POST HARVEST DISEASES OF FRUITS AND VEGETABLES AND THEIR MANAGEMENT BY BIOCONTROL AGENTS

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DEDICATED
TO
PARENTS
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INTRODUCTION
1. INTRODUCTION

Fruits and vegetables constitute an essential part of the daily diet and are in great demand round the year from most sections of the population. Perishables, reservoirs of vitamins and essential mineral nutrients, antioxidants, bioflavonoids, flavor compounds and dietary fibre, easily fall victim to abiotic and biotic adversities. Once detached from the parent plant at harvest, they get deprived of essential ingredients as hormones, nutrients, water and other requisites which enhance the ability of these products to overcome the adverse effects. Apart from rendering the products unfit for human consumption they reduce the aesthetic appeal and also bring about substantial decrease in their food value and organoleptic quality.

Population trends and dietary changes have greatly increased the importance of fruits and vegetables. Although India is a major producer of horticultural crops, many inhabitants are unable to get their daily requirement of fruits and vegetables and thus the Human Development Index (HDI) is very low (Asia Pacific Organisation, 2006). There has been concern in recent years regarding the efficiency of marketing of fruits and vegetables, which in turn is leading to high and fluctuating consumer prices and so only a small share of the consumer rupee reaches the farmers.

Fruits and vegetables are highly perishable products especially during the post-harvest phase, when considerable loss due to microbial diseases, disorder, transpiration and senescence can occur. Marketing of horticultural crops is complex especially because of their perishable nature, seasonal occurrence. Considerable quantities of fruits and vegetables produced in India go waste owing to improper postharvest operations and insufficient procedures for processing. This leads to escalating price rate which results in
a considerable gap between gross food production and net availability to the plate of common man.

Increasing the postharvest life of fruits and vegetables became an international issue, being a focal point of research addressed it poses a big challenge for food technologist throughout the world. Postharvest decay of fruits and vegetables can be traced to infections that occur either between flowering and fruit maturity or during harvesting and subsequent handling and storage (Droby, 2006). In the former case, preharvest infections remain quiescent until the fruit becomes senescent shortly after harvest or during prolonged storage. Conversely, vast majority of postharvest infections occur through surface wounds inflicted during harvest and subsequent handling. Many of the fungi which cause postharvest diseases belong to the phylum Ascomycota and the associated Fungi Anamorphici (Fungi Imperfecti). In the case of the Ascomycota, the asexual stage of fungus (the anamorph) is usually encountered more frequently in postharvest diseases than the sexual stage of the fungus (the teleomorph). Important genera of anamorphic postharvest pathogens include Penicillium, Aspergillus, Geotrichum, Botrytis, Fusarium, Alternaria, Colletotrichum, Dothiorella, Lasiodiplodia and Phomopsis. Some of these fungi also form ascomycete sexual stages. In the phylum Oomycota, the genera Phytophthora and Pythium are major postharvest pathogens, while Rhizopus and Mucor are also important genera of postharvest pathogens in the phylum Zygomycota. Rhizopus stolonifer is a common wound pathogen of a very wide range of fruits and vegetables, causing a rapidly spreading watery soft rot. Genera within the phylum Basidiomycota are generally not important causal agents of postharvest disease, although fungi such as Sclerotium rolfsii and Rhizoctonia solani, which have basidiomycete sexual stages, can cause significant postharvest losses of vegetable crops such as tomato and potato.
The loss resulting from these infections have been reduced primarily by applying fungicides in the field or after harvest. Number of chemical pesticides, such as Benzimidazoles, Imazalil, organic and inorganic sulphur compounds and oxidizing materials were introduced to control the disease but in most cases because of environmental problems, toxicity to humans, development of resistant race and sometimes high costs of such combinations, their use is not recommended (Rahemi, 2003). Presently, there are still no cost effective methods of controlling disease in fruits and vegetables, as spraying programmes are not effective once the fungi have invaded the core. Further, deregistration of some of the more effective fungicides has generated interest and provided impetus in the development of safer, more balanced, cost effective and eco-friendly alternatives that are effective and pose less risk to human health and environment.

Recent years, both the public and health authorities have become increasingly concerned about the presence of pesticides in our food supply and the environment. As a direct result of this mounting concern, research efforts for the development of alternative methods for the control of postharvest diseases of fruits and vegetables have been intensified.

Several non-chemical treatments have been proposed for fungal decay control. Although these approaches have been shown to reduce postharvest rots of fruits and vegetables, each has its own limitations that can affect their commercial applicability.

The exploitation of natural products to control decay and prolong storage life of perishables has received considerable attention. Biologically active natural products have the potential to replace synthetic fungicides. Thus, exploitation of some natural products such as flavour compounds, acetic acid, jasmonates, glucosinolates, propolis, fusapyrone, deoxyfusapyrone, chitosan, essential oils and plant extracts for the management of fungal
rotting of fruit and vegetables prolongs the shelf life of these commodities (Tripathi & Dubey, 2004).

The use of biologicals to control postharvest diseases of fruits and vegetables has grown in the past 20 years from a novel discovery to a full-fledged science. Biological control an innovative, cost effective and eco-friendly approach offers a viable option. The use of naturally occurring microbial antagonists has emerged as one of the most promising alternatives, either alone or as part of an integrated control strategy to reduce synthetic fungicide inputs (Fan & Tian, 2001). Using microorganisms to suppress plant disease is a viable alternative to the use of synthetic chemicals. Biological control is also likely to be more robust than disease control that is based on synthetic fungicides (Emmert & Handelsman, 1999). Increasing the abundance of a particular strain in the vicinity of a plant can suppress disease without producing organisms in the ecosystem. The complexity of the organismal interactions, the involvement lasting effects on the rest of the microbial community or other of numerous mechanisms of disease suppression by a single microorganism and the adaptiveness of most biocontrol agents to the environment in which they are used all contribute to the belief that biocontrol will be more durable than synthetic chemicals (Cook, 1993; Benbrook et al., 1996).

The greatest view for biological approach (using a broad definition of biological control) lies in a further understanding of the mechanisms of action of microbial antagonists and natural products, innate and induced resistance in the host, and the biology of decay pathogens. It is expected that this knowledge will lead to new, innovative approaches for controlling decay in harvested commodities and presents the best hope for the future of the biological control of postharvest disease (Diagram 1).

In the postharvest environments, yeasts appear to be particularly promising because production of antibiotics does not seem to be involved in their activity (Droby &
Diagram 1: A Diagram shows problems and remedy of postharvest management of perishables
Chalutz, 1994). Antagonistic yeasts have been selected mainly for their capability to rapidly colonize and grow in surface wounds and subsequently out compete pathogens for nutrients and space (Spadaro et al., 2002; Sharma et al., 2009) and parasitize postharvest pathogens directly through strong attachment to their hyphae (Droby et al., 2003; Wan & Tian, 2005). As the yeasts used in the experiment were originally isolated from fruit surfaces after or near harvest, they might be tolerant of the field conditions and adversely affected by pre harvest application of fungicides (Fan & Tian, 2001; Tian et al., 2002b). Some yeasts can colonize plant surfaces or wounds for long periods under dry conditions, and can produce extracellular polysaccharides that enhance their survival and restrict pathogen colonization sites (Wisniewski & Wilson, 1992; Chand-Goyal & Spotts, 1996).

Apart from antagonistic yeasts, biologically active essential oils represent a rich potential source of an alternative and perhaps environmentally more acceptable disease management compounds. With a broad range of natural fungicidal plant volatiles, numerous opportunities exist to explore their usefulness in controlling post-harvest diseases. The general antifungal activity of essential oils is well documented (Meepagala et al., 2002) and there have been some studies on the effects of essential oils on post-harvest pathogens (Bishop & Thornton, 1997). The advantage of essential oils is their bioactivity in the vapour phase, a characteristic which makes them attractive as possible fumigants for stored product protection. There are various essential oils have been reported for its antifungal activity against post harvest pathogens.

Apple and tomato have been selected as fruit and vegetable commodity for study. During the survey of local markets (2009-2013), Alternaria rot was recognized as major post- harvest disease, therefore responsible Alternaria sp. was chosen as target pathogen of commodities. Keeping in view the aforesaid need to develop an alternative control as
biological treatment, with suitable edible coatings which is eco-friendly, safe for human beings, economical and easy to use against postharvest pathogens of apple and tomato, the present investigation was done. The aspects of study are as follows:

- Survey of local markets for the collection of diseased apple and tomato.
- Isolation, identification, purification and maintenance of fungal isolates.
- To test the pathogenicity of pathogen in fruits and vegetables.
- To study the effect of bio control agents on postharvest pathogens.
- To explore the mechanism of action of biologicals.
- To select suitable coatings, that can protect the fruits and vegetables from diseases.
- Evaluation of nutritional status of fruits and vegetables.
REVIEW OF LITERATURE
2. REVIEW OF LITERATURE

2.1. Fruits and vegetables: an overview

India: a developing country is characterized by an exponential growth in population. The horticultural sector has established its credibility for improving land use, generating employment and nutritional security. Over the years, horticulture has emerged as an indispensable part of agriculture in India. It offers a wide range of choices to the farmers for crop diversification and contributes about 24.5 per cent of the GDP.

India is the second largest producer of fruits & vegetables in the world after China. Food & Agriculture Organization (FAO) data shows that India produced 76424.2 thousand tonnes of fruits; 156325.5 thousand tonnes of vegetables and 388269.2 thousand tonnes of food grains during 2011 (Kumar, 2011; www.nhb.gov.in). The government has stated that nearly 35-40% of the fruits and vegetables produced in the country are wasted. But as per the Indian Council on Agricultural Research (ICAR), the maximum loss in fruits and vegetables ranges between 12.4 to 18% (post harvest).

The fruit and vegetable sector has grown substantially both in volume and in variety of outputs traded globally. Rising incomes, falling transportation costs, improved technologies and evolving international agreements, have all contributed to this level of growth. This increased level of fruit and vegetable production has, unfortunately, not been matched by developments in supply chain management, or by vertical integration of production with processing in many developing countries.

There has been a significant increase in the production of horticultural crops in recent years in India. Crops; covering fruit, vegetables and spices; occupy an area of 17.7 million hectares, producing 218.7 million tonnes of horticultural produce.
Fruits and vegetables are responsible for 92.3 per cent of total horticultural production. The area under fruit cultivation is 6.6 million hectares with a total production of 75.8 million tonnes (Kumar, 2011; www.nhb.gov.in).

As per the study by the Central Institute for Post Harvest Engineering & Technology, Ludhiana (published in 2010) post-harvest losses of major agricultural produces including fruits and vegetables at National level were estimated to the tune of about Rs. 44,000 crore per annum (Mahant, 2012; www.pib.nic.in).

Fruits and vegetables typically constitute an essential part of the daily diet in India and they are in great demand round the year from most sections of the population being processed into a variety of products such as juices and concentrates, pulp, canned and dehydrated products, jams and jellies, pickles and chutneys etc.

A large variety of fruits are grown in India. Some important ones are banana, mango, citrus, papaya, guava, grape, sapota, pomegranate, pineapple, aonla, litchi, pear, plum and walnut. India accounts for 13 percent of the total world production of fruits and leads the world in the production of mango, banana, papaya, sapota, pomegranate, acid lime and aonla. The leading fruit growing states are Maharashtra which accounts for 16.0 per cent of production followed by Andhra Pradesh (13.0%), Gujarat (10.0%), Karnataka (9.0%), Uttar Pradesh (8.0%), Tamil Nadu (7.0%) and Bihar (5.0%), which altogether contributes for about 68.0 percent of the total fruit production in the country. Banana is the major fruit accounting for 35 per cent of total production followed by mango (21%), citrus (11%), papaya (6%), guava (3.3%), grapes (3%), apple (3%) and others (17.7%) in the country. It may also be mentioned that in the Himalayan states of Himachal and Jammu & Kashmir, the GDP from apples, plums, pears and stone fruits (State of Indian Agriculture, 2012-2013).
Vegetables also, constitute the most important and inexpensive component of a balanced diet, which people now realize due to their high nutritive values, indispensable for the body. There are reports about the increasing demand of brassicaceous vegetables in market (Shanmugasundram, 2005).

Vegetable crops are an important part of the horticultural industry in India and occupy an area of 8.2 million hectares with a total production of 137.7 million tonnes (Mahant 2012, www.pib.nic.in). During the last two decades considerable emphasis has been laid on increasing production of vegetable crops in India (Chadha, 2000).

Consumption of vegetables is only 210 g/person/day against the minimum requirement of 285 g/person/day for a balanced diet as recommended by Nutrition. Advisory Committee of ICMR considered the place of vegetables for nutrition and food security. Besides, meeting the domestic requirement, there is a great demand for vegetable export because vegetables have shown to earn 20-30 times more foreign exchange per unit area than cereals.

More than 40 kinds of vegetables belonging to different groups are grown in India in tropical, sub tropical and temperate regions. Important vegetable crops grown in the country are potato, tomato, onion, brinjal, cabbage, cauliflower, peas, okra, chillies, beans, melons, etc. The leading vegetable growing states are West Bengal which accounts for 15% of production followed by Uttar Pradesh (12%), Bihar (10%), Andhra Pradesh (8%), Madhya Pradesh (6.5%), Gujarat (6.4%), Tamil Nadu (5.8%), Maharashtra (5.7%), Karnataka (5.0%) and Haryana (3%), which altogether contributes about 83.4% of the total vegetable production in the country. Among vegetables, potato is the major vegetable accounting for 27.0% followed by tomato (12%), onion (11%), brinjal (8%), cabbage (5.4%), cauliflower (4.7%), okra (4%), peas (2.5%) and others (25.4%) in the country. India is the second largest producer of vegetables after China and
is a leader in production of vegetables like peas and okra. Besides, India occupies the second position in production of brinjal, cabbage, cauliflower and onion and third in potato and tomato in the world. Vegetables such as potato, tomato, okra and cucurbits are produced abundantly in the country (State of Indian Agriculture, 2012-2013).

Apples (Malus pumila auct.) of the family Rosaceae popularly known as ‘hill fruit’ or ‘cold region fruit’ originated in Middle East nearly 4000 years ago and are grown in temperate climates. Apple is called ‘seb’ in Hindi and ‘malus’ in Latin. Some varieties of apple grown in the country are Red Delicious, Golden Delicious, Baldwin, Ambri Kashmiri, Maharaji, Hazaratbali, Jonathan, Rome Beauty, Granny Smith, King of Pippins, Winter Banana, Richard, Starking Delicious, Yellow Newton, Golden Pippin, Early Shanburry and Irish Peach. Indian apple production averaged nearly 1.4 million tons during 2002-2004, making it the sixth largest apple producer in the world. Its area is estimated to be second largest in the world while its average yield, about 5.5 tons per square hectare, is the lowest of the major world producers. Nearly all of India’s apples are grown in three mountainous states in north India- Himachal Pradesh, Jammu and Kashmir, and Uttarakhand where they typically grow at an altitude of 4,000 to 11,000 feet. Jammu and Kashmir has the highest average yield and accounts for about two thirds of total production. The remaining small amounts of production occur in the hill regions of north eastern India, in the states of Arunachal Pradesh and Nagaland (Kumar 2011, www.nhb.gov.in).

Apart from their taste apples have great nutritional value. Nutrient level per 100g of apple consists of sugars (10.39 g), dietary fibers (2.4 g), fat (0.17 g), protein (0.26 g), vitamin A (3 µg), thiamine (0.017 mg), riboflavin (0.026 mg), niacin (0.091 mg), pantothenic acid (0.061 mg), vitamin B₆ (0.041 mg), folate (3µg), vitamin C (4.6 mg), calcium (6 mg), iron (0.12 mg), magnesium (5 mg), phosphorus (11 mg), potassium (107
mg), zinc (0.04 mg), energy (50 Kcal) (USDA nutrient database). Raw apples generally contain a small quantity of starch, which gets wholly converted into sugar during the process of ripening. The malic acid content of the apple which is completely utilized by the body is beneficial to the bowels, liver and brain. The skin of the fruit besides containing more vitamin C also contains five times more vitamin A than the flesh. Apples are invaluable in maintenance of good health and in the treatment of many ailments as the active medicinal principle of apple i.e. pectin, aids in elimination of certain harmful substances from the body. It also helps to prevent decomposition of protein matter in the alimentary canal. The consumption of apple helps in prevention of anemia, constipation, stomach disorders, hypertension, rheumatic afflictions etc. Apples are about 85% water. Apples can get rid of fat that unhealthy foods put into a body. Apples contain pectin, which are fibers that are soluble and can lower the cholesterol level. Apples have phenolic antioxidants in them that block cholesterol damage and oxidation. The contents inside an apple inhibit early reactions that lead to plaque in blood vessels and too much plaque in your blood vessels could result in a heart attack. Apples in this way help to escape from heart attack and make immune system work harder (Courtney, 2002).

Apples can be canned, juiced and optionally fermented to produce apple juice, cider, vinegar, apple wine and pectin. Apples are an important ingredient in many desserts such as apple cake. Apples are also used in apple butter and apple jelly. Consumption of fresh apple is already considered to be very important with a saying that “an apple a day keeps the doctor away.”

Tomato (Lycopersicon esculentum Mill.) is a highly cherished solanaceous crop native to the west coast of South America in the area of Peru and Ecuador (Olson et al.,
Tomato is cultivated mainly in all parts of the world. In India, Punjab, Rajasthan, Maharashtra are major states of tomato cultivation.

Tomato is one of the most important vegetable crops in India, accounting for about 8.23 per cent of the total vegetable production in the country. Tremendous progress has been made in tomato production during the past four and half decades. Tomato production has increased by almost 15-times, from a mere 0.54 Mt in 1961 to about 8.2 Mt in 2005 (FAO, 2007).

Tomatoes are nutritionally valuable for their high pro-vitamin A and vitamin C content. The Nutritional Information for 1 serving (about ¾ cup) of Simple Tomato Salad Calories (100), Calories from Fat 80, Total Fat (8 g), Saturated Fat (1.5 g), Cholesterol (0 mg), Sodium (60 mg), Total Carbohydrate (4 g), Dietary Fiber (1 g), Sugar (3 g), Protein (3 g), Vitamin A (50) RAE [Retinol Activity Equivalents], Vitamin C (14 mg), Calcium (75 mg), Iron (0 mg).

2.2. Post-harvest diseases and pathogens

Post-harvest diseases are characterized by latent infections of the fruits and vegetables that are asymptomatic throughout the growing season and only develop on mature fruit after storage (Mantyka, 2010). Postharvest decay of fruits and vegetables limits the period of storage, compromises marketing and consumers acceptability, and causes substantial losses. Gray mold, Green mold, blue mold, and sour rot caused by Botrytis cinerea (Pers.) Fries, Penicillium digitatum (Pers.) Sacc., Penicillium italicum Wehmer, and Geotrichum candidum Link, respectively, are the most common pathogens during storage and transportation of fruits. Likewise, soft rot, Fusarium rot, and Sclerotium rot caused by Rhizopus oryzae Went & Prinsen and R. stolonifer Ehrenb. ex Fr., Fusarium oxysporum Schlecht and F. solani M.Sacc., and Corticiu rolfsii Curzi, respectively, are the common postharvest pathogens of vegetables, roots, and tubers (Snowdown, 1990).
Fresh apples are stored after harvest to provide nutritious fruit throughout the year (He et al., 2003). More than 90 fungal species have been described that cause decay of apples during storage (Leibinger et al. 1997). Postharvest fungal pathogens cause severe losses on apples during postharvest storage and commercialization. The most aggressive pathogens are *Penicillium expansum*, *Botrytis cinerea*, *Monilinia* sp., *Mucor piriformis*, *Rhizopus* sp., *Phlyctaena vagabunda* (*Gloeosporium album*) and *Alternaria* sp. even in production areas where the most advanced storage technologies are available (Eckert & Ogawa, 1988).

However, the major factors responsible for low production of solanaceous vegetables are the diseases caused by *Alternaria*. The blights are serious destroyers and disease development is so fast that whole crops are lost in a few days. Therefore, the problem deserves immediate and effective measures of control.

The ubiquitous genus *Alternaria* includes both saprobes and plant pathogens which have been reported worldwide infecting crops in the field and causing post harvest decay of many plant products (Thomma, 2003). Besides yield losses, *Alternaria* spp. are responsible for spoilage of commodities during transport, storage and in processing, which may lead to the reduction of technological quality and serious economic losses.

European Food Safety Authority (EFSA) provided a scientific opinion on the risks for animal and human health related to the presence of *Alternaria* toxins in food and feed (EFSA, 2011). The conidiophores are allergens and can cause serious allergic respiratory diseases (asthma, chronic sinusitis, and rhinitis). It can also cause cutaneous mycoses of the skin and of the scalp. Its mycotoxins are responsible for leukopenia. It is also a phytopathogenic commonly found on several fruits and vegetables. *A. alternata* was the most frequent pathogen producing the disease called mouldy heart. Infection
occurred in the orchard and growth was favoured by low temperature and long periods of storage (Stinson et al., 1981).

There is a growing concern of Alternaria spp. due to their ability to produce secondary metabolites with different toxicological properties, which are harmful for human and animal health. Mycotoxin contamination in certain agricultural systems has been a serious concern for human and animal health. Mycotoxins are toxic substances produced mostly as secondary metabolites by fungi that grow on seeds and feed in the field, or in storage.

Many Alternaria species are mycotoxin producers with different toxicological properties. The most important Alternaria toxins are alternariol (AOH), alternariol monomethyl ether (AME), altenuen (ALT), tenuazoic acid (TEA) and altertoxins (ATX-I, II, III) (Logrieco et al., 2009).

2.2.1. Alternaria rot of Apple:

Alternaria sp. is the predominant fungal pathogen responsible for mouldy-core in the apple fruit. Spores infect the open calyx of young fruits (presumably through the open calyx tube), and mycelia reach the seed and carpel wall during storage (Miller 1959), which provides a point of entry for an invading pathogen. Alternaria sp. are the most commonly isolated fungi from core rots. Alternaria mali Roberts was first identified in the United States in 1924, but the fungus was not considered an important pathogen of apple (Roberts 1924; Sawamura 1990) in Former Yugoslavia (Bulajic et al., 1996), China, India, Japan, Taiwan and Turkey (Sawamura 1990). While A. alternata is, in Europe, a very minor fruit rotting fungus on apples, affecting only fruits which are already damaged, A. mali is important in the Far East because it causes both leaf and fruit disease. In the United States, the disease is only important in North Carolina where Asian A. mali has been introduced (Sawamura 1990). By 1993, growers in nine counties
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in southern and central Virginia reported seeing this problem, some with as much as 50 to 60 percent defoliation on ‘Delicious’. The disease was noticed first time in West Virginia in 2008 (Yoder & Biggs, 1998). This is the first report of Alternaria leaf blotch on apples caused by A. mali in Iran. This could be a damaging disease of apple orchards in the north-western area of Iran (Soleimani & Esmailzadeh, 2007) and potentially elsewhere.

Pathogenic Alternaria isolates that infect apple (e.g. Alternaria mali) occur on ornamental Malus species as inconspicuous, circular, black spots, only 1–3 mm in diameter. These spots are superficial, centred on lenticels and do not cause extensive rotting (while Alternaria species can cause storage rots of apple, e.g. mouldy core). Imported planting material, either as lesions on leaves or shoots, or as latent infections in lenticels, wounds or dormant buds, also imported infected fruits, if these coincide with young spring growth (Hutton 2000).

In recent years, levels of moldy-core in Red Delicious have caused significant losses in Israel. The incidence of fruits infected with moldy-core, according to samplings from a packaging house in northern Israel, averaged from 7% to 12% until 2002, but in 2003 there was a significant increase in disease incidence: up to 40% in some orchards. In general, when more than 9% of the fruits are infected with moldy-core, they can only be sold to industry at very low prices. The Red Delicious cultivars constitute about one-third of the apple trees planted in Israel; therefore, moldy-core is an important factor that reduces apple fruit quality and can become an economically important problem.

2.2.2 Alternaria rot of tomato:

Alternaria rot of tomato caused by Alternaria alternata (Fr.) Keissler has been considered as the most common disease of tomato fruits and causes heavy losses in quantities of the fruits, thus rendering large quantities of tomato fruits unfit for consumption. The disease
was reported by Douglas (1922) from California. The causal organism was isolated from dead leaves of potato and afterward it was reported from decaying tomato fruits. Samuel (1932) reported that *A. solani* was mainly responsible for the tomato decay and typical symptoms occurred on stem were brown to black, depressed and usually with distinct rings. Warner (1936) reported that the spot range in size from minute pin-heads to areas extending completely across the surface of the fruit giving it a flattened appearance.

The fungal rot was also reported from India (Thomas 1944; Agarwal *et al.* 1950). Barkai and Fauchs (1980) and Hassan (1996) have reported that *Alternaria* is main decay causing organism of post harvest tomato fruits while responsible for black rot lesions on tomato fruits. The major factors responsible for low production of cucurbitaceous, brassicaceous and solanaceous vegetables are the diseases caused by *Alternaria*.

### 2.3. The genus *ALTERNARIA*

*Alternaria* Nees. ex Fr. belongs to the sub-division Deuteromycotina, class Hyphomycetes, family Dematiaceae. Species of the genus are cosmopolitan, surviving both as saprophytes as well as weak parasites. The genus is characterized by the formation of polymorphous conidia either singly or in short or longer chains and provided with cross, longitudinal as well as oblique septa and having long or short beaks. The spores of these polyphagous fungi occur commonly in the atmosphere and also in soil. The telomorphs (sexual stage) are known in very few species and placed in the genus *Pleospora* of class Loculoascomycetes of sub-division Ascomycotina, in which sleeper-shaped, muriform ascospores are produced in bitunicate asci. The genus *Alternaria* was first recognised by Nees in 1817 (Nees & Esenbeck, 1817). In 1836, Berkeley (Berkeley 1836) identified the causal fungus on plants belonging to family Brassicaceae as *Macrosorum brassicace* Berk., which was later renamed as *Alternaria brassicace* (Berk.) Sacc. (Saccardo 1886). Thereafter, Elliot studied the taxonomy of...
Alternaria in detail (Elliott, 1917). Wiltshire pioneered the basic studies of this group of hyphomycetes. His descriptive literature was fundamental to the prevailing concepts of Alternaria, Macrosporium and Stemphylium (Wiltshire, 1933). Later, Neergaard made an extensive study on the taxonomy, parasitism and economic significance of this genus (Neergaard, 1945). The morphological variations of Alternaria species were described by Joly (1959) and later he divided these in three sections and proposed a simple key for identification and determination of the most common species (Joly, 1964).

In India, the first report of Alternaria was made from Pusa (Bihar) on a herbarium material of Sarson (Brassica sp.) (Mason, 1928). In U.P. the appearance of Alternaria spp. was noticed by Dey (1948). A comprehensive account of distinguishing characters of the Indian species of Alternaria has been described by Subramanian (1971). The characteristic features of a number of Alternaria species are described in “Dematiaceous Hyphomycetes” (Ellis, 1971) and “More Dematiaceous Hyphomycetes” (Ellis, 1976).

The genus Alternaria Nees ex Wallr. (Macrosporangium) belonging to class Moniliales includes a diverse assemblage of species that occur worldwide in a variety of habitats. Many species are commonly saprobes causing decay and are ubiquitous (Rotem 1994). As plant pathogens, more than 4000 Alternaria-host associations are recorded in the USDA Fungal Host Index and genus ranks 10th among nearly 2000 fungal genera listed, based on host records (Farr et al., 1989).

2.4. Pathogenicity and inoculum concentration of pathogen

In a study, it was found that $10^8$ conidia ml$^{-1}$ of A. alternata resulted in 93.4% infection recorded after 7 days of incubation. It was evident that $10^6$ conidia ml$^{-1}$ could serve as the inoculum potential of A. alternata (Verma, 2004).
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Yousefi & Hagian Shahri (2009) conducted pathogenicity tests by inoculating slightly wounded plant tissue with conidial suspension adjusted to $1.5 \times 10^4$ conidia ml$^{-1}$ of *Alternaria alternate* using a hemocytometer that was capable of causing infection within two to four days.

Kakvan *et al.* (2012) evaluated that pathogenicity of *Alternaria* isolates was capable of causing infection on citrus leaves from suspension of conidia ($10^5$ conidia /ml) prepared from 5-7 days old PDA cultivated isolates.

2.5. Morphological character of *Alternaria* sp.

In Europe, *Alternaria tenuis* has been isolated from the leaves of lilac, forsythia, blackberry, from the fruits of apple, cranberry, and blueberry which grew on artificial media and studied. All these forms, while resembling one another in many respects, revealed many points of difference. In some species the hyphae were almost colorless, forming a thin scarcely perceptible crust over the surface of a plate of corn-meal agar with an exceedingly scant production of conidia and no aerial hyphae. Others formed a dense, nearly black crust with a copious production of conidia and no aerial hyphae. Still others produced a black crust with abundant conidial production at its surface and an abundance of more or less flocculent aerial mycelium or a greenish gray aerial mycelium so dense as to form a thick carpet like growth over the surface of the culture medium. A few produced an abundance of dirty white aerial hyphae with very scant growth in the medium or along its surface and very scant production of conidia. In the species studied, the conidia conformed to the generic description that is, they were ovate, obclavate, or elongate. In most of these obclavate conidia predominated, but ovate conidia were found in cultures of all except one form which produced elongate forms exclusively. In the species producing practically no aerial hyphae, elongate conidia predominated. While the
surfaces of the spores were usually smooth, in some forms verrucosity was quite common. An entire conidium or only a part of it often possessed a verrucose surface. If only a part of the conidial wall was verrucose, it was in a strip or band at right angles to the long axis of the conidium. Verrucosity or absence of verrucosity was not so much a difference between conidia as between chains of conidia, the conidia of individual chains usually being all verrucose or all smooth-walled (Roberts, 1924).

In literature review, only A. alternata and Alternaria brassicicola produced by chains are listed as member of the Longicatenate, but A. brassicicola produces sooty black colonies (Rotem, 1994) and differs from produced colonies of A. alternata which are grayish to olive-green, velvety to cottony. Therefore, A. alternata was distinguished in dormant buds. On PDA plates, the primary conidiophore is comparatively short, 41.22×4 μm; it remains simple or may become branched or geniculated, with corresponding number of primary conidial chains (commonly 3-4), pale browning or brown; the first conidia in a chain usually remain long-elliptical as they mature; conidia produced latter in the chain become ovoid, ellipsoid, or subsphaeroid and obclavate; 27×11.55 μm with 3 transverse septa and sometimes with 1 to 2 oblique septa. Some conidia produced secondary conidiophore that may be long to reach 30 μm. On the basis of morphological characteristics of conidia and conidiophores the results in the experiments of species diagnosis were confirmed by Simmons (1999) reports.

2.6. Growth of pathogen on different media

Reddy and Gupta, (1981) tested 6 media out of which Potato dextrose broth medium was found best for the growth and sporulation of Alternaria helianthi while Mohanthy et al., (1981) reported Richard’s B medium as the best. Neergaard, (1945) reported the optimal growth of Alternaria alternata at 20-30°C (max: 23-32°C to min: 2.5-6.5°C).
Misaghi (1978) studied A. alternata morphology on CMA (Corn Meal Agar), PDA, acidified PDA, V8 agar [(20% V/V) V8 juice, 0.25% CaCl\(_2\) and 1.5 % Bacto agar], glucose nitrate agar [1.5% glucose, 1% NaNO\(_3\) and 1.5 % Bacto agar in 0.05 M phosphate buffer], and yeast peptone agar (YMPG) [0.5% yeast extract, 0.5% meat extract, Difco Protease Peptone, and 1% bacto agar], found Alternaria grew best on CMA at 27°C had the largest diameter. PDA, oatmeal agar, Czapek Dox, Richards, Walks manns agar medium, water agar and Martins Rose Bengal Agar media were also studied by Hubballi (2010), maximum growth was observed on PDA.

2.7. Factors affecting infection and growth of pathogens

Quality of fruit during storage to a great extent depends on the storage environment and fruit microorganisms, since the activity of microorganisms can cause fruit decay, as a result reducing their quality (Juhneviča, 2011). The role of environmental factors on the development of storage and postharvest diseases need hardly be emphasized. Amongst them the temperature and relative humidity are most important as they not only influence the germination of inoculum but also the penetration, establishment and spread of the pathogen (Wood, 1967).

Considerable postharvest losses of fruit and vegetables are brought about by decay caused by fungal plant pathogens. Fruits due to their low pH, higher moisture content and nutrient composition are very susceptible to attack by pathogenic fungi, which in addition to causing rots may also make them unfit for consumption by producing mycotoxins (Phillips, 1984; Moss, 2002).

The combination of high temperature and relative humidity favours the growth of postharvest pathogens and can contribute to the development of disease at the retail end (Fitzell & Muirhead, 1983). The data obtained from Sharma and Kaul (1990) revealed
clearly that storing apple at 0 °C was most effective in controlling postharvest fungi. Storage beyond 10°C caused rapid decay and maximum rotting was observed at 30°C followed by 25°C (31.10%). The fruit kept at 20, 25 and 30°C started rotting early and 12.55 to 15.50% fruits were spoiled after one month. Temperature and water activity significantly influenced the growth rate of *A. alternata* (Sempere & Santamarina, 2007).

The time and intensity of rot incidence depended on cultivar, harvest date and climatic conditions. Cold and wet weather during the ripening period determined an earlier and significantly higher occurrence of fungus during storage (Valiuskaite et al., 2006). The optimum temperature favouring the progress of fruit rot can often be higher than the optimum temperature for growth of the pathogen in artificial culture. This is because of the fact that higher temperature enhances the process of ripening of the tropical fruits resulting in the increase in the sugar content and decrease in acidity as well as the firmness of the fruit tissue.

At optimum temperatures, infection occurs with 5.5 hours of wetting, and lesions can appear in the orchard two days after infection, causing a serious outbreak (Yoder & A.R. Biggs, 1998). Tandon and Ghosh (1962) found that optimal temperature was 20-25°C for the development of *Alternaria tenuis* Nees ex Pers. on pears. Tomato fruits infected by *A. solani* (Ellis and Mart) Jones and Grout and *A. tenuis* Nees ex Pers it occurred at 25°C (Mehta et al., 1975). *A. alternata* requires 25-30°C temperatures for its optimal growth. The maximum temperature at which growth has been reported is ≤ 31-32°C while the minimum is within 2.5-6.5°C (Neergaard, 1945). Saad and Hagedorn (1970) and Akhtar *et al.* (1999) have reported is cessation of growth at ≥ 36°C. Disease development by *A. mali* in apple is favoured by wet weather and very high temperatures (25–30°C) (Hutton, 2000).
The pH of the medium or substrate is very important parameter as far as the growth and activities of fungal mycelium are concerned (Ram & Pant, 2001). Mishaghi (1978) mentioned that colony diameter of *A. alternata* was not affected by the pH of media. *A. alternata* grew and sporulated readily at all pH values from (4.0 to 8.0).

Pandey *et al.* 2002 reported the growth of *Alternaria lini* from 5 to 45°C with optimum growth and sporulation at 25°C followed by 30°C and in pH range of 3.5 to 9.0 with optimum at 6.5 followed by 6.0. Sucrose, peptone and nitrates were observed to best for the growth of *Alternaria alternata* by Verma (2004).

Hubballi (2010) observed effect of different pH levels, temperature, light intensity and media were tested against the growth of *A. alternata* under *in vitro* conditions. The results of experiment indicated that the growth of *A. alternata* was maximum in pH range of 6.00- 6.50 and temperature range of 25 - 30°C.

### 2.8. Efficacy of biological control agents against fungal pathogens

The use of biocontrol agents as an alternative to synthetic, chemical fungicides that are presently used to control postharvest pathogens, better understanding of the mode of action of postharvest biocontrol agents, relationships between infection levels occurring in the field and development of postharvest decay, along with basic information on microbial ecology and survival mechanisms of biocontrol agents on fruit surfaces, is critical for the advancement of successful implementation of postharvest biocontrol technology.

Earliest efforts to control postharvest diseases involved the use of *Bacillus subtilis* (Pusey & Wilson, 1984), *Trichoderma* and *Rhodotorula* (Sadfi *et al.*, 2002), and *Pseudomonas cepacia* (Janisiewicz & Roitman, 1988). All of these antagonists could colonize wound sites and elaborate antimicrobial substances, which prevent development
of pathogens in apple, strawberry, cherry, peach, plum, etc. (Ippolito & Nigro 2000; Sharma et al., 2009). Since then, several other antagonists (yeasts, fungi and bacteria) have been identified and used for controlling various postharvest diseases of fruits and vegetables. Meanwhile, some biocontrol products, *i.e.*, Aspire (*Candida oleophila* strain 182), Biocoat (*Candida saitoana*+chitosan), Bio-cure (*C. saitoana*+antifungal lytic enzyme), BioSave (*Pseudomonas syringae*), and Yield Plus (*Cryptococcus albidus*) were registered in recent years and are commercially available for the control of postharvest decay of fruits and vegetables (Janisiewicz & Korsten, 2002).

In the present study, some yeasts and essential oils for controlling postharvest diseases have been selected and different yeasts (*Table 1*) and essential oils (*Table 3*) that were already used against post harvest pathogens have been reviewed.

### 2.8.1. Yeast

Application of many types of yeasts as postharvest antagonists appears to be quite a promising agent. Janisiewicz (1987), Chalutz *et al.* (1988), and Tian *et al.* (2005) have made several positive points in recommending yeasts as potential microbial agents for controlling the postharvest diseases of fruits and vegetables, including

(a) Yeasts can colonize the wound surface for long periods even under dry conditions.

(b) Yeasts produce extracellular polysaccharides, which enhance their survivability and restrict the growth of pathogen propagules.

(c) They can use nutrients rapidly and proliferate at a faster rate; and

(d) They are the least affected by the pesticides.

Of the various yeasts, *Candida sake*, *C. oleophila*, *Debaryomyces hansenii*, *Pichia anomala*, and *Pichia guilliermondii* have exhibited a wide spectrum of biological activity against many postharvest pathogens (Wilson & Chalutz, 1989; Karabulut & Baykal, 2003). However, recent research has been focussed on the use of several other yeasts (*i.e.*
Candida albidus, Saccharomyces cerevisiae, Issatchenkia orientalis, Metschnikowia pulcherrima, C. laurentii, etc.) for controlling postharvest diseases of fruits and vegetables (Kinay & Yildiz, 2008; Zhang et al., 2008).

Recent studies have focussed on yeast-like fungus, Aureobasidium pullulans, as the most effective antagonist against postharvest plant pathogens, because it has the ability to survive and increase its population under a variety of field conditions and during cold storage (Leibinger et al., 1997; Schena et al., 1999). In an earlier study, Schena et al. (1999) found that isolates of A. pullulans at high concentrations (10^7 and 10^8 cells ml^{-1}) were able to control Penicillium digitatum on grapefruit, Botrytis cinerea, R. stolonifer, and Aspergillus niger on grapes, and B. cinerea and R. stolonifer on cherry tomatoes. Other reports on biocontrol activities of A. pullulans against postharvest diseases include blue mold in stored apple caused by Penicillium expansum (Bencheqroun et al., 2007), monilinia rot in banana (Wittig et al., 1997), and grapes by Monillinia taxa (Barkai-Golan, 2001), etc.

2.8.1.1. Efficacy of yeast at certain concentrations

Rapid colonization of fruit wound by the antagonist is critical for decay control, and manipulations leading to improved colonization enhance biocontrol (Mercier & Wilson, 1994). Thus, microbial antagonists should have the ability to grow more rapidly than the pathogen. Similarly, it should have the ability to survive even under conditions that are unfavorable to the pathogen (Droby et al., 1992). The biocontrol activity of microbial antagonists with most harvested commodities increased with the increasing concentrations of antagonists and decreasing concentrations of pathogen. e.g. C. saitona was effective at a concentration of 10^7 CFU ml^{-1} for controlling P. expansum on apples (McLaughlin et al. 1990a, b). In another study, El-Ghaouth et al. (1998) reported that for C. saitona (10^8 CFU ml^{-1}) was better in controlling blue mold (P. expansum) on apples.
This qualitative relationship, however, is highly dependent on the ability of the antagonists to multiply and grow at the wound site. This was demonstrated by using a mutant of *P. guilliermondii*, which lost its biocontrol activity against *P. digitatum* on grapefruit and against *B. cinerea* on apples, even when applied to the wounds at concentrations as high as $10^{10}$ CFU ml$^{-1}$ (Droby *et al.*, 1991). The cell population of this mutant remained constant at the wound sites during incubation period, while that of the wild type increased 10- to 20-fold, within 24 h.

### 2.8.1.2. Mode of action of biocontrol Agents

Biological control by antagonistic microorganisms uses naturally occurring mechanisms to suppress harmful organisms. The modes of action are competition for nutrients (Bencheqroun *et al.*, 2007; Spadaro & Gullino, 2004) and space (Piano *et al.*, 1998; Fan & Tian, 2000), parasitism (Wan & Tian, 2002), induced resistance (Fan *et al.*, 2002), and antibiosis (Bull *et al.*, 1998). In general, more than one mechanism is implicated, but in no case was a single mechanism found to be responsible for biological control (Janisiewicz & Korsten, 2002).

#### 2.8.1.2.1. Competition for nutrients

Competition for nutrients is the most promising mode of action for several postharvest micro antagonists. Yeasts appear to be particularly promising as biocontrol agents against the postharvest decay of fruits and vegetables (Droby & Chalutz, 1994). Meanwhile, most postharvest fruit pathogens are necrotrophs needing nutrients for spore germination and initiation of the pathogenic process, which can be an effective mechanism of biological control. This hypothesis plays a major role in the mode of action of *P. guilliermondii* against *P. digitatum* in citrus (Droby *et al.*, 1992; Arras, 1996); *P. anomala* against *Penicillium* spp. in banana (Lassois *et al.*, 2008); *D. hansenii* against *B. cinerea* in grapes (Chalutz *et al.*, 1988); and *A. pullulans* against *P. expansum*...
in grapes (Castoria et al., 2001) and apple (Bencheqroun et al., 2007), etc. Attachment by microbial antagonist to the pathogen hyphae appears to be an important factor necessary for competition for nutrients as shown by *P. guilliermondii* and *P. italicum* (Arras et al., 1998). *In vitro* studies conducted on such interactions revealed that due to direct attachment, antagonistic yeasts and bacteria take nutrients more rapidly than target pathogens and thereby prevent spore germination and growth of the pathogens (Droby et al., 1989). Nonpathogenic species of *Erwinia*, such as *E. cypripedii*, showed antagonistic activity against various isolates of *Erwinia caratovora* sub sp. *caratovora*, the causal agent of soft rot of many vegetables such as carrot, tomatoes, and pepper, primarily by competing for nutrients (Janisiewicz et al., 2000). In a recent study, *A. pullulans* strain Ach1-1 was selected for its effectiveness against blue mold caused by *P. expansum* on stored apple fruit (Bencheqroun et al., 2007). The possible involvement of competition for nutrients in the biocontrol activity of this antagonistic strain was investigated both *in vitro* and *in situ*. For *in vitro* assays, the effect of strain Ach1-1 on germination percentages of *P. expansum* conidia was evaluated after a 24 h incubation period in the presence of increasing apple juice concentrations (0–5%) using a system allowing the physical separation of both agents. In the absence of strain Ach1-1, conidial germination was strongly promoted by apple juice whatever the concentration. However, germination was significantly reduced by the presence of strain Ach1-1 except at the highest juice concentration. For *in situ* assays, strain Ach1-1 was very protective against *P. expansum* on postharvest wounded apples. However, the application of high concentrations of exogenous sugars, vitamins, and most particularly amino acids significantly reduced such protection. Time-course analysis of apple amino acids at the wound site revealed that these compounds were more depleted in wounds treated with strain Ach1-1 alone and especially in those treated with both agents (strain Ach1-1 and *P. expansum*)
compared to wounds treated with *P. expansum* alone or to untreated ones. Exogenous amino acids, applied at high concentrations on apple wounds as a mixture of specific amino acid groups or as individuals, significantly decreased strain Ach1-1 efficacy against *P. expansum*. This study provided *in vitro* and *in situ* evidence that competition for apple nutrients, most particularly amino acids, may be a main mechanism of the biocontrol activity of *A. pullulans* strain Ach1-1 against blue mold caused by *P. expansum* on harvested apple fruit (Bencheqroun *et al*., 2007).

### 2.8.1.2.2. Competition for space

Competition for space is the competition for infection sites, which may occur if antagonists are able to occupy the specific places where recognition mechanisms between host and pathogen take place. If these places are no more available for pathogens, the necessary procedure of recognition cannot take place and infection does not occur (Janisiewicz *et al*., 2000). Thus, microbial antagonists should have the ability to grow more rapidly than the pathogen. Similarly, it should have the ability to survive even under conditions that are unfavorable to the pathogen (Droby *et al*., 1992). Wound competence under environmental conditions may be an important character for the evaluation of microbial agents with commercial potential.

Roberts (1990) found that *C. laurentii*, an effective antagonist against gray mold (*B. cinerea*) and blue mold (*Penicillium sp.*) of apple, could rapidly colonize the wounds of apple fruit at temperatures ranging from 5 to 20°C and even under cold storage conditions (1–2°C). The yeast also exhibited rapid increase in population dynamics on apple fruit wounds. Similar results were obtained in controlling postharvest decay caused by *B. cinerea* of apple fruit by *A. pullulans* (Ippolito *et al*., 2000). Biological control of *P. digitatum* on orange fruits with *Candida famata* was reported by Arras (1996). Scanning electron microscope observations of the mode of action of the antagonist
against the pathogen revealed rapid colonization of the fungal mycelium and the wounds, with lytic and phagocytic activity against the hyphae.

2.8.1.2.3. Populations of the microbial antagonist

Initial concentration of antagonist plays a significant role in the microbial antagonists when applied on the wound site and the ability of the antagonist to rapidly colonize the wound site (Spadaro et al., 2002). Microbial antagonists (i.e., C. saitoana, C. oleophila, E. caratovora, C. laurentii, etc.) are most effective in controlling postharvest decay on fruits and vegetables when applied at a concentration of $10^6$–$10^9$ CFU ml$^{-1}$ (El-Ghaouth et al., 2004; Zhang et al., 2007a; Cao et al., 2008), and rarely, higher concentrations are required. $10^7$ CFU ml$^{-1}$ concentration of C. saitoana was effective for controlling P. expansum on apples (McLaughlin et al., 1990a, b).

In another study, El-Ghaouth et al. (1998) reported that for $10^8$ CFU ml$^{-1}$ concentration of C. saitoana, was better in controlling blue mold (P. expansum) on apples. Similarly, for C. laurentii ($10^9$ CFU ml$^{-1}$), when challenged with P. italicum, applied at $10^4$ spores ml$^{-1}$, the blue mold decay of oranges completely inhibited during 5 days of incubation at 20°C (Zhang et al., 2005). This qualitative relationship, however, is highly dependent on the ability of the antagonists to multiply and grow at the wound site. This was demonstrated by using a mutant of P. guilliermondii, which lost its biocontrol activity against P. digitatum on grapefruit and against B. cinerea on apples, even when applied to the wounds at concentrations as high as $10^{10}$ CFU ml$^{-1}$ (Droby et al., 1991). The cell population of this mutant remained constant at the wound sites during incubation period, while that of the wild type increased 10 to 20 fold, within 24 h.

2.8.1.2.4 Direct parasitism

Antagonist and pathogen can also interact through direct parasitism. Wisniewski et al. (1991a, b) observed a strong adhesion in vitro of P. guilliermondii antagonist cells to B.
cinerea mycelium, perhaps due to a lectin link. After yeast cells were dislodged from the hyphae, the hyphal surface appeared to be concave and there was partial degradation of the cell wall of B. cinerea at the attachment sites. In a recent study, Zhao et al. (2010) using scanning electron microscopy unveiled that P. guilliermondii multiplied rapidly on tomato fruit wounds and its cells had a storage capability of adhesion to the hyphae of R. stolonifer. Moreover, P. guilliermondii shows a high activity of b-1,3-glucanase enzyme that could result in the degradation of the fungal cell walls (Jijakli & Lepoivre, 1998). A. pullulans in apple wounds produces extracellular exochitinase and b-1,3-glucanase, which could play a role in the biocontrol activity (Castoria et al., 2001). Through ultrastructural and cytochemical studies, El-Ghaouth et al. (1998) found that C. saitoana yeast cells, when cultivated together with B. cinerea mycelium, were associated with fungal hyphae showing cytological damage, such as papillae and other protuberances in the cell wall, and degeneration of the cytoplasm. Bonaterra et al. (2003) reported that direct parasitism was a major factor that permitted P. agglomerans to control M. laxa and R. stolonifer decay on stone fruits.

2.8.1.2.5. Production of cell-wall Lytic Enzymes

Microbial antagonists also produce lytic enzymes such as glucanase, chitinase, and proteinases that help in the cell-wall degradation of the pathogenic fungi (Lorito et al. 1993; Castoria et al. 1997, 2001; Chernin & Chet 2002).

In recent years, exocellular lytic enzymes (β-1, 3-glucanase) produced by yeasts such as P. anomala, A. pullulans, Rhodotorula glutinis, and C. laurentii have also been studied to obtain a better understanding of the biocontrol of postharvest diseases of fruits (Castoria et al., 1997; Jijakli & Lepoivre, 1998).
2.8.1.2.6. Antibiosis

Production of antibiotics is other important mechanism by which microbial antagonists suppress the pathogens (antibiosis) of harvested fruits and vegetables. In the case of bacterial antagonists, it has been suggested that their biocontrol activity may be partly associated with the production of antibiotics, such as iturins (a powerful antifungal peptide) produced by *B. subtilis*, pyrollnitrins produced by *P. cepacia*, and trichothecenes produced by *Myrothecium roridium* (Bull *et al.*, 1998; Golubev *et al.*, 2001). The main concern, related to the use of antibiotics in food products, is the development of human pathogens resistant to these compounds and the possible development of resistance in fruit pathogens. Even if antibiotic producers appear to be able to control wound infections established before antagonist application, at the moment, there are no such biocontrol agents registered for use on fruits and vegetables. Although, antibiosis might be an effective tool for controlling postharvest diseases in a few fruits and vegetables, at present emphasis is being given for the development of non-antibiotic producing microbial antagonists for the control of postharvest diseases of fruits and vegetables (El-Ghaouth *et al.*, 2004; Singh & Sharma, 2007).

2.8.1.2.7. Induced resistance

Several evidences have indicated that microbial antagonists may elicit defense mechanism of the host as well (Ippolito *et al.*, 2000). For example, El-Ghaouth *et al.*, (2001) investigated the ability of *C. saitoana* to induced systemic resistance in apple fruit against *Botrytis cinerea*. To differentiate antagonistic activity of *C. saitoana* from the ability to induce resistance, the antagonist and the pathogen were applied on separate wounds in fresh apple. When *C. saitoana* was applied 0 and 24 h before inoculation of *B. cinerea*, no effect on lesion development was found. But, when applied 48 or 72 h post inoculation with *B. cinerea*, *C. saitoana* reduced lesion diameter by more than 50 and
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70%, respectively, in comparison to wounding. Further, it was evident that *C. saitoma* was capable of inducing systemic resistance in apple fruit and increased chitinase and β-1, 3-glucanase activities with a higher accumulation in fresh than in stored apples. Similarly, *A. pullulans* causes a transit increase in the activity of β-1, 3-glucanase, peroxidase, and chitinase enzymes in apple wounds, which stimulated wound healing process and induce defense mechanisms against *P. expansum* (Ippolito *et al.*, 2000). Efficacy of *C. laurentii* was maintained when applied simultaneously or prior to inoculation with *P. italicum*, causing blue mold of oranges (Zhang *et al.*, 2005). Efficacy was reduced when *C. laurentii* was applied after inoculation. The yeast *C. laurentii* was also reported to control a postharvest disease of jujube fruit by producing β-1, 3-gucanase, a cell-wall degrading enzyme involved in plant host defense (Tian *et al.*, 2007). Similarly, Fan *et al.* (2002) demonstrated that *Pichia membranifaciens* and *Candida guilliermondii* two antagonistic yeasts could produce chitinase and β-1, 3-glucanase in vitro and induce an increase in β-1, 3-glucanase and chitinase activities in the wounds of nectarine fruit, resulting in an effective decrease in decay caused by *R. stolonifer*. In peach fruit infected by *P. expansum*, the yeast *P. membranifaciens* induced a number of proteins related to host defense mechanisms (Chan *et al.*, 2007). Furthermore, the yeast *P. membranifaciens* induced production of H$_2$O$_2$-metabolizing enzymes and total protein synthesis and reduced oxidative stress in harvested sweet cherry fruit (Chan & Tian, 2006). *P. guilliermondii* strain R13, yeast isolated from Thai rambutan, has been shown to suppress the fungal pathogen *Colletotrichum capsici* in harvested chili (Nantawanit *et al.*, 2010). The pretreatment of chili with the yeast antagonist, physically separated from the fungus by known distances, significantly reduced disease incidence and lesion diameter caused by *C. capsici*. Compared to the controls, the yeast treatment also significantly enhanced the activities of chitinase and β-
1, 3-glucanase and the accumulation of capsidiol phytoalexin in chilli tissue (Nantawanit et al. 2010). Induction of disease resistance was also reported in avocado, citrus, peach, and pineapple fruits (Rodov et al., 1994; Fan et al., 2002).

Microbial antagonist-induced disease resistance in fruits was also manifested by the production phenylalanine ammonia-lyase activity as in grapefruit (Droby et al., 2001, 2003a,b) and chilli (Nantawanit et al., 2010) and the accumulation of phytoalexin such as scoparone and scopoletin in orange fruits (Arras et al., 1998). The biosynthesis of scoparone in particular, 4 days after inoculation with the C. famata (F35) only, was 124 mg/g fresh weight of the fruit, 12 times higher than that in the non-inoculated wound tissues, while it decreased to 47 mg/g when F35 strain was inoculated at the same time as P. digitatum, and to 37 mg/g when the pathogen only was inoculated (Arras, 1996).

2.9. Essential oils

Essential oils are volatile, natural, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. The secondary metabolites can be classified into three major heads:

(i) Isoprenoids

(ii) Aromatic compounds

(iii) Alkaloid components

They are liquid, volatile, limpid and rarely colored, lipid soluble and soluble in organic solvents with a generally lower density than that of water. They can be synthesized by all plant organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes.

2.9.1. Chemical composition of essential oil

Essential oils are very complex natural mixtures which can contain about 20–60 components at quite different concentrations (Table 2). They are characterized by two or
three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts. For example, carvacrol (30%) and thymol (27%) are the major components of the *Origanum compactum* essential oil, linalol (68%) of the *Coriandrum sativum* essential oil, a- and b-thuyone (57%) and camphor (24%) of the *Artemisia herba-alba* essential oil, 1,8-cineole (50%) of the *Cinnamomum camphora* essential oil, a-phellandrene (36%) and limonene (31%) of leaf and carvone (58%) and limonene (37%) of seed *Anethum graveolens* essential oil, menthol (59%) and menthone (19%) of *Mentha piperita* essential oil. Generally, these major components determine the biological properties of the essential oils. The components include two groups of distinct biosynthesitical origin (Pichersky *et al.*, 2006). The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight.

**2.9.2. Fungitoxicity of essential oils of plants against post-harvest pathogens**

The general antifungal activity of essential oils is well documented (Meepagala *et al.*, 2002) and there have been some studies on the effects of essential oils on postharvest pathogens. These essential oils are thought to play a role in plant defense mechanisms against phytopathogenic micro-organisms. The various oils were tested against *Aspergillus flavus* and *Aspergillus parasiticus* by different workers. Thanaboripat *et al.* (2007) studied the effects of 16 essential oils from aromatic plants against mycelia growth of *A. flavus* IMI 242684. The results showed that the essential oil of white wood (*Melaleuca cajeputi*) gave the highest inhibition followed by the essential oils of cinnamon (*Cinnamomum cassia*) and lavender (*Lavandula officinalis*), respectively. In the *in vitro* studies Jardim *et al.* (2008) reported antifungal activity of essential oil from the Brazilian epazote (*Chenopodium ambrosioides* L.) evaluated by the poison food
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assay at concentrations of 0.3, 0.1 and 0.05 % against postharvest deteriorating fungi (A. flavus, A. glaucus, A. niger and A. ochraceous). Kumar et al. (2009) studied the efficacy of essential oil from Mentha arvensis L. to control storage moulds of Chickpea. The oil effectively reduced mycelia growth of A. flavus. Kumar et al. (2010) studied the efficacy of O. sanctum essential oil and found efficacious in checking growth of A. flavus and also inhibited the aflatoxin B1 production completely at concentration of 0.2 and 0.1 μg ml$^{-1}$, respectively. Similarly the effect of oils of was tested against other storage fungi enumerated in Table 3. In vitro efficacy of citrus oil against A. niger was tested by Sharma and Tripathi (2006) Citrus sinensis essential oil caused complete growth inhibition of A. niger on agar plates. Higher concentration of oil was found to be lethal. The oil showed fungistatic activity at low concentration. The essential oil significantly reduced the growth of A. niger in a dosage response manner. Citrus sinensis is a result of attack of oil on the cell wall and retraction of cytoplasm in the hyphae and ultimately death of the mycelium.

The advantage of essential oils is their bioactivity in the vapour phase, a characteristic that makes them attractive as possible fumigants for stored product protection. Most of the essential oils have been reported to inhibit postharvest fungi in in vitro conditions (Bellerbeck et al., 2001; Hidalgo et al., 2002). However, the in vivo efficacy and practical activity of only a few of the essential oils have been studied. Some of the essential oils have been reported to protect stored commodities from biodeterioration. There are also some reports on essential oils in enhancing storage life of fruit and vegetables by controlling their fungal rotting. Dubey and Kishore (1988) found that the essential oils from leaves of Melaleuca leucadendron, Ocimum canum and Citrus medica were able to protect several stored food commodities from biodeterioration caused by A. flavus and Aspergillus versicolor. These oils were active at
between 500 and 2000 µg ml$^{-1}$. The potential of using essential oils by spraying or dipping to control postharvest decay has been examined in fruit and vegetables (Dixit et al., 1995).

Thymol is an essential oil component from thyme (*Thymus capitatus*) and has been used as medicinal drug, food preservative, and beverage ingredient (Mansour et al., 1986). Fumigation of sweet cherries with thymol was effective in controlling postharvest grey mold rot caused by *B. cinerea* (Chu et al., 1999), and brown rot caused by *M. fructicola* (Chu et al., 2001). Fumigation with thymol at 30 mg l$^{-1}$ reduced the incidence of grey mold rot from 35% in untreated fruit to 0.5%. Liu et al. (2002) also found that thymol was more effective for controlling brown rot symptoms on apricots, and fumigation of plums with relatively low concentrations such as 2 or 4 mg l$^{-1}$ can greatly reduce postharvest decay without causing any phytotoxicity. The US Food and Drug Administration lists thymol, thymol essential oil and thyme (spice) as food for human consumption, as well as food additives. Thymol was initially registered as a pesticide in the US in 1964.

Recently, carvone, a monoterpane, isolated from the essential oil of *Carum carvi* has been shown to inhibit sprouting of potatoes during storage and it also exhibited fungicidal activity in protecting the potato tubers from rotting without altering taste and quality of the treated commodity, and without exhibiting mammalian toxicity (Oosterhaven, 1995). It has been introduced under the trade name TALENT in The Netherlands. The essential oil of *Salvia officinalis* has also shown practical potency in enhancing the storage life of some vegetables by protecting them from fungal rotting (Bang, 1995).

Treatment of oranges with the essential oils of *Mentha arvensis, O. canum* and *Zingiber officinale* has been found to control blue mold, thereby enhancing shelf life.
The fungitoxic potency of the essential oils may be due to synergism between their components. Thus, there would be negligible chance of development of resistant races of fungi after application of essential oils to fruit and vegetables.

Although the fungitoxic properties of the volatile constituents of higher plants have been reported, little attention has been paid to the fungitoxic potency of these substances when combined. This information is desirable since the fungitoxic potency of most of the fungicides has been reported to be enhanced when combined (Migheli et al., 1988). The enhancement of fungitoxic potential of mixtures of the oils may be due to the joint action of two or more substances present in the oils (Scardavi, 1966). This synergism would be beneficial in postharvest protection because the pathogen would not easily produce resistance against the components. However, more work on synergistic action of plant products in in vitro and in vivo conditions is required. The literature is also silent on the mode of action of the essential oils when used as postharvest fungitoxicants.

**2.9.3. Essential oil: mechanism of action**

Because of the great number of constituents, essential oils seem to have no specific cellular targets (Carson et al., 2002). As typical lipophiles, they pass through the cell wall and cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids and permeabilize them. Cytotoxicity appears to include such membrane damage (Di Pasqua, 2007). Essential oils can coagulate the cytoplasm (Gustafson et al., 1998) and damage lipids and proteins (Burt, 2004). Damage to the cell wall and membrane can lead to the leakage of macromolecules and to lysis (Oussalah et al., 2006).

In eukaryotic cells, essential oils can provoke depolarisation of the mitochondrial membranes by decreasing the membrane potential, affect ionic Ca$^{2+}$ cycling (Vercesi et al., 1997) and other ionic channels and reduce the pH gradient, affecting (as in bacteria)
the proton pump and the ATP pool. They change the fluidity of membranes, which become abnormally permeable resulting in leakage of radicals, cytochrome C, calcium ions and proteins, as in the case of oxidative stress and bioenergetic failure. Permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (Armstrong, 2006). It seems that chain reactions from the cell wall or the outer cell membrane invade the whole cell, through the membranes of different organelles like mitochondria and peroxisomes. These effects suggest a phenolic like prooxidant activity (Barbehenn et al., 2005). Scanning and transmission electron microscopy observations reveal cell ultrastructural alterations in several compartments such as plasma membrane, cytoplasm (swelling, shrivelling, vacuolations, leakage) and nucleus (Santoro et al., 2007). Analyses of the lipid profiles by gas chromatography and of the cell envelope structure by scanning electron microscopy of several bacteria treated by some essential oil constituents showed a strong decrease in unsaturated and an increase in saturated fatty acids, as well as alterations of the cell envelopes (Di Pasqua et al., 2007). Light micrograph and Scanning electron micrograph reveals the mechanism of action of oil. The biological activity attributed to the action of oils could be cytotoxicity, phototoxicity, nuclear mutagenicity and cytoplasmic mutagenicity of the oil components (Bakkali et al., 2008).

In particular, recent work in the yeast S. cerevisiae, has shown that the cytotoxicity of some essential oils, based on colony forming ability, differed considerably depending on their chemical composition; essential oil treated cells in stationary phase of growth showed 50% lethality at 0.45µl ml⁻¹ of Origanum compactum essential oil, 1.6 µl ml⁻¹ of Coriandrum sativum essential oil, >8 µl ml⁻¹ of Cinnamomum camphora, Artemisia herba-alba and Helichrysum italicum essential oils (Bakkali et al., 2005). Moreover, it depended also on the state of cell growth, dividing cells being much more sensitive.
probably because essential oils penetrated more efficiently at the budding sites. In
general, the cytotoxic activity of essential oils is mostly due to the presence of phenols,
aldehydes and alcohols (Bruni et al., 2003; Sacchetti et al., 2005).

2.9.4. Role in postharvest

Essential oils possess a wide spectrum of different impressive qualities including
antiphlogistic, spasmolytic, antinociceptive and antioxidant activity. Moreover, they
exert immunomodulant, psychotropic, acaricide and expectorant effects (Pisseri, 2008).
Due to their multifunctionality, they find a huge application area in medicine and
aromatherapy.

The general antifungal activity of essential oils is well documented (Meepagala et
al., 2002) and there have been some studies on the effects of essential oils on postharvest
pathogens. These essential oils are thought to play a role in plant defense mechanisms
against phytopathogenic micro-organisms.

Most of the essential oils have been reported to inhibit postharvest fungi in in vitro
conditions (Bellerbeck et al. 2001; Hidalgo et al. 2002). Combrinck (2011) reported
effect of eighteen essential oils against postharvest fruit pathogens. Thyme oil proved to
be the best inhibitor against all of the pathogens tested, like Lasodiplodia theobromae,
Colletotrichum gloeosporioide, Alternaria citrii, P. digitatum and B. cinerea. In vitro
efficacy of citrus oil against A. niger was tested by Sharma and Tripathi (2008) Citrus
sinensis essential oil caused complete growth inhibition of A. niger on agar plates.
Higher concentration of oil was found to be lethal. The oil showed fungistatic activity at
low concentration. The essential oil significantly reduced the growth of A. niger in a
dosage response manner. Citrus sinensis is a result of attack of oil on the cell wall and
retraction of cytoplasm in the hyphae and ultimately death of the mycelium.
The biological activity attributed to the action of oils could be cytotoxicity, phototoxicity, Nuclear mutagenicity and Cytoplasmic mutagenicity of the oil components (Bakkali 2008).

Srivastava et al (2008) concluded that, in general, the inhibitory action of natural products on fungal cells involves cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of synthesis of intracellular enzymes.

Essential oils of basil (Ocimum basilicum L.) coriander (Coriandrum sativum L) caraway (Carum carvi L.) and rosemary (Rosmarinus officinalis L) were found inhibitory for A. flavus at 1000 ppm (Deabes et al., 2011). Recently, the essential oil of Cinnamomum camphora (Singh et al 2008a) Thymus vulgaris (Kumar et al. 2008) and Pelargonium graveolens (Singh et al 2008b) have also reported to suppress aflatoxin B1 secretion by different toxigenic strains of A. flavus. The antifungal activity of ginger oil has been reported by Singh et al (2008c) against different Aspergillus spp. viz A. flavus, Alternaria solani, Aspergillus oryzae, A. niger and Fusarium moniliforme.

Our experimentation with ginger oil has also given positive results against various postharvest floras. The small amount of oil applied to the pathogens gave a promising result to use them in vivo to manage the storage fungi. The incorporation of essential oils into fruit coatings, primarily applied to retain moisture, has gained popularity. The advantage of using coatings amended with essential oils, rather than vapour, is that there is closer contact between the essential oils and fruit surfaces, allowing exposure of each fruit to similar concentrations of inhibitor over a longer period.

Amiri et al. (2008) applied different formulations amended with eugenol oil (Eugenia caryophyllata) to two apple cultivars and successfully reduced the disease incidence after cold storage. Trans (isomerized) jojoba oil was applied by Ahmed et al.
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Regnier et al. 2008 successfully achieved pathogen control, without any observed physiological breakdown, by applying commercial coatings amended with L. scaberrima essential oil to mango fruit. Further general observation was that amended coatings yielded fruit exhibiting no shriveling or browning even after ten days of storage. Other alternatives such as the inclusion of sachets impregnated with essential oils such as thymol oil during 35 days packaging reduced the development of moulds of table grapes (Martinez-Romero et al. 2005).

E. Bosquez-Molina et al. (2010) reported for the first time the use of essential oils of thyme and Mexican lime in reducing C. gloeosporioides and R. stolonifer infection of papaya fruit. In addition, another positive effect of thyme and Mexican lime is that they do not have the strong flavor that characterizes other essential oils. Coating application extends the storage life of horticultural commodities since they cover the fresh produce by providing physical barriers to reduce loss of water vapor and aroma volatiles and delay the side effects of respiration. Some studies have concluded that whole essential oils have greater antimicrobial activity than the major components mixed, which suggests that the minor components are critical to the activity and may have a synergistic effect or potentiating influence. This is the case of Salvia officinalis and certain species of Thymus and Origanum vulgari.

Apart from fruit losses, there is a chronic problem of decaying grains due to improper storage facilities, which provide a conducive environment for the fungal growth. The fungal contamination of cereals like maize by toxin producing Fusarium graminearum can be effectively cured by lemon grass essential oil. (Marin et al., 2004). Besides lemon grass, a huge number of oils have been tested for fungal control or
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mycotoxin prevention on maize. It has been confirmed that essential oils from seeds of Azadirachta indica (500 and 1000µg g⁻¹) and leaves of Morinda lucida (500µg g⁻¹) completely inhibited A. flavus aflatoxin synthesis in maize grains.

Wheat treated with essential oils of C. citratus, Ocimum gratissimum, Zingiber cassumunar and Caesalia axillaris showed complete inhibition of A. flavus, whereas a concentration of 100µg/ml of Chenopodium ambrosioides oil used as fumigant, was found effective to control A. flavus, A. niger, A. parasiticus, A. terreus, A. candidus and P. citrinum during 12 months of storage in wheat (Kumar, 2007). The use of Hyptis suaveolens oil to control Fusarium gladioli (Sharma & Tripathi, 2008) has provided a clue of use of oils in protection of ornamentals also.

2.9.5. Current status of essential oils

In the quest of bioefficacious, economical and environmental friendly alternative to postharvest pathogens, the essential oils can be ideal candidates for use as agrochemicals (Macias et al. 2002). It is often thought that the existing biodiversity of natural products is not sufficient. The generation of diversity as well as enhancement in the yield of the oils can be achieved by biotechnological tools, which may bring about specific and site directed mutagenesis. This can be applied to develop variants and employed in postharvest management by subsequent screening. The advance tools like the recombinant DNA technology (genetic engineering) have provided a platform for generation of novel secondary metabolites. Bringing a natural substrate in contact with enzyme from another cell may result into an array of entirely new natural product.

Several plant essential oils are marketed as fungicides for organic farming (Dayan et al., 2009) these include E-Rase™ from jojoba (Simmondsia californica) oil, Sporan™ from rosemary (Rosmarinus officinalis) oil, Promax™ from thyme (T.
vulgaris) oil, Trigoly™ from neem (A. indica) oil and GC-3™ being a mixture of cotton seed (Gossypium hirsutum) oil and garlic (Allium sativum) oil.

2.9.6. Future prospects

Since the Second World War, traditional agricultural practices have included the use of synthetic chemicals for the management of plant pathogens. This has undoubtedly increased crop production but with some deterioration of environment and human health. Essential oils are considered as GRAS (Generally Recognized As Safe) and thus their application for the protection of fruit, vegetables and cereals should not pose regulatory problems. But practical application of oils still remains expensive, which limits their use in the preservation of fruit and vegetables. The incorporation of oils into coating formulations appears to be a good strategy to reduce application costs since its quantities can be reduced.

Postharvest treatment by this oil would prevent the development of both latent preharvest and postharvest infections. Certain plant essential oils and/or their constituents have a broad spectrum of activity against insect and mite pests, plant pathogenic and other fungi, and nematodes. As such, they have considerable potential as crop protectants and for pest management in other situations (e.g. urban pest control). Current information indicates that they are safe to the user and the environment, with few qualifications.

Like other alternative pest management products, essential oil-based pesticides will not be a panacea for crop protection, but there should be substantial market niches, particularly where there is a premium on worker safety and environmental protection, in which these types of products will have wide acceptance among growers.
Table 1: Antagonistic yeasts used for the successful control of postharvest diseases of fruits and vegetables

<table>
<thead>
<tr>
<th>Fruits/vegetables</th>
<th>Disease</th>
<th>Pathogen</th>
<th>Yeast antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gray mold rot</td>
<td><em>Botrytis cinerea</em></td>
<td><em>Cryptococcus albidus</em> (Saito) Skinner (Fan &amp; Tian, 2001), <em>Pichia guilliermondii</em> (McLaughlin <em>et al.</em>, 1990a), <em>Metschnikowia pulcherrima</em> (Spadaro <em>et al.</em>, 2004b), <em>Rhodotorula glutinis</em> (Zhang <em>et al.</em>, 2009)</td>
</tr>
<tr>
<td></td>
<td>Rhizopus rot</td>
<td><em>Rhizopus nigricans Ehrenberg</em></td>
<td><em>Candida sake</em> (CPA-1) (Vinas <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td></td>
<td>Bitter rot</td>
<td><em>Glomerella cingulata</em></td>
<td><em>Cryptococcus laurentii</em> (Blum <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>Banana</td>
<td>Monilinia rot</td>
<td><em>Monilinia laxa</em></td>
<td><em>Aureobasidium pullulans</em> (Wittig <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td></td>
<td>Crown rot</td>
<td><em>Colletotrichum musae</em></td>
<td><em>Pichia anomala</em> (Hansen) (Lassois <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td></td>
<td>Sour rot</td>
<td><em>Geotrichum candidum</em></td>
<td><em>Debaryomyces hansenii</em> (Chalutz &amp; Wilson 1990)</td>
</tr>
<tr>
<td>Cherry</td>
<td>Brown rot,</td>
<td><em>Monilinia</em></td>
<td><em>Cryptococcus laurentii</em> (Tian <em>et al.</em></td>
</tr>
<tr>
<td>Crop</td>
<td>Disease</td>
<td>Pathogen</td>
<td>Reference</td>
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<tr>
<td>Grape</td>
<td>Botrytis rot</td>
<td><em>Botrytis cinerea</em></td>
<td><em>Aureobasidium pullulans</em> (Schena et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Soft rot</td>
<td><em>Monilinia laxa</em></td>
<td><em>Aureobasidium pullulans</em> (Barkai-Golan, 2001)</td>
</tr>
<tr>
<td></td>
<td>Rhizopus rot</td>
<td><em>Rhizopus stolonifer</em></td>
<td><em>Pichia guilliermondii</em> (Chalutz et al., 1988)</td>
</tr>
<tr>
<td>Jujube</td>
<td>Alternaria rot</td>
<td><em>Alternaria alternata</em></td>
<td><em>Cryptococcus laurentii</em> (Qin &amp; Tian, 2004)</td>
</tr>
<tr>
<td></td>
<td>Penicillium rot</td>
<td><em>Penicillium expansum</em></td>
<td><em>Rhodotorula glutinis, Cryptococcus laurentii, Rhodotorula glutinis</em> (Tian et al., 2005)</td>
</tr>
<tr>
<td>Loquat</td>
<td>Anthracnose rot</td>
<td><em>Colletotrichum acutatum</em></td>
<td><em>Pichia membranifaciens</em> (Cao et al., 2008)</td>
</tr>
<tr>
<td>Mango</td>
<td>Anthracnose</td>
<td><em>Colletotrichum gloeosporioides</em></td>
<td><em>Candida membranifaciens Hansen</em> (Kefialew &amp; Ayalew, 2008), <em>Meyerozyma caribbica</em> (Rosales et al., 2013)</td>
</tr>
<tr>
<td>Papaya</td>
<td>Anthracnose</td>
<td><em>Colletotrichum gloeosporioides</em></td>
<td><em>Candida oleophila</em> (Gamagae et al., 2003)</td>
</tr>
<tr>
<td>Peach</td>
<td>Gray mold</td>
<td><em>Botrytis cinerea</em></td>
<td><em>Candida oleophila</em> (Karabulut &amp; Baykal, 2004), <em>Cryptococcus laurentii</em> (Zhang et al., 2007)</td>
</tr>
<tr>
<td>Rhizopus</td>
<td>Rot</td>
<td><em>Rhizopus stolonifer</em></td>
<td><em>Debaryomyces hansenii</em> (Mandal et al., 2007)</td>
</tr>
<tr>
<td>Brown</td>
<td>rot</td>
<td><em>Monilinia fructicola</em></td>
<td><em>Cryptococcus laurentii</em> (Yao &amp; Tian, 2005)</td>
</tr>
<tr>
<td>Blue</td>
<td>rot</td>
<td><em>Penicillium expansum</em></td>
<td><em>Cryptococcus laurentii</em> (Zhang et al., 2007a)</td>
</tr>
<tr>
<td>Pear</td>
<td>Bitter rot</td>
<td><em>Glomerella cingulata</em></td>
<td><em>Candida sake (CPA-1)</em> (Torres et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Mucor rot</td>
<td><em>Mucor piriformis</em></td>
<td><em>Cryptococcus flavus, Cryptococcus albidos</em> (Saito) Skinner (Roberts, 1990)</td>
</tr>
<tr>
<td>Fruit/Plant</td>
<td>Disease</td>
<td>Pathogen 1</td>
<td>Pathogen 2</td>
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<tr>
<td>Gray mold rot</td>
<td>Botrytis cinerea</td>
<td>Rhodotorula glutinis (Zhang et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>Blue mold</td>
<td>Penicillium expansum</td>
<td>Rhodotorula glutinis (Zhang et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>Strawberry</td>
<td>Gray mold</td>
<td>Botrytis cinerea</td>
<td>Rhodotorula glutinis (Zhang et al. 2007b)</td>
</tr>
<tr>
<td>Tomato</td>
<td>Gray mold</td>
<td>Botrytis cinerea</td>
<td>Candida guilliermondii, Candida oleophila (Saligkarias et al. 2002), Cryptococcus laurentii (Xi &amp; Tian 2005), Pichia guilliermondii (Chalutz et al. 1988)</td>
</tr>
<tr>
<td></td>
<td>Alternaria rot</td>
<td>Alternata alternata</td>
<td>Pichia guilliermondii (Chalutz et al. 1988)</td>
</tr>
<tr>
<td></td>
<td>Rhizopus rot</td>
<td>Rhizopus nigricans</td>
<td>Pichia guilliermondii (Zhao et al. 2008)</td>
</tr>
<tr>
<td>Chillies</td>
<td>Anthracnose</td>
<td>Colletotrichum capsici (Syd.) Butler &amp; Bisby</td>
<td>Pichia guilliermondii (Chanchaichaovivat et al. 2007)</td>
</tr>
</tbody>
</table>
Table 2: A general chemical composition of essential oils

<table>
<thead>
<tr>
<th>Categories</th>
<th>Sub categories</th>
<th>Examples</th>
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<tbody>
<tr>
<td>Terpenes and terpenoids</td>
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<tr>
<td>Alcohols</td>
<td>Geraniol, Linalol, Citronellol, Lavandulol, Nerol, Menthol, a-terpineol, Carveol, Borneol, Fenchol, Chrysanthanol etc.</td>
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<tr>
<td>Aldehydes</td>
<td>Geranial, Neral, Citronellal.</td>
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<tr>
<td>Carbures</td>
<td>Myrcene, Oicimene, Terpinenes, p-cimene, Phellandrenes, Pinenes, 3-Carene, Camphene, Sabinene.</td>
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<tr>
<td>Ketone</td>
<td>Tegetone, Menthones, Carvone, Pulegone, Piperitone, Camphor, Fenchone, Thuyone</td>
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<tr>
<td>Esters</td>
<td>Linalyl acetate or propionate, Citronellyl acetate, Menthyl or a-terpinyl acetate</td>
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<tr>
<td>Ethers</td>
<td>1,8-cineole, Menthofurane,</td>
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<tr>
<td>Peroxydes</td>
<td>Ascaridole, etc</td>
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<tr>
<td>Phenols</td>
<td>Thymol, Carvacrol</td>
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</tr>
<tr>
<td>Aldehyde</td>
<td>Cinnamaldehyde</td>
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<tr>
<td>Alcohol</td>
<td>Cinnamic alcohol</td>
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<tr>
<td>Methoxy derivatives</td>
<td>Anethole, Elemicine, Estragole, Methyleugenols</td>
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<tr>
<td>Methylene dioxy compounds</td>
<td>Apiole, Myristicine, Safrole</td>
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<td>Phenols</td>
<td>Chavicol, Eugenol</td>
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### Table 3: Antimycotic activity of some essential oils

<table>
<thead>
<tr>
<th>Plant name</th>
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<tr>
<td><em>Allium cepa; Allium sativum</em>; <em>Aspergillus niger,</em> <em>Penicillium cyclopium</em> and <em>Fusarium oxysporum</em></td>
<td></td>
<td>Benkeblia (2004)</td>
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<tr>
<td><em>Allium sativum</em> (Bulb), <em>Amomum subulatum</em> (Seeds), and <em>Azadirachta indica</em> (Seeds), <em>Syzygium aromaticum</em> (Flower buds)</td>
<td><em>Aspergillus fumigatus,</em> <em>Penicillium italicum</em></td>
<td>Thind &amp; Dahiya (1977)</td>
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<tr>
<td><em>Calocedrus macrolepis</em> var. <em>formosana</em> (Taiwan incense cedar)</td>
<td><em>Fusarium oxysporum</em> f.sp. <em>ciceri,</em> <em>Fusarium oxysporum</em> f.sp. <em>lycopersici,</em> <em>Fusarium oxysporum</em> f.sp. <em>melonis</em></td>
<td>Behtoei (2012)</td>
</tr>
<tr>
<td><em>Carum carvi</em> (Caraway)</td>
<td><em>Alternaria alternata,</em> <em>Colletotrichum corchori,</em> <em>Curvularia lunata,</em> <em>Diplodia sp., Fusarium equiseti,</em> <em>Macrophomina phaseolina,</em> <em>Colletotrichum coccodes,</em> <em>Diaporthe helianthi,</em> <em>D. phaseolorum var. cauliwora,</em> <em>Fusarium oxysporum,</em> <em>F. subglutinans,</em></td>
<td>El-Zemity &amp; Ahmed (2005); Begum et al. (2008); Ćosić et al. (2010)</td>
</tr>
<tr>
<td>Disease Causing Organisms</td>
<td>Biocontrol Agents</td>
<td>Reference</td>
</tr>
<tr>
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<tr>
<td>Post-harvest diseases of fruits and vegetables and their management by biocontrol agents</td>
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<td>Verticillioides, Helminthosporium sativum, Phomopsis longicolla, P. viticola, Rhizoctonia solani</td>
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<tr>
<td><em>Cestrum nocturnum</em></td>
<td>Botrytis cinerea, Colletotrichum capsici, Fusarium oxysporum, F. solani, Phytophthora capsici, Sclerotinia sclerotiorum</td>
<td>Al-Reza et al. (2010)</td>
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<td><em>Chenopodium ambrosioides</em></td>
<td>Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Botryodiplodia theobromae, Fusarium oxysporum, Sclerotium rolfsii, Macrophomina phaseolina, Cladosporium cladosporioides, Helminthosporium oryzae and Pythium debaryanum</td>
<td>Kumar et al. (2007)</td>
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<tr>
<td><em>Cinnamomum verum</em> (C. zeylanicum) (Cinnamon)</td>
<td>Alternaria alternata, Alternaria sp., Aspergillus niger, Aspergillus sp., Botrytis cinerea, Colletotrichum coccodes, C. gloeosporioides, Diaporthe helianthi, D. phaseolorum var. cauliemora, Fusarium avenaceum, F. oxysporum, Fusarium oxysporum f.sp. ciceri, F. oxysporum f.sp. lycopersici, F. oxysporum f.sp. melonis, F. proliferatum, F.</td>
<td>Velluti et al. (2003); Gupta et al. (2008); Sukatta et al. (2008); Siripornvisal et al. (2009); Ćosić et al., (2010); Behtoei (2012),</td>
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<tr>
<td>Fruit/Plant Name</td>
<td>Diseases/Agents</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>-------------------------</td>
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<tr>
<td>Citrus hystrix (Bergamot)</td>
<td>Colletotrichum coccodes, Helminthosporium sativum, Alternaria brassicicola, Aspergillus flavus, Bipolaris oryzae, Fusarium proliferatum, F. verticillioides, Pyricularia grisea, Rhizoctonia solani</td>
<td>Thobunluepop et al. (2009)</td>
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<td>Curcuma longa (Rhizome)</td>
<td>Fusarium oxysporum f. sp. dianthi, Alternaria dianthi, F. oxysporum f. sp. gladioli and Curvularia trifolii f. sp. gladioli</td>
<td>Babu et al. (2007)</td>
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<tr>
<td>Cuminum cyminum (Seeds)</td>
<td>Aspergillus flavus and A. niger</td>
<td>Kumar &amp; Tripathi (2002)</td>
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<td>Cymbopogon martinii</td>
<td>Alternaria solani</td>
<td>Babu et al., (2000)</td>
</tr>
<tr>
<td>Cinnamomum camphora (leaves)</td>
<td>Aspergillus flavus</td>
<td>Mishra et al., (1991)</td>
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<tr>
<td>Daucus carota (Seeds)</td>
<td>Aspergillus flavus</td>
<td>Dwivedi et al. (1991)</td>
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<td>Eucalyptus citriodora (Lemon-scented eucalyptus)</td>
<td>Cryponectria parasitica, Fusarium circinatum, Phytophthora cactorum</td>
<td>Lee et al. (2008)</td>
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<tr>
<td>Plant Name</td>
<td>Diseases Reported</td>
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<td><em>Foeniculum vulgare</em> (Fennel)</td>
<td>Alternaria alternata, Aspergillus flavus, Botrytis cinerea, Cladobotryum sp., Fusarium oxysporum, Mycogone perniciosa, Verticillium fungicola var. fungicola</td>
<td>Tanović <em>et al.</em> (2009)</td>
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<td>Abdolahi <em>et al.</em> (2010), Javed <em>et al.</em> (2012)</td>
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<td><em>Hyptis suavelens</em></td>
<td>Rhizoctonia solani, Sclerotium rolfsii, Sclerotinia sclerotiorum, Fusarium oxysporum var. gladuli, Aspergillus and Penicillium sp.</td>
<td>Singh &amp; Handique, 1997; Tripathi &amp; Sharma, 2013</td>
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<td><em>Lantana camara var. aculeata</em> (Seeds)</td>
<td>Curvularia lunata</td>
<td>Avadhoot &amp; Verma, 1978</td>
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<td><em>Laurelia sempervirens</em> (Laurel)</td>
<td>Fusarium oxysporum, Phragmidium violaceum, Pythium irregulare, Rhizoctonia solani</td>
<td>Bittner <em>et al.</em> (2009)</td>
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<td><em>Laureliopsis philippiana</em> (Tepa)</td>
<td>Fusarium oxysporum, Phragmidium violaceum, Pythium irregulare, Rhizoctonia solani</td>
<td>Bittner <em>et al.</em> (2009)</td>
</tr>
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<td><em>Lavandula angustifolia</em> (Lavender)</td>
<td>Helminthosporium sativum, Phomopsis viticola Botrytis cinerea, Colletotrichum capsici, Fusarium oxysporum, F. solani, Phytophthora capsici, Sclerotinia</td>
<td>Ćosić <em>et al.</em> (2010)</td>
</tr>
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<td><em>Lavandula officinalis</em> (Lavender)</td>
<td>Cladobotryum sp., Mycogone perniciosa, Verticillium fungicola var. fungicola</td>
<td>Tanović <em>et al.</em> (2009)</td>
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<td><em>Leptospermum petersonii</em></td>
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<td>Plant</td>
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<td><em>Melaleuca alternifolia</em></td>
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<td>(Tea tree)</td>
<td><em>Fusarium proliferatum</em>, <em>Fusarium verticillioides</em></td>
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<td><em>Mentha arvensis</em>, <em>Ocimum canum</em> and <em>Zingiber officinale</em></td>
<td><em>Penicillium italicum</em></td>
<td>Tripathi et al., 2004</td>
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<td><em>Murraya koenigii</em></td>
<td><em>Rhizoctonia bataticola</em>, <em>Helminthosporium oryzae</em> and <em>Rhizoctonia solani</em></td>
<td>Srivastava &amp; Singh, 2001</td>
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<td><em>Myristica fragrans</em></td>
<td>Some storage fungi</td>
<td>Gopal et al., 1986</td>
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<td><em>Ocimum basilicum</em> (Basil)</td>
<td><em>Alternaria brassicicola</em>, <em>Aspergillus flavus</em>, <em>Bipolaris oryzae</em>, <em>Botrytis cinerea</em>, <em>Fusarium proliferatum</em>, <em>Fusarium verticillioides</em>, <em>Pyricularia grisea</em></td>
<td>Abdolahi et al., 2010</td>
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<tr>
<td><em>Ocimum gratissimum</em> (Sweet fennel)</td>
<td><em>Alternaria brassicicola</em>, <em>Aspergillus flavus</em>, <em>Bipolaris oryzae</em>, <em>Fusarium proliferatum</em>, <em>Fusarium verticillioides</em>, <em>Pyricularia grisea</em></td>
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<td><em>Pelargonium graveolens</em> (Geranium)</td>
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<td>Tanović et al., 2009</td>
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<td><em>Petroselinum aetheroleum</em> (Parsley)</td>
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<td>Tanović et al., 2009</td>
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<td><em>Peumus boldus</em> (Boldo)</td>
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<td>Bittner et al., 2009</td>
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<td>Plant Type</td>
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<td><strong>Pinus species</strong></td>
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<td>Baranowska et al., 2002</td>
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<td><strong>Syzygium aromaticum</strong></td>
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<td>Javed et al., 2012</td>
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<td><strong>(Clove)</strong></td>
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<td><strong>(Thyme)</strong></td>
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<td><strong>Trachyspermum ammi</strong></td>
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<td>Siripornvisal (2010), Behtoei (2012)</td>
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<td><strong>(syn. Carum coticum)</strong></td>
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<td><strong>(Ajowan, Bishop's weed)</strong></td>
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<td>Post-harvest diseases of fruits and vegetables and their management by biocontrol agents</td>
<td>Review of Literature</td>
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<td>oxysporum f. sp. lycopersici, F. oxysporum f.sp. melonis</td>
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<td>Zingiber officinale (rhizome)</td>
<td>Bacillus subtilis, Pseudomonas aeruginosa, Candida albicans, Trichoderma spp, Aspergillus niger, Pencillium spp.</td>
<td>Menon &amp; Sasidharan, 2010</td>
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MATERIALS
&
METHODS
3. MATERIALS AND METHODS

The experiments were carried out at the Mycology and Plant Pathology Laboratory of Botany Department, Lucknow University, Lucknow during October 2009-December 2013. The experimental techniques, procedures and the formulas were adopted during the research work are discussed below:

3.1. Survey, collection, isolation, identification and disease incidence of pathogens of apple and tomato fruit

3.1.1. Geographic status of survey spot

Postharvest diseases of apple (*Malus pumila* auct. var. Ambri kashmiri) and tomato (*Lycopersicon esculentum* Mill. var. Banglore) were surveyed in different market spots.

Lucknow, Bahraich and Shravasti districts in Uttar Pradesh were selected for the study of postharvest pathogens of these fruits. The study area *i.e.* Lucknow falls in central part of U.P. and lies between 26.30° & 27.10° North latitude and 80.30° & 81.13° East longitude. Bahraich (in eastern part of U.P.) is located between 28.24° & 27.4° latitude and 81.65° & 81.3° East of longitude. Shravasti district is located at latitude 27.5° and longitude 82.0°. Shravasti district is sharing borders with Bahraich district to the West, Balrampur district to the East.

3.1.2. Loss estimation

The disease symptoms produced on fruits and vegetables were observed under natural conditions of infection. Infected fruits and vegetables selected were those which showed characteristic of different rots caused by fungal pathogens.
Survey was conducted market of Nishatganj, Aliganj market, Kapoorthala, Daliganj, Raidas lane, Bhootnath market Indiranagar and also fruit and vegetable market of tarai belts like Bahraich and Shravasti district (U.P.) of India during 2009-2013.

Random sampling techniques were applied in sample collection, made from September to May for apple and all over the year for tomato.

3.1.3. Isolation, purification and identification of pathogens

Sample of rotted and diseased fruits and vegetables collected from markets were used for the isolation of pathogens. The fruits and vegetables showing the initial and distinct characteristic symptoms were selected for isolation of pathogen by using the method described by K.R. Aneja in his book (Experiments in Microbiology, Plantpathology and Biotechnology). The selected fruits were washed with running water in order to remove the dust particles. The fruits and vegetables were dipped in 3 per cent solution of sodium hypochlorite for 30 seconds and washed thoroughly three to four times with sterilized water to remove the traces of sodium hypochlorite, after that the infected fruit was wiped with a cotton swab dipped in 70 per cent ethanol followed by lightly flaming the tissue. By using flamed scalpel, diseased sample were peeled, exposing tissue not previously exposed to contaminants. Small pieces of tissue (1/8 to 1/2" sizes) were dug out with cooled flamed scalpel or forceps from freshly exposed area of the advancing margin of infection. Three or four pieces were placed on per Petriplate (90 mm diameter) which was already poured with acidified Potato dextrose agar medium (PDA: Potato-200.0g, dextrose-20.0g, streptomycin-0.3g and agar-20.0g per litre). Petriplates were incubated at 25°C±2C, in an inverted position, for 5-7 days in BOD incubator. As soon as mycelial growth and spores of fungus became visible, bits of agar containing mycelia from the
edge of developing colonies were transferred to slants for identification and further use (Aneja, 2009).

To obtain single spore isolate, method described by Manandhar et al. (1995) was followed. Petri dish containing the fungus was flooded with sterile distilled water and serial dilution was made. One ml of the suspension (50 conidia/ml) was spread on Petri dish containing water agar (25 ml). The inoculated Petri dishes were incubated at 25±2°C for 12-24 h. The colony formed by single spore was picked and sub-cultured on fresh medium for further studies. The purified pathogen was identified up to genus level based on cultural character and type of conidia produced. The culture was purified using hyphal-tip method and maintained on PDA slants in the refrigerator at 6-8°C for further studies (Brown, 1934). The culture was multiplied and sub-cultured on to fresh medium after every one month. Purified cultures were maintained on potato dextrose agar (PDA) medium in refrigerator for further studies. For microscopic observations, different fungal isolates were stained with lactophenol cotton blue and observed under the microscope (Nikon ECLIPSE E200, Japan) at different magnifications.

Fungi were identified on the basis of their morphological and cultural characteristics with the help of available literature (Gilman, 1967; Ellis, 1976). The cultural characters like type and colour of growth on Potato dextrose agar medium as well as pathogenic behaviour towards the host were recorded. Morphological characters taken into account were:

- Colony: Color and growth
- Mycelium: Color, branching pattern, Septation and width
- Colony: Color and growth
- Conidiophores: Color, size, and septation
3.1.4. Disease incidence

In all the shops, samples were stored in cartons, each containing 10 kg of fruit and vegetable sample. Cartons were placed in racks. For collecting data, the samples were spread over the floor and the diseased samples from each carton were sorted out. Disease incidences were calculated using the following formula.

\[
\text{\% Infection/Disease incidence} = \frac{\text{Number of infected particular in a commodity}}{\text{Total number of particulars in a commodity}} \times 100
\]

The fruit showing typical symptoms of disease were brought to the laboratory for isolation, identification and further studies.

3.2. Pathogenicity test

Pathogenicity test was proved by Koch’s postulate. After isolation of pathogen cultures were purified and maintained. Healthy apples and tomatoes, of the same size were washed after being surface sterilized with disinfectant rinsed with sterile distilled water. By using alcohol dipped and flamed cork-boarer, fruits and vegetables were punctured with single wound centrally near stem end. Fruits and vegetables were inoculated with a mycelial disc cut from the margin of growing pathogen culture. Test samples were incubated at 25±2°C for 7 days. Re-isolation of the pathogen was carried out.

3.3. Effect of inoculum concentration of pathogens on disease development

Apple and tomatoes of the same size after being surface sterilized with ethanol were wounded with cork borer at one site, subsequently inoculated by placing spore suspension which were adjusted with a hemocytometer to obtain concentration of
pathogen containing $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$ and $10^8$ conidia ml$^{-1}$. The wounds were immediately covered with sterilized wet cotton swabs. The fruits were placed in trays, covered with sterilized polythene and incubated at 25±2°C for 18 days. Lesion diameter was measured to observe the disease development. Single wound was made and inoculated by placing a disc of 3 mm diameter taken from the growing edge of pure culture of the test fungus. In all the experiments further conducted, mycelial disk of 3 mm diameter taken from growing edge of 14-day-old fungal culture served as source of inoculum unless stated (Jadeja & Bhatt, 2008; Gholamnejad et al., 2009). The experiment was repeated three times.

3.4. In vitro studies

3.4.1. Growth of pathogens on different medium

The fungal organism requires different types of nutrition for growth so, the test organism was cultured on a variety of media. Seven media i.e. Czapek dox agar (CzDA), Asthana Hawker’s medium (AHM), Malt yeast extract agar (MYEA), Potato dextrose agar (PDA), Host extract agar (HEA) Richard’s medium (RM) and Sabouraud’s agar (SbA) (Appendix) were used to study the radial growth of test fungus. The pH of the different culture media was adjusted to 7.0 and they were sterilized at 15 p.s.i. for 20 minutes. About 25 ml of each media was poured in Petri dishes and allowed to solidify. Fresh mycelia discs (5 mm) of the pathogen were cut with the help of sterile cork borer from the growing edge of one week old culture and inoculated on the assay plates. The plates were incubated at 25±2°C in BOD incubator. After 7 days of inoculation, the radial growth was measured and compared with different medium selected for the test. Three replications were maintained and the experiment was repeated twice.
For measuring the dry weight of the test pathogen the broth of same medium were prepared but without agar. 40 ml of sterilized medium was poured in sterilized conical flasks (150 ml). After cooling, the flasks were inoculated with an equal amount of fungal inoculum and incubated at 25±2°C for 15 days. Each treatment was replicated three times. After the completion of the incubation period, the mycelial mat was filtered using Whatman's filter paper No. 42. Filter paper retaining fungal mat was washed thoroughly with distilled water to remove the traces of chemicals associated with the mycelial mat and then oven dried at (65°C) warm condition for 48 hours, subsequently cooled in desiccators having anhydrous calcium chloride and accurately weighed on electric balance. The dry weight of actual fungal mat was calculated in milligram (mg) by subtracting the weight of the filter paper. Average dry weight of the three replicates was taken as standard value for comparing the growth in different media.

3.4.2. Growth of pathogen at different temperatures

The effect of different temperatures was evaluated for radial growth of the pathogen. Potato dextrose agar media was used for this experiment. 20 ml of medium was poured in Petri plates (90 mm diameter) and then were allowed to solidify. Mycelial disc (3 mm) of pathogen was removed from the 7 day old culture with the help of sterile cork borer and aseptically inoculated in the center of the Petri dishes. Petri dishes were incubated at 10, 15, 20, 25, 30, and 35°C after inoculation. The radial growth was recorded in mm in two directions at right angles to each other and the average was calculated.

For measuring the dry weight of the test pathogen the broth of PDA medium were prepared without agar. 40 ml of sterilized medium was poured in sterilized conical flasks (150 ml). After cooling, the flasks were inoculated with an equal amount of fungal inoculum (20µl of test pathogens at the concentration 10^6 spores ml^-1 at 10, 15, 20, 25,
30, and 35°C for 15 days. Each treatment was replicated three times. After the completion of the incubation period, the mycelial mat was filtered through previously dried and weighed Whatman's filter paper No. 42. Filter paper retaining fungal mat was washed thoroughly with distilled water to remove the traces of chemicals associated with the mycelial mat and then oven dried (65°C) at warm condition for 48 hours, subsequently cooled in desiccators having anhydrous calcium chloride and accurately weighed on electric balance. The dry weight of actual fungal mat was calculated in mg by subtracting the weight of the filter paper. Average dry weight of the three replicates was taken as standard value for comparing the growth under different temperatures.

3.4.3. Growth of pathogens at different pH

The effect of different pH was evaluated for radial growth of the pathogens. Potato dextrose agar media were used for this experiment. Before sterilization, the pH of the medium was adjusted in the range of pH-4 to pH-8 by using 1M solution of HCl and NaOH and sterilized at 15 p.s.i. for 20 minutes. 20 ml of medium was poured in Petri plates (90 mm diameter) and then were allowed to solidify. Three replicates of each pH range were maintained. Mycelial disc (3 mm) of pathogen was removed from the 7 day old culture with the help of sterile cork borer and aseptically inoculated in the centre of the Petri dishes. Petri dishes containing PDA of different pH were incubated at 25±2°C. The radial growth from the edge was measured regularly. The radial growth was recorded in mm in two directions at right angles to each other and the average was calculated.

For measuring the dry weight of the test pathogen the broth of PDA medium were prepared without agar. 40 ml of sterilized medium was poured in sterilized conical flasks (150 ml). After cooling the flasks were inoculated with an equal amount of
fungal inoculums (20µl of target pathogens at the concentration $10^6$ spores ml$^{-1}$) and incubated at 25±2°C for 15 days. Each treatment was replicated three times. After the completion of the incubation period, the mycelial mat was filtered through previously dried and weighed Whatman’s filter paper No. 42. Filter paper retaining fungal mat was washed thoroughly with distilled water to remove the traces of chemicals associated with the mycelial mat and then oven dried (65°C) at warm condition for 48 hours, subsequently cooled in desiccators having anhydrous calcium chloride and accurately weighed on electric balance. The dry weight of actual fungal mat was calculated in milligram (mg) by subtracting the weight of the filter paper. Average dry weight of the three replicates was taken as standard value for comparing the growth at different pH.

3.5. Study of Biocontrol agent

3.6.1. Primary screening of biocontrol agent against A. alternata and A. mali

The four antagonists were selected of *Candida utilis*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* were isolated from the carposphere, however, *Rhodotorula phylloplana* was shopped from Microbial type culture collection & gene bank (MTCC). Four yeast bioagent were tested for their efficacy as biocontrol agent against *A. alternata* and *A. mali* in tomato and apple respectively viz. *R. phylloplana, S. cerevisiae, C. utilis and D. hansenii*. The method described by Janisiewicz (1987) was used to select microorganisms capable of reducing disease caused by *A. alternata* and *A. mali* in tomato and apple respectively. All the four isolates were tested *in vivo* for their efficacy to check the decay in tomato and apple. Surface sterilized fruits were wounded at blossom end. The wounds were 3mm×3mm. All the fruits were inoculated with 20µl of
cell suspension of the yeast. The fruits were then challenged with spore suspension of
their respective pathogens. The tomatoes were incubated at 15±2°C and the apple at
20±2°C. The fruits were evaluated daily for lesion size and percent decay was calculated
at the end of 7 days for tomatoes and end of 10 days for apple, by cutting and weighing
the rotten portion of the fruit and subtracting it from the weight of the intact fruit. Three
fruits constituted a single replicate and each treatment had three replicate and was
repeated three times. The most effective isolate was selected for further studies.

### 3.6.1.2. Maintenance of biocontrol agent

The yeast *Rhodotorula phylloplana* strain R6 MTCC 2748 used in the experiment were
previously isolated from apple fruit peel. Cultures were maintained at 4°C on malt yeast
extract media (MYEA: 3 g malt extract; 3 g yeast extract; 5 g peptone; 10 g dextrose in
1L distilled water). Liquid cultures were grown in 250 ml Erlenmeyer flasks containing
50 ml of malt yeast extract broth (MYEB), inoculated with sterile loopful of the yeast
starter culture. Flasks were incubated overnight at 25°C in a rotary shaker at 130 rpm.
Following incubation, the yeast cells were centrifuged at 3000 × g for 10 min and
washed twice in SDW to remove growth medium. The pellets were re-suspended in
SDW or an aqueous solution of coating liquids and adjusted to different concentrations
(cells ml\(^{-1}\)) depending on the requirements of experiments. Yeast cell counts were
determined by yeast plate count.
3.7. Biocontrol efficacy of potential antagonist

3.7.1. *In vitro* bioactivity of yeast *R. phylloplana*

3.7.1.1. Determination of yeast cell concentration

The *in vitro* efficacy of yeast cell concentration was measured as per the method given by Ray (2002) with slight modifications. For measuring the dry weight of the test pathogen the potato dextrose broth (PDB) was prepared in the manner mentioned in appendix. 40 ml of sterilized medium was poured in sterilized conical flasks (150 ml). After cooling, the flasks were inoculated with 20 µl of antagonist $10^6$ CFU ml$^{-1}$ and $10^8$ CFU ml$^{-1}$ concentration of yeast, an equal amount of spore suspension (*Alternaria mali* and *Alternaria alternata* at the concentration $10^6$ conidia ml$^{-1}$) was inoculated and incubated at $25\pm 2^\circ C$ for 7 days. After the completion of the incubation period, the mycelial mat was filtered through previously dried and weighed Whatman's filter paper No. 42. Filter paper retaining fungal mat was washed thoroughly with distilled water to remove the traces of chemicals associated with the mycelial mat and then oven dried (65$^\circ$C) at warm condition for 48 hours, subsequently cooled in desiccators having anhydrous calcium chloride and accurately weighed on electric balance. The dry weight of actual fungal mat was calculated in mg by subtracting the weight of the filter paper for comparing the growth under different treatments.

3.7.1.2. Efficacy of yeast for controlling *A. mali* and *A. alternata*

40 ml of cooled PDB poured sterilized conical flasks (150 ml) were inoculated with 20 µl of antagonist at the effective concentration. An equal amount of spore suspension of *A. mali* and *A. alternata* at the concentration $10^6$ conidia ml$^{-1}$ were inoculated in previously yeast inoculated PDB conical flasks after 0, 24, 48 and 72h successively,
incubated at 25±2°C for 7 days. There were three replicates for each treatment and the experiment was conducted three times (Ray, 2002).

3.8. **In vivo efficacy of potential antagonist**

3.8.1. **Determination of effective concentration of yeast**

To determine the minimum effective concentration of *R. phylloplana* against *A. alternata* and *A. mali*, surface-sterilized fruits were wounded. Then 25 µl of aqueous suspension of *R. phylloplana* (10⁶ and 10⁸ CFU ml⁻¹) were applied to each wound. After 1h, the wounds were inoculated with 20 µl of an aqueous suspension of both pathogens (10⁶ conidia ml⁻¹) separately. Three fruits constituted a single replicate and each treatment had three replicates and the experiment was repeated three times. Lesion diameters were measured after 14 days of incubation, at 22±2°C (Janisiewicz, 1987).

3.8.2. **Effect of inoculation time of antagonist and pathogen**

Aliquots (20µl) of a 1×10⁸ CFU/ml suspension of *R. phylloplana* were pipetted on to the wound in apple and tomato 0, 24, 48 and 72 h before and after counter inoculation of respective *Alternaria* spore suspension (10⁶ CFU/ml). The fruits were incubated at 22±2°C. Decay development was determined 7 days after inoculation of pathogens. The treated fruit were sealed in polyethylene-lined plastic boxes to retained high humidity. There were replicates of 5 fruits for each treatment and the experiment was conducted three times.

3.9. **Effect of *Rhodotorula phylloplana* on cellulose and pectinase in apple infected with *A. mali* and tomato with *A. Alternata***

3.9.1. **Sample preparation**
Post harvest diseases of fruits and vegetables and their management by bio control agents

Materials & methods

*R. phylloplana* treated Apple and tomato (Y1, Y2), challenged with respective pathogens *A. mali* and *A. Alternata* (P1, P2), also counterchallenged with yeast and pathogens where yeast coating before 72 hrs. to pathogens (YP1, YP2) were used as study the enzyme activities. After different intervals (2, 4 and 6 day) infected tissue (1 g) has been taken and ground in a electric blender in 10 mL sterile distilled water for 20 min., centrifuged at 10,000rpm for 15min at 4°C. The supernatant obtained was used as a crude enzyme preparation.

3.9.2. **Cellulase activity**

1mL of the crude enzyme extract was incubated with 9mL of 0.55% CMC in 0.055M citrate buffer pH 5 for 1 h at 45°C. The amount of reducing sugar was determined by combining 1mL of the filtrated CMC reaction mixture with 3mL of dinitrosalicylic acid reagent, boiling the mixture for 3min and determining the absorbance at 550nm using a spectrophotometer. The reaction mixture of the uninoculated control was used to adjust absorbance at zero. The absorbance of a standard solution of D-glucose of various concentrations (0–100 mg/mL) was determined and used to prepare a curve of absorbance related to mg of glucose per mL. The amount of reducing sugar produced by 1mL of fungal filtrate acting on the CMC-citrate substrate for 1 h at 45°C was calculated from the glucose standard graph. The cellulolytic activity of the tissue-free filtrates was expressed in terms of total reducing sugars and was expressed as mg reducing sugars (RS) per mL/h. (Reese & Mandels, 1963)

3.9.3. **Pectinase activity**

A reaction mixture was prepared containing 0.4mL of 0.1M sodium acetate buffer (pH 5) and 0.2 mL crude enzyme. The reaction mixture was incubated for 1 h at 40°C. The reaction mixture of filtrate was boiled in a water bath for 20min and also incubated in a similar manner and used as a control. The amount of reducing sugar was then determined.
by combining 0.5mL of the reaction mixture with 0.5mL of Fehling’s solution and boiling the mixture for 20 min and determining the absorbance at 520 nm. The absorbance of a standard solution of D-glucose of various concentrations (0–100 mg/mL) was determined using the method of Nelson (1944) and Somogyi (1952). The amount of reducing sugar produced by 1mL of tissue-free filtrate acting on sodium polypectate at 40°C for 1 h was calculated from the glucose standard graph as related to mg of glucose per mL. The exo-PG activity of the filtrate was expressed in terms of total reducing groups per mL/h.

3.10. Population dynamics: survival of antagonist growing in wounded apple and tomato

To study the populations of *R. phylloplana* in apple and tomato were wounded (5×5×3 mm³) with sterile cork borer and each wound was inoculated with 20µl of *R. phylloplana* (10⁶CFU ml⁻¹). Populations of *R. phylloplana* were monitored at 24, 48, 72, 96, 120, 144 and 168 h in fruits stored at 20±1°C. Wounds were excised at above stated time periods using a 5 mm diameter sterile cork borer and placed in 10 ml sterile distilled water (SDW), mashed thoroughly with the glass rod and vortexed. Washings were serially diluted and suitable dilutions were plated on MYEA plates. Colonies were counted after 3 days of incubation at 25±2°C.

Sample tissue (1g) was taken with a knife. Tissues were shaken in 200 ml sterile phosphate buffer (pH 6.5) on a rotatory shaker for 20 min at 150 rpm and then sonicated for 10 min in an ultrasonic bath. Serial dilutions of the washings were made and plated on MYEA [malt extract, 3g l⁻¹; yeast extract, 3g l⁻¹; dextrose, 10g l⁻¹ and agar, 15 g l⁻¹] supplemented with colonies, and counted after incubation at 25±2°C in the dark for 48 h. Population was expressed as Log₁₀ CFU/g.
3.11. Study of fungal and yeast interaction

3.11.1. Light microscopic study

The interaction of yeast with fungal hyphae was evaluated in Petri dishes (8.5 mm in diameter method of Chan and Tian (2005), followed with slight modifications. Plugs of agar containing seven day old sporulating mycelium of *A. alternata* were placed on acidified agar plate at one side. After 48 h at 25±2°C, 40µl of yeast suspension (1×10^8 CFU/ml) was placed at the margin of fungal growth as a strip. The dual culture was incubated at the 25±2°C until the margins of fungal mycelia and yeast cell interacted to each other. The dual culture were washed with a stream of distilled water for 30-60 seconds and viewed with light microscopy.

3.11.2. Scanning electron microscopic study

The dual culture were studied in yeast–pathogen direct interactions, two or three samples, size 3–5mm block, were cut and fixed in 5% cold buffered glutaraldehyde for 24 h. The samples were washed with sodium cacodylate buffer for 30 min and then fixed in 1% osmium tetroxide for 1 h. The samples were washed again in the same buffer again for 30 min each and dehydrated by using an ascending grade of ethanol 30, 50, 70, 90% for 1 h and 100% for 24 h and then amylacetate for 24 h also. After that, the samples were dried in a critical point drainer using liquid carbon dioxide, and each sample stuck on a metallic block using silver paint. In a Gold Sputter Apparatus, the samples were evenly gold coated to a thickness of 15 nm. The samples then were examined at scanning electron microscope and photographed.
3.12. Screening of botanicals for biocontrol activity against post harvest test pathogen

Oil from different parts of seven angiospermic plants viz., *Mentha arvensis* L., *Tagetes minuta* L., *Cymbopogon citrates* (D.C.) Stapf., *Coriandrum sativum* L. *Callistemon lanceolatus* DC., *Chenopodium album* L. and *Zingiber officinale* Roscoe. were assayed for their fungitoxicity against *A. alternata* and *A. mali* a causal organisms responsible for Alternaria rot of tomato and apple. The essential oils were assayed for its antifungal activity against the test fungus on the basis of percent spore germination inhibition.

3.13. Use of ginger (*Zingiber officinale* Roscoe.) oil in post harvest pathogen control of apple and tomato)

3.13.1. Plant preparation

Ginger rhizomes were purchased from a local market in Lucknow (India). Ginger was cleaned with distilled water to remove soil and dust, cleaned ginger was chopped into small pieces.

3.13.2. Extraction of essential oil

The fresh ginger (5 kg) was hydro-distilled for 6-7 hrs. in a Clevenger type apparatus Voucher specimens (no. BDLU 77/03). The oil was dried over anhydrous sodium sulphate. To isolate the oil from the aqueous portion, the oil was extracted with solvent ether in a separating funnel. The ether was removed at reduced pressure which resulted in an oily residue that was added to the oil collected earlier. The remaining aqueous fraction, free from smell was stored separately. Thus, the hydrodistilled volatile fraction
from the ginger rhizome was separated into two fractions an oil fraction and an aqueous fraction. The essential oil obtained was kept in sealed glass tube at 4°C until analysis.

3.13.3. Recovery of essential oil from the rhizome of Zingiber officinale Roscoe.

1000 g ginger rhizome was separately subjected to usual hydro-distillation and the per cent recovery of the amount of the oil extracted was calculated.

3.13.4. Physico-chemical characterization of the essential oil of Zingiber officinale Roscoe. rhizome

The oil was qualitatively standardized by their various physico-chemical properties. Ginger oil was standardized by determining its various physico-chemical properties viz., acid value, ester value, saponification number, phenolic test, solubility in organic solvents and water and colour using the technique of Langenau (1948).

3.13.4.1. Acid value

Acid value is defined as the number of milligrams of potassium hydroxide (KOH) required to neutralized the free acids present in 1 g of oil.

One g of the essential oil was weighed accurately and dissolved in 5 ml of 95% ethanol in a conical flask. In this 0.2 ml of 1% phenolphthalein indicator solution was added to the contents of the flasks and titrated for free acidity with 0.1M sodium hydroxide solution. The first appearance of pink colouration that did not fade within 10 seconds was considered as the end point. Another set, without the oil was run parallel to treatment and the difference in the amounts of alkali used while titrating the treatment
and the set without oil gave the amount of alkali consumed for determination of the acid number of the oil. The acid number was calculated using the following formula:

\[
\text{Acid Value (AV)} = 5.61 \frac{V}{m}
\]

Where \( V \) = volume in milliliters of 0.1 M potassium hydroxide required to neutralize the acid, \( m \) = Weight of 1 ml of essential oil in g*

*(The weight of 1 ml essential oil of *Zingiber officinale* Roscoe. was 1.845 g)

### 3.13.4.2. Ester value

The ester value of an essential oil is the number of milligrams of potassium hydroxide required to neutralize the acids resulting from the complete hydrolysis of 1 g of the oil. For reagent blank, a blank determination was carried out by boiling 5 ml of ethanol, 25.0 ml of 0.5 M ethanolic potassium hydroxide and 0.2 ml of phenolphthalein indicator solution under reflux for 1 hr, cooling, adding 20 ml of water and 0.5 ml of phenolphthalein indicator solution, and immediately titrating with 0.5 M hydrochloric acid.

5 g of the essential oil was taken into saponification flask and in this 5ml of ethanol and 0.2 ml of phenolphthalein indicator solution were added. Then titration was done for free acidity with 0.1 M ethanolic potassium hydroxide. To neutralize liquid, 25.0 ml of 0.5 M ethanolic potassium hydroxide was added and boiled under reflux for 1 hr, cooled and 20 ml of water and 0.5 ml of phenolphthalein indicator solution was added and immediately titrated the excess of alkali with 0.5 M hydrochloric acid. The difference between the titration and that of the blank represents the volume of 0.5 M ethanolic potassium hydroxide required to saponify the esters.
The Ester Value (EV) = 28.05 \( (B-V)/m \)

Where \( B = \) volume, in milliliters of 0.5 M hydrochloric acid required for the blank, \( V = \) volume in milliliters of 0.5 M hydrochloric acid required to neutralize the excess of the alkali after hydrolysis, \( m = \) mass in grams of the oil taken.

### 3.13.4.3. Saponification value

Saponification value is defined as the number of milligrams of KOH required to Saponify (Hydrolyse) ester present in 1 g of oil. 1.5 ml of essential oil was added to 10 ml of 0.5 N alcoholic sodium hydroxide in to a 100 ml saponification flask. A glass air, cooled condenser (1.0 meter in length and 1.0 cm diameter) was attached to it. The mixture was refluxed for 1 h on a water bath and then allowed to cool down to room temperature. The contents were then tritrated against 0.5N aqueous hydrochloric acid using 0.2 ml of 1% phenolphthalein solution as the indicator. Another set, without oil was run parallel to the treatment sets and the differences in the amounts of acids used in titrating the treatment and the set without oil gave the amount of acid consumed for the determination of saponification number of the oil. The saponification number was calculated using the following formula

\[
\text{Saponification number} = \frac{\text{Volume of 0.5 N acid consumed} \times 28.05}{\text{weight of 1ml essential oil}}
\]

### 3.13.4.4. Test for total phenolic content

One ml of the oil was taken in sterilized beaker containing 1 ml of 95% ethanol and three drops of alcoholic ferric chloride (FeCl\(_3\)) solution were added to it. The appearance of brown colour indicated the presence of phenols in the oil.
3.13.4.5. **Solubility in ethanol, acetone, solvent ether, methanol and carbon tetra chloride and water**

A 10 ml glass stoppered cylinder (Calibrated to 0.1 ml) was taken. One ml of the oil was introduced in the cylinder followed by adding ethanol drop by drop and shaking the cylinder after each addition. Appearance of a clear solution indicated the solubility of the oil in the alcohol. Likewise, miscibility of the oil with acetone, solvent ether, ethanol, methanol, carbon tetra chloride and water was also determined.

3.13.4.6. **pH and colour**

pH of oil was calculated using pH meter. The colour of the oil was also observed and was found light yellow.

3.14. **GC-MS analysis of essential oil**

Gas Chromatographic analysis followed by mass spectra was carried out in Perkin Elmer Autosystem XL Packed mode. Column used for analysis was OV-1, 100% Methyl gum (10 feet). The conditions were as follows: Temperature programming from 4°C-220°C, hold at 75°C for 20 minute. Injection temperature 250°C and detector temperature was 255°C. Carrier gas was N₂ at a flow rate 14 ml/min.

The identification of individual compound is based on their retention time’s relatives to those of authentic samples and matching spectral beaks available with NIST mass spectral libraries.
3.15. *In vitro* efficacy of essential oil treatments and antifungal activity measurements against Target pathogens

### 3.15.1 Poisoned food technique (PF)

The fungi-toxicity of the oil was evaluated against the test fungi by the poisoned food technique of Grover and Moore (1962). PDA (20ml) was poured into sterilized Petri dishes and measured amount of oil was added to give desired concentrations 25 ppm to 1000 ppm. In medium 0.05 % Tween-80 was also added for even distribution of the oil in the medium. For control sets, the medium was supplemented with the same amount of distilled water instead of oil and 0.05 % Tween-80. Plates were incubated at 25±1°C. The growth of the test fungi were recorded for seven days and percent inhibition was computed after comparison with the control.

Fungitoxicity was expressed in terms of percentage of mycelia growth inhibition and calculated as per formula of Pandey *et al.* (1982).

\[
\text{Percentage of mycelial growth inhibition} = \left( \frac{dc - dt}{dc} \right) \times 100
\]

Where \( dc \) = Average diameter of fungal colony in control

\( dt \) = Average diameter of fungal colony in treatment

### 3.15.2. Volatile activity assay (VA)

Tests for the volatile activity of oil were carried out by inverted Petri plate method in the 90mm Petri plates (Borosil) containing 20 ml of solidified PDA. A 5 mm diameter disc of the test fungus, cut from the periphery of an actively growing culture, was placed on the agar in each Petri plate and the plates were kept in inverted position.
Sterilized cotton swab was placed on upper lid of each inverted plates. Different concentrations (25 ppm to 1000 ppm) of oil were pipetted on cotton swab and were sealed by parafilm to check the release of volatile oil. For each corresponding control equal amount of water was pipetted on the sterilized cotton swab. The dose of essential oil was calculated as ppm volatile present in air in the Petri plate [Total volume of 90 mm Petri plate was 95.46 cm$^3$, in this 20 ml (20 cm$^3$) PDA was added so the volume of air left was 75.46 cm$^3$]. The inverted Petri plates were incubated at 25±1°C for 7 days. Fungitoxicity was expressed in terms of percentage of mycelia growth inhibition and calculated as per formula of Pandey et al. (1982a).

Percentage of mycelial growth inhibition =\[\frac{(dc-dt)}{dc}\times100\],

where dc= Average diameter of fungal colony in control and dt = Average diameter of fungal colony in treatment.

### 3.15.3. Minimum inhibitory concentration (MIC)

The minimum concentration of oil needed to inhibit the mycelial growth completely of the test fungi was determined by different assays. MIC was found different for different assays.

### 3.15.4. Determination of mycelial weight

To determine the effect of essential oil on the dry weight of the test fungus different concentrations of oil in Potato Dextrose Broth (PDB) medium were prepared in Erlenmeyer flask and inoculated with $10^6$ spores /ml of target pathogens. Spore population was counted using haemocytometer. In the corresponding control equal
amount of distilled water was added. After fifteen days dry weight of mycelium was
determined. Flasks containing mycelia were filtered through Whatman filter no. 1 and
then washed with distilled water. The mycelia were allowed to dry at 60° C for 6 hr
and then at 40° C over night. The filter paper containing dry mycelia were weighed.
Percent growth inhibition on the basis of dry weight was calculated as:

\[
\text{Percent growth inhibition} = \left( \frac{\text{Control weight} - \text{Sample weight}}{\text{Control weight}} \right) \times 100.
\]

3.15.5. Spore germination assay

Thirteen concentrations of oil (25 ppm to 1000 ppm) were tested for conidial germination of the test fungus. Fungal conidia obtained from 10-day-old cultures were taken and placed on glass slides in triplicate. Slides containing the conidia were incubated in a moist chamber at 25±1° C for 24 hrs. Each slide was then fixed in lactophenol-cotton blue and observed under the microscope for conidial germination. About 200 conidia were counted and the number of conidia germinated was scored using haemocytometer to calculate the percentage of conidial germination (Surender et al. 1987). The conidium was found considered germinated when the length of germ tube was apparently equal to that of conidia.

3.15.6. Nature of toxicity of essential oil

The fungitoxicity of a substance against a fungus may be temporary (fungistatic) allowing the latter to regain its vitality when the former is removed from its vicinity or the toxicity of the substance may be permanent (fungicidal) not allowing the fungus to regain its vitality even if the former is removed from the vicinity of latter. The fungitoxicity (fungistatic / fungicidal) of the essential oil was tested by using the
technique of Thompson (1989). Experiments were carried out to ascertain the nature of toxicity of oil at its MIC and hyper MIC doses by poisoned food technique and volatile activity assay. On the 7th day the inhibited fungal discs of treatment sets were taken out, washed with sterilized water and reinoculated, separately in petriplates containig the fresh medium. The plates were incubated similarly and the observation for the revival of growth of the reinoculated fungal discs was recorded on the 7th day as presence or absence of the mycelial growth.

3.15.7. Effect of temperature and autoclaving on toxicity of oil

Experiments were performed to determine the thermostable or thermolabile nature of the oil. Different glass vials containing five ml oil each were subjected to different temperature treatments for three hours in incubators already adjusted to 40, 60, 80 and 100°C. Antifungal activity of oil was also tested after autoclaving it at 121°C for 15 min. The glass vials were then allowed to cool down to room temperature and the fungitoxicity of the treated oil from each set was tested at its MIC against the test fungus using the poisoned food technique and volatile activity assay.

3.15.8 Effect of storage or self-life of the oil

Experiments were undertaken to ascertain the duration for which the oil can be stored without losing its fungitoxicity. Five ml of essential oil was stored in an air tight glass vial at room temperatures (20°C to 38°C ± 2°C). The fungitoxicity of the stored oil at its MIC was tested at regular intervals of 2 months using the poisoned food technique and volatile activity assay.

3.16. Mechanism of toxicity of essential oil of Z. officinale Roscoe. oil
3.16.1 Light microscopy

A sample of mycelium was taken from the periphery of the colony grown on PDA with 25, 50, 100, 200 and 500 ppm treatments of oil after four days of incubation. The samples were fixed in lacto-phenol-cotton blue and examined under the microscope (Nikon ECLIPSE E200, Japan) at 40-100 X to examine structural abnormalities. Samples from control plates without oil were also stained and observed. Photographs were taken with the help of computer attached Samsung COLOR CAMERA SAC-410PA.

3.16.2 Scanning electron microscopy

Fifteen day old fungal cultures on PDA treated concentrations 25ppm, 50ppm, 100ppm, 200ppm, 500ppm of oil was used for all scanning electron microscopic (SEM) observations. 5x10mm segments were cut from cultures growing on potato dextrose plates and promptly placed in vials containing 3% glutaraldehyde in 0.05M phosphate buffer (pH 6.8) at 4°C.

Samples were kept in this solution for 48 h for fixation and then washed with distilled water three times for 20 min each. Following which they were dehydrated in an ethanol series (30%, 50%, 70%, and 95%), for 20 min in each alcohol dilution and finally with absolute ethanol for 45 min. Samples were then critical point dried in liquid carbon dioxide. Fungal segments were placed in desiccators until further use. Following drying, samples prepared were mounted on standard 1/2 in Cambridge SEM stubs using double-stick adhesive tabs and coated with gold-palladium electroplating (60 s, 1.8 mA, 2.4 kV) in a Polaron SEM Coating System sputter coater. All samples were viewed in a Cambridge LEO S-430 SEM operating at 15 kV at various levels of magnification (300X to 5 k X).
3.17. Effect of increased inoculum on fungitoxicity of essential oil

The effect of increased inoculum density of the test fungus on the fungitoxicity of the essential oil was studied by the poisoned food technique using potato dextrose broth medium. The inoculum density in the treatment and the control sets was increased by (1) increasing the number of inoculum discs in arithmetical progression of 5 mm up to a maximum of 14 discs, (2) increasing the diameter of disc in arithmetical progression of 5 mm up to a maximum diameter of 25 mm and (3) increasing the number of conidia in the broth medium from $2 \times 10^6$ spores to $14 \times 10^6$ in arithmetical progression. The treatment and control assay plates with increased inoculum were incubated and observed on the 7th day of incubation for the presence or absence of growth of the test fungus.

3.18. *In vivo* investigation of the oil in the form of fungicidal spray:

The study was designed to observe the activity of the oil in the form of fungicidal spray applied on fruit surface for manage the rot of apple and tomato by different methods given by Shahi *et al.*, 2013 with slight modifications.

For *in vivo* study, both pre and post inoculation treatments (fungicidal fumes) were applied to the fruits. In the pre inoculation treatment, two set were prepared treatments as well as controls.

*In vivo* experiments for postharvest storage were performed on the apparently disease free tomato were obtained from the garden of Botany Department (Lucknow University). Untreated apples were purchased from an orchard of Jammu (India) Uniform apple fruits, in size and free from physical damage or fungal infection, were harvested at $\frac{3}{4}$ surface color stage, packed in field box and transported to the lab. Fruits were stored overnight at 0°C.
Apples and tomatoes were surface sterilized for 5 min in 4% (w/v) Sodium hypochlorite and washed three times with sterile water before experiment. In the pre inoculation treatment, two set were prepared treatments as well as controls. In treatment set fruits were sprayed in known tolerable concentrations (200-600ppm/ml) of oil. Before treatments of essential oil, apples and tomatoes were wounded by pin pricking on different sides (approximate 5-10 per fruit, each of 1 mm depth using pin 0.5 mm in diameter tapering to points) and were inoculated by dipping fruits and vegetables in conidial suspension (10^6 conidia ml^-1) of pathogen *A. mali* and *A. alternata* for 5 min and incubated at 25 ±2°C for 24 hr. There were three replicates of each treatment and experiment was repeated twice.

For treatment with essential oil, 50 pre-inoculated apples and tomatoes were placed on a grid in plastic trays (dimensions 45 × 30 × 16 cm). Under the grid, two glass Petri-dishes were placed, each holding definite amount of oil as per concentration of 200, 400, 500 and 600 ppm of the volatile oil. The trays were sealed with cellophane sheet to obtain conditions which promote fungal growth (high relative humidity) and stored in the dark at 25±2°C. The volatiles were allowed to diffuse into the atmosphere in which the fruits were kept. After a storage period of one or two weeks in the presence of the volatiles, the trays were opened and the apples and tomatoes were scored for fungal infection. Corresponding control fruits in absence of oil were placed in trays like treated fruits and vegetables. Subsequently, the fruits and vegetables were stored at 4°C in refrigerator in the absence of the essential oil.

In post inoculation treatment, apple and tomato fruits were first wounded with a sterilized needle and spore suspensions of *Alternaria mali* and *Alternaria alternata* were placed over the wounded areas. After 24h of incubation, samples were kept in separate
trays fumed with different concentrations (500ppm/ml) of oil preparation separately. In controls, fruits were sprayed with distilled water. Inoculated fruits were incubated at 20±2°C and the observations were recorded on seventh day. The data were average of 5 replicates and repeated twice.

After pre and post treatment of essential oil, population dynamics of pathogen spores in fruits and vegetables were calculated in terms of $\log_{10} (\text{CFU/g})$.

3.19. **In-vitro comparison of the Zingiber officinale Roscoe. oil and antagonistic yeast with selected fungicides:**

The efficacy of essential oil of Z. officinale Roscoe. and yeast (R. phylloplana, $10^8$ CFU ml$^{-1}$) were compared with that of prevalent fungicides namely: Bavistin, Blitox, Captaf, Indofil, and Ridomil. For essential oil and all fungicides concentration of 500 ppm was selected and at this ppm percent inhibition of test fungus was evaluated using volatile activity assay for essential oil and poisoned food technique for fungicides. The concentration of fungicides was calculated with respect to their active ingredient.

3.20. **Quality parameters**

To evaluate the effect of yeast antagonist and essential oil both on postharvest quality of pears, fruit were treated with these treatments, Quality parameters were measured after storage. Quality measurements were made on three replicates of three fruit each, and performed at ambient temperatures at 4°C and 20°C.

The testing methods used are described below.

- *Mass loss*
The mass of the fruit was measured by Shimadzu electric balance (±0.01 g) before treatment (A) and after storage (B), respectively, and the mass loss was calculated Therefore fruits were weighed at the beginning of the coating with antagonist and essential oil and then weight after storage of the fruits.

- **General appearance:**

General appearance was obtained by submitting samples to 5-member panel experienced in judging sensory analysis of vegetables. Samples were identified with random numbers and arranged on individual plates. Each sample was rated using score system as follows: 9 = excellent, 7 = good, 5 = fair, 3 = poor and 1 = unsalable) as described by Kader et al. (1973). This scale describes fresh appearance, fresh calyx, change of color and 2 decay.

- **Soluble solid content (SSC)**

SSC was measured by an Erma, Japan (0–32%) hand refractometer. The results have been represented in °Brix.

- **Titrable acidity**

Acidity was determined in fruit tissue extract. 10g of fruit tissue were ground in a pestle and mortar using 30 ml deionised water. The fruit juice was then suspended with H₂O upto a volume of 100 ml. 25 ml of fruit extract was titrated with 0.1 N NaOH to an end point of pH 8.1(AOAC, 1980). Titrable acidity is expressed as mg of citric acid per 100 g fruit tissue.

\[
\text{Percent titrable acidity} = \frac{T \times N \times 9.2 \times \text{Eq.wt} \times 50\text{ml} \times 100}{W \times V \times s \times 1000}
\]

\[
T = \text{Titre value}
\]
Ascorbic acid concentration was estimated by the visual titration method based on the reduction of 2, 6-dichlorophenol indophenol dye (Mahadevan & Sridhar 1980). 2.0g tissue was ground in distilled water and final volume was made to 50 ml. 25 ml of the extract was pipetted to a flask and titrated against the 2,6-dichlorophenol indophenol reagent (standardized against 0.1 N oxalic acid) until the solution became pink and the colour persisted for at least 15 second. The ascorbic acid content of the extract is expressed as mg g⁻¹ fresh weight and was calculated by using the following formula:

\[
\frac{1 \times S \times D \times A \times 100}{W}
\]

- \( S \) = mg of ascorbic acid reacting with 1ml of the reagent
- \( D \) = volume of the extract in ml
- \( A \) = the aliquot titrated in ml
- \( W \) = the weight of the sample in g
• **Analysis of total phenolic content**

The spectrophotometric determination method with Folin-Ciocalteu agent was used for determination of total phenol content. Pipetting out 1ml of the extract in a test tube and 1ml of Folin-Ciocalteu reagent followed by 2ml of Na$_2$CO$_3$ 20% solution. Shaken test tubes were heated in boiling water bath for exactly 1 minute. After that test tubes were cooled under a running tap. The blue solutions were diluted up to 25 ml with water and absorbance was measured at 650 nm in spectrophotometer. If there is any precipitate form, should be filtered or centrifuged the solution at 2000 rpm for 15 minute before measuring the absorbance. A standard curve was made from different concentrations of gallic acid. A blank containing all the reagents minus tissue extract should be used to adjust the absorbance to zero (Mahadeven & Sridhar, 1980).
RESULTS
4. RESULTS

The investigations on rot diseases of apple and tomato caused by *A. mali* and *A. alternata* carried out during 2009-2010, 2010-2011, 2011-2012, 2012-2013 and the results thus obtained are presented below:

4.1. Survey for incidence of postharvest diseases of apple and tomato and collection of diseased specimen

A comprehensive survey was conducted to assess the losses of apple and tomato caused by postharvest pathogens. In Lucknow, the selected five locations *viz.* Nishatganj, Raidas lane, Kapoorthala, Aliganj, Daliganj and Bhootnath were surveyed every month. Fruit and vegetable market of Bahraich and Shravasti (district of Tarai belt) were also considered as survey spot during the study. It was found that markets of Bhootnath and Bahraich were severely infected with different fungal rots (34.66% and 33% respectively) followed by Shravasti (32.66%), Daliganj (28.33%), Aliganj (26.33%), Nishatganj (25.75%), Raidaslane (15.27%), and Kapoorthala (13.66%) in case of tomatoes. With reference to apple, Shravasti and Bahraich were severely infected (25.83% and 19.27% respectively) followed by Raidaslane (17.22%), Aliganj (17.10%), Bhootnath (17.02%), Nishatganj (15.95%), Kapoorthala (14.4%) and Daliganj (10.33%) (Figure 1).
Figure 1: Percentage decay of apple and tomato by post harvest pathogens in different market spots.
4.2 Isolation, purification, identification and disease incidence of pathogens

The isolation and purification of fruit rot pathogens was carried out as per the method described in materials and methods. The pathogens, which were responsible for diseases on apple and tomato observed during survey, and the symptoms they produced as well as their cultural characteristics, they have been presented in Plate 1a, 1b, 2, 3a, 3b and 4. The effect of different seasons (2009-2013) on fungal pathogens isolated from apple and tomato fruits during survey were also studied and are presented in Table 4, 5 and Plate 1a, 1b, 2, 3a, 3b and 4. The results demonstrate that A. mali, P. expansum and A. niger were reported to be of maximum occurrence (Plate 1a, 1b, 2) during the survey period of apple which extended from September to May (Table 4).

Table 4: Disease incidence (%) of apple fruit from September to May (2009-2013)

<table>
<thead>
<tr>
<th>Months*</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
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<td>33</td>
<td>30</td>
<td>24</td>
<td>23</td>
<td>27</td>
<td>33</td>
<td>39</td>
<td>40</td>
</tr>
<tr>
<td>Min.</td>
<td>25</td>
<td>20</td>
<td>14</td>
<td>12</td>
<td>8</td>
<td>11</td>
<td>16</td>
<td>21</td>
<td>25</td>
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<tr>
<td>Humidity (%)</td>
<td>88</td>
<td>86</td>
<td>89</td>
<td>92</td>
<td>94</td>
<td>75</td>
<td>80</td>
<td>62.5</td>
<td>57</td>
</tr>
<tr>
<td>Rainfall (mm)</td>
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<td>28</td>
<td>10</td>
<td>14.3</td>
<td>15.3</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>SN</td>
<td>Pathogens</td>
<td>Disease Incidence** (%)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>A. mali Roberts</td>
<td>7.5 10 11 22.1 12.5 14.7 11.5 8.5 6.5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>A. niger Van Tiegh</td>
<td>11.7 12.8 5.6 1.7 1.0 0.6 0.9 1.3 1.0</td>
<td></td>
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<tr>
<td>3</td>
<td>P. expansum Link.</td>
<td>12.8 14.9 20.7 20.9 6.7 0.3 1.8 2.8 5.0</td>
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<tr>
<td>4</td>
<td>M. piriformis Fisher.</td>
<td>2.8 6.7 7.0 2.0 1.0 1.9 0.9 0.6 8.2</td>
<td></td>
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<tr>
<td>5</td>
<td>R. stolonifer (Ehr. ex Fr.) Lind</td>
<td>4.0 2.8 0.9 1.0 0.5 0.9 0.8 0.7 1.2</td>
<td></td>
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<tr>
<td>6</td>
<td>Trichoderma sp.</td>
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<tr>
<td>7</td>
<td>T. roseum Lin. Ex. Fr</td>
<td>2.9 1.0 1.0 0 0 0 0 0 0</td>
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Table 5: Disease Incidence (%) of tomato

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<td>Humidity (%)</td>
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<td>Rainfall (mm)</td>
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<td>107.1</td>
<td>236.9</td>
<td>196</td>
<td>211</td>
<td>28</td>
<td>10</td>
<td>14.3</td>
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<tr>
<td>SN</td>
<td>Pathogens</td>
<td>Disease Incidence** (%)</td>
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<tr>
<td>1.</td>
<td><em>A. alternata</em> (Fr.) Keissler</td>
<td>25</td>
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<td>2.</td>
<td><em>A. niger</em> Van Tiegh.</td>
<td>1.0</td>
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<td>3.</td>
<td><em>H. carposaporum</em></td>
<td>3.4</td>
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<td><em>P. italicum</em> Wehmer</td>
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<td>6.</td>
<td><em>P. destructiva</em> Plowright</td>
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<td>7.</td>
<td><em>M. piriformis</em> Scop.</td>
<td>1.0</td>
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<tr>
<td>8.</td>
<td><em>R. stoloniwer</em> (Fr.) Ehr.</td>
<td>0.5</td>
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<tr>
<td>9.</td>
<td><em>S. rolfsii</em></td>
<td>0.4</td>
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<td>10.</td>
<td><em>T. roseum</em> (Pers.) Link</td>
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*The meteorological data of Lucknow city has been taken from the website of World Weather Online.

* *The values are mean of four years viz., 2009-2010, 2010-11, 2011-12 and 2012-2013.
PLATE – 1a

PLATE -1: Symptom, culture plate and microphotograph of *Rhizopus stolonifer* (A, B, C); *Penicillium expansum* (D, E, F); *Aspergillus niger* (G, H, I); *Mucor piriformis* (J, K, L).
PLATE – 1b: Symptom, culture plate and microphotograph of *Rhizoctonia bataticola* (M, N, O); *Trichoderma* sp. (P, Q, R); *Trichotheceum roseum* (S, T, U)
PLATE – 2

PLATE -2: Fig: A, Fig: B. Symptoms of alternaria rot on apple, Fig: C. Culture plate and Fig: D. Microphotograph of A. mali
PLATE – 3a: Symptom, culture plate and microphotograph of *Fusarium roseum* (A, B, C); *Phoma destructiva* (D, E, F); *Helminthosporium carposaprum* (G, H, I); *Trichothecium roseum* (J, K, L); *Geotrichum candidum* (M, N, O)
PLATE – 3b: Symptom, culture plate and microphotograph of Aspergillus niger (A, B, C); Mucor piriformis (D, E, F); Rhizopus stolonifer (G, H, I); Penicillium italicum (J, K, L)
PLATE 4

PLATE 4: Fig: A, B. Symptoms of Alternaria rot on tomato, Fig: C. Culture plate & Fig: D. Microphotograph of Alternaria alternata
Tomato berries were found to be infected with *A. alternata*, *A. niger*, *Fusarium roseum*, *Geotrichum candidum*, *Phoma destructiva*, *Rhizopus stolonifer*, *T. viride* and *Trichothecium roseum* (Plate 3a, 3b, 4) throughout the year (2009-2013), however, *Cladosporium fulvum* and *Penicillium italicum* (Plate 3b) were not frequent (Table 5).

The average disease incidence (2009-2013) of apple and tomato was found to be maximum for *Alternaria* disease. The average loss in tomato due to *A. alternata* was maximum (25.0%) in month of January. In case of *Alternaria* rot of apple, disease incidence was calculated as 22.1% followed by blue mold rot (20.7%) in month of December.

Therefore, *A. alternata* and *A. mali* were selected as test organism for further studies (Plate 2; Plate 4).

**4.3. Morphological characters of test pathogens:**

**4.3.1. Alternaria rot of apple (A. mali Roberts):**

Moldy-core on tomato is characterized by inconspicuous, circular, black spots, sometimes only 1–2 mm in diameter, on the carposphere. The growth of the hyphae can be seen within the locules, with or without penetration into the mesoderm.

The colonies developed on PDA plates incubated at 25±2 °C. Gray to almost black mycelium developed within 3 to 4 days (Plate 2; Fig C). Dark brown conidiophores were formed singly or in small groups, short, less than 95 μm long and 3 μm wide, sometimes flexuous and occasionally geniculate. The obclavate conidia were brown to black, pyriform, 21.8–36.0 μm long and 8.55–17.0 μm wide, with three to four transverse septae and one to three longitudinal or oblique septae and a short conical beak (Plate 2; Fig D). Chains of conidia three to nine spores long were sometimes branched, appearing as bushy heads.
4.3.2. Alternaria rot of tomato [A. alternata (Fr.) Keissler]

The lesions frequently developed near the stem-end or alternatively at the blossom-end of the fruit. The infected tissue initiated decaying and decayed tissue was firm, dry, and brown to black in colour. The decaying extended deep into the fruit. Internal cavities were formed and were lined with dark gray mould. Humid conditions favoured pathogen growth on the surface of the fruit (Plate 4; Fig A).

Dark olive-green to black colonies on PDA developed. The fertile hyphae were well developed, articulate, 3-5µm in diameter and showed recemose branching. Conidia arising from conidiophores were distinctly obclavate, oblong, lageniform, black-green. The size of conidia was variable with maximum length in ranging 10-50µm. It was 3-7 septate and muriform (Plate 4; Fig C, D).

4.4. Pathogenicity test

Pathogenicity test showed severe decay of apple and tomato inoculated with their respective pathogens under laboratory conditions. On the infected fruits, fungal growth on fruit surface was clearly visible. These symptoms were similar to those observed under natural condition. The fruits inoculated with distilled water (control) remained free from infection. Re-isolations confirmed Koch’s postulates for pathogenicity. In apples inoculated with A. mali, (Plate 5, Fig A) symptoms appeared after 72 h as small brown lesions which gradually increase in size. Similarly, the initial symptoms of Alternaria rot on tomato was also appeared after 72h of inoculation as small light brown spots, which also increased in size. Later on, fungal mycelium of A. alternata grew on surface as dark greenish colonies (Plate 5, Fig B).
PLATE - 5

PLATE - 5: Fig (A) Effect of different inoculum concentrations of *A. mali* on disease severity in apple; Fig (B) Effect of different inoculum concentrations of *A. alternata* on disease severity in tomato, treated with $10^2$, $10^4$, $10^6$ and $10^8$ spores ml$^{-1}$. 
4.5. **Effect of inoculum concentrations of the pathogen**

The concentration of inoculum spore load affected the disease severity. As evident (Table 6; Plate 5), it was found that as the concentration of spore/conidia increased, the severity of disease also increased. At the lower concentration $10^2$ spores ml$^{-1}$, the lesion diameter was 5.5 and 8.6 mm whereas at higher concentration $10^8$ spores ml$^{-1}$, it was 12.33mm and 20.33mm for *A. mali* and *A. alternata* respectively.

**Table 6:** Effect of inoculum concentrations of *A. mali* and *A. alternata* on severity of infection (Lesion diameter) on apple and tomato fruits, experimental setup was incubated at 22±2°C for 18 days.

<table>
<thead>
<tr>
<th>SN</th>
<th>Inoculum concentration (Spore ml$^{-1}$)</th>
<th>Lesion diameter (mm)</th>
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<tr>
<td></td>
<td></td>
<td><em>A. mali</em></td>
<td><em>A. alternata</em></td>
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<tr>
<td>1</td>
<td>$10^2$</td>
<td>5.5±1.20</td>
<td>8.6±2.40</td>
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<tr>
<td>2</td>
<td>$10^4$</td>
<td>6.1±1.52</td>
<td>12±2.08</td>
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<tr>
<td>3</td>
<td>$10^6$</td>
<td>8±3.05</td>
<td>19.67±1.67</td>
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</tr>
<tr>
<td>4</td>
<td>$10^8$</td>
<td>12.33±2.9</td>
<td>20.33±1.67</td>
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</tbody>
</table>

Each value is mean of three replicates and ± SE are given along the mean values in case of lesion diameter

4.5. **In vitro studies**

4.5.1 **Growth of pathogens on different medium**

*A. mali* and *A. alternata* were grown on seven different media in order to find the best medium suitable for the growth of pathogens (Table 7, 8; Plate 6, 7). Out of seven medium selected for the growth of the test pathogen, Potato dextrose agar (PDA) medium
was found to be the best medium as the radial growth of *A. mali* on PDA was maximum (80.0 mm). The radial growth of *A. mali* on Malt yeast extract agar (73 mm) followed by Czapek’s Dox agar (63.3mm), Sabouraud Dextrose agar (53mm), Asthana & Hawker agar medium (41.66mm), Richard Medium (31.67 mm). Minimum radial growth was recorded in Host extract agar (25.0 mm). Potato Dextrose Agar also supported the growth of *A. alternata*, (74mm) followed by Malt yeast extract agar (69.67mm), Sabouraud agar (62.67mm), Czapek’s Dox agar (57mm), Asthana & Hawker agar medium (56.67), Host extract agar (44.33 mm). The least growth supporting medium was Richard’s agar (25.67mm). Thus, Potato Dextrose Agar medium was selected for the further studies of the test fungi (*Plate 6 Fig A; Table 7*).

Maximum dry weight of *A. mali* was obtained in Potato dextrose broth (421.66 mg) followed by Malt yeast extract broth (396.67 mg), Czapek’s Dox broth (360 mg), Sabouraud medium (298.33mg) Asthana & Hawkers broth medium (296.67 mg) and Richard’s broth (286.66 mg). While least in Host extract broth (205.67 mg). Best growth of *A. alternata* in terms of dry weight was recorded in Potato dextrose broth (410.67 mg) followed by Malt yeast extract broth (378.33 mg), Czapek’s Dox broth (347.67mg), Asthana and Hawkers broth (312.33), Sabouraud medium (303.67mg) and Host Extract Broth (297 mg) while lowest dry weight of *A. alternata* was recorded in Richard’s broth (198.0 mg) (*Table 8*).

**Table 7:** Effect of different media on radial growth of *Alternaria mali* and *Alternaria alternata* after 18 days at 25±2°C.

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<td><em>A. mali</em></td>
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<td>1.</td>
<td>Asthana &amp; Hawker (AHM)</td>
<td>41.66±1.7</td>
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<tr>
<td>2.</td>
<td>Czapek’s Dox agar (CzDA)</td>
<td>63.3±1.7</td>
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Results

<table>
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<tr>
<th>SN</th>
<th>Media</th>
<th>Dry weight(mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Host extract agar (HEA)</td>
<td>25±2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44.33±2.3</td>
</tr>
<tr>
<td>4</td>
<td>Malt yeast extract agar (MYEA)</td>
<td>73±3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69.67±0.33</td>
</tr>
<tr>
<td>5</td>
<td>Potato Dextrose agar (PDA)</td>
<td><strong>80±2.9</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>74±1.00</strong></td>
</tr>
<tr>
<td>6</td>
<td>Richard Medium (RM)</td>
<td>31.67±1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.67±0.33</td>
</tr>
<tr>
<td>7</td>
<td>Sabouraud Dextrose agar (SbA)</td>
<td>53±2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.67±1.33</td>
</tr>
</tbody>
</table>

Table 8: Effect of different media on dry weight of *Alternaria mali* and *A. alternata* after 18 days at 25±2°C.

<table>
<thead>
<tr>
<th>SN</th>
<th>Media</th>
<th>A. mali</th>
<th>A. alternata</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Asthana &amp; Hawker</td>
<td>296.67±3.33</td>
<td>312.33±1.45</td>
</tr>
<tr>
<td>2</td>
<td>Czapek’s Dox broth</td>
<td>360 ±2.89</td>
<td>347.67±1.15</td>
</tr>
<tr>
<td>3</td>
<td>Host extract</td>
<td>205.67±0.88</td>
<td>297±1.52</td>
</tr>
<tr>
<td>4</td>
<td>Malt yeast extract broth</td>
<td>396.67±3.33</td>
<td>378.33±1.66</td>
</tr>
<tr>
<td>5</td>
<td>Potato Dextrose broth</td>
<td><strong>421.66±1.66</strong></td>
<td><strong>410.67±0.67</strong></td>
</tr>
<tr>
<td>6</td>
<td>Richard’s medium</td>
<td>286.66±3.33</td>
<td>198±1.52</td>
</tr>
<tr>
<td>7</td>
<td>Sabouraud medium</td>
<td>298.33±3.33</td>
<td>303.67±0.89</td>
</tr>
</tbody>
</table>

Each value is the average of three replicates and ± SE are given along the mean values.

### 4.5.2 Growth of pathogens at different pH

Effect of different pH on growth of *A. mali* and *A. alternata* were studied and the results are presented in Table 9, 10 and Plate 6, 7 Fig B. Optimum growth of *A. mali* (83 mm) at pH 7.0 and *A. alternata* (80 mm) at pH 6.0 were obtained in terms of radial diameter. At pH 4 and 8 the radial growth of *A. mali* was 63.0 mm and 31.67 mm respectively and mycelial weight produced were 384.35 mg and 293.33 mg respectively. *A. alternata* grew well at pH 4.0 to 7.0. As the pH increased, growth was reduced and but not fully arrested at pH 8. At pH 4.0 and 8.0 the radial growth of *A. alternata* was 75.67 mm and 38.33 mm and biomass produced was recorded 386.67 mg and 251 mg (Table 10). The growth of
PLATE – 6: **Fig A.** Growth of *A. mali* on different media, **Fig B.** at different pH, **Fig C.** Growth at different temperature (°C).
PLATE – 7: **Fig A** Growth of *A. alternata* on different media, at different pH **Fig B**, at different temperature (°C) **Fig C**.
Alternaria spp. was sensitive to higher pH range. At very low and very high pH the growth of test fungus was slow and morphologically differed showing compact colonies having irregular margins. Best growth was observed at pH 6 (A. alternata) and pH 7 (A. mali).

Table 9: Effect of different pH on radial growth of Alternaria mali and Alternaria alternata after 18 days at 25±2°C.

<table>
<thead>
<tr>
<th>SN</th>
<th>pH</th>
<th>A. mali</th>
<th>A. alternata</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>63±1.52</td>
<td>75.67±0.33</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>62.67±1.85</td>
<td>75.33±1.20</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>82.33±1.45</td>
<td>80±0.58</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>83±1.52</td>
<td>78.33±0.33</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>31.67±0.88</td>
<td>38.33±1.67</td>
</tr>
</tbody>
</table>

Each value is the average of three replicates and ± SE are given along the mean values.

Table 10: effect of different pH on dry weight of A. mali and A.alternata after 18 days at 25±2°C

<table>
<thead>
<tr>
<th>SN</th>
<th>pH</th>
<th>A. mali</th>
<th>A. alternata</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>384.35±4.40</td>
<td>386.67±0.67</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>357.67±4.33</td>
<td>352.67±0.33</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>411.33±0.66</td>
<td>415±0.58</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>414±1.00</td>
<td>411.67±0.33</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>293.33±1.66</td>
<td>251±0.58</td>
</tr>
</tbody>
</table>

Each value is the average of three replicates and ± SE are given along the mean values.
4.5.3 Growth of pathogens at different temperatures

The test pathogens were grown at six different temperatures in order to find out the optimum temperature best suited for their growth. The optimum temperature for radial growth and bio mass of *A. mali* and *A. alternata* appeared to be between the range of 25 to 30°C (*Table 11, 12* and *Plate 6, 7, Fig C*). With decrease and increase in temperature, growth of *A. mali* and *A. alternata* were found to be reduced. It is evident from the results *Alternaria* spp. sensitive to low as well as high temperature.

Table 11: Effect of different temperatures on radial growth of *A. mali* and *A. alternata* after 18 days at 25±2°C.

<table>
<thead>
<tr>
<th>SN</th>
<th>Temperature (°C)</th>
<th>Radial growth(mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>A. mali</em></td>
</tr>
<tr>
<td>1.</td>
<td>10</td>
<td>23.33±0.88</td>
</tr>
<tr>
<td>2.</td>
<td>15</td>
<td>27.33±0.67</td>
</tr>
<tr>
<td>3.</td>
<td>20</td>
<td>51.67±0.88</td>
</tr>
<tr>
<td>4.</td>
<td>25</td>
<td>73±1.52</td>
</tr>
<tr>
<td>5.</td>
<td>30</td>
<td>76.67±0.33</td>
</tr>
<tr>
<td>6.</td>
<td>35</td>
<td>31.67±0.88</td>
</tr>
</tbody>
</table>

Table 12: Effect of different temperatures on dry weight of *A. mali* and *A. alternata* after 18 days at 25±2°C.

<table>
<thead>
<tr>
<th>SN</th>
<th>Temperature (°C)</th>
<th>Dry weight(mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>A. mali</em></td>
</tr>
<tr>
<td>1.</td>
<td>10</td>
<td>147.33±4.67</td>
</tr>
<tr>
<td>2.</td>
<td>15</td>
<td>167±1.52</td>
</tr>
<tr>
<td>3.</td>
<td>20</td>
<td>200.33±4.40</td>
</tr>
<tr>
<td>4.</td>
<td>25</td>
<td>315.33±0.33</td>
</tr>
<tr>
<td>5.</td>
<td>30</td>
<td>403.33±3.33</td>
</tr>
<tr>
<td>6.</td>
<td>35</td>
<td>293.33±1.66</td>
</tr>
</tbody>
</table>
4.6. Efficacy of biological control agents against pathogens

4.6.1. Primary screening of biological control agents against A. alternata and A. mali

The four biological control agents were selected, which demonstrated significant bioactivity against the pathogens of tomato and apple. The bio activity of three *C. utilis*, *D. hansenii* and *Saccharomyces cerevisiae* had already been proved in the laboratory (Sharma et al., 2006), however the activity of *R. phylloplana* was examined for the first time on apple and tomato (Table 13). The reduction in lesion diameter was maximum in case of *R. phylloplana* in tomato and apple fruits whereas *C. utilis* could arrest the growth of the pathogen at the wound site as compared to control significantly in apple and tomatoes but was less in comparison to *R. phylloplana*. The efficacy of *D. hansenii* was average in case of apple and tomato in controlling lesion diameter than *R. phylloplana* but less than the other two biocontrol agent. *S. cerevisiae* could not control the pathogen and more than 50% fruits decayed. Although the antagonists were effective in reducing the decay but *R. phylloplana* was found to be the best biocontrol agent. Thus *R. phylloplana* was selected as potential biocontrol agent for further investigations.

Table 13: Primary screening of biocontrol agents

<table>
<thead>
<tr>
<th>Biocontrol agents</th>
<th>Lesion diameter</th>
<th>Decay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AM</td>
</tr>
<tr>
<td><em>Rhodotorula phylloplana</em></td>
<td>03.0±0.000</td>
<td>03.0±0.000</td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td>07.0±0.096</td>
<td>07.0±0.096</td>
</tr>
<tr>
<td><em>Debaryomyces hansenii</em></td>
<td>12.0±0.181</td>
<td>25.0±0.119</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>40.0±0.040</td>
<td>Entire fruit</td>
</tr>
</tbody>
</table>
The values is mean of five replicates and represented as mean± SE
AA= Alternaria alternata, AM= Alternaria mali

4.6.2. Maintenance of antagonist

The antagonist used as biocontrol agent in experiments was *R. phylloplana* MTCC 2748 (Plate 8; Fig A, B, C, D, E, F). These cultures were grown in Malt Yeast Extract broth.

4.6.3. *In vitro* efficacy of *R. phylloplana*

4.6.3.1. Determination of cell concentration of bioagent for bioactivity

Dry weight of *A. mali* and *A. alternata* was reduced in broth inoculated with *R. phylloplana* as compared to control. Antagonist significantly reduced the dry weight of pathogens as compared to control. *In vitro* experimental set up was kept at 25±2°C for 15 days (*Table 14; Plate 9 Fig A, C*).

**Table 14: *In vitro* efficacy of yeast antagonist against *A. mali* and *A. alternata***

<table>
<thead>
<tr>
<th>Conc. of <em>R. phylloplana</em></th>
<th>Dry weight of mycelial mat (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. mali</em></td>
</tr>
<tr>
<td>Control</td>
<td>399.33±0.88</td>
</tr>
<tr>
<td>10^6 CFU ml^-1</td>
<td>193.67±2.18</td>
</tr>
<tr>
<td>10^8 CFU ml^-1</td>
<td>33±1.52</td>
</tr>
</tbody>
</table>

One way ANOVA was made, based on mycelia weight of *Alternaria* sp. Data were found significantly different at level of 5% significance. Values in the same column followed by different superscript’s letters are significantly different (P<0.05) according to Duncan’s Multiple Range Test. The values followed by same superscript letters are not statistically significant. Each value is mean of three replicates and ±SE are given along the mean values.

4.6.3.2. Efficacy of yeast against *A. mali* and *A. alternata* at different time interval

*R. phylloplana* inhibited growth of *A. mali* at 48 h whereas of *A. alternata* at 72 h in PDB after 7 day incubation at 25±2°C (*Table 15; Plate 10 fig A; Plate 11 fig A*).
PLATE – 8: Fig (A) Liquid culture (MYEB) of *Rhodotorula phylloplana*, (B) *R. phylloplana* on MYEA (Malt Yeast Extract Agar), (C) in culture tube, (D) in culture tube (enlarged), (E) Light Microscopy, Bar 10µm, magnification 100X (F) Scanning electron micrograph of *R. phylloplana* grown on MYEA, Bar 1µm, magnification 8 KX.
Table 15: Bioactivity of *R. phylloplana* against *A. mali* and *A. alternata* at different time interval

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Dry weight of mycelial mat (mg)</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. mali</em></td>
<td></td>
<td>345.02 ±1.00</td>
<td>104.03 ±0.66</td>
<td>0 d</td>
<td>0 d</td>
</tr>
<tr>
<td><em>A. alternata</em></td>
<td></td>
<td>340.92 ±0.33</td>
<td>195.00 ±0.33</td>
<td>100.07 ±1.00</td>
<td>0 d</td>
</tr>
</tbody>
</table>

One way ANOVA was applied, based on the mycelia weight of *Alternaria* sp. Data were found significantly different at level of 5% significance. Values in the same column followed by different superscript’s letters are significantly different (P<0.05) according to Duncan’s Multiple Range Test. The values followed by same superscript letters are not statistically significant. Each value is mean of three replicates and ±SE are given along the mean value.

4.6.4. In vivo efficacy of *R. phylloplana*

4.6.4.1. Efficacy of effective concentrations of yeast against target pathogens

*R. phylloplana* effectively inhibited the lesion diameter of *A. mali* and *A. alternata*. The lesion diameter in all treated commodities was significantly lower than control. The results showed that the higher the concentration of the antagonist, smaller the lesion diameter. In the treatment having CFU of $10^8$ ml$^{-1}$ the lesion diameter was 5.33 mm while lesion diameter of 34.33 mm was observed in untreated tomato fruits. Apple fruits treated with similar concentration of antagonist resulted in negligible growth as compared to 26 mm lesion in control. At $10^6$ CFU ml$^{-1}$ concentration lesion diameters were significantly reduced as compared to control in both the fruits challenged by their respective pathogens (Table 16; Plate 9; Fig B, D). *In vivo* experimental set up was kept at 22±2° C for 15 day.
**Determination of antagonist concentration:** Fig (A) *In vitro* and Fig (B) *In vivo* efficacy of yeast antagonist against *A. mali* at increasing concentration; Fig (C) *In vitro* and Fig (D) *In vivo* efficacy of yeast antagonist against *A. alternata* at increasing concentration.
Table 16: *In vivo* efficacy of yeast antagonist against *A. mali* and *A. alternata*

<table>
<thead>
<tr>
<th>Conc. of <em>R. phylloplana</em></th>
<th>Lesion diameter (mm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Alternaria mali</em></td>
<td><em>Alternaria alternata</em></td>
</tr>
<tr>
<td>Control</td>
<td>26±1.00</td>
<td>34.33±2.33</td>
</tr>
<tr>
<td>10⁶CFUml⁻¹</td>
<td>13±1.00</td>
<td>19.33±1.20</td>
</tr>
<tr>
<td>10⁸CFUml⁻¹</td>
<td>0ᵃ</td>
<td>5.33³±0.166</td>
</tr>
</tbody>
</table>

Each value is the average of three replicates and ± SE are given along the mean values. One way ANOVA was made, based on the lesion diameter of *Alternaria* sp. Data were found significantly different at level of 5% significance. Values in the same column followed by different superscript’s letters are significantly different (P<0.05) according to Duncan’s Multiple Range Test. The values followed by same superscript letters are not statistically significant. Each value is mean of three replicates and ±SE are given along the mean values.

### 4.6.4.2. Effect of inoculation time of antagonist and pathogen

Complete disease control was achieved by *R. phylloplana* at 48 h and 72 h in pre inoculation treatments of respective *A. alternata* and *A. mali* spore suspension. The biocontrol activity was significantly reduced when the antagonist was applied after the pathogen inoculation. The wounds were 100% infected when the pathogens were inoculated 24 h after wounding, the lesion diameter 12.33 mm for *A. mali* and 13.50 mm for *A. alternata* when the pathogens were inoculated 48 h after wounding, the lesion diameter 13.59 mm for *A. mali*, 14.70 mm for *A. alternata* after 8 day incubation at 22 ± 2°C. The lesion diameter were significantly lower for *R. phylloplana* pretreated apple and tomato when inoculated with *A. mali* and *A. alternata* at the duration of 24, 48 and 72 h. At 48 h, *A. mali* was completely controlled whereas *A. alternata* was controlled at 72 h. (Plate 10, 11; Fig B, C; Table 17).
Efficacy of yeast antagonist: Fig (A) *In vitro* and (B) *In vivo* efficacy of yeast antagonist against *A. mali* at regular time interval
Efficacy of yeast antagonist Fig (A) *In vitro* and (B) *In vivo* efficacy of yeast antagonist against *A. alternata* at regular time interval
Table 17: Effect of time of application of antagonist and pathogen

<table>
<thead>
<tr>
<th>Treatment time (h)</th>
<th>Lesion diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. mali</td>
</tr>
<tr>
<td></td>
<td>Pre</td>
</tr>
<tr>
<td>0</td>
<td>9.1±0.57</td>
</tr>
<tr>
<td>24</td>
<td>5.2±2.18</td>
</tr>
<tr>
<td>48</td>
<td>0a</td>
</tr>
<tr>
<td>72</td>
<td>0a</td>
</tr>
</tbody>
</table>

Each value is the average of three replicates and ± SE are given along the mean values. All values are means of three replications per treatment of three experiments. Values in each column followed by same superscript are not significantly different according to Duncan’s multiple range test (P< 0.05).

4.6.2.2.2. Effect of R. phylloplana on cellulase and pectinase in apple infected with A. mali and tomato infected with A. alternata

4.6.2.2.2.1. Cellulase activity

Cellulase was produced in high quantities in apple and tomato infected with A. mali and A. Alternata after 6 days were 314.33 and 353.33 mg/g fresh weight/h respectively (Figure 2). Cellulase activities were reduced in R. phylloplana yeast treated samples in comparison to infected apple (73.33 mg/g fresh weight/h) and tomato (73.33 mg/g fresh weight/h). It was found that in fresh (uninoculated) apple and tomato, cellulose produced in minute quantities 52.33 and 56.33 mg/g fresh weight/h.

4.6.2.2.2.2. Pectinase activity

Exo-polgacturonase (exo-PG) was assayed in apple and tomato infected with A. mali and A. alternata and treated with R. phylloplana. Data plotted in Figure 3, reveal that the pathogen produced exo-PG in infected fruit in relatively low quantities during first two
day infection. However, the enzyme was detected in large quantities after 6\textsuperscript{th} day of incubation 44.33 and 50 mg/g fresh weight/h in apple and tomato respectively.

4.7. Surface population of *Rhodotorula phylloplana*

Population dynamics of *R. phylloplana* with in fruit or on the fruit surface was evaluated. Tomatoes and apples were treated with *R. phylloplana* solution (10\textsuperscript{8} CFU ml\textsuperscript{-1}) by dip treatment method and were stored at 20°C for 7 days and surface population was quantified at interval of 24 days and results were shown in Table 18.

The population of *R. phylloplana* increased progressively to reach maximum population (Log\textsubscript{10} 9.150 CFU/g) after 168 h of storage in apple while population on tomato also increased after 48h (Log\textsubscript{10} 8.647/g) as compare to initial population (Log\textsubscript{10} 8.280 CFU/g) but after 48 h population increased progressively and reach maximum (Log\textsubscript{10} 9.045CFU/g) after 168 h.

Table 18: Surface population (Log\textsubscript{10}CFU/g) of *R. phylloplana* on apple and tomato fruits during storage at 20±2°C

<table>
<thead>
<tr>
<th>SN</th>
<th>Storage(h)</th>
<th>Apple(Log\textsubscript{10}CFU/g)</th>
<th>Tomato(Log\textsubscript{10}CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>24</td>
<td>8.424\textsuperscript{a}</td>
<td>8.280\textsuperscript{c}</td>
</tr>
<tr>
<td>2.</td>
<td>48</td>
<td>8.732\textsuperscript{b}</td>
<td>8.647\textsuperscript{a}</td>
</tr>
<tr>
<td>3.</td>
<td>72</td>
<td>8.828\textsuperscript{ac}</td>
<td>8.755\textsuperscript{d}</td>
</tr>
<tr>
<td>4.</td>
<td>96</td>
<td>8.831\textsuperscript{ab}</td>
<td>8.823\textsuperscript{a}</td>
</tr>
<tr>
<td>5.</td>
<td>120</td>
<td>9.017\textsuperscript{b}</td>
<td>8.935\textsuperscript{ac}</td>
</tr>
<tr>
<td>6.</td>
<td>144</td>
<td>9.096\textsuperscript{c}</td>
<td>8.993\textsuperscript{d}</td>
</tr>
<tr>
<td>7.</td>
<td>168</td>
<td>9.150\textsuperscript{d}</td>
<td>9.045\textsuperscript{c}</td>
</tr>
</tbody>
</table>

The effect of storage hour on population of yeast was subjected to analysis of variance using MS- excel sheet 2007. Data were found significantly different at level of 5% significance. Values followed by different superscript’s letters are significantly different (P<0.05) according to Duncan’s Multiple Range Test.
4.8. Study of fungal and yeast interaction

4.8.1. Light Microscopy

To investigate the mechanism behind yeast pathogen interaction light microscopy was used. The studies prepared from the dual cultures of the antagonist and target pathogens revealed that the yeast cells gathered around the hyphae of *A. mali* and *A. alternata* forming a sort of biofilm. However, tenacious attachment of yeast cells to the hyphal surface, despite extensive rinsing of samples with distilled water, could not be separated (Plate 12; fig D, & E).

4.8.2. Scanning Electron Microscopy

The observations (Plate 14; fig D) done after SEM revealed as if the cells of *R. phylloplana* have sunken into the hyphal wall. To enhance dislodgement of *R. phylloplana*, some samples were rinsed with 2% CaCl$_2$ prior to preparation. These treatments were mildly effective in dislodging the yeast from some areas of hyphae. When these areas of hyphae were observed from where yeast cells presumably had either become detached on their own were dislodged during sample preparation, irregularities in wall confirmation were noticed (Plate 13; Plate 14 fig E). Discrete areas of hyphae cell wall appeared concave, giving the hyphal strand a general appearance of being pitted (Plate 13; Plate 14 fig C, D). In some areas pitting was quite extensive and deep. Similar results have been seen in both cases *A. alternata* and *A. mali*. 
PLATE – 12: Mode of action: (A) Microphotograph of *R. phylloplana*, (B) *A. mali* (C) *A. alternata* (D) Light microscopy of dual culture of *R. phylloplana* with *A. mali* and (E) with *A. alternata*
PLATE – 13: Scanning electron micrographs of (A) antagonist *R. phylloplana* Bar 1µm, Mag = 8 KX (B) pathogen *A. mali*, Bar 3µm, Mag = 5 KX. Dual culture of antagonist and pathogen on PDA(C) Embedding of yeast into hyphal wall and (D) pitting of the surface of hyphal cell wall (arrow) is visible, Bar 10µm, Mag = 3 KX.
PLATE – 14: Scanning electron micrographs of (A) antagonist *R. phylloplana*, Bar 1µm, Mag = 8 KX (B) pathogen *A. alternata* grown on PDA, Bar 3µm, Mag = 5 KX, Dual culture of antagonist and pathogen on PDA (C) Embedding of yeast into hyphal wall and (D) pitting of the surface of hyphal cell wall (arrow) is visible, Bar 10µm, Mag = 2.12 KX (E) ruptured hyphal wall in comparison of smooth hyphae of pathogen (B).
4.9. Screening of some higher plant part for their fungitoxicity against test fungi

Essential oil was extracted from the 30 screened plant materials for the evaluation of the antifungal activity against the test fungus. Screening of fungitoxic potential was based on the ability of essential oils to inhibit the spore germination of the test fungus at 500 ppm concentration. It is evident from Table 19 that out of seven screened plants most of the species showed either poor (below 50%) or moderate (above 50% and below 100%) antifungal activity.

Among seven plant species Chenopodium album L. (30%, 27%) and Callistemon lanceolatus DC. (38%, 39%) showed poor antifungal activity whereas Coriandrum sativum L. (50%, 56%), Mentha arvensis L. (59%, 58%), Cymbopogon citrates (D.C.) Stapf. (75%, 77%), Tagetes minuta L.( 85%, 89%) exhibited strong fungitoxic activity but did not completely check the spore germination of the test fungi A. mali and A. alternata. Among all the screened plant part, rhizome of Z. officinale exhibited the absolute toxicity, inhibiting completely spore germination of A. mali and A. alternata at 500 ppm of Z. officinale essential oil, and therefore it was selected for the further detailed investigations. None of the plant species showed any stimulatory activity against the test fungi (Table 19).

4.10. Study of essential oil

4.10.1. Characterization of the essential oil

Various physico-chemical properties of oil are shown in Table 20. It is evident from the physico-chemical studies that the oil have acid number 8.7, saponification number 38.11
Table 19: Screening of essential oils from angiospermic plant part for their fungitoxicity against test fungi

<table>
<thead>
<tr>
<th>SN</th>
<th>Plant name</th>
<th>Local Name</th>
<th>Family</th>
<th>Plant Part used</th>
<th>Percent inhibition of spore germination at 500 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AM</td>
<td>AA</td>
</tr>
<tr>
<td>1.</td>
<td><em>Callistemon lanceolatus</em> DC.</td>
<td>Lal Bottle Brush</td>
<td>Myrataceae</td>
<td>Leaf</td>
<td>38</td>
</tr>
<tr>
<td>2.</td>
<td><em>Chenopodium album</em> L.</td>
<td>Bathua</td>
<td>Chenopodiaceae</td>
<td>Whole plant</td>
<td>30</td>
</tr>
<tr>
<td>3.</td>
<td><em>Coriandrum sativum</em> L.</td>
<td>Dhaniya</td>
<td>Umbelliferae</td>
<td>Whole plant</td>
<td>50</td>
</tr>
<tr>
<td>4.</td>
<td><em>Cymbopogon citrates</em> (D.C.) Stapf.</td>
<td>Neembu Ghas</td>
<td>Poaceae</td>
<td>Leaf</td>
<td>75</td>
</tr>
<tr>
<td>5.</td>
<td><em>Mentha arvensis</em> L.</td>
<td>Podina</td>
<td>Lamiaceae</td>
<td>Leaf</td>
<td>59</td>
</tr>
<tr>
<td>6.</td>
<td><em>Tagetes minuta</em> L.</td>
<td>Genda</td>
<td>Asteraceae</td>
<td>Leaf</td>
<td>85</td>
</tr>
<tr>
<td>7.</td>
<td><em>Zingiber officinale</em> Roscoe.</td>
<td>Adrak</td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
<td>100</td>
</tr>
</tbody>
</table>
and ester number was 33.56. There was no phenolic content. The pH was 3.4 and the
colour was golden yellow.

**Table 20: Properties of the essential oil of Zingiber officinale**

<table>
<thead>
<tr>
<th>Properties</th>
<th>Ginger oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil yield (% g/1000g)</td>
<td>0.204</td>
</tr>
<tr>
<td><strong>Organoleptic</strong></td>
<td></td>
</tr>
<tr>
<td>Taste</td>
<td>Pungent</td>
</tr>
<tr>
<td>Odour</td>
<td>Characteristic spicy smell</td>
</tr>
<tr>
<td>Colour</td>
<td>Golden Yellow</td>
</tr>
<tr>
<td>Appearance at room temperature (30°C)</td>
<td>Homogeneous, transparent liquid, lighter than water</td>
</tr>
<tr>
<td><strong>Solubility</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soluble in acetone, solvent ether, ethanol, methanol and CCl₄</td>
</tr>
<tr>
<td><strong>Chemical properties</strong></td>
<td></td>
</tr>
<tr>
<td>Acid value</td>
<td>8.7</td>
</tr>
<tr>
<td>Ester value</td>
<td>33.56</td>
</tr>
<tr>
<td>Saponification value</td>
<td>38.11</td>
</tr>
<tr>
<td>Phenolics</td>
<td>43.9µgGAE/ml</td>
</tr>
<tr>
<td>pH</td>
<td>3.4</td>
</tr>
</tbody>
</table>

**4.10.2. GC-MS analysis of Z. officinale Roscoe. essential oil and its components**

The chemical composition of essential oil of *Z. officinale* was elucidated employing GC-MS analysis. *Z. officinale* oil consisted of 16 compounds. The main monoterpenene (C₁₀H₁₆) compound was camphene which was 23.9% followed by sesquiterpene (C₁₅H₂₄) zingiberene 12.2%, whereas 1, 8- cineole reported as major component (27.9%) which was an oxygenated ether compound. Other hydrocarbons were α- pinene 7.2%, α-farnesene 1.1%, α- seliene 0.9%, ar- curcumene 0.8%, Camphor 0.4%, Tricyclene 0.1%. The main oxygenated compounds were Geranial 2.8, Nerolidol 1%, Neral 0.9%, Elemol 0.9 %, Zingiberenol 0.9% and Octanel 0.1%.
Table 21 various mass spectrums of different components of the essential oil of *Zingiber officinale* are shown in Plate (15) and Figure 4-14.

Figure 4: Gas chromatogram of *Zingiber officinale* essential oil
PLATE – 15: (A) Ginger (*Z. officinale* Roscoe.) rhizome, (B) Ginger oil (C) Clvenger’s type apparatus: Hydrodistillation unit
<table>
<thead>
<tr>
<th>SN</th>
<th>Retention time</th>
<th>Area</th>
<th>Compound</th>
<th>IUPAC name</th>
<th>Molecular weight</th>
<th>Molecular formula</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>4.580</td>
<td>0.09</td>
<td>Tricyclene</td>
<td>2,2,3-Trimethyl-3,5-cyclobicyclo[2.2.1]heptane</td>
<td>136.23</td>
<td>C_{10}H_{16}</td>
<td>0.1</td>
</tr>
<tr>
<td>2.</td>
<td>7.748</td>
<td>0.01</td>
<td>Octanel</td>
<td>Octanal</td>
<td>128</td>
<td>C_{8}H_{16}O</td>
<td>0.1</td>
</tr>
<tr>
<td>3.</td>
<td>19.241</td>
<td>0.09</td>
<td>Camphor</td>
<td>4,7,7-trimethylbicyclo[2.2.1]heptan-3-one</td>
<td>152</td>
<td>C_{10}H_{16}O</td>
<td>0.4</td>
</tr>
<tr>
<td>4.</td>
<td>21.237</td>
<td>7.96</td>
<td>α-pinene</td>
<td>4,6,6-trimethylbicyclo[3.1.1]hept-3-ene</td>
<td>136</td>
<td>C_{10}H_{16}</td>
<td>7.2</td>
</tr>
<tr>
<td>5.</td>
<td>22.087</td>
<td>31.40</td>
<td>Camphene</td>
<td>3,3-dimethyl-2-methylidenebicyclo[2.2.1]heptane</td>
<td>136</td>
<td>C_{10}H_{16}</td>
<td>23.9</td>
</tr>
<tr>
<td>6.</td>
<td>23.699</td>
<td>3.94</td>
<td>Geranial</td>
<td>(2E)-3,7-dimethylocta-2,6-dienal</td>
<td>152</td>
<td>C_{10}H_{16}O</td>
<td>2.8</td>
</tr>
<tr>
<td>7.</td>
<td>24.836</td>
<td>0.06</td>
<td>Neral</td>
<td>(2Z)-3,7-dimethylocta-2,6-dienal</td>
<td>152</td>
<td>C_{10}H_{16}O</td>
<td>0.9</td>
</tr>
<tr>
<td>8.</td>
<td>26.271</td>
<td>47.47</td>
<td>1,8- cineole</td>
<td>2,2,4-trimethyl-3-oxabicyclo[2.2.2]octane</td>
<td>154</td>
<td>C_{10}H_{16}O</td>
<td>27.9</td>
</tr>
<tr>
<td>9.</td>
<td>29.844</td>
<td>0.10</td>
<td>sabinene</td>
<td>4-methylidene-1-propan-2-ybicyclo[3.1.0]hexane</td>
<td>136</td>
<td>C_{10}H_{16}</td>
<td>0.05</td>
</tr>
<tr>
<td>10.</td>
<td>29.844</td>
<td>0.09</td>
<td>ar-curcumene</td>
<td>1-methyl-4-[(2R)-6-methylhept-5-en-2-yl]benzene</td>
<td>202</td>
<td>C_{15}H_{22}</td>
<td>0.8</td>
</tr>
<tr>
<td>11.</td>
<td>33.661</td>
<td>0.25</td>
<td>α-farnesene</td>
<td>(3E,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene</td>
<td>204</td>
<td>C_{15}H_{24}</td>
<td>1.1</td>
</tr>
<tr>
<td>12.</td>
<td>34.830</td>
<td>0.07</td>
<td>α-selinene</td>
<td>5,8a-dimethyl-3-prop-1-en-2-yl-2,3,4,4a,7,8-hexahydro-1H-naphthalene</td>
<td>204</td>
<td>C_{15}H_{24}</td>
<td>0.9</td>
</tr>
<tr>
<td>13.</td>
<td>36.707</td>
<td>5.81</td>
<td>α-Zingiberene</td>
<td>(5S)-2-methyl-5-[(2R)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene</td>
<td>204</td>
<td>C_{15}H_{24}</td>
<td>12.2</td>
</tr>
<tr>
<td>14.</td>
<td>38.027</td>
<td>0.46</td>
<td>Elemol</td>
<td>2-[(1R,3S,4S)-4-ethenyl-4-methyl-3-prop-1-en-2-ylcyclohexyl]propane-2-ol</td>
<td>222</td>
<td>C_{16}H_{26}O</td>
<td>0.9</td>
</tr>
<tr>
<td>15.</td>
<td>48.229</td>
<td>0.16</td>
<td>Zingiberenol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.9</td>
</tr>
<tr>
<td>16.</td>
<td>48.920</td>
<td>0.10</td>
<td>Nerolidol</td>
<td>(6E)-3,7,11-trimethyldodeca-1,6,10-trien-3-ol</td>
<td>222</td>
<td>C_{16}H_{26}O</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Mass Spectra of some important chemical components of Ginger essential oil

Figure: Mass spectra of (5) 1, 8-cineole, (6) camphor
Figure: Mass spectra of (7) camphene, (8) α- zingiberene
Figure: Mass spectra of (9) Sabiene, (10) Nerolidol
Figure: Mass spectra of (11) α-pinene, (12) α-farnesene
Figure: Mass spectra of (13) Geranial, (14) Neral
**Figure 2:** Effect of application of *R. phylloplana* on cellulase productivity in apple and tomato fruits infected with *A. mali* and *A. alternata* at 25±2°C for 6th day of incubation of samples (C1=fresh apple, P1= infected apple with *A. mali*, Y1= *R. phylloplana* treated apple, YP1= counterchallenged with *R. phylloplana + A. mali*, C2=fresh tomato, P2= infected tomato *A. alternata*, Y2= *R. phylloplana* treated apple, YP2= counterchallenged with *R. phylloplana + A. alternata*.)

**Figure 3:** Effect of application of *R. phylloplana* on pectinase productivity in apple and tomato fruits infected with *A. mali* and *A. alternata* at 25±2°C for 6th day of incubation of samples (C1=fresh apple, P1= infected apple, Y1= *R. phylloplana* treated apple, YP1= counterchallenged with *R. phylloplana + A. mali*, C2=fresh tomato, P2= infected tomato *A. alternata*, Y2= *R. phylloplana* treated apple, YP2= counterchallenged with *R. phylloplana + A. alternata*.)
4.11. *In vitro* efficacy of essential oil treatments and antimycotic activity against *A. mali* and *A. alternata*

4.11.1 *Poisoned food technique (PF), volatile activity assay (VA), nature of toxicity, minimum inhibitory concentration (MIC) and spore germination assay*

The fungitoxicity of the oil was measured by percent radial growth inhibition using PF and VA. In the PF 700 ppm oil was required to completely inhibit the growth of the fungus after 7 days of incubation. Radial growth of *A. alternata* and *A. mali* were significantly reduced in response to various concentrations of *Z. officinale* oil ranging from 25 ppm to 700 ppm. In PF at 900 ppm oil showed its fungicidal nature (*Table 2* and *Plate 16a Fig A, Fig B*).

In VA at 500 ppm of the essential oil concentration, fungal development was completely inhibited after 7 days of incubation. Studies on nature of toxicity of oil revealed that the oil was fungicidal at 700 ppm. As it is evident from the *Table 2* and Figure A, B (Plate 16b) that volatile activity assay was more promising that of poisoned food technique. In PF the MIC was 700 ppm whereas in VA the MIC was 500 ppm. So it is evident that oil is more fungitoxic in its volatile phase.

Determination of conidial germination in presence of oil was also done and it was found that the oil exhibited 100% inhibition of conidial germination at 200 ppm concentration (*Table 2*). It was also observed that those spores which germinated in presence of low concentrations of oil produced small germ tube as compared to the control.
Table 22: *In vitro* effects of different concentrations of *Zingiber officinale* oil on per cent radial growth inhibition, nature of toxicity and per cent conidial germination of *A. mali* and *A. alternata* after 7 days of incubation at 25±2°C

<table>
<thead>
<tr>
<th>Conc. of oil (ppm)</th>
<th>Percent radial growth inhibition (PF)</th>
<th>Nature of toxicity (Reinoculated) (PF)</th>
<th>Percent radial growth inhibition (VA)</th>
<th>Nature of toxicity (Reinoculated)(VA)</th>
<th>Percent Conidial germination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM</td>
<td>AA</td>
<td>AM</td>
<td>AA</td>
<td>AM</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>13.6</td>
<td>13.2</td>
<td>NT</td>
<td>NT</td>
<td>20.7</td>
</tr>
<tr>
<td>50</td>
<td>21.7</td>
<td>21.3</td>
<td>NT</td>
<td>NT</td>
<td>27.7</td>
</tr>
<tr>
<td>100</td>
<td>35.9</td>
<td>35.2</td>
<td>NT</td>
<td>NT</td>
<td>50.1</td>
</tr>
<tr>
<td>200</td>
<td>75.8</td>
<td>75.2</td>
<td>NT</td>
<td>NT</td>
<td>65.7</td>
</tr>
<tr>
<td>500</td>
<td>90.7</td>
<td>90.2</td>
<td>NT</td>
<td>NT</td>
<td>100</td>
</tr>
<tr>
<td>600</td>
<td>98.1</td>
<td>97.9</td>
<td>NT</td>
<td>NT</td>
<td>100</td>
</tr>
<tr>
<td>700</td>
<td>100</td>
<td>100</td>
<td>95.6(S)</td>
<td>94.6(S)</td>
<td>100</td>
</tr>
<tr>
<td>800</td>
<td>100</td>
<td>100</td>
<td>98.7(S)</td>
<td>98.5(S)</td>
<td>100</td>
</tr>
<tr>
<td>900</td>
<td>100</td>
<td>100</td>
<td>100(C)</td>
<td>100(C)</td>
<td>100</td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
<td>100</td>
<td>100(C)</td>
<td>100(C)</td>
<td>100</td>
</tr>
</tbody>
</table>

AA= *A. alternata*, AM= *A. mali*

All values are means of three replications per treatment of two experiments. (PF)= poisoned food technique, (VA) = volatile activity assay NT= Not tested, (-) = Reduced percent radial growth inhibition of reinoculated mycelial disc as compared to their respective treated plates. (S) = Fungistatic toxicity of essential oil allowing the fungus to regain its vitality on fresh medium. (C) = Fungicidal toxicity of essential oil not allowing the fungus to regain its vitality on fresh medium
PLATE – 16a

PLATE- 16a: Antifungal activity (Poisoned food Technique) of *Z. officinale* Roscoe. Oil against Fig (A) *A. mali* and Fig (B) *A. alternata* at increasing concentrations
PLATE – 16b: Antifungal activity (Vapour Toxicity method) of *Z. officinale* Roscoe. Oil against Fig (A) *A. mali* and Fig (B) *A. alternata* at increasing concentrations.
4.11.2. Determination of mycelial weight

Effect of essential oil on the yield of mycelial weight was observed in liquid medium Potato Dextrose Broth and it was found that insignificant growth occurred at 200 ppm concentration. At 500 ppm negligible mycelial growth was recorded and 100% inhibition was observed (Table 23). On the basis of per cent inhibition of dry weight it was found that the oil was more effective in liquid medium than solid medium.

Table 23: Effect of different concentrations of Z. officinale oil on dry weight (after 15 days) of A. mali and A. alternata at 25±2°C.

<table>
<thead>
<tr>
<th>Conc. of oil (ppm)</th>
<th>Dry weight (mg ± SE)</th>
<th>Per cent growth inhibition on the basis of dry weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. mali</td>
<td>A. alternata</td>
</tr>
<tr>
<td>Control</td>
<td>410±5.77</td>
<td>407±1.66</td>
</tr>
<tr>
<td>25</td>
<td>382.33±2.33</td>
<td>372±0.33</td>
</tr>
<tr>
<td>50</td>
<td>281.67±1.66</td>
<td>275±1.33</td>
</tr>
<tr>
<td>100</td>
<td>113.67±0.88</td>
<td>111.67±0.88</td>
</tr>
<tr>
<td>200</td>
<td>21±0.33</td>
<td>19±5.77</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>600</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>700</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

All data shown are averages and standard errors from three determinations of experiment.
4.11.3. Effect of temperature on toxicity of oil

The thermostability of the oil was tested under PF and VA at MIC and it was found that at temperature ranging from 40-100°C and even after autoclaving (121°C for 15 min) the oil, its activity was not altered in both the techniques tested (Table 24).

Table 24: Effect of different temperature treatments and autoclaving on fungitoxicity of oil (at MIC) against A. alternata and A. mali using poisoned food technique (PF) and volatile activity assay (VA) incubated at 25±2°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Percent inhibition of mycelial growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poisoned food technique (PF)</td>
</tr>
<tr>
<td></td>
<td>AM</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>autoclaving (121°C for 15 min)</td>
<td>100</td>
</tr>
</tbody>
</table>

AM = A. mali and AA = A. alternata
Table: 25. Effect of different storage periods on fungitoxicity of Z. offinale oil at MIC (500 ppm at VA and 700 ppm at PF) against Alternaria sp. incubated at 25±2°C.

<table>
<thead>
<tr>
<th>Storage period (months)</th>
<th>Percent inhibition of mycelial growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poisoned food technique (PF)</td>
</tr>
<tr>
<td></td>
<td>AM</td>
</tr>
<tr>
<td></td>
<td>AM</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
</tr>
</tbody>
</table>

4.12. Mechanism of toxicity of essential oil

4.12.1. Light microscopy

The observations of A. mali and A. alternata examined under light microscope at 100X magnification, after treatment with different concentrations (Control, 200ppm, 500ppm) of Ginger oil are presented in Plate 17a. In addition to inhibited growth, mycelial colonies grown in the presence of essential oil seemed to consistently exhibit distinct morphological alterations when compared to control. These variations included lack of sporulation, visible loss of pigmentation and aberrant development of conidiophores. In microscopic examination of untreated mycelium hyphae had homogenous, clear cytoplasm and elongated conidiophores bearing conidia which were black and elongated clearly visible (Plate 17, Fig. A & B) The alterations in hyphal structures started with budding of hyphal tip, anomalous structures such as swelling, localized along the hyphae.
PLATE - 17a

PLATE - 17A Effect of essential oil on the growth of pathogen: Light Microscopy Fig (A) A. mali and (B) A. alternata grown on PDA as Control set incubated at 25±2°C, homogenous structure of mycelia taken at 100 X. (C) at 500ppm conc. of oil loss of cytoplasm and pigmentation and distortion of conidiophores and conidia in A. mali (D) Complete loss of cytoplasm flattened and ribbon like hyphae in A. alternata
Scanning electron microscopy: Fig. A. (A. mali) & D. Control (A. alternata), magnification 5 kx, Bar 3 µm B. (A. mali) & E. (A. alternata) Showing distortion of conidia (200 ppm) at magnification 2.08 kx, Bar 10 µm C. (A. mali) & F. (A. alternata) treatment at 500 ppm of oil/ml, causing cell wall disruption, squashed and degrading hyphae clearly visible, 2.08 kx, Bar 10 µm.
and at their extremities with the apexes showing an irregular growth in dimension with irregular shape. Higher concentration of 500ppm clear zonation of cytoplasm from hyphae and ultimately hyphae without cytoplasm were found (Fig. C & D).

4.12.2. Scanning electron microscopy

The effect of Z. officinale essential oil on the morphology of A. mali and A. alternata examined by SEM is shown in Plate 17b (A & C). The A. alternata mycelium grown on PDA medium as control showed the characteristic morphology with lengthened, regular, homogenous hyphae of constant diameter with smooth external surface and with rounded apex. After 7 days of incubation in the treatments (control, 200 ppm, 500 ppm) fungal mycelium showed alterations in the morphology of the hyphae. Treatment with essential oil at 500 ppm clearly showed distorted mycelium, squashed and flattened conidiophores bearing damaged conidia (Plate 17b, Fig. C, F). At 200 ppm, we can see the spores becoming distorted there was clear decrease in cytoplasmic content, with clear separation of cytoplasm from cell wall in hyphae and apical tip without cytoplasm were observed (Plate 17b, Fig B, E). Flattened empty hyphae with the presence of undulations along the hyphal borders were clearly visible which look like ribbon (Plate 17b, Fig. C, F). On increasing the concentration up to 500 ppm complete cell wall disruption and squashed hyphae were found.

4.13. Effect of increased inoculum on fungitoxicity of essential oil

The results related to effects of increased inoculum of pathogen on fungitoxicity of essential oil showed that the essential oil at its MIC was able to inhibit the growth of all the fourteen discs (each of 5 mm diameter) as well as the growth of single mycelial disc up to 25 mm diameter, the maximum size taken into account in the present study. The
fungitoxicity of essential oil also showed its positive result when numbers of conidia were increased up to $14 \times 10^6$ in arithmetical progressions in broth medium. Thus, the fungitoxic potential of the oil appears to sustain heavy inoculum density (Tables 26, 27 & 28).

Table 26: Effect of increased inoculum on fungitoxicity of oil of *Zingiber officinale* at MIC against *A. alternata* and *A. mali* using poisoned food technique in potato dextrose broth medium.

<table>
<thead>
<tr>
<th>Number of inoculum discs (5 mm diameter)</th>
<th>Mycelial growth in potato dextrose broth medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM</td>
</tr>
<tr>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
</tr>
</tbody>
</table>

*AM* = *A. mali* and *AA* = *A. alternata*

Table 27: Effect of inoculums size on fungitoxicity of oil of *Z. officinale* at MIC against *A. alternata* and *A. mali* using poisoned food technique in potato dextrose broth medium.

<table>
<thead>
<tr>
<th>Diameter of inoculum disc (mm)</th>
<th>Mycelial growth in potato dextrose broth medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM</td>
</tr>
<tr>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 28: Effect of increased inoculum concentration on fungitoxicity of oil of Z. officinale at MIC against A. alternata and A. mali using poisoned food technique in potato dextrose broth medium.

<table>
<thead>
<tr>
<th>Number of conidia in media</th>
<th>Mycelial growth in potato dextrose broth medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM Treatment</td>
</tr>
<tr>
<td>2 × 10^6</td>
<td>-</td>
</tr>
<tr>
<td>4 × 10^6</td>
<td>-</td>
</tr>
<tr>
<td>6 × 10^6</td>
<td>-</td>
</tr>
<tr>
<td>8 × 10^6</td>
<td>-</td>
</tr>
<tr>
<td>10 × 10^6</td>
<td>-</td>
</tr>
<tr>
<td>12 × 10^6</td>
<td>-</td>
</tr>
<tr>
<td>14 × 10^6</td>
<td>-</td>
</tr>
</tbody>
</table>

Where, + = Presence of mycelial growth, - = Absence of mycelial growth.


In all the treatments of oil with 200, 400, 500 and 600 ppm concentration drastic reduction in log_{10} transformation of CFU of A. alternata and A. mali was found. With 600 ppm oil treatment at both 48 h and 72 h no CFU was found after storage of 4 weeks in case of pre-treatment. Pretreatment was found more effective than post-treatment in
respect of apple and tomato. Treatment times were different in apple and tomato experiment (Table 28, 29).

Table 29: Effect of Z. officinale essential oil treatment on colony forming units g\(^{-1}\) apple fruit having artificially inoculated A. mali infection after storage of 4 and 8 weeks.

<table>
<thead>
<tr>
<th>Conc. of oil (ppm)</th>
<th>Period of oil treatment (h)</th>
<th>Colony forming units of A. mali (log(_{10}) CFU g(^{-1}) fruit) in apple after essential oil treatment at different storage period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-treatment 4 weeks</td>
</tr>
<tr>
<td>200</td>
<td>72</td>
<td>8.21(^a)</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>6.52(^b)</td>
</tr>
<tr>
<td>300</td>
<td>72</td>
<td>4.92(^b)</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>2.61(^a)</td>
</tr>
<tr>
<td>400</td>
<td>72</td>
<td>0.66(^a)</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.32(^a)</td>
</tr>
<tr>
<td>500</td>
<td>72</td>
<td>0.00(^a)</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.00(^a)</td>
</tr>
<tr>
<td>600</td>
<td>72</td>
<td>0.00(^a)</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.00(^a)</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>46.22(^c)</td>
</tr>
</tbody>
</table>

One way ANOVA was made, based on the population of Alternaria spores at different concentration of oil. Data were found significantly different at level of 5% significance.

Values in the same column followed by different superscript’s letters are significantly different (P<0.05) according to Duncan’s Multiple Range Test. The values followed by same superscript letters are not statistically significant.
Table 30: Effect of *Z. officinale* essential oil treatment on colony forming units g\(^{-1}\) tomato having artificially inoculated *A. alternata* infection after storage of 1 and 3 weeks.

<table>
<thead>
<tr>
<th>Conc. of oil (ppm)</th>
<th>Period of oil treatment (h)</th>
<th>Colony forming units of <em>A. alternata</em> (log(_{10}) CFU g(^{-1}) fruit) in tomato after essential oil treatment at different storage period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 weeks</td>
</tr>
<tr>
<td>200</td>
<td>48</td>
<td>9.22(^a)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>7.82(^b)</td>
</tr>
<tr>
<td>300</td>
<td>48</td>
<td>5.12(^b)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3.67(^a)</td>
</tr>
<tr>
<td>400</td>
<td>48</td>
<td>0.86(^a)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.49(^c)</td>
</tr>
<tr>
<td>500</td>
<td>48</td>
<td>0.00(^a)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.00(^a)</td>
</tr>
<tr>
<td>600</td>
<td>48</td>
<td>0.00(^a)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.00(^a)</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>49.22(^d)</td>
</tr>
</tbody>
</table>

One way ANOVA was made, based on the population of *Alternaria* spores at different concentration of oil. Data were found significantly different at level of 5% significance. Values in the same column followed by different superscript’s letters are significantly different (P<0.05) according to Duncan’s Multiple Range Test. The values followed by same superscript letters are not statistically significant.
4.15. *In-vitro* comparison of the *Z. officinale* Roscoe. oil and yeast with selected fungicides

*In vitro* comparison of fungicides, essential oil of *Z. officinale* (500 ppm) and *R. phylloplana* (10^8 CFU ml⁻¹) was done. It was found that approximate 100 per cent inhibition of test fungus was recorded at 500 ppm of essential oil same per cent inhibition was also recorded by fungicide, Indofil M45 and *R. phylloplana*. Rest of the fungicides showed less than 50 per cent inhibition of the test fungus at 500 ppm was recorded for the remaining fungicides. From the results it was observed that essential oil have the similar fungitoxic ability to Indofil M45 out of five fungicides tested (*Figure 15*).

4.16. Semi commercial test: efficacy of biological control agents against fungal pathogens

Apple and tomato fruits were coated with *R. phylloplana* suspension (10^8 CFU ml⁻¹) and ginger oil (500 ppm). Application of yeast and ginger oil resulted in average decay incidence on fruit after storage at 20°C for 7 d and 20°C for 15d followed by 4°C for 60 d, natural decay incidence on apple 3.6 % (yeast treated), 2.17% (ginger oil treated) in comparison to control (24.3%) and tomato treated with yeast and ginger oil was 5.1 % or 4.23% respectively with 24.3% in the control fruit. The decay incidence on apple and tomato were compared with each other. Essential oil was found better in comparison with yeast. (*Figure 16*)
Figure 15: Comparative percent inhibition of radial growth of *A. mali* and *A. alternata* due to effect of different fungicides at 500 ppm with yeast (10^8 CFU ml⁻¹) and essential oil (500 ppm).

Figure 16: Effect of Yeast antagonist and essential oil treatment on natural infection of apple and tomato (A= Apple, RP= *R. phylloplana*, EO= Essential oil, T= tomato), each value is the mean of three experiments. Bar represent the standard error of the mean.
4.16. Effect of yeast and ginger essential oil on post-harvest quality of apple and tomato

Yeast antagonist and essential had no significant effect on fruit colour, taste, mass loss, TSS, ascorbic acid, titratable acidity and phenolics compared to control fruit, regardless of whether the fruit were stored at 20°C for 7 d and 20°C for 15d followed by 4°C for 60 d (Figure 17, 18, 19, 20, 21 and 22).
Effect of Yeast antagonist and essential oil treatment on Quality parameters of apple and tomato

**Figure 17:** Effect of yeast antagonist and essential oil treatment on General appearance Score (GAO) of apple and tomato (A= Apple, RP= *R. phylloplana*, EO= Essential oil, T= tomato)

**Figure 18:** Effect of yeast antagonist and essential oil treatment on mass loss (%) of apple and tomato (A= Apple, RP= *R. phylloplana*, EO= Essential oil, T= tomato)
**Figure 19:** Effect of Yeast antagonist and essential oil treatment on Total Soluble Solids (TSS %) of apple and tomato (A= Apple, RP= *R. phylloplana*, EO= Essential oil, T= tomato)

**Figure 20:** Effect of Yeast antagonist and essential oil treatment on Titratable Acidity (TA mg/100g) of apple and tomato (A= Apple, RP= *R. phylloplana*, EO= Essential oil, T= tomato)
Figure 21: Effect of Yeast antagonist and essential oil treatment on Ascorbic acid (mg/100g) of apple and tomato (A= Apple, RP= R. phylloplana, EO= Essential oil, T= tomato)

Figure 22: Effect of Yeast antagonist and essential oil treatment on Phenol (GAE mg/100g) of apple and tomato (A= Apple, RP= R. phylloplana, EO= Essential oil, T= tomato)
DISCUSSION
5. DISCUSSION

It is an established fact that the fruits and vegetables constitute commercially and nutritionally important indispensable food commodities, playing a vital role in human nutrition by supplying the necessary growth factors such as vitamins and essential minerals in human daily diet that can help to keep a good and normal health. One of the limiting factors that influence the perishables, economic value is the relatively short shelf-life which is further reduced due to pathogens attack.

Apple and tomato being succulent and rich in nutrients are susceptible to attack by a variety of microorganisms during various phases of marketing. Both fungi and bacteria are responsible for the post-harvest losses but fungi are the major pathogens in resulting deterioration (Singh & Sumbali, 2004). In India, occurrence of post-harvest pathogens on pomes is a serious problem resulting in loss of food calories and nutritive elements, and in the production of a wide variety of biologically active compounds, which pose a danger of poisoning for the consumer (Agrawal & Roy, 1994).

The conventional approach to the control of fungi has been the use of synthetic antifungal compounds. However, the development of fungicide resistance by pathogens and increasing environmental concern over fungicidal residues in fruits and vegetables has stimulated a search for alternative measures for disease control (Fan & Tian, 2001). The present study was undertaken with the view that fungicides be banned and sensibly replaced by ecofriendly, cheap, safe, easy to use and effective alternatives.

During survey (2009-2013) of local markets, apples and tomatoes were found to be severely infected with Alternaria sp. although blue mold in apple and sour rot in tomato caused by Penicillium expansum (20.9%) followed by Geotrichum candidum
(15.6%) respectively were other dominant pathogens. Dry core rot (DCR) of apple, which mainly infects the Red Delicious varieties, has in the past been linked to a single species, *A. alternata* (Miller, 1959, Combrink *et al.*, 1985b), while several *Alternaria* spp. have been associated with mouldy core of apples (Ellis & Barrat, 1983). It was observed that *Alternaria mali* (causal agent of mouldy-core) was responsible for the maximum loss within the span of four years in apple (ranging from 6.5 to 22.1% disease incidence). Brown and Hendrix (1978) reported that *A. Alternata* was the predominant fungal pathogen responsible for mouldy-core in the Red Delicious strains of apple. Nearly 6 to 8% loss of this fruit gets deteriorated by *A. mali*. (Combrink *et al.*, 1984)

In recent years, levels of moldy-core in Red Delicious have caused significant losses in Israel. The incidence of moldy core was reported 9% according to sampling size due to *Alternaria alternata* (Reuveni, 2006). However, in the present investigation it was recorded that *A. mali* was responsible for Alternaria rot or core rot of apple.

Mouldy core is considered to be of minor economic importance in fruit for immediate consumption, as the flavour of these fruit is unimpaired (Carpenter, 1942). However, as this disease is often a precursor to DCR during storage (Ellis & Barrat, 1983; Combrink *et al.*, 1985a, b), it should also be regarded as serious. Symptoms of both diseases are generally not externally visible, which is why DCR is such a great nuisance to producers and consumers, alike, It is only once the apple is cut open through the core that grey mycelium (mouldy core) or even dry, spongy, black core rot (DCR) is noticed in and around the carpel chambers (Plate 2 fig B). During storage, this lesion may extend further into the fruit fresh until the whole fruit gets rotted (Brien, 1937). Presently, there are still no cost effective methods of controlling this disease in South Africa (Fugler, 1990), as spraying programmes are not effective once the fungi have invaded the core. Same problem was also noticed in India.
Besides *Alternaria mali* other fungi isolated during survey were *P. expansum*, *Botrytis cinerea*, *Rhizopus* sp. and *Aspergillus* sp. Teixido et al. (1998) also isolated dominant filamentous fungi in stored Golden Delicious apples *viz.* *Penicillium* spp. (mainly *P. expansum*), *Alternaria* spp. (*Alternaria alternata*) and *Cladosporium* spp. (*C. herbarum* and *C. cladosporioides*). Other genera and species isolated occasionally included *Fusarium*, *Acremonium* and occasionally *Epicoccum nigrum*, *Gleosporium*, *Aspergillus* (*A. flavus* and *A. niger*), *Trichoderma*, *Botrytis* and *Rhizopus*. Kaul and Munjal (1982) reported that many fungi are associated with the decay complex but *P. expansum*, *Monilinia fructigena*, *Trichothecium roseum*, *Glomerella cingulata* and *R. stolonifer* were the principal pathogens of apples grown in Himachal Pradesh.

Tomato is another most important vegetable crops grown in India. However, in comparison to many other countries of the world, its production in India is hampered due to diseases in the fields, which happen to cause heavy losses. Moreover, information is largely lacking regarding diseases during transit and storage. Therefore, it is not only necessary to improve the average production of this crop, but also to protect the produce from postharvest losses due to infection (Verma, 2004). During survey (2009-2013), it was found that the pathogens causing postharvest losses of tomato fruits mainly include *Alternaria* rot caused by *Alternaria alternata*, a serious problem in U.P. India.

Survey conducted during a span of 4 years extending from 2009-2013 at different sites of local markets revealed that, under natural condition, black mold rot of tomato had highest disease incidence of 25 % in January month.

*Alternaria* rot of tomato caused by *A. alternata* (Fr.) Keissler was reported from U.P. by Srivastava and Tandon (1966) and Tandon and Chaturvedi (1965). In India, sour rot disease caused by *G. candidum* had been reported by Nema (1952) from Nagpur and Srivastva and Tandon (1966) from U.P. *Rhizopus* soft rot, which is caused by *Rhizopus*
nigricans has been reported by Ratnam and Nema (1967) from Jabalpur (India). *Alternaria mali* and *A. alternata* were the pathogens which were responsible for heavy loss in apple and tomato commodity during survey conducted in the local markets of Lucknow.

*Alternaria* spp. is widely distributed in soil and on aerial plant surfaces. Studies on symptomology of diseases on fruits revealed that *Alternaria* rot appears as small brown coloured and sunken irregular spots. These spots are mostly circular with fungal mycelium growing on the surface and later on the conidiophores dark greenish conidia were developed in tomato but in apple, *Alternaria mali* was confined to core then increased via mesoderm to fruit surface, hardly 1-4 mm circular and concentric ring were observed. These symptoms are in resemblance with the symptoms described by Snowdown (1979) and Kallo (1987). The pathogenicity test was performed using fungal disk as inoculums (Jadeja & Bhatt, 2008) with slight modifications and placing them in wounds made on single wounding of stem end. The fungus was readily and invariably isolated from the affected portion of the fruits, confirming the postulates given by Robert Koch.

*Alternaria alternata* has an important place among species of this genus, because of wide range of hosts including garden plants, field crops, vegetables, and ornamentals. Inoculum potential of $10^6$ conidia ml$^{-1}$ (*A. alternata*) was found to be were sufficient to cause 100% infection. In case of *A. mali* $5 \times 10^4$ conidia ml$^{-1}$ concentration was found to be effective to cause infection on apple in an earlier study but in the present studies $10^6$ CFU ml$^{-1}$ were effective to cause infection. Similar results were reported earlier by Reuveni (2006).
Behaviour of fungal pathogens depends upon their nutritional responses and may vary within races or strains isolated from different sources (Punithaligam, 1980). Fungi exhibit varying response to physiological factors like temperature, pH, light and nutrition (Quimio 1973; Rewal & Grewal, 1989). These factors also influence certain taxonomic characters in fungi (Hewitt et al., 1971). Different isolates express a similarity in broader behaviour for their basic nutritional needs yet, they maintain their individuality for the choice of specific substrates (Webster et al., 1971).

The observations on the growth of the *A. alternata* and *A. mali* were made on different liquid media and solid media. Best growth was observed in Potato dextrose broth medium at pH 7 and pH 8. Malt extract supported the best growth of the fungus at pH 4, 5, 6 while Good growth was recorded on Sabouraud medium, Malt yeast extract broth medium, Potato dextrose broth medium while poor growth was recorded on Host extract medium, Czapek (Dox) broth and Richards’s medium. The present observation are not in accord with findings of Mohanthy et al. (1981), who have reported that the growth and sporulation of *A. helianthi* was best on Richard’s medium. However Reddy and Gupta (1981) tested 6 liquid media out of which Potato dextrose medium was found best for growth and sporulation of *A. helianthi*. These observations are similar to the results obtained for *A. alternata* and *A. mali* in the presented study. Morphological characteristics of the cultures on potato carrot agar and V8 agar media after 7 days exhibited typical characteristics of *Alternaria mali* (Simmons, 1999). The best growth of the *A. alternata* was recorded on Potato dextrose agar followed by Malt extract and Malt yeast extract. The present findings are in accord with the observations made by Sharma and Singh (1994).
Several pathogenic microorganisms develop on the fruit and vegetable surface during storage. pH and temperature are very important factor which are responsible for health of commodity and also for survival of their respective pathogen.

Temperature was found to influence the growth and sporulation of *A. alternata* and *A. mali*. It was observed that sporulation occurred at a temperature below that required for optimal growth. The optimal temperature appears to be specific for a particular fungal species; it was observed that optimal temperature for best growth of *A.* and *A. mali* were between 25-28°C. However, both the genera showed fairly good growth within the range, 10-35°C. While the results for growth are in accordance with the other earlier reports (Tandon, 1967; Mehta *et al.* 1975; Tandon and Ghosh, 1962), but those for sporulation are contradictory to these reports.

pH is also considered as an important factor influencing the growth of fungus. We observed that both *Alternaria* sp. grew well at 4-8 pH range. Optimum pH for the growth of *A. alternata* and *A. mali* was found to be pH 6 and pH 7. Similarly, Mishaghi (1978) mentioned that colony diameter of *A. alternata* was not affected by the pH of media. *A. alternata* grew and sporulated readily at all pH values from (4 to 8). Optimum pH for radial growth was observed to be pH 7 followed by pH 9 (Sharma, 2011). However Pandey *et al.* (2002) reported optimum pH for the growth of *Alternaria lini* as 6.5.

The quality of fruit during storage depends on the storage environment and fruit microorganisms, since the activity of microorganisms can cause fruit and vegetable decay (Reuveni, 2006). *A. alternata* is the most common *Alternaria* species in harvested fruits and vegetables, and is the most important mycotoxin-producing species. *A. mali* also a pathotype of *A. alternata* and almost same morphologically and shows similar
pathogenic activity. Due to their growth even at low temperature, *Alternaria* species are also responsible for spoilage of these commodities during refrigerated transport and storage. Moreover, *Alternaria* spores are considered to be one of the most prolific fungal allergens, which has been associated with respiratory allergies and skin infections (Pavon *et al.*, 2010).

Disease control is achieved mainly through the use of fungicides, benomyl and iprodione were available for postharvest treatment to reduce decay and extend the shelf-life of stone fruits (Fan & Tian, 2000). Many of the fungicides such as benzimidazole and dicarboximide fungicides that are still available for use are losing their effectiveness because of the development of resistance in many postharvest pathogens (Lennox & Spotts, 2003). Although maneb, mancozeb, chlorothalonil, or copper fungicides are available for tomato disease management, but consumers are demanding less chemical residue on produce, and many fungi are developing resistance to commonly used fungicides (Conway *et al.*, 2004). A recent (October 21, 2003) report by the Environmental Working Group ([www.ewg.org](http://www.ewg.org)) indicated that apples are among the top four fruits and vegetables that are the most consistently contaminated with pesticides. The use of fungicides is becoming more restricted due to health concerns (Ragsdale & Sisler, 1994). It is therefore necessary to develop alternatives to synthetic chemical control to reduce environmental risks and raise consumer confidence.

Elucidating non-chemical control methods to reduce postharvest decay is becoming increasingly important. Thus, biological control has emerged as an effective strategy to combat major postharvest decays of fruits (Janisiewicz & Korsten, 2002) and vegetables. Microbial biocontrol agents have shown great potential as an alternative to synthetic fungicides for the control of postharvest decay of fruit and vegetables.
Post–harvest diseases of fruits and vegetables and their management by bio control agents

Discussion

(Wisniewski & Wilson, 1992). Bio-control, especially using antagonists (mainly yeasts, bacteria and plant based natural products) of the fungal plant pathogens, has gained considerable attention and appears to be promising as a viable option to chemical control (Spadaro & Gullino, 2004).

Yeast strains including Candida sake, Galactomyces geotrichum, Trichosporon pullulans, Candida pulcherrina, Rhodotorula glutinis, Cryptococcus albidus and Aurobasidium pullulans Candida oleophila strain I-182 (Aspire, Ecogen, Langhorne, PA) and Candida guilliermondii (strains 101 and US 7), can colonize plant surface and control plant diseases by pathogens (Saligkarias et al., 2002).

Rhodotorula species are in general considered as safe for humans, although some species have been reported to cause opportunistic infections, especially in tropical and subtropical climates (Preney et al., 2003). Rhodotorula has been used for the production of carotenes and it have been shown that oral feeding of frozen whole cells of Rhodotorula gracilis did not produce any toxic effects on male and female rats. (Bhosale & Gadre, 2001; Naidu et al., 1999)

Yeasts of the genus Rhodotorula have been reported as good biocontrol agent against P. expansum, in apples and pears (Benbow & Sugar, 1999) and against Botrytis cinerea in strawberry (Helbig, 2001) when preharvest and/or postharvest applications are made. The antagonist yeast Rhodotorula glutinis (Fresenins) Harrison has been also proposed for the postharvest biological control of green mold decay of oranges (Zheng et al., 2005) and gray mold decay of apples (Sansone et al., 2005), as well as postharvest diseases on sweet cherries (Tian et al., 2004).

In present study, Rhodotorula phylloplana has been explored as new (not previously known) biocontrol agent against A. mali and A. alternata, which were proved
as predominant pathogens (Alternaria rot) during the survey conducted in the local market.

Pre-treatment of wounded apple and tomato fruits with *R. phylloplana*, effectively reduced developments of lesions of *A. mali* and *A. alternata*. This enlargens upon the activity of *R. phylloplana* as a potential biocontrol agent for the control of postharvest diseases of contains alternaria rot of apple and tomato respectively. *In vivo* and *in vitro* experiments strongly indicate that it could control natural development of decay of apple and tomato mainly caused by these pathogens. As yeast antagonist develops before infection, presumptive evidence that biocontrol is achieved when actively multiplying populations of *R. phylloplana* are present in wounds.

Albeit, the concentrations of antagonist had significant effects on biocontrol effectiveness, the higher the concentration of *R. phylloplana*, the better biocontrol activity of the antagonist had. The inoculum concentration of *R. phylloplana* at 1×10⁸ CFU/ml, proved that the disease could be managed, the occurrence of decay was put off compared with other concentration treatments and prolonged the storage period. However, *R. phylloplana* increases in co-cultures and dual cultures, but this does not provides evidence for nutrition, as it as the main mode of action presents a different dimension when its interaction with pathogen was observed in Light and Scanning electron microscopy.

These results above suggested that the main mode of action of *R. phylloplana* was considered as the attachment to pathogen hyphae. Individual yeast cells of *R. phylloplana* (Plate 14 fig D) gave the appearance of having sunk into the hyphal cell wall. 2% CaCl₂ treatment was mildly effective in dislodging the yeast from some areas of hyphae. Discrete areas of hyphae cell wall appeared concave, giving the hyphal strand a general
appearance of being pitted (Plate 14 fig C, D). Pitting was quite extensive in both cases of *A. alternata* and *A. mali*.

The microscopic examinations revealed that hyphae of *A. mali* and *A. alternata* were heavily colonized by *R. phylloplana*. Heavy yeast colonization was observed near and around the hyphal tips and the extracellular matrices had accumulated around the hyphae. The colonized hyphae had undergone some swelling and beading. The attachment of *R. phylloplana* to the fungal hyphae indicated a pitting appearance in the hyphal cell wall, resulting in the concave appearance of the hyphal surface under the attached yeast cell. Eventually, the hyphae of *A. mali* and *A. alternata* were totally penetrated and destroyed by cells of the antagonistic yeast. Similar results were obtained by Chan and Tian (2005). They indicated that *P. membranefaciens* had a stronger capability for attachment to the fungal hyphae of *Monilinia fructicola*, *Penicillium expansum* and *Rhizopus stolonifer* than did *Candida albidos*.

Depending on these observations, it can be interpreted that the active mechanism through which yeast could antagonize *A. mali* and *A. alternata*, are by the production of fungal cell wall degrading enzymes or lethal toxins. This hypothesis is supported by findings of the production of killer toxin by, *R. phylloplana* that have been found to be inhibitory to a wide range of pathogenic fungi (Buzzini & Martini, 2001; Freudlund *et al.*, 2002). Grevesse *et al.* (2003) have suggested that exo-β-1, 3-glucanase activity is involved in the lethal action of this *P. anomala* toxin. Izgu *et al.* (2006) have found that high exo-β- 1, 3-glucanase activity and the reasonable pH and thermostability of the *P. anomala* NCYC 432 lethal toxin highlights its potential for use against human and animal fungal infections and as a biocontrol agent in the food industry.
The mode of action of *P. guilliermondii* against some fungal pathogens such as *Penicillium digitatum* and *B. cinerea* has been studied by other researchers who have suggested that they compete for nutrients along with secretion of cell wall degrading enzymes (Droby *et al.*, 1990; Wisniewski *et al.*, 1990). Fan *et al.* (2002) indicated that *P. membranefaciens* and *Candida guilliermondii* were able to produce significant levels of chitinase *in vitro*. These control mechanisms are acceptable for use in the biological control of moulds in foods and feeds since they do not involve the secretion of potentially hazardous antibiotics.

Antagonist–pathogen interaction has been shown to play a major role in their biological activities (Cook, 2002). El-Ghaouth *et al.* (2003) found that *Candida saitoana* gets attached to *Botrytis cinerea* hyphae and restricted the proliferation of *B. cinerea*. An earlier study by Wisniewski *et al.* (1991a,b) showed that *P. guilliermondii* protected apples from postharvest fruit rotting fungi *Botrytis cinerea* and *Penicillium expansum*. Light microscopy revealed a general attachment of the isolate to the fungal pathogens. Scanning electron microscopy indicated that *P. guilliermondii* tenaciously attaches to the hyphae of *B. cinerea*. The observations were confirmed using transmission electron microscopy. They concluded that tenacious attachment, along with secretion of cell wall degrading enzymes, may play a role in the biocontrol activity of this yeast antagonist.

In this study, we found that the *R. phylloplana* yeast showed different attachment capabilities to the hyphae of the fungal pathogen *in vitro*. These results indicate that attachment potential might universally exist between the interactions of yeast cells to fungal hyphae, and play a role in their biocontrol activity.

Scientists have indicated the production of fungal cell wall degrading enzymes such as chitinase and exo-β-1, 3-glucanase by *P. membranefaciens*, *Candida guilliermondii* and *C. albidos in vitro* in different quantities. This was correlated with
their attachment capabilities to the hyphae of pathogens (Chan & Tian, 2005). Wisniewski et al. (1991a,b) indicated that in the presence of the pathogen, the yeast cells produced lytic enzymes that could enhance the attaching ability of yeast to the hyphae of pathogens. Cellulase and pectinase enzymes are the two major cell wall degrading enzymes produced by the fungal pathogens. It is clear that the reduction in activity of these enzymes reflects inhibition or destruction of the pathogen.

The results of studies undertaken also indicate that cellulase and pectinase enzymes which were excreted by A. alternaria and A. mali were reduced significantly when the R. phylloplana yeast strain were applied. However, R. phylloplana was responsible for the highest reduction in the activity of both enzymes. Similarly, Hashem and Alamri (2009) found that the production of cellulase and pectinase enzymes was significantly inhibited in guava fruit infected with B. theobromae when yeast strains of P. anomala were applied.

Microbial antagonists also produce lytic enzymes such as gluconase, chitinase, and proteinases that help in the cell wall degradation of the pathogenic fungi (Lorito et al. 1993; Castoria et al., 1997, 2001; Jijakli & Lepoivre, 1998; Chernin & Chet, 2002). Bonaterra et al. (2003) reported that direct parasitism was a major factor that permitted Pantoea agglomerans (Ewing & Fife) to control Monilinia laxa (Aderh. & Ruhl.) Honey or Rhizopus stolonifer decay on stone fruits. Thus, strong attachment of microbial antagonist with enhanced activity of cell wall degradation enzymes may be responsible for enhancing the efficacy of microbial agents in controlling the postharvest diseases of fruits and vegetables (Wisniewski et al., 1991). And, attachment of the microbial antagonists to a site enhances their potential activity for the utilization of nutrients at the invasion site; it partly affects the access of the pathogen to nutrients as well (El-Ghaouth et al., 2004).
Fruits and vegetables are often kept at low temperature to maintain the freshness of the product. *R. phylloplana* significantly reduced the natural development of decay of apple when stored at 20°C for 7 days, 15 days and followed by 4°C for 60 days. The biocontrol ability of antagonist under normal shelf-life conditions (including a low temperature stage and an ambient temperature stage) suggest that *R. phylloplana* can be used in cold storage to enhance disease control. As, *R. phylloplana* did not impair quality parameters of apple and tomato under commercial conditions, it can be suggested that *R. phylloplana* has potential for commercialization. Wilson and Wisniewski (1994) summarised following characteristics of an ideal antagonist: genetic stability, efficacy at low concentrations and against a wide range of pathogens on various fruit products, simple nutritional requirements, survival in adverse environmental conditions, growth on cheap substrates in fermenters, lack of pathogenicity for the host plant and no production of metabolites potentially toxic to humans and resistance to the most frequently used pesticides. *R. phylloplana* seems to possess a good number of the above-mentioned features, and, has the potential as an ideal antagonist.

Natural products are an excellent alternative to synthetic pesticides as a means to reduce negative impacts to human health and the environment. The move toward green chemistry processes and the continuing need for developing new crop protection tools with novel modes of action makes discovery and commercialization of natural products as green. More recently, the encouragement of use of products from natural resources and even the extremely biodegradable synthetic and semisynthetic products in pest management has been considered to constitute the umbrella of green pesticides (Dhaliwal & Koul, 2007; Koul, 2008).

However, it will be beyond the scope of any research work to discuss all of them at one place. Here we shall like to emphasize on some recent developments (Post harvest
fumigants for perishables) where essential oils have been projected as safe. Particular interest focused on the potential application of plant essential oils and extracts from plants have recently been of great interest. Their possible use as natural additives emerged from a growing tendency to replace synthetic antimicrobial agents with natural ones.

Phyto-compounds are expected to be far more advantageous than synthetic pesticides for sheer magnitude of complexity, diversity and novelty of chemicals, reactions and phenomenon (Sharma, 1998) as they are bio-degradable in nature, non-pollutant and possess no residual or phytotoxic properties (Tewari, 1990; Badei et al. 1996; Bishop & Thornton, 1997). During the past years there has been growing interest in testing natural compounds of different origins as defense for cultivated plants against phytopathogenic fungi (Muller-Riebau et al. 1995; Wilson et al. 1997; Bowers & Locke, 2000, Sharma & Tripathi, 2006). Essential oils have shown a substantial potential to controlling the post harvest decay of fruits and vegetables and might act as alternatives to synthetic fungicides. Essential oils produced by different genera are, in many biologically active, endowed with antimicrobial, allelopathic, antioxidant and bioregulatory properties. The essential oils extracted from various scent plants have been shown to have significant antifungal properties (Ahmet et al. 2005).

The general antifungal activity of essential oils is well documented (Reuveni et al. 1984; Meepagala et al. 2002). In particular, essential oils were seen to exert good antifungal activities both in vitro and in vivo (Baruah et al. 1996; Bhaskara Reddy et al., 1998; Tripathi et al. 2004; Sharma & Tripathi, 2006).

Ginger, the rhizome of *Zingiber officinale* Roscoe. is one of the most widely used spices and a traditional remedy in Indian, Chinese and Oriental medicine against pain, inflammation and gastrointestinal disorders. Ginger oil is produced from fresh rhizomes
of *Zingiber officinale*. It possesses the aroma and flavor of the spice but lacks the pungency. The essential oil of ginger has been found to possess antibacterial, antiviral and antifungal properties (Singh *et al*., 2005a,b,c; Koch *et al*., 2008). Essential oils are mixtures of organic substances of heterogenous groups, the level of which may vary with plants growing in different ecological situations. Hence the quality of biologically active essential oil must be standardized in order to get the reproducible results (Dube *et al*., 1991).

The hyphenated technique, GC–MS, is a useful tool in modern food, medicine and biological research aiming at the separation and identification of components of organic mixtures, and this method has already been applied successfully for the analysis of terpenoids, especially mono- and sesquiterpenes, in various essential oils (Delazar *et al*., 2004). Essential oils are very complex natural mixtures which can contain about 20–60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts (Bakkali, 2007). It appears that the fungicidal/fungitoxic nature of the *Z. officinale* Roscoe. oil is due to 1, 8- cineole which is an ether compound and due to other monoterpenes camphene and sesquiterpene zingiberene (Sharma & Tiwari, 2013).

In literature, many variations have been found in chemical composition of ginger oil. Agrawal *et al*., (2001) reported curcumene as the major constituent in the fresh ginger rhizomes, while Menut *et al*., (1994) identified citral as the main constituent of ginger oil. In other previous report on ginger oil (Singh *et al*., 2005a, b, c; Singh *et al*., 2008c), α-zingiberene was found to be the major constituent. But in our study we found 1, 8- cineole as major component (27.9%) followed by α-zingiberene (23.9%). These differences in the chemical composition of the oil from the same plant/plant part could
be due to the environmental, developmental, genetic or some other factors. Yield and composition of oil differ widely with the production conditions (Blair et al., 2001), variety, cultivars or population (Galambosi & Peura, 1996) and on climatic and soil factors.

This cytotoxic property is of great importance in the applications of essential oils not only against certain human or animal pathogens or parasites but also for the preservation of fruits, vegetables and other agricultural or marine products. Ginger essential oil is indeed effective against several mycotoxin producing fungi of stored commodities. There are various instances of antimicrobial activity of ginger essential oils and their effects on various plant pathogens in in vitro conditions are well documented. Phytotoxicity of ginger oil has been reported by Singh et al. (2008c) against different Aspergillus spp., viz. Aspergillus flavus, Aspergillus solani, Aspergillus oryzae, Aspergillus niger and Fusarium moniliforme. Sasidharan & Nirmala Menon (2010) also reported the antimicrobial activity of ginger oil against B. subtilis, Pseudomonas aeruginosa, Candida albicans, Trichoderma sp, A. niger, Pencillium spp. and Saccharomyces cerevisiae, was assessed by disc diffusion method. The MIC values of the oils ranged from 10μg mL\(^{-1}\) to 1 μg mL\(^{-1}\) which is very significant. The study shows a wide application of ginger oil in the treatment of many bacterial and fungal diseases.

In the present study the minimum inhibitory concentration of essential oil of ginger in poisoned food technique and volatile activity assay was 700 ppm and 500 ppm effective against target pathogens Alternaria alternata and Alternaria mali respectively, which is less than many of essential oils previously tested by different workers (Pandey et al., 1982; Antonov et al., 1997; Beg and Ahmad 2002; Daferera et al., 2003; Nguefack et al., 2004; Singh et al., 2008a,b,c;Menon & Sasidharan, 2010). The fungicidal nature of
oil strengthens its exploitation as an economical source of fungitoxicant and there was no report of its use as a fungitoxic fumigant. Keeping in view the residual toxicity and the side effects of synthetic fungicides, the essential oil of *Z. officinale* may be exploited as fungitoxicant in management of Alternaria rot pathogen of apple and tomato. Similarly Shahi & Shahi (2013) also reported pesticidal activity of ginger oil as fungitoxicant against post harvest pathogen *A. alternata* of apple, both *In vivo* and *In vitro* condition.

In present investigation, it was found that *Z. officinale* oil was more effective in volatile phase, as evident by the decreased MIC values in volatile activity assay. This property was utilized in concentrating the oil in treatment of diseases by fumigation technique. Based upon the present study it could be concluded that fungitoxic activity of volatile oil from *Z. officinale* oil is worth exploiting for eco-friendly preservation of apple and tomato. The essential oil significantly reduced the growth of *A. alternata* and *A. mali* in a dosage response manner. After calculating the percent inhibition with respect to radial growth (PDA) and dry weight (PDB) basis we found that the oil was more effective in liquid as well as solid medium.

The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism is known as the MIC. The determination of the MIC of oil is necessary for prescribing its appropriate dose. Clearly, unnecessarily high doses of oil increase wastage and may cause considerable harm to the quality of the commodity treated. Such variation may be due to the use of different test fungi or different technique adopted.

A fungitoxicant may act as a fungistat or a fungicide inhibiting the growth of fungus temporarily or permanently respectively (Sharma & Tripathi, 2007). Ginger oil exhibited a capability to be fungitoxic even at high doses of inoculum, thereby,
indicating the possibility of its exploitation as an ideal fungitoxicant. A fungicide should be able to retain its activity over a long period of shelf life. The essential oil of *Z. officinale* was found to retain its fungitoxic activity for up to two months for apple and one month for tomato, which was the maximum period for storage of fruits and vegetables, which it was tested, thus showing that this oil possess another attribute of an ideal fungicide.

A fungicide must not be affected by extremes of temperature. Only a few workers have studied the effect of temperature on antifungal activity of the oils, but the oil of *Pepromia pellucida* was reported to be active up to 80°C (Singh et al., 1983); in the present study the oil of *Z. officinale* Rosc. retained activity up to 100°C. In this case the fungitoxic activity of the oil was found to be thermostable up to 100°C and even after autoclaving. Storage time did not affect the fungicidal nature of oil. Similar results were found in the study of *Hyptis suaveolens* oil (Tripathi & Sharma, 2013).

The mode of action and morphological alterations were not studied yet. Here we explored not only antifungal activity of ginger oil against *A. alternata* but also observed the biological effects of essential oil and its uses in storage of fruits and vegetables. Based on the present study, it could be concluded that essential oil from *Z. officinale* Roscoe. possess fungitoxic activities inhibiting the growth of *A. alternata* leading to irreversible deleterious morphological alterations and thus it is worth exploiting for the bio management of mycotoxin producing *Alternaria* sp.

*In vitro* light microscopic study and scan electron microscopic study of the mechanism of action of *Z. officinale* essential oil revealed that mycelium treated with oil showed alterations in the morphology of hyphae, which appeared severely collapsed, loss
of conidiation and degradation of the cytoplasm. SEM photograph clearly demonstrate the ribbon like flattened hyphae without cytoplasm of both *Alternaria* sp.

Several studies have been conducted to understand the mechanism of action of essential oils (Chang *et al.*, 2001), however it is still unclear. The literature is also silent on the mode of action of the essential oils when used as postharvest fungitoxicants (Tripathi & Dubey, 2004). Because of the great number of constituents, essential oils seem to have no specific cellular targets (Carson *et al.*, 2002). As typical lipophiles, they pass through the cell wall and cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids and permeabilize them. Cytotoxicity appears to include such membrane damage.

The literature reveals that the essential oils have been found to exhibit a narrow or wide range of activity (Dubey *et al.*, 1983), but in the present study the *Z. officinale* Roscoe oil exhibited a broad antifungal spectrum. Antifungal active oils derived from plants are generally nonphytotoxic (Tripathi *et al.*, 1983). In the present study, the oil was found to be nonphytotoxic at morphological level. Additionally, in preliminary *in vivo* trials, it has also been found effective in the control of Alternaria rot of apple and tomato.

A chemical should be tested under both *in vitro* and *in vivo* conditions in order to prove its potential as promising antifungals for the control of disease. Since, detailed *in vitro* studies on the essential oil of *Zingiber officinale* Rosc indicate its potential as ideal antifungal compounds against post harvest spoilage fungi; it was further subjected to *in vivo* investigation so as to confirm their efficacy as a natural product for the control of rotting in fruits. The present study clearly demonstrates that oil of *Zingiber officinale* Roscoe holds a good promise as an antifungal against post harvest spoilage on account of
their following characteristics viz., strong efficacy against fungi with fungicidal action, potentiality against heavy fungal inoculum, long shelf life, thermostable, wide range of antifungal activity and absence of any phytotoxic effects and better result during in vivo trials. The oil in the form of fungicidal spray can be exploited commercially after undergoing successful completion of wide range of field trial and to find out their economic viability.

Encouraging results on the use of natural products to control postharvest fungal rotting indicate that natural fungicides could be developed that would be as effective as synthetic fungicides, and presumably safer for man and the environment. Although about 10,000 secondary plant metabolites have been chemically defined for their role as anti-pathogenic chemicals, the total number of plant chemicals available could amount to 400,000 or more. Still the exploitation of natural products to protect the postharvest decay of perishable products is in its infancy, these products have the potential to be safe fungicides and will replace the synthetic ones. Proper organoleptic tests are also necessary before any recommendation. The product should be effective even for short duration treatments due to the limited postharvest life of fruit. The treatment should not have an effect on quality parameters such as acidity, flavour and aroma. The lowest suitable dose of the chemicals for practical application should also be determined. Keeping in view the merits of the botanicals as postharvest fungitoxicants, the products which are found efficacious during in vitro testings, should be properly tested for their practical potency based on in vivo trials, organoleptic tests and safety limit profile.

Our research goal was to bring bio-control strategy in broad way including yeast and essential oil. These alternatives have been proved highly effective and reliable in
reducing postharvest decay of apples and tomatoes. One thing is also notable that these treatments did not impair the quality parameters of apple and tomato.

In conclusion, our results show that *R. phylloplana* and ginger oil can be used as a non-chemical alternative treatment against postharvest diseases of apple and tomato. Future research will be aimed at developing the technology to be used under large-scale operations and investigating about the mode of action of *R. phylloplana* and ginger oil to control postharvest diseases of fruits and vegetables precisely.
REFERENCES
6. REFERENCES


oil and their constituents from *Cinnamomum osmophloeum*. *Journal of Ethnopharmacology* 77, 123–127.


Courtney, H. (2002). Research project, “Which apple type changes in sugar content most after removal from cold storage?”


Post-harvest diseases of fruits and vegetables and their management by biocontrol agents


EFSA Panel on Contaminants in the Food Chain (2011) Scientific opinion on the risks for animal and public health related to the presence of Alternaria toxins in feed and food. EFSA Journal 9, 2407.


Post-harvest diseases of fruits and vegetables and their management by biocontrol agents

References


177.


Miller, P. M. (1959) Open calyx tubes as a factor contributing to carpel discoloration and decay of apples. *Phytopathology* **49**(8), 520-523.


Post-harvest diseases of fruits and vegetables and their management by biocontrol agents

References

1. College Magazine 26, 15.


Pitarokili, D., Tzakou, O., Couladis, M. & Verykoidou, E. (1999) Composition and


Saad, S. & Hagedorn, D.J. (1970) Growth and Nutrition of an *Alternaria* pathogenic to
Snapbeans, **Phytopathology** 60, 903-906.


Post-harvest diseases of fruits and vegetables and their management by biocontrol agents

References


Sharma, S. (2011) Ph.D thesis titled Use of Stress Tolerant Bacterial Strain for Promoting Plant Growth Botany Department, Lucknow University, Lucknow.


Molecular Plant Pathology 4 (4), 226-236.


Wisniewski, M., Biles, C. & Droby, S. (1991a) The use of yeast *Pichia guilliermondii* as a biocontrol agent: characterization of attachment to *Botrytis cinerea*. In: Wilson, C.L., Chalutz, E. (Eds.), *Biological Control of Postharvest Diseases of Fruit and
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References


postharvest blue mold decay of pears with hotwater treatment and *Rhodotorula glutinis*. *Postharvest Biology and Technology* 49, 308–313.


APPENDIX

Composition of different culture media

1. Potato Dextrose Agar
   - Potato (peeled): 200.0g
   - Dextrose: 20.0g
   - Distilled water: 1000ml

2. Asthana & Hawker’s
   - Glucose: 5.0g
   - KNO₃: 3.5g
   - KH₂PO₄: 1.75g
   - MgSO₄·7H₂O: 0.75g
   - Distilled water: 1000ml

3. Czapek’s (Dox) Agar
   - KNO₃: 3.0g
   - KH₂PO₄: 1.0g
   - MgSO₄·7H₂O: 0.5g
   - KCl: 0.5g
   - FeSO₄·7H₂O: 0.01g
   - Sucrose: 30.0g
   - Distilled water: 1000ml

4. Sabouraud Medium
   - Peptone: 10.0g
   - Dextrose: 40.0g
   - Distilled water: 1000ml

5. Malt Yeast Extract Agar
   - Malt: 3.0g
   - Yeast extracts: 3.0g
   - Peptone: 5.0g
   - Glucose: 10.0g
   - Distilled water: 1000ml

6. Richard’s Medium
   - KNO₃: 10.0g
   - KH₂PO₄: 5.0g
   - MgSO₄·7H₂O: 2.5g
   - FeCl₃: 0.02g
   - Sucrose: 50.0g
   - Distilled water: 1000ml

7. Host extract agar
   Host extract- 50g of host tissue was crushed in 100 ml distilled water, filtered through two layer of cheese cloth. The filtrate was centrifuged at 2000 rpm for 30 min. Volume was made up to 1000 ml.
   **Note:** 2 percent agar was used for preparation of solid media.
PUBLICATIONS & PRESENTATIONS
PUBLICATIONS

International research papers


National research papers


Presentations


**Workshop attended**

Hands on training workshop in “Recent techniques in molecular & cell biology” March 11-13, 2013, Dept.of Zoology, Lucknow University, Lucknow.
RESEARCH ARTICLE

BIOLOGICAL EFFECTS OF GINGER (ZINGIBER OFFICINALE ROSCOE) ESSENTIAL OIL ON ALTERNARIA ALTERNATA (FR.) KEISSL

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ABSTRACT

Essential oil compounds and their derivatives are considered to be an alternative means of controlling many bacterial and fungal pathogens. In present study biological effects of essential oil extracted from Ginger (Zingiber officinale Roscoe.) that belongs to family Zingiberaceae was evaluated on common bio-deteriorating storage fungus Alternaria alternata. The essential oil has been extracted from ginger. The chemical components were analyzed by GC-MS. The oil yield was 2.04±0.03%. 1, 8-Cineole (27.9%) has been found to be major component followed by camphene (23.9%) and Zingiberene (12.2%). The MIC value was determined by volatile activity assay. Minimum inhibitory concentration (MIC) of oil was found to be effective showing results at 500ppm of oil in respect to fungus. The main changes observed under light and scanning electron microscopy after oil treatment were loss of cytoplasm in fungal hyphae at higher concentration of oil (500ppm), whereas we can observe malformation in Alternaria spore at the dose 200ppm. The hyphal wall and its diameter became markedly thinner, distorted and resulted in cell wall disruption with flattened and empty hyphal tips.

INTRODUCTION

The ubiquitous genus Alternaria includes both saprobes and plant pathogens which have been reported worldwide infecting crops in the field and causing post harvest decay of many plant products (Thomma, 2003). Beside yield losses, Alternaria spp. are responsible for spoilage of commodities during transport, storage and in processing, which may lead to the reduction of technological quality and serious economic losses. There is a growing concern of Alternaria spp. due to their ability to produce secondary metabolites with different toxicological properties, which are harmful for human and animal health. Over the past years, numerous chemical pesticides, such as Benzimidazoles, Imazalil, organic and inorganic sulfur compounds and oxidizing materials were introduced to control the disease but in most cases because of environmental problems, toxicity to humans, creation resistant race and sometimes high costs of such combinations, is not recommended (Rahemi, 2003).

Biologically active essential oils represent a rich potential source of an alternative and perhaps environmentally more acceptable disease management compounds. With a broad range of natural fungicidal plant volatiles, numerous opportunities exist to explore their usefulness in controlling post-harvest diseases. The general antifungal activity of essential oils is well documented (Meepagala et al. 2002) and there have been some studies on the effects of essential oils on post-harvest pathogens (Bishop and Thornton 1997). The advantage of essential oils is their bioactivity in the vapour phase, a characteristic which makes them attractive as possible fumigants for stored product protection.

Zingiber officinale Roscoe. (Family Zingiberaceae) is well known in Asia. The ginger has been listed in Generally Recognized as Safe (GRAS) document of USFDA and has antimicrobial and antymycotoxigenic effects (Tatsadjieu et al., 2009) because of its aroma and taste has been used for culinary purposes from ages. It has also been reported in use for medicinal purposes for more than 2500 years. Ginger rhizome is widely used as an ingredient in food, pharmaceutical, cosmetic and other industries. Ginger is also known to possess antioxidant properties. Ginger contains a unique flavour derived from both nonvolatile and volatile oils. The antifungal activity of its oil is well documented (Ficker et al. 2003).

Ginger is found to be rich in bioactive compounds and various researches have been carried out to explore the beneficial properties of ginger and its extracts. (Grzanna et al., 2005). Essential oils of ginger are of interest because of its richness in various functional compounds mostly terpene, monoterpene and sesquiterpenes, which gives the oil its biological activity (Daferera et al, 2002).

Thus objective of this study was to analyze the essential oil composition of ginger and its biological effects on storage rot causing fungus Alternaria alternata. Here we will elucidate the morphological alterations in Alternaria alternata due to exposure of ginger oil.

MATERIALS AND METHODS

Plant preparation

Ginger rhizomes were purchased from a local market in Lucknow (India). Ginger was cleaned with distilled water to remove soil and dust, peeled and chopped.

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**Extraction of essential oil**

The fresh ginger (5 kg) was hydro-distilled for 6-7 hrs. in a Clevenger type apparatus. The oils were dried over anhydrous sodium sulphate. To isolate the oil from the aqueous portion, the oil was extracted with solvent ether in a separating funnel. The ether was removed at reduced pressure which resulted in an oily residue that was added to the oil collected earlier free from smell was stored separately. Thus, the hydrodistilled volatile fraction from the ginger rhizome was separated into two fractions an oil fraction and an aqueous fraction. The essential oil obtained was kept in sealed glass tube at 4°C until analysis.

**GC-MS analysis**

Gas Chromatographic analysis followed by mass spectra was carried out in Perkin Elmer Autosystem XL Packed mode. Column used for analysis was OV-1, 100% Methyl gum (10 feet). The conditions were as follows; Temperature programming from 4°C-220°C, hold at 75°C for 20 minute. Injection temperature 250°C and detector temperature was 255°C. Carrier gas was N2 at a flow rate 14 ml/min.

The identification of individual compound is based on their retention time’s relatives to those of authentic samples and matching spectral peaks availability with NIST mass spectral libraries.

**Test Fungus**

Strain of *Alternaria alternata* (Fr.) Keissl. MPPLU from the collection of Mycology and Plant Pathology Division, Botany Department, University of Lucknow was used. The fungus was maintained on Potato Dextrose Agar (PDA) at 25 ±1°C. The Petriplates were kept at 25±2°C for 24 h. Each slide was then fixed in lacto-phenol-cotton blue and observed under the microscope for spore germination. About 200 spores were counted and the number of spores germinated was scored using haemocytometer to calculate the percentage of spore germination (Surender et al., 1987).

**Volatile activity measurements**

Tests for volatile activity were carried out in 90mm Petriplates containing 20ml of solidified potato dextrose plate. A 5mm diameter disc of inoculum of the test species cut from the periphery of an actively growing culture on PDA plates was placed on the agar in each Petriplate and then Petriplates were kept in inverted position. In the lid of each Petriplate a sterilized cotton swab was placed on to which a different concentration of oil was poured. The Petriplates were sealed with parafilm to check the release of volatile oil. For each corresponding control an equal amount of sterile distilled water was poured on sterilized cotton swab.

The Petriplate were kept at 25±2°C for 30 days. Fungitoxicity was expressed in terms of percentage of mycelial growth inhibition and calculated as per formula of Pandey et al., (1982).

\[
\text{Percentage of mycelial growth inhibition} = \frac{d_c - d_t}{d_t} \times 100
\]

Where,

- \(d_c\) = average diameter of fungal colony in control.
- \(d_t\) = average diameter of fungal colony in treatment.

**Determination of mycelial weight**

To determine the effect of essential oil on the dry weight of the test fungus, different concentrations of oil in potato dextrose broth (PDB) medium were prepared in Erlenmeyer flask and inoculated with 10⁷ spores/ml of spore population was counted using haemocytometer. In the corresponding control equal amount of distilled water was added. After 15 days dry weight of mycelium was determined. Flasks containing mycelia were filtered through Whatman filter no. 1 and then washed with distilled water.

The mycelia were allowed to dry at 60± 1°C for 6h and then at 40 ±1°C over night. The filter paper containing dry mycelia were weighed. Percent growth inhibition on the basis of dry weight was calculated as

\[
\left(\frac{\text{Control weight - Sample weight}}{\text{Control weight}}\right) \times 100
\]

**Spore germination assay**

Five concentrations of oil (25ppm, 50ppm, 100ppm, 200ppm, 500ppm) were tested for spore germination of the test fungi. Fungal spores obtained from 10-day-old cultures of the fungi were taken and placed on glass slides in triplicate. Slides containing the spores were incubated in a moist chamber at 25±2°C for 24 h. Each slide was then fixed in lacto-phenol-cotton blue and observed under the microscope for spore germination. About 200 spores were counted and the number of spores germinated was scored using haemocytometer to calculate the percentage of spore germination (Surender et al., 1987).

**Nature of toxicity**

The fungitoxicity (fungistatic/fungicidal) of the essential oil was tested by using the technique of Thomson (1989).

**Light microscopy**

A sample of mycelium was taken from the periphery of the colony grown on PDA with treatments of oil 25ppm, 50ppm, 100ppm, 200ppm, 500ppm after seven days of incubation. The samples were fixed in lactophenol- cotton blue and examined under the microscope (Nikon ECLIPSE E200, Japan) at 40 X-100Xto examine structural abnormalities. Samples from control plates without oil were also stained and observed. Photographs were taken with the help of computer attached Samsung COLOR CAMERA SAC-410PA.

**Scanning electron microscopy**

Fifteen day old fungal cultures on PDA treated concentrations 25ppm, 50ppm, 100ppm, 200ppm, 500ppm of oil was used for all scanning electron microscopic (SEM) observations. 5x10mm segments were cut from cultures growing on potato dextrose plates and promptly placed in vials containing 3% glutaraldehyde in 0.05M phosphate buffer (pH 6.8) at 4°C. Samples were kept in this solution for 48 h for fixation and then washed with distilled water three times for 20 min each. Following which they were dehydrated in an ethanol series (30%, 50%, 70%, and 95%), for 20 min in each alcohol dilution and finally with absolute ethanol for 45 min. Samples were then critical point dried in liquid carbon dioxide. Fungal segments were placed in desiccators until further use. Following drying, samples prepared were mounted on standard 1/2 in Cambridge SEM stubs using dou ble-stick adhesive tabs and coated with gold-palladium electroplating (60 s, 1.8 mA, 2.4 kV) in a Polaron SEM Coating System sputter coater. All samples were viewed in a Cambridge LEO S-430 SEM operating at 15 kV at various levels of magnification (300X to 5 k X).
RESULTS

The chemical components of the oils

In present study 81.25% of the compounds were identified in oil. The chemical composition of oil was given in Table 1 and Figure 1. The main monoterpenene compound was camphene which was 23.9% followed by sesquiterpene zingiberene 12.2%, whereas 1,8-cineole was reported as major component (27.9%). Other hydrocarbons were α-pine 7.2%, α-seline 0.9%, α-farnesene 1.1%, ar-curcumene 0.8%, Camphor 0.4%, Tricyclene 0.1%. The other oxygenated compounds were Geranial 2.8, Neral 0.9%, Elemol 0.9% and Nerolidol 1%.

Table 2 Effect of different concentrations of Ginger oil on dry weight(15days), radial growth (7days) percentage and spore germination percentage(7days) of Alternaria alternata at 25±1°C

<table>
<thead>
<tr>
<th>Conc. (ppm)</th>
<th>Dry weight (mg±SE)</th>
<th>Radial growth (%)</th>
<th>Spore germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>410±5.77</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>382.3±23.3</td>
<td>6.66</td>
<td>74.2</td>
</tr>
<tr>
<td>50</td>
<td>281.67±1.66</td>
<td>34.12</td>
<td>36</td>
</tr>
<tr>
<td>100</td>
<td>113.67±0.88</td>
<td>76.89</td>
<td>15</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Percent inhibition on the basis of radial growth and dry weight was calculated and it was found that the oil was equally effective in liquid medium than solid medium (Table 2). The oil exhibited 100% inhibition of spore germination at 500ppm concentration, while at 50ppm concentration approximately 50% inhibition of spore germination was observed (Table 2).

Light microscopy

The observations of A. alternata examined under light microscope at 100X magnification, after treatment with different concentrations (Control, 200ppm, 500ppm) Ginger oil are presented in Figure 2. In addition to inhibited growth, mycelial colonies grown in the presence of essential oil seemed to consistently exhibit distinct morphological alterations when compared to control. These variations included lack of sporulation, visible loss of pigmentation and aberrant development of conidiophores. In microscopic examination of untreated mycelium hyphae had homogenous, clear cytoplasm and elongated conidiophores bearing conidia which were black and elongated clearly visible (Fig. 2A) while treatment with 200ppm concentration clearly showed reduction in conidial heads, poorly developed spores become malformed, elongated to round shape of (Fig. 2B) conidiation and conidiophores becoming distorted (Fig. 2C). The alterations in hyphal structures started with budding of hyphal tip, anomalous structures such as swelling, localized along the hyphae and at their extremities with

Inhibitory effect of Ginger oil on test fungus

Inhibitory activity of the essential oil in agar medium is presented in Table 2. Radial growth and biomass of A. alternata was significantly reduced in response to various concentrations of ginger oil ranging from 25ppm to 500ppm. At 500ppm concentration fungal development was completely inhibited after 7 days of incubation. 500ppm concentration of ginger oil was found to be fungicidal against A. alternata which was also the MIC for the test fungus. At 100ppm, growth was reduced to about half that of the control (Table 2). Determination of mycelial weight was done and it was found that insignificant growth occurred at 500ppm concentration.
the apexes showing an irregular growth in dimension with irregular shape. Higher concentration of 500ppm clear zonation of cytoplasm from hyphae and ultimately hyphae without cytoplasm were found (Figs. 2B and 2C).

Scanning electron microscopy

The effect of Zingiber officinale essential oil on the morphology of A. alternata examined by SEM is shown in Fig. 2(D, E and F). The A. alternata mycelium grown on PDA medium as control showed the characteristic morphology with lengthened, regular, homogenous hyphae of constant diameter with smooth external surface and with rounded apex. After 7 days of incubation in the treatments (control, 200ppm, 500ppm) fungal mycelium showed alterations in the morphology of the hyphae. Treatment with essential oil at 500ppm clearly showed distorted mycelium, squashed and flattened conidiophores bearing damaged conidia (Fig. 2E). At 200ppm, we can see the spores becoming distorted there was clear decrease in cytoplasmic content, with clear separation of cytoplasm from cell wall in hyphae and apical tip without cytoplasm were observed (Fig 2C, 2F). Flattened empty hyphae with the presence of undulations along the hyphal borders were clearly visible which look like ribbon (Fig. 2C, 2F). On increasing the concentration up to 500ppm complete cell wall disruption and squashed hyphae were found (Fig. 2F).

DISCUSSION

Essential oils are very complex natural mixtures which can contain about 20–60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts (Bakkali, 2007). In literature, many variations have been found in chemical composition of ginger oil. Agrawal et al. (2001) reported curcumene as the major component in the fresh ginger rhizomes, while Menut et al. (1994) identified citral as the main constituent of ginger oil. In other previous report on ginger oil (Singh et al., 2005a, b, c), α-zingiberene was found to be the major constituent. But in our study we found 1, 8-cineole as major component. These differences in the chemical composition of the oil from the same plant/plant part could be due to the environmental, developmental, genetic or some other factors. Yield and composition of oil differ widely with the production conditions (Blair et al., 2001), variety, cultivars or population (Galambosi and Peura, 1996) and on climatic and soil factors. This cytotoxic property is of great importance in the applications of essential oils not only against certain human or animal pathogens or parasites but also for the preservation of agricultural or marine products. Ginger essential oil is indeed effective against several mycoxin producing fungi of stored commodities. It appears that the fungicidal/ fungitoxic nature of the oil is due to 1, 8-cineole which is an ether compound and due to other monoterpenes camphene and sesquiterpene zingiberene. The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism is known as the MIC. The determination of the MIC of oil is necessary for prescribing its appropriate dose. Clearly, unnecessarily high doses of oil increase wastage and may cause considerable harm to the quality of the commodity treated. It is noteworthy that in some instances, the oil of a plant investigated by different workers has shown variation in the MIC (Singh and Handique, 1997 and Pandey et al., 1982). Such variation may be due to the use of different test fungi or different technique adopted. However, in the present work the MIC of ginger oil 500ppm was effective against all test fungi Alternaria alternata. A fungitoxicant may act as a fungastat or a fungicide inhibiting the growth of fungus temporarily or permanently respectively (Sharma and Tripathi, 2007). The essential oil significantly reduced the growth of Alternaria alternata in a dosage response manner. After calculating the percent inhibition with respect to radial growth (PDA) and dry weight (PDB) basis we found that the oil was more effective in liquid as well as solid medium. The present study is dedicated to confirm the antifungal activity of Ginger oil already described in literature that Ginger Oil had higher biological activity towards Aspergillus niger, Candida albicans and Bacillus subtilis (Nirmala menon, 2010) but the mode of action and morphological alterations were not studied yet. Here we explored not only antifungal activity of ginger oil against Alternaria alternata but also observed the biological effects of essential oil. Based on the present study, it could be concluded that essential oil from Zingiber officinale Roscoe. possess fungitoxic activities inhibiting the growth of Alternaria alternata leading to irreversible deleterious morphological alterations and thus it is worth exploiting for the bio management of Alternaria alternata.

References


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