic (BEM) field (Korotkov, 1998) of groundnut leaves.

This experiment was designed to detect the potential of optical method like GDV technique, in detecting the level of pathogenecity in the leaves of groundnut. The plant stress hormone ABA was also measured from the same leaves to access the mark of comparison and efficacy of the GDV technique.

MATERIALS AND METHODS

Sample Collection

Groundnut plants were collected from a field near Rajkot. Random sampling of leaves was done, based on their morphological and visual characters, from different plants in ten replicates. The leaves were divided into five categories:

1- Leaves with no infection and completely green

2- Green leaves with the initiation of infection

3- Leaves with medium infection and turning yellow in color

4- Yellow leaves with heavy infection turning to black lesions

5- Completely dried and infected leaves.

Detection of fungal infection

This was done by following the Koch's postulates. The leaves were washed with tap water for 1 h followed by methanol for 30 seconds and then three washes with sterile distilled water were given. The infected portions were placed on the plates filled with Potato Dextrose Agar (PDA) media at different sites. These plates were incubated for 24 h at 28 ^{o}C temperature.

GDV instrument and GDV Analysis

The images of the leaves (adaxial surface) were captured using a specialized GDV camera instrument i.e. the Crown TV instrument (Plate 8) (Kirlionics Technologies International, St. Petersburg, Russia) (Bell *et al*., 2003) at an electrode voltage of 12.5 kV with the impulse duration of 10 mcs, repetition frequency 1024 Hz and exposure for 1 second. Leaves were placed on the visualization electrode, grounded and recorded. These recorded GDV images were analyzed using GDV analysis software and data for 9 different numerical parameters of GDV image were obtained. From each category ten leaves were selected, recorded and measured.

Raising of polyclonal antibodies against ABA

Preparation of ABA-BSA (Bovine serum albumin)/Casien conjugates

ABA is a hapten, a low molecular weight compound and therefore it is not immunogenic. It has to be tagged with a high molecular weight carrier molecule, like protein. The ABA-BSA conjugate was prepared according to Gokani *et al*., 1998. ABA (132 mg) (Hi-media laboratories, Mumbai) was dissolved into 3 ml mixture of distilled water and DMF (Dimethyl formamide) (2:1) and pH was adjusted to 8.0 with 1N NaOH. This solution was then added drop wise with gentle stirring to 250 mg BSA dissolved in water and its pH was readjusted to 8.5. In this solution, N-ethyl-N (3-dimethylaminopropyl) carbodiimide-hydrochloride (EDC, 210 mg) was added and stirred in dark at 4 ^{o}C for nearly 20 h. Conjugate was finally dialyzed against water for 4 days and stored in deep freezer.

Immunization

ABA-BSA conjugate was mixed thoroughly in equal volume of Freund's complete adjuvant. Two rabbits were immunized by intramuscular route at a regular interval of 15 days, till sufficient titer was achieved and serum was collected.

Purification of antiserum

Purification of antiserum was carried out in order to separate IgG from other serum proteins by ion-exchange chromatography using DEAE (diethylaminoethyl) cellulose cake. For every 10 ml of serum, 50 g wet weight of cellulose was taken. To lower the ionic strength of serum, every 10 ml of serum was mixed with 30 ml of distilled water, mixed with cellulose cake and kept at 4 ^{o}C. It was stirred thoroughly after every 10 min at 4 ^{o}C for 1 h in order to equilibrate the mixture. The supernatant containing IgG was collected in 0.01 M phosphate buffer (pH 8.0) and concentrated.

Extraction of ABA from the leaves

The leaves were crushed in 80% methanol for the extraction of hormone (ABA). The mixture was centrifuged at 10 000 g for 10 min. Pellets were washed thrice with methanol to ensure the complete extraction of ABA. Pooled supernatants were mixed and kept for evaporation in dark. After complete evaporation of methanol the residue was mixed with phosphate buffer saline (PBS, pH 7.2) and 10 ml of the final volume was prepared.

Procedure for ELISA

To avoid cross-reactivity with conjugated protein, in this method, ABA-casein conjugate was used to immobilize antigen on a polystyrene microtiter plates (96 well, flat bottom plates, Polylab, A. K. Scientific Industries, Delhi, India). The ABA-casein conjugate was diluted with coating buffer (10 mM carbonate buffer, pH 9.7). The plate was incubated at 37 ^{o}C for 3 h and then it was washed several times with washing buffer (phosphate buffer saline, pH 7.2 containing 0.05% Tween-20). The plate was blocked with blocking buffer (washing buffer mixed with 0.5% egg albumin). Various dilutions of antiserum were added in the wells and incubated for 1 h at 37 ^{o}C. The unbound rabbit serum was washed away by repeated washing with washing buffer. Second antibody, anti-rabbit Goat IgG conjugated to peroxidase (1:2500 in phosphate buffer, pH 7.2) was added in each well, and incubated for 1 h at 37 ^{o}C. All wells were washed again and substrate solution containing 20 mM OPD (ortho phenelene diamine), H_2O_2 (3%) and 0.03 M phosphate buffer (pH 5.0) was added. The reaction was terminated by the addition of 6 N Sulphuric acid. The color developed was read at 492 nm with ELISA reader (µ Quant, Bio-tek Instruments, USA). For the estimation of ABA from groundnut leaves samples, different concentrations of samples were mixed with antibody (with or without internal standards) prior to addition into the wells. To test sensitivity of the assay, each sample was mixed with known concentration of ABA (200 ng) as an internal standard before reacting with the antibodies.

Relative binding values were calculated as B/Bo, where B and Bo are the values of absorbance in the presence and absence of internal standard hormone or sample, respectively. A standard curve was prepared for each particular plate and the values falling on the curve were taken. The estimations were done in triplicates and mean values were calculated.

Leaf area measurements

The area of leaves was measured with the help of specialized software known as "Leaf Area Meter". This software was recently developed in our laboratory by Mucchadia and Thaker (© Mucchadia & Thaker, 2006). It has high sensitivity (0.035 mm^2), easy to use and less time consuming. The area of infected portions distinguished from healthy portions was also measured by this software.

Statistical Analysis

The data were subjected to Analysis Of Variance (ANOVA) and multiple comparisons were done with the help of Tukey test at $p < 0.05$ to detect significant differences in the GDV parameters between all the selected leaf categories. Correlation studies were carried out between GDV parameters and the physiological parameters of tested five categories of leaves. All analysis is presented as means ± SD.

RESULTS AND DISCUSSION

In the present work, groundnut leaves with five different categories were selected randomly based on their visual symptoms of infection (Plate 9). The first category contained the leaves with no visual symptoms of infection; they were mature, fully expanded and healthy as appeared from their complete green color. Second category contained green leaves with initiation of infection. Third category of leaves selected were half infected with black color appearance, few portions were yellow and rest of the portion was green. The fourth category consisted of the completely yellow leaves with the infection that had turned to lesions and partially dried. The last category of leaves was completely black with lesions and dried out.

The leaves were scanned and their total area was measured with the help of Leaf Area Meter (Fig. 17). Fresh weight of the leaves was measured (Fig. 18) which showed that maximum weight was occupied by 1st category leaves after which no significant difference between weight of other category leaves was observed.

The pathogen infecting the leaves was isolated according to the Koch's postulates from the leaves showing similar lesions. It was found that the same fungus appeared on the PDA media from all the leaves categories (data not presented). The fungus was same as appeared from its morphological characters (but it was not identified) so no probability remained to be misguided in the analysis and thus it gave an additional emphasis to use GDV technique. ABA levels were measured and explained on leaf fresh weight basis (Fig. 19). The level of ABA rose from 1st category leaves to the 2nd category being the maximum and then it gradually declined with the increase in infectious levels as appeared from the visual symptoms (Plate 9) in rest of the three categories.

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Enhanced levels of ABA have been reported from plant pathogen interactions (Flors *et al*., 2005). Also it is indicated that the rise in the level of ABA in infected leaf is dependent on the presence of the pathogen in the host tissue. Role of ABA in influencing the outcome of plant-pathogen interactions is controversial, with most research pointing into the direction of increased susceptibility, recent results have shown that ABA can also be involved in rendering plants more resistant to pathogen attack (Flors *et al*., 2005). Increased endogenous ABA levels have been reported from various plant species in response to infection with fungi, bacteria and viruses (Kettner and Dorffling, 1995).

Here the high levels of ABA in the first two categories of leaves may represent the initiation of infection, which is in agreement with the previous reports. High endogenous concentration of ABA at the moment of pathogen infection can contribute to develop susceptibility. Later on the ABA level decreased gradually with the minimum found in the 5th category leaves. This might be because the leaves were completely dried up. It has been recorded that ABA decreases when plants recognize the pathogen (Flors *et al*., 2005). Earlier, two virulent strains of *Botrytis cinerea* were used to study the biosynthesis and metabolism of ABA in infected isolated leaves (Kettner & Dorffling, 1995).

Also, ABA can induce resistance by involving itself in the plant defense mechanisms where numerous signaling pathways, producing a web of interactions of remarkable complexity and subtlety are activated (Cutler, 2005). The changes in ion channels have been studied in detail by means of electrophysiology (Cutler, 2005). Thus we have taken this hormone, involved in the interaction between phytopathogenic microorganism and their host plants, as one of the physiological parameters to study and support the GDV analysis of the leaves from the stages of interaction to infection.

The GDV instrument, with the help of special electrode system, creates a high intensity electric field around the object, which produces a visible gas discharge around the object whose glow can be seen and measured (Korotkov, 1998). The electric field reaches high enough intensity in relation to surface or volume heterogeneity of the object, and to the gaseous composition in the immediate environment (Korotkov, 1998). The ignition points of the avalanche are related, both to the characteristics of the internal conductive structure, and to the properties of gases emitted through the pores and thus could yield information on the physiological state of leaves and their reactions to various influences (Korotkov, 1998). The image is transferred to a computer using built in CCD aided video capture card.

All the nine parameters obtained by using the GDV analysis software from the GDV grams (Plate 10) are presented in Figure 20. These parameters are: area, noise, fractality (form coefficient), form fractal dimension, brightness, brightness deviation, number of fragments, fragments average area and deviation (Sadikov, 2000). All of these parameters exhibited a significant difference ($p < 0.001$) amongst all the five categories of leaves (Fig. 20). Maximum significance was observed with brightness followed by brightness deviation, no. of fragments, noise, fractality, form fractal dimension, area, fragments average area and fragments deviation, respectively. Previous studies have shown that GDV can differentiate reliably between drops of different electrolyte solutions and distilled water (Korotkov & Korotkin, 2001). They also reported that fractality was the GDV image parameter with the best stability and sensitivity across different concentrations of inorganic solutes in distilled water solution. Many previous studies included the analysis of form coefficient (fractality), mean image area, image brightness and fragments average area. In this also, brightness, brightness deviation and form coefficient exhibited highest significant differences amongst all the categories of leaves as evident from the Tukey test (Fig. 20).

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The correlation worked out amongst GDV parameters and ABA levels suggested a highly positive correlation with form coefficient (fractality) whereas, highest negative correlation with brightness deviation (Table 8). It has been reported previously that the form coefficient is the most universal parameter, which shows good combination of stability and sensitivity properties (Korotkov & Korotkin, 2001).

Maximum positive correlation was observed between brightness deviation and infected area and area of necrotic lesions of the leaves (Table 9). However highest negative correlation was observed between form coefficient and infected area and area of necrotic lesions of the leaves (Table 9). It has been reported previously that parameters like area, noise, no. of fragments and fragment average area are dependent on the area of leaf and the most important parameter they found was area per fragment deviation (Sadikov, 2000). Thus infection levels showed positive correlation whereas ABA showed negative correlation with the brightness deviation but it was opposite in case of form coefficient. In a previous study (Skočaj *et al*., 2000) it was recorded that the numerical parameters calculated from the coronas of grape berries can be used successfully to classify berries according to infection and sort.

GDV technique provides indirect judgments about the level of energy resources at the molecular level of functioning in structural – protein complex (Korotkov *et al*., 2004). GDV images for yeast cultures, grains, plant leaves and seedlings have been reported (Korotkov, 1998). It reveals the distribution and type of energy in an organism (Korotkov, 1998). It is also possible to detect and find useful information in Bioelectromagnetic fields of seeds of plants (Korotkov, 1998). GDV images may be effectively used for quantitative investigation of different biological objects. It opens a wide perspective for research and practical applications in agriculture, biophysics and ecology (Korotkov, 1998).

This technique can also provide us with additional information and can be used alongside standard agronomical indicators. This computer-based instrument is absolutely safe for using, meets the basic electro technical, and informative demands (Korotkov, 1998). It provides a whole set of gas discharge parameters of an object and finds a lot of unique practical applications (Korotkov, 1998). This technique is non invasive, less expensive, gives real time images, easy to use and less time consuming.

Thus GDV analysis may help us to predict diseased condition of plant and may help to give an idea about applying the control measures before disease symptoms appear. From the present work it is concluded that ABA level is directly correlated to form coefficient while infection level is directly correlated to brightness deviation with a maximum value. This study is further subject of investigation with other plant systems interacted with pathogens. In a single plant system, stages from uninfected to infected, needs to be studied consistently.

Perhaps this preliminary experiment based on the leaves physiological parameters like weight, area, infected area, area of necrotic lesions and ABA measurement during interaction and infection till the visual symptoms appeared, provides the best study tool to the researchers to predict disease before infection. GDV analysis do have enormous potential significance, it helps in understanding the internal state of an object and thus is useful technique for plant biologists, plant pathologists and perhaps may inspire new ideas and new contributions to the field of disease predictions.

TABLE 8

Correlation between nine parameters of GDV technique and ABA levels (degrees of freedom - 4)

Parameter	r- value
Area	0.342
Noise	0.327
Form coefficient	**0.898 ***
Form fractal dimension	0.040
Brightness	0.265
Brightness Deviation	**-0.920 **
Fragments	0.048
Fragments-Average area	-0.316
Deviation	0.654

P level significant at: * 0.05 and **0.01

TABLE 9

Correlation between nine parameters of GDV technique/infected area of leaves and GDV technique/area of necrotic lesions leaves (degrees of freedom - 4)

Parameter	r- value (infected area of leaves)	r- value (area of necrotic lesions of leaves)
Area	-0.478	-0.329
Noise	-0.365	-0.519
Form coefficient	**-0.850 ***	**-0.908 ** **
Form fractal dimension	0.080	-0.088
Brightness	-0.761	-0.846 *
Brightness Deviation	**0.909 ** **	**0.959 ** **
Fragments	0.077	-0.084
Fragments-Average area	0.162	0.318
Deviation	-0.635	-0.759

P level significant at: * 0.05 and **0.01
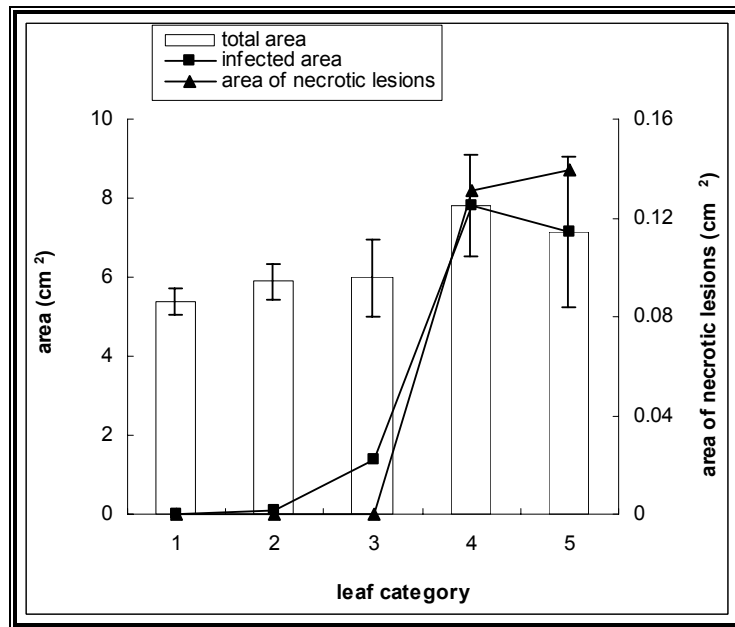
Figure 17

Total area, infected area and area of necrotic lesions found in the
groundnut leaves
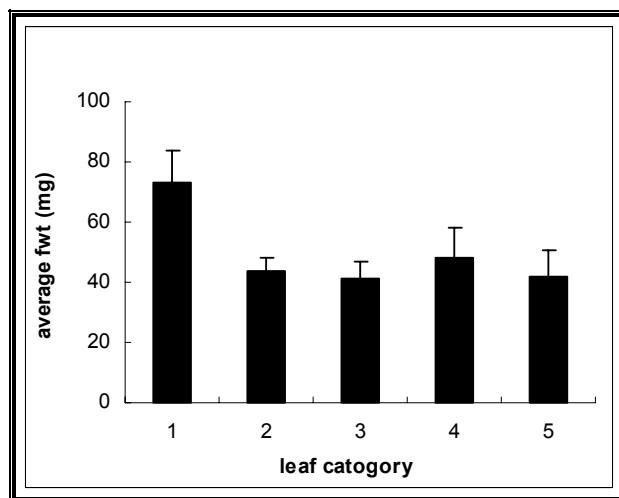
Figure 18

Fresh weight/leaf of groundnut leaves
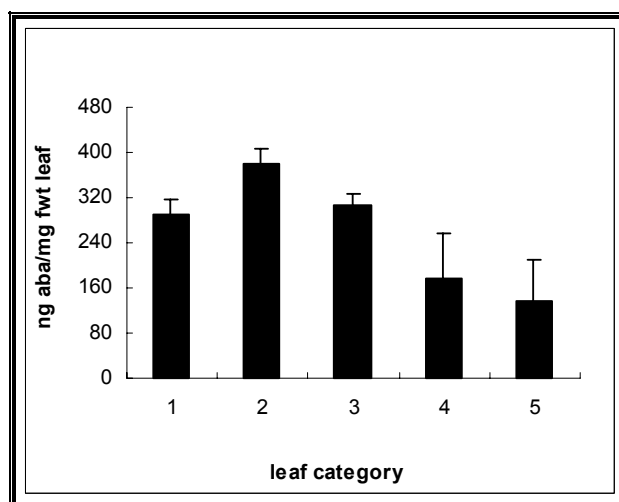
Figure 19

ABA level of groundnut leaves
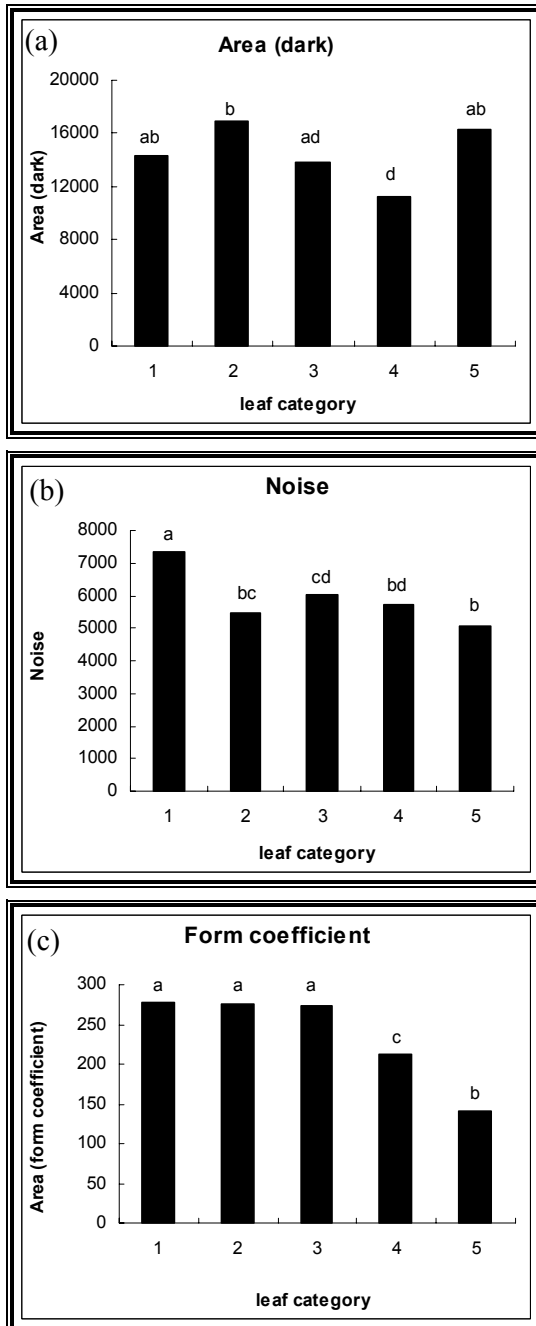
Figure 20 …..

The GDV grams of all the nine parameters obtained by GDV analysis software

The columns (means of ten replicates) with different letters differ significantly
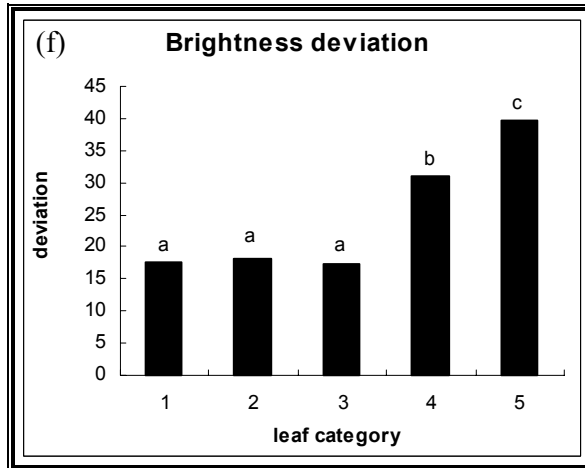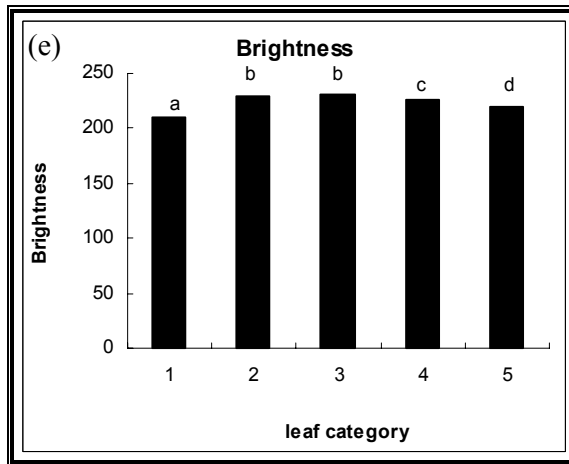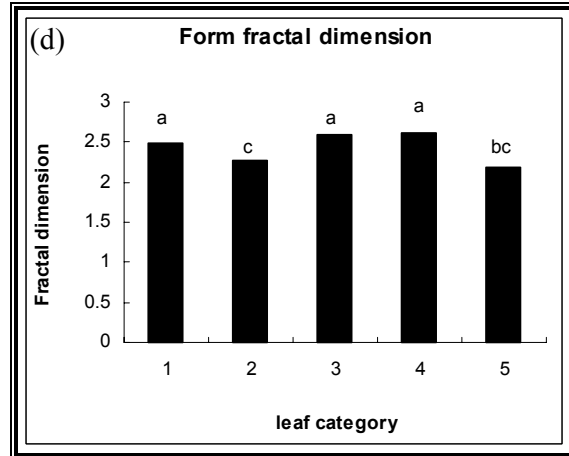
(p < 0.05, Tukey test)

….. Figure 20

The GDV grams of all the nine parameters obtained by GDV analysis software

The columns (means of ten replicates) with different letters differ significantly
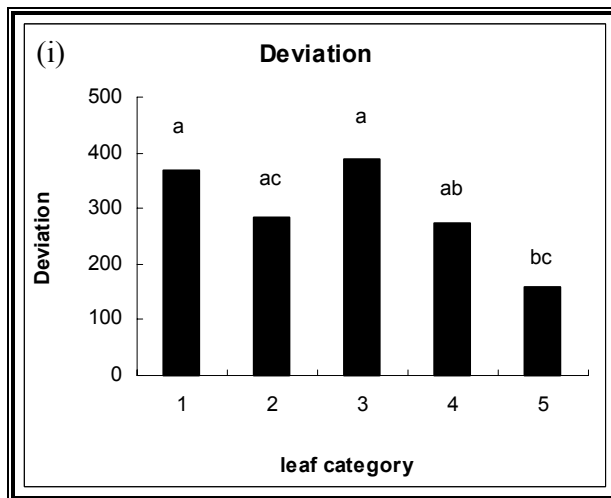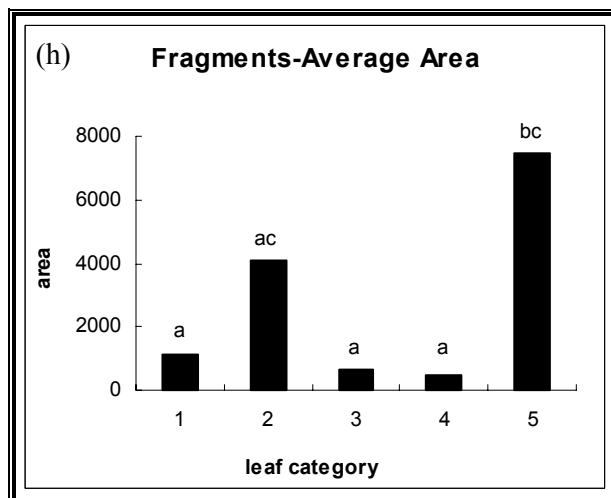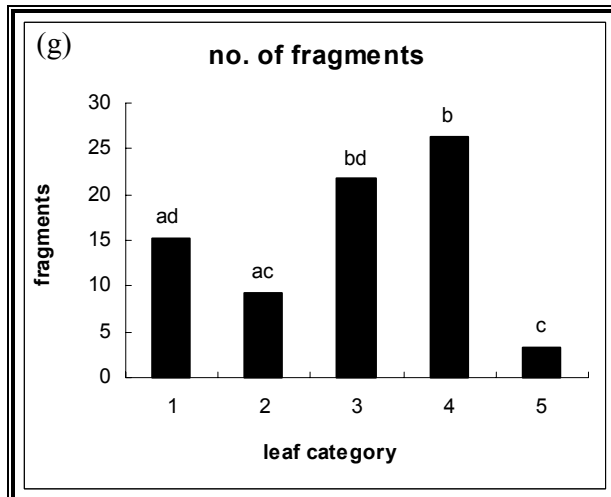
(p < 0.05, Tukey test)

…..Figure 20

The GDV grams of all the nine parameters obtained by GDV analysis software

The columns (means of ten replicates) with different letters differ significantly

(p < 0.05, Tukey test)
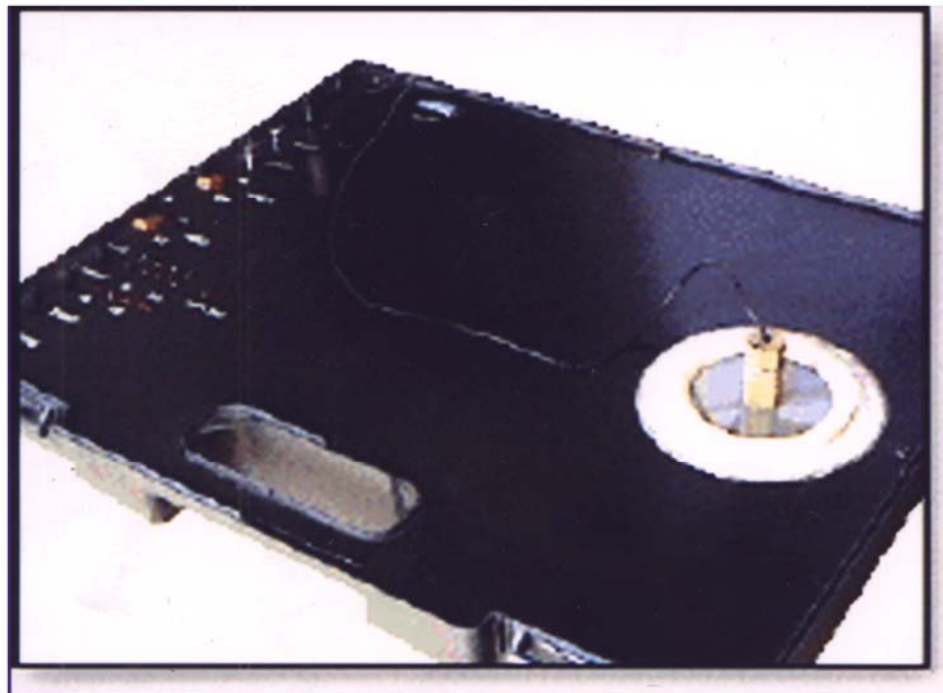
Plate 8
GDV computerized Kirlian Camera
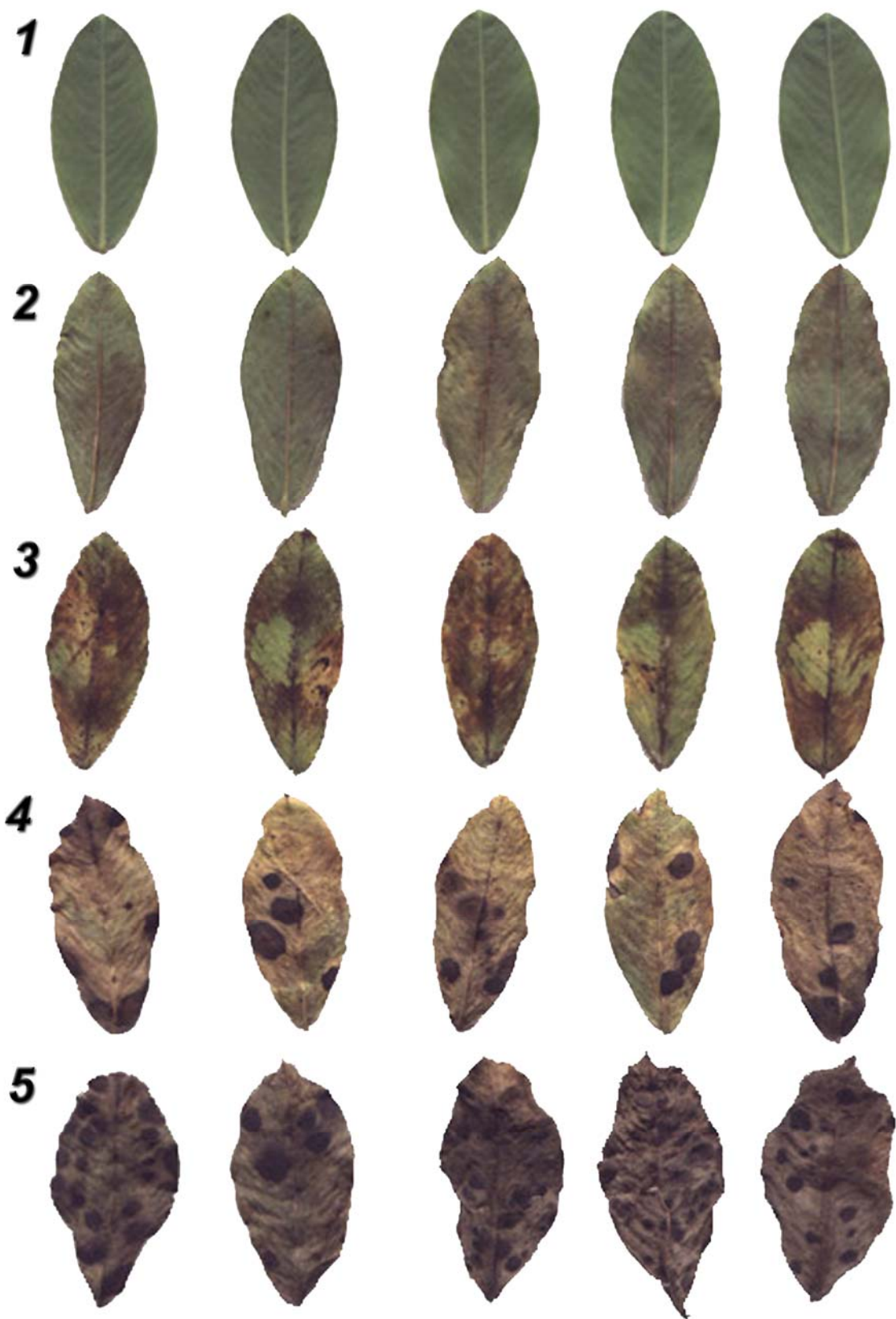
Plate 9. Groundnut leaves with different categories
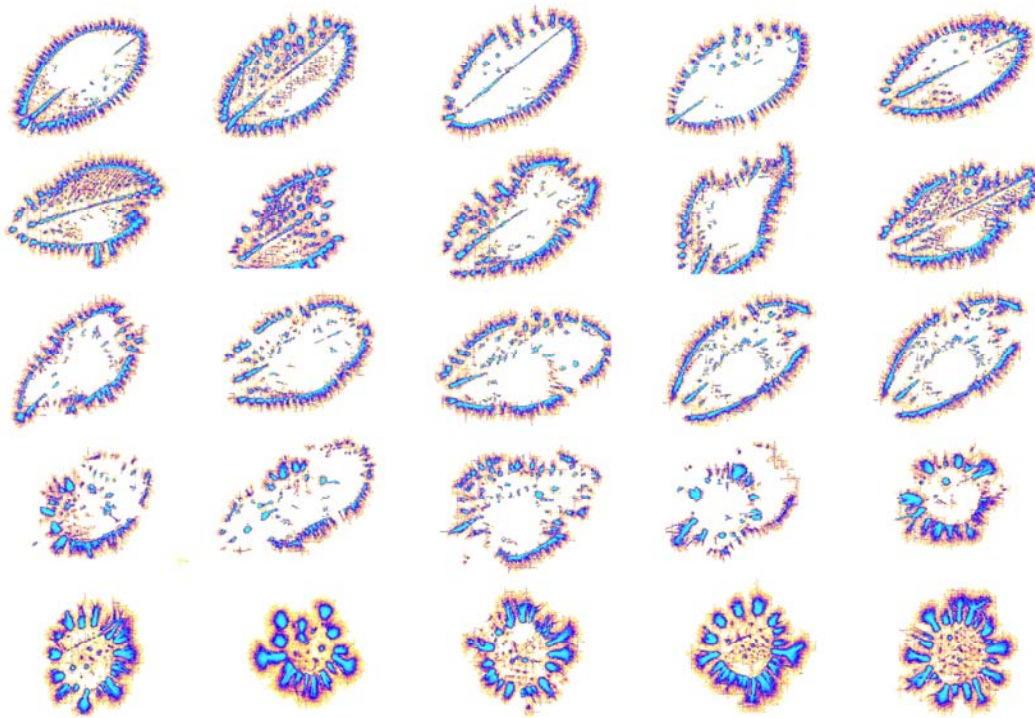
Plate 10. GDV grams of groundnut leaves with different categories

CHAPTER 6

GENERAL DISCUSSION

Today, of the total crop loss of 42% due to various biotic causes, nearly 13% is caused by plant pathogens alone (Mayee & Chakrabarty, 2005). Plant pathogens pose an enormous problem for global food security. They cause damage to both storage and production of food. Plants are subject to disease both in the field and postharvest, the major groups of pathogens being viruses, bacteria, fungi, nematodes and parasitic plants (Strange & Scott, 2005).

Fungi are of great economic importance to man and play an important role in the disintegration of organic matter and are most important as the agents of decay (Alexopoulos & Mims, 2004). They are responsible for a large number of diseases of plants and animals including man (Mehrotra & Aggarwal, 2004). They cause serious economic losses to agronomically important species. Moreover, a large number of fungi are responsible for diseases of stored fruits and vegetables (Mehrotra & Aggarwal, 2004).

Plant disease is responsible for significant losses of global crop production every year and thus has major impact on the world's agricultural productivity (Nölke *et al*., 2004). Numerous strategies have been developed and different approaches may be used in attempts to prevent, mitigate or control plant diseases (Pal & Gardener, 2006). Traditional approaches are based on the avoidance of sources of infection, vector management, modification of cultural practices, the use of resistant varieties obtained through conventional breeding, cross protection and chemical control (Mehrotra & Aggarwal, 2004; Nölke *et al*., 2004).

Despite the continual development of new resistant cultivars and pesticides, annual crop losses caused by plant pathogens, insect pests and weeds, have steadily increased to 42% worldwide, causing damage estimated at $500 billion in the process (Oerke *et al*., 1994).

Furthermore some pesticides are being withdrawn form the market because of their undesirable effects on the environment. New strategies for disease control are therefore urgently required (Nölke *et al.*, 2004) and amongst them, biological control is of paramount importance (Spurrier, 1990; Mendgen *et al.*, 1992).

The present work has been focused on three different aspects:

1. Biocontrol of plant pathogenic fungi using essential oils and antibodies
2. Mechanism of infection by investigating some plant cell wall degrading enzymes and also optimizing conditions for their exploitation
3. Non invasive and non destructive method of detecting diseased condition in plant tissue using a biophysical approach

Since the inception of civilization humans have been using herbs against various mycotic infections in plants as well as in human beings. This fact is based on the knowledge that plants have their own defense system against microbial infections in genera and mycotic infections in particular. It is assumed that in the twenty first century plant derived antimycotics will create a revolution in the field of a new generation of ecofriendly fungicides for the control of plant as well as human mycotic diseases. With the revitalization of use of traditional antimycotic extracts essential oils, which form the basis of the ancient science of life i.e. Ayurveda were used in this piece of research work.

The odorous, volatile compounds, responsible for the essence of plant, are called essential oils (EOs) (Nakajima *et al.*, 2005). They are soluble in alcohol, ether and other lipid solvents and practically insoluble in water. They are secreted in special structures such as ducts, cells, lysigenous glands, trichomes, etc. (Tyler *et al.*, 1981). They are pure, natural and extremely concentrated substances.

They are antibacterial, anti-inflammatory, antiviral, and antifungal and are powerful stimulants in cellular processes. The plants create these oils to help protect them from fungus and bacteria, attract pollinators and help to heal wounds in their bark. EOs contain a tremendous number of other powerful chemical groups, including alcohols, phenols, esters, ethers, oxides, coumarins, sesquiterpernes, terpinols, ketones and aldehydes. Most essential oils are complex mixtures involving a few to several hundred compounds (Charlwood & Charlwood, 1991; Bertoli *et al*., 2004). EOs have been extensively investigated for their activity against a number of storage fungi, plant pathogens, bacteria and other harmful microorganisms (Njenga *et al*., 2005).

We had about 120 different EOs provided by VIRSACO but only 75 were selected based on the criteria that they were extracted either from flowers or spices. EOs can be best used for postharvest or stored crop protection, for their qualities that they are effective at very low concentration and also due to their volatile nature; they are highly diffusible and hence react quickly. Seventy five essential oils were used for their antimycotic activity against *Aspergillus niger*, a common postharvest pathogen. It was observed that *A. niger* was highly sensitive to the essential oils of Cinnamon species. Lemongrass, Cumin, Rose and Bergamot oils showed very high antifungal activity. These 75 EOs were also evaluated for their antifungal activity against the vascular pathogen *Fusarium oxysporum* f.sp. *cicer* (*FOC)* and saprophytic parasite *Alternaria porri.*

The other 4 phytopathogenic fungi were studied only against the most potent 10 EOs as obtained by the initial screening from anti *A. niger* activity. The GC-MS of these 10 EOs was accomplished to investigate the major constituents responsible for high antifungal activity. Different chemical constituents were present in these oils and it was inferred from their antifungal activities that they act synergistically. The activity of all the EOs for different fungi varied, as it appeared from the anti *FOC* and anti *A. porri* activities especially.

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The oil of Cumin was most potent against *A. porri*, *Curvularia, FOC* and *A. niger*. This oil constituted cumic aldehyde and cuminal. Almost all the fungi showed inhibition against the EO of Cinnamon. Eugenol and cinnamaldehyde in Cinnamon oils may be responsible for such higher activity against the tested fungi. Linalool, methyl eugenol, citronellal, geraniol were some of the compounds commonly present in the essential oils exhibiting higher activity. Eugenol was also found to be the major component of Clove oil and Basil oil, which might be responsible for their antifungal activity. Active constituents against all the fungi were different but they gave potent inhibition when acted synergistically.

The biological activity of EO of Clove has been recently reviewed in which its antimicrobial, antioxidant, antifungal, antiviral, anti-inflammatory, cytotoxic, insect repellent and anaesthetic properties are described. The main constituents of Clove oil in this review described are carvacrol, thymol, eugenol and cinnamaldehyde (Chaieb *et al*., 2007). The essential oils from *Ocimum gratissimum, Monodora myristica* and *Thymus vulgaris* have been shown to have strong antifungal properties; significantly reducing the mycological growth of *F. moniliforme* (Nguefack *et al*., 2004). The fungitoxic activity against *Penicillium digitatum, P. italicum*, *Botrytis cinerea*, and *A. citri* of 12 EOs distilled from medicinal plants has also been reported (Arras & Usai, 2001).

EOs penetrate tissue roughly 100 times faster than water and 10,000 times faster than salts (Römmelt, 1974). They are active synergistically and can be used in both pre and postharvest conditions. Little amount of EO is highly effective and generally regarded as safe. This means that essential oils that are registered food grade materials could be used as alternative anti-fungal and anti-bacterial treatments for fresh produce. They are cheaper than the economic loss caused due to low crop yield. Especially they may be effective in postharvest conditions because of their volatile nature, high permeability, non wetness, and non hazardous properties.

These properties of EOs provoked their use as an antifungal agent in the present investigation. Mostly the EOs of Cinnamon, Cumin and Lemongrass were found highly effective. Moreover as they are the spices which people consume routinely, are safe to use and do not cause any hazards to animals, humans and environment. Thus essential oils are the best preservative measure for postharvested crops which are stored in a closed chamber and where essential oils can be used economically.

As fungi are a major problem in agricultural crops, along with other pests, many insecticides and pesticides are available in market for various pests. However, they are very costly and also specific towards the agent to be inhibited. Apart from the use of plant based antimycotics another promising approach is the expression of recombinant antibodies that bind to and neutralize essential components of the pathogen and therefore interfere with its infection cycle (Nölke *et al*., 2004).

Taking these points into consideration, an experiment was designed to produce antibodies (Abs) against the whole cell antigen of *A. niger*. To study the mechanism of pathogenecity of fungi an immunological approach was applied. Abs were produced against *A. niger* whole cell antigen and were checked against its growth. These polyclonal antibodies were tested in broth as well as radial growth assay was performed. It was found that the Abs suppressed the growth of *A. niger* for 7 days.

These Abs were also checked against all the other fungi which were inhibited drastically. It was speculated that there might be some common mechanism of action functioning for the growth of fungi and spore germination which is responsible for the development of fungi. These Abs cross-reacted with all the tested fungi and showed a physiological lag in their growth period. Maximum inhibition was found with *Curvularia sp., Rhizoctonia bataticola, Botryodiplodia theobromae, A. porri, FOC, F. solani* and *A. niger* respectively.

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However, antibodies with a higher titer increased the lag phase of fungi for more than fifteen days. The inhibition was tested by liquid as well as solid assay. Previously, an ELISA test was developed for the quantitative detection of the obligate parasite *Polymyxa betae*, the vector of Beet necrotic yellow vein virus (BNYVV), in infected sugarbeet roots (Kingsnorth *et al.*, 2003).

Spores need to break the cell wall and then the germ tube appears so if the wall is blocked the degradative enzymes needed to break wall may not act. The Abs produced may be against protein(s) or carbohydrates. Our aim was only to block the spore germination which succeeded but a along with that, few more fungi were also inhibited. Two of these fungi do not sporulate even. So it was inferred that there might be any mechanism common for hyphae vegetative growth and spore growth. The common mechanism of action of Abs might help in detecting the factor responsible for pathogenecity and growth of fungi. There might be some common metabolite or key enzyme which if blocked may help to inhibit fungal growth thereby decreasing its pathogenic activities. Abs may prove to be a potent future molecule for the production of plantibodies.

The basis of antibody-based resistance is the neutralization of invading pathogens through interactions between high-affinity antibodies and critical pathogen proteins, thus preventing pathogen entry, replication and systemic spread (Nölke *et al.*, 2004). Various molecular approaches have been used to generate disease-resistant plants, focusing on antibody-based resistance, which involves the expression of cloned antibody genes that neutralize target pathogens by interfering with their life cycle (Schillberg *et al.*, 2001). Thus far, antibodies have been raised against a number of fungal antigens including conidia proteins, secreted proteins and other compounds, cell wall fragment and cell surface antigens of mycelia (Pain *et al.*, 1992; Robert *et al.*, 1993, Goebel *et al.*, 1995; Murdoch *et al.*, 1998).

Antibodies have been used to detect various plant pathogens. Polyclonal antibodies were used previously to test *Polymyxa betae* (Mutasa-Göttgens *et al.*, .2000). The *in vitro* studies showed that the development of disease symptoms in avocado, mango and banana infected with *Colletotrichum gloeosporioides* was inhibited if the inoculum was first mixed with polyclonal antibodies specific for the fungal pectate lyase (Wattad *et al.*, 1997). The application of antibodies to prevent fungal infection in plants has succeeded *in vitro* but still work at a great extent is needed for the demonstration of effective protection in transgenic plants.

After the study of inhibitory activities of essential oils and antibodies on the plant pathogenic fungi an enzymatic study was conducted. This study was conducted only for *A. niger* because apart from its postharvest pathogenic character it is widely used in industries for the production of many enzymes.

This study was divided into two parts:
- Study of acid phosphatase and invertase enzymes which are not only responsible for fungal growth and development but also are potential industrially important enzymes.
- Study of few cell wall degrading enzymes which help in the penetration of fungal hyphae in the host to initiate infection.

A. niger conidia are used as an inoculum in industry. It is an omnipresent fungi and well known for its pathogenecity, no matter it would be in plants or humans. The black *Aspergilli* have a number of characteristics which make them ideal organisms for industrial application, such as good fermentation capabilities and high levels of protein secretion. Moreover its GRAS (generally regarded as safe) status, rapid growth on inexpensive media and a relatively well-studied genetic background (Rose & van Zyl, 2002) also makes it best suited for the study. Toxins of *Aspergillus* are known to be toxic to plants (Vinokurova *et al.*, 2003). Extracts from culture liquid of *Aspergillus sp.* and mycelium cause fading of cuttings and seedlings, canker, constriction of plant vessels, etc. (Vinokurova *et al.*, 2003).

Aspergillus is an active producer of proteolytic enzymes and can utilize various nutrients due to the fact that it possesses diverse hydrolytic enzymes. Therefore, it widely occurs in nature and is easy to cultivate, which makes it promising object for studies (Blieva *et al*., 2003).

Influence of different concentrations of sucrose as a carbon source in a defined media on growth and development of *A. nige*r was studied. Generally protein extraction from undefined media is extensive, time consuming and contaminating so a defined media was used in this study. Production of enzymes by microorganisms is closely related to the main conditions determining culture growth and development, first and foremost, the composition of the nutritive medium (Blieva *et al*., 2003). Moreover, an operational classification of enzyme activities as cytoplasmic, wall bound and extracellular had been utilized. Total carbohydrate and protein consumption were also evaluated in this study.

Fungi releases digestive enzymes into the external environment which breakdown large and relatively insoluble molecules such as carbohydrates, proteins and lipids into smaller and more soluble molecules that then can be absorbed (Alexopoulos & Mims, 2004). Moreover they are able to utilize almost any carbon source as food (Alexopoulos & Mims, 2004).

Among fertilizers constituents, phosphorous is one of the major nutrients for plants and it plays an important role in plant metabolism by supplying energy required for metabolic processes. Acid phosphatase hydrolyzes phosphate esters to which the cells are impermeable. Alternatively, they may play a role in recognition and / or infection processes, as has already been suggested for such enzymes in other biological systems. The activity of invertase an enzyme that hydrolyzes sucrose, a vital plant metabolite, has often been investigated in diseased plants (Storr & Hall, 1992).

Saccharose and starch play an important role in satisfying the saccharide requirements of leaf and leaf-borne pathogens (Pius *et al*., 1998).Both of these enzymes are important in carbon and phosphate metabolism. These enzymes are also important in the process of pathogenecity but their use in industrial exploitation is less studied.

In this study it was observed that, *A. niger* produces extracellular acid phosphatase and invertase in a maximum amount under defined media and all the other controlled conditions. These enzymes were found maximum in the lowest sucrose concentration. In low sucrose concentration the pH turned alkaline so it was inferred that alkaline pH might be stressful for growth and development of *A. niger* and hence to survive in such conditions it might be producing more enzymes to fulfill the nutritional requirements. Thus under stress the fungus produces extra enzymes at the initial hours which can be exploited industrially.

Also, concentration of sucrose influences the growth, pH and enzyme production of *A. niger.* There was no statistically significant difference between 3% and 5% sucrose concentrations both in weight and pH but in 1% the weight was less and pH alkaline. It was observed that pH was inversely proportional to weight and concentrations of sucrose had direct influence on weight and pH. Sucrose might have influence on division of fungal network hence weight of mycelia had increased. The natural autolysis of fungi takes place at values of pH between 6 to 7 (Perez-Leblic *et al*., 1982). Also the lytic enzymes which attack the cell wall and the reserve carbohydrates actuate at these pH values (Gomez *et al*., 1977). This might be the reason for low mycelial weight of *A. niger* in lower sucrose concentration where pH turned alkaline.

Microbial pathogens of plants secrete enzymes capable of degrading the polysaccharides of plant cell walls. The pectic polysaccharide polygalacuronic acid, hemicellulose xylan and glycosides are present in the plant cell wall. The pathogen has to degrade these polysaccharides both for penetration as well as for satisfying their nutritional needs. They have to deal with plant cell walls which function as physical barriers, providing host cell resistance to both biotic and abiotic stresses (Douaiher *et al*., 2007).

Several recent studies have provided new genetic evidence for the importance of cell wall polysaccharides in the host-pathogen interaction outcome (Reiter, 2002; Vorwerk *et al*., 2004). Xylanase and polygalacturonase when grown in a liquid medium, correlation tests revealed significance between the production of both the enzymes and pathogenecity components (Douaiher *et al*., 2007). These enzymes were also shown to be correlated with the pathogenecity of *P. nodorum* isolates (Lalaoui *et al*., 2000). Moreover, fungal polygalacturonases have shown to be inhibited by plant polygalacturonase - inhibiting proteins (PGIPs) (Lorenzo *et al*., 2001).

The degradation of host cell walls by pathogenic fungi is based on the coordinated excretion of a number of enzymes that depend on the parasitic features of the pathogens (Kang & Buchenauer, 2000). Two xylanase genes from the ergot fungus *Claviceps purpurea*, were detected *in planta* during the wheat infection process (Giesbert *et al*., 1998). In the case of *Fusarium culmorum*, polygalacturonase activity has been reported to enable both epidermal penetration and entry in the middle lamella (ten Have *et al*., 2002). Glycosidases are a group of hydrolases widely existing in various sources such as bacteria, fungi, plant and animal tissues (Yan & Lin, 1997; Scigelova *et al*., 1999). Moreover, they are useful products in pharmaceutical, food, cosmetic and fine chemical industries (Yu *et al*., 2007).

Amongst all the three carbon concentrations tested, the lowest one showed highest enzyme activity. Polygalacturonase was the most abundantly produced enzyme. Xylanase was also found to be produced at a maximum level during initial growth phase of the fungus. All the three glycosidases were also found highest in the extracellular fraction but in the later hours. Polygalacturonase produced maximum compared to all the enzymes tested, imply that it might be the most useful enzyme in initial penetration activities. Xylanase may be functional for colonization in the host tissue and glycosidases for later degradation of the tissue. Polygalacturonase is the first enzyme produced during wall degradation because it was found maximum at the initial hours of *A. niger* growth.

In the present study, the extent to which polygalacturonase and xylanase are produced can be used in the industrial exploitation also. The potential applications of xylanases include the bioconversion of lignocelluloses to sugar, ethanol and other useful substances, clarification of juice and wine, and nutritional value improvement of silage and green feed (dos Reis *et al*., 2003). They have potential application in biopulping (Rao *et al*., 2002), nutritional improvement of feedstock, production of ethanol, methane, other products and in the processing of food (Butt *et al*., 2002). Thus the enzymatic studies of *A. niger* might help to explore the host-pathogen interaction, the enzymes involved in pathogenecity, their mechanism in degrading cell wall, their role in providing nutrition to the fungus and fungal establishment in the host for generating the diseased condition. These enzymes are produced highest in initial hours of *A. niger* growth at the lowest sucrose concentration in the defined media. This property could be used in exploiting these enzymes industrially.

For successful management of a plant disease, it is critical to correctly identify the cause of the disease in its early stages. Delaying this can result in extensive crop damage and financial loss to farmers. Some diseases can be diagnosed quickly by visual examination although sometimes, visual detection at the plant level is usually only possible after major damage to the crop has been done, by which time it is too late (Mehrotra & Aggarwal, 2004).

Once the disease is diagnosed the management becomes easy. In the initial research work of this thesis the inhibition of plant pathogenic fungi using the biocontrol strategy were carried out, after which the biochemical strategy to study the mechanism of pathogenecity was investigated. However, this part of the research work was planned to confer a new technique to detect the diseased condition of a plant before visual symptoms appear.

The machine learning technique used was Gas Discharge Visualization (GDV) technique. In this technique the biological emission and optical radiation stimulated by electromagnetic field amplified by gaseous discharge is visualized by computer processing. Bioelectromagnetic field were recorded with the help of computer coupled camera (Korotkov, 1998). The groundnut leaves were selected with a range from no visual symptoms to the appearance of visual symptoms and were divided into five categories. The leaf area and the area of the lesions were calculated with the help of leaf area meter and levels of ABA were measured by ELISA. ELISA is relatively quick and easy, without the need for expensive laboratory equipment, and it can be automated for rapid on-line testing (Kingsnorth *et al*., 2003). Observations were made to check whether any correlation is there between measured ABA levels and GDV parameters.

The GDV instrument, with the help of special electrode system, creates a high intensity electric field around the object, which produces a visible gas discharge around the object whose glow can be seen and measured (Korotkov & Korotkin, 2001). Nine parameters obtained by using the GDV analysis software were: area, noise, fractality (form coefficient), form fractal dimension, brightness, brightness deviation, number of fragments, fragments average area and deviation (Sadikov, 2000).

All of these parameters exhibited a significant difference ($p < 0.001$) amongst all the five categories of leaves. Also maximum correlation was observed between the form-coefficient and ABA levels. The correlation worked out amongst GDV parameters and ABA levels suggested an extremely positive correlation with form coefficient (fractality) whereas, highest negative correlation with brightness deviation.

Optical methods have two main advantages, high sensitivity and non invasiveness. The aim of this method was to develop an optical method for assessing the pathogenecity in the leaves of groundnut through GDV technique, a non destructive method. In recent years a great deal of theoretical and experimental research on this subject has been continued (Korotkov, 1998). Thus GDV analysis may help us to predict diseased condition of plant and may help to give an idea about applying the control measures before disease symptoms appear. This study is further subject of investigation with other plant systems interacted with pathogens.

MAJOR CONCLUSIONS

- The essential oils can be successfully used as a biocontrol agent to prevent the growth of postharvest pathogen like *A. niger*. Also it can be used to control vascular, saprophytic and other plant pathogenic fungi. They may be cheaper than the total crop yield loss. The difference in the fungitoxic activity depends upon the chemical constituents of the oils tested and toxicity for the particular fungi.

- The polyclonal Abs raised, inhibited all the tested fungal pathogens. Antibodies with a higher titer increased the lag phase of fungi for more than fifteen days. Thus there might be some common key metabolite(s) which if blocked may help to inhibit the fungal growth.

- Amongst the various enzymes degrading plant cell wall, polygalacturonase enzyme is the first one to be produced during the plant pathogenic activities and it is produced maximum in the initial stages of *A. niger* growth. Moreover, acid phosphatase and invertase enzymes secreted by *A. niger* in controlled conditions in a defined medium can be used as a principle to exploit these enzymes industrially. The results obtained could be used for better productivity of enzymes. The results found in the present work also indicate that the concentration of sucrose influences on growth and enzyme production.

- The GDV technique may prove to be an important tool in detection and prediction of pathogen in agricultural crops.

"THE STUDY OF FUNGI IS NEVER TIRING,

RATHER IT IS REFRESHING"

Plant Pathogenic Fungi

Biocontrol

Antifungal efficacy of EOs (75) against 3 phytopathogenic fungi

Study of inhibition of phytopathogenic fungi by Abs (all 7 fungi)

GC-MS of 10 most potent EOs

Liquid Assay

Radial growth measurement in solid media

Rest 4 fungi tested against most potent 10 EOs

Disease detection by Gas Discharge Visualization technique

Study of groundnut leaves from interaction to infection to symptomatic stages

GDV analysis

Comparison with ABA levels

Enzymatic study from *A. niger* (Mechanism & Exploitation) (Ontogenic changes in defined media with various sucrose concentrations)

Extracellular

Cytoplasmic

Wall - bound

Acid phosphatase

Invertase

Polygalacturonase

Xylanase

α - Glucosidase

β - Galactosidase

α - Galactosidase

Proteins

Sugars (Reducing & Total sugars)

SUMMARY OF THE WORK DONE IN THIS THESIS

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