The Effect of Ozone on the Growth and Development of Selected Food Spoilage Fungi

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ABSTRACT

Fungal spoilage during the storage of fresh produce is a major concern in the food industry. Several methods have been used in order to improve and increase the shelf-life of food such as pesticide treatment, but the safety issues and concerns about these methods has been questioned due to their observed ill effects on consumer health and environment. Thus, it leads to emergence of a novel alternative residue free treatment. Ozone is known to be a powerful oxidising agent having the ability to break down into harmless oxygen and interestingly leaves no residue. The potential of ozone to reduce food spoilage organisms is well documented and the use of ozone against food spoilage has been approved by US-FDA. The impact of ozone exposure on macroscopic development and morphological characters of such organisms are yet to be understood. This study was carried out to elucidate the effect of continuous gaseous (180 - 200 ppb) ozone exposure on fungal development of different strains. Four different genera (*Penicillium, Fusarium, Mucor* and *Cladosporium)* were identified from fourteen different strains by using molecular techniques which were aseptically isolated from various types of produce. The effects of continuous ozone exposure on radial growth, spore production and micromorphological modifications were observed with both spore and mycelial inoculum. Variations in growth pattern were observed due to behavioural differences of different species under ozone induced oxidative stress. However, significant differences were observed with reduced fungal growth in most of the isolates especially in *Penicillium* sp. whereas, suppressed spore production (> 50%) in eight isolates were observed. In addition, morphological modifications were also observed in the isolates grown under ozone. Therefore, ozone can be efficiently used for controlling and inhibiting food spoilage during storage. Hence, more research needs to be done to reveal the reason for these variations among species.

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CHAPTER-1

INTRODUCTION & LITERATURE REVIEW

1.1. Introduction: Food spoilage

Food spoilage is defined as “the change in the characteristics of a food that makes it unacceptable” (Curtis *et al.,* 2003). The cause of these changes can be attributed to physical or biological process. Biological process includes insect infestation or microbial action (Curtis *et al.,* 2003). Food spoilage leads to changes in food that makes it unsafe and thus reducing its acceptability to the consumer for its original purpose (Harrigan and Park, 1991). The changes in food produced by the microbes and other pests vary widely, including severe changes in the quality (including appearance and odour) of fresh produce (Stewart and Amerine, 1982).

A food is considered spoilt when the changes are detectable and the microbial population has reached to ca. 10^{7-9} /ml, /g, or /cm^2. These changes are due to the catalytic actions of a large number of microbial enzymes (Ray and Bhunia, 2008). The microbial enzymes are usually of two types: *intracellular enzymes* or *extracellular enzymes.* The intracellular enzymes act on the nutrients that can be transported inside the cells through several transport mechanisms. The extracellular enzymes (after synthesis) either remain bound to the cell surface or are excreted in the food environment (Ray and Bhunia, 2008). Even with the modern preservation techniques, an excessive amount of foods are lost and damaged due to microbial spoilage. It has been estimated that between 10 - 20% more food would be made available if the spoilage could be prevented (Fox and Cameron, 1972).

Food spoilage is one of the most important issues in the field of the food technology. The problem of the food spoilage is now catching up the eyes of the technologist as the present preservation methods are not able to cope up with the vast increase in the microbial spoilage of food. The economic cost of microbial food spoilage is difficult to estimate, but is certainly billions of pounds a year (Adams and Moss, 2000). Despite the benefits of modern preservation and storage technology, large quantities of food (25%) have to be discarded every year as a result of microbial attack and over-ripening (Adams and Moss, 2000). Microbial food spoilage is also important in many foods as a warning of the possible growth of pathogens. In the United States and other countries, where foods are produced and procured from many countries more than the need, food spoilage is not considered a serious problem. However, in countries where the food production is not efficient, spoilage can adversely affect the availability of food. Therefore, it is necessary to understand the factors associated with the food spoilage for recognizing the cause as well as to develop an effective means of control.

This review will focus on filamentous fungi which is a main cause of food spoilage. Though, bacterial contamination is also known to be a major cause of food spoilage but is outside the scope of this review. The factors affecting growth of micro-organisms in fruit spoilage, current methods of preservation of fresh produce and their drawbacks will be discussed in this review. This review will focus on the use of ozone to reduce fungal growth in order to control food spoilage considering its limitations.

1.2. Causes of food spoilage

Micro-organisms are the major cause of the spoilage of food (Bell *et al.,* 2007) and are responsible for these chemical changes (Garbutt, 1997). Fresh fruits and vegetables easily get contaminated by micro-organisms present in soil, water, air, other environmental sources and various plant pathogens (Ray and Bhunia, 2008). Microbes grow more rapidly in damaged or cut vegetables. Loss of fruit and vegetable texture occurs mainly from reactions initiated by enzyme such as β-glycosidase, pectin methyl esterase and polygalacturonase (Lidster *et al.,* 1986). Loss of water by transpiration is an important factor affecting storage life and quality of fruits and vegetables after harvest. The common cause of food spoilage in fruits is *'filamentous fungi'* because of their ability to grow and survive in low pH environments (Moss, 2008).

1.2.1 Moulds/Filamentous fungi

The term *'mould'* is used industrially for mycelial fungus which is a part of genus ascomycetes (Garbutt, 1997). These are the microscopic organisms which do not possess chlorophyll. They grow in filaments or fine threads which extend in length and form a mass visible as *"mould growth"*. Moulds form spores or seeds which float through air currents and infect other foods and start their growth cycle again. They generally need oxygen for their growth and development and this is the main reason that they are usually found on the food surfaces (Fox and Cameron, 1972) but can also be found in anaerobic environments (Bell *et al.,* 2007). The growth of moulds has different morphological forms based on their morphology and mode of reproduction and these groups include basidiomycetes, dueteromycetes, zygomycetes and ascomycetes (Garbutt, 1997). The identification of the species can be done based on their spore growth, arrangement of spores and attachment to the mycelium (colour and appearance of mould colony) (Garbutt, 1997). The growth of moulds usually begins with spore germination, hyphae production and then branching into mycelium (Bell *et al.,* 2007).

Moulds can easily grow in high acid foods such as fruits, vegetables, pickles, bread, cake, cured meats, sweet foods and other fermented dairy products (Stewart and Amerine, 1982). The changes caused by moulds in food are off-color, off-flavor and unsightly appearance (Stewart and Amerine, 1982). Mould growth usually does not occur above the normal body temperature. Thus, by processing high acid foods at high temperature (100°C) for two or more days destroys the germination of spores (Fox and Cameron, 1972). Eating mouldy food (bad taste and smell) may cause vomiting or nausea in consumers. A very large number of moulds produce toxic substances known as "*Mycotoxins*". There are 14 known mycotoxins and they can have carcinogenic and mutagenic properties. The most common mycotoxins are

Aflatoxin, Alternaria toxins, Citrinin, Ochratoxins, Patulin, Penicillic acid, Sterigmatocystin and *Fumonisins* (Jay *et al.,* 2005).

Some examples of the fungal spoilage in the fresh produce are:

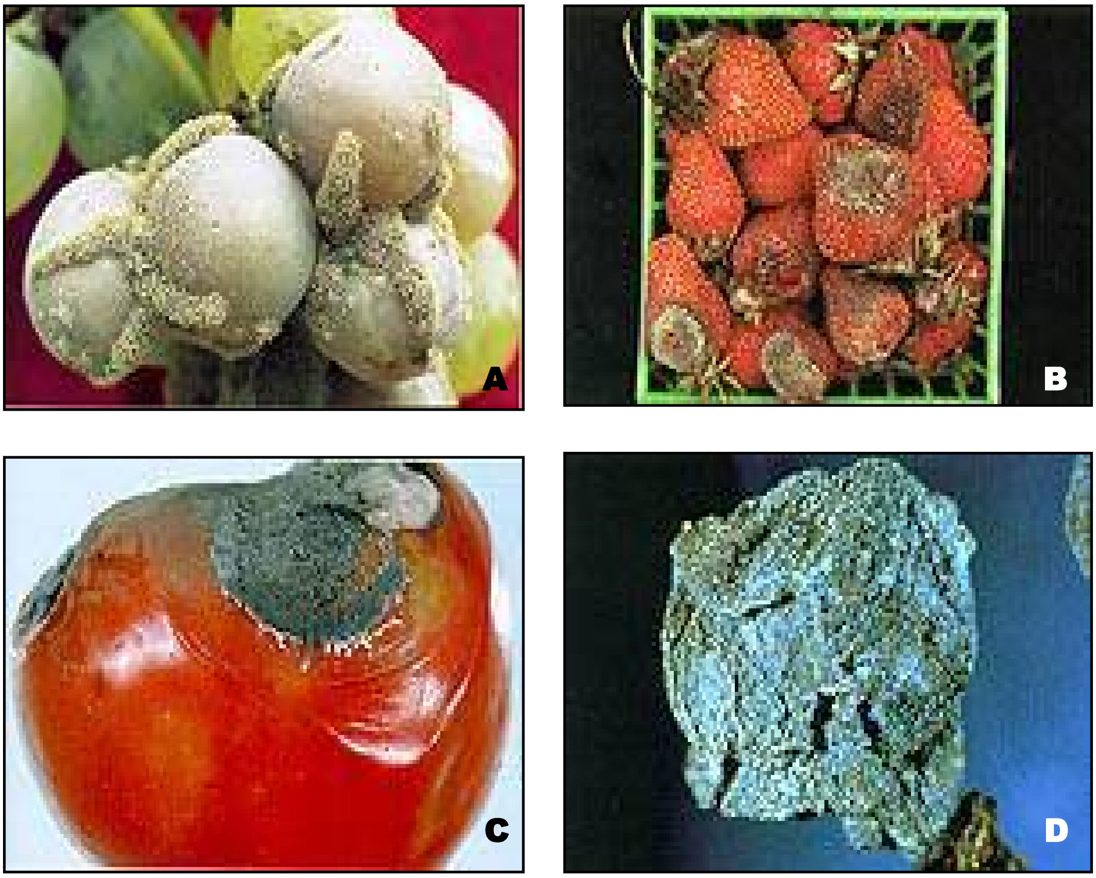

Figure 1.1. Fungal spoilage in fresh produce **(A).** storage rot in grapes caused by *Botrytis cinerea*; **(B).** storage rot in strawberry caused by *Botrytis cinerea;* **(C).** blue mould rot caused by *Penicillium* sp. (also by *Fusarium* sp.); **(D).** black mummy rot of grapes caused by *Guignardia bidwelli.*

Source: Yu *et al.* (Chemistry Project "Food Preservation")

1.3. Fungi spoiling fresh produce

The microflora of freshly harvested fruits and vegetables is diverse (De Roever, 1998). The presence of numerous genera of spoilage bacteria, moulds, yeasts and occasionally pathogens on fresh produce has been recognized for many years (Beuchat, 1996). The presence of food pathogens on fresh produce are particularly important concern as they are usually eaten raw. Pathogenic micro-organisms associated with fresh produce include *Penicillium italicum*, *Penicillium digitatum* found on oranges and *Penicillium* sp. is also found on garlic, mangoes, apple, cheese and several other citrus fruits (Filtenborg *et al.,* 1996; Fallik *et al.,* 1996; Palejwala *et al.,* 1989), *Botrytis cinerea* commonly found on grapes and strawberries (Stewart and Amerine, 1982), *Fusarium* sp. in cucumber, *Mucor circinelloides* (Smith *et al.,* 1979), *Cladosporium fulvum* (Coleman *et al.,* 1997) and *Penicillium expansum* (Harwig *et al.,* 1979) are found in tomato. The most common spoilage is caused by different types of moulds from the genera *Penicillium, Phytopthora, Alternaria, Botrytis, Rhizopus* and *Aspergillus*. Microbial vegetable spoilage is generally described by a term *"rot",* with the changes in appearance such as black rot, gray rot, pink rot, soft rot and stem end rot (Ray and Bhunia, 2008).

Type of Spoilage	Micro-organisms Involved
Soft rot	*Rhizopus, Erwinia*
Gray mould rot	*Botrytis*
Black mould rot	*A. niger*
Pink yeast (rot)	*Rhodotorula*

Table 1.1. Four different types of microbial spoilage and the micro-organisms involved in the fungal spoilage (Ray and Bhunia, 2008).

1.4. Factors affecting the growth of micro-organisms in foods

The factors affecting the microbial growth in foods are divided into four main categories:

1.4.1. *Intrinsic factors*

The physical, chemical and biological properties of the food such as pH (around 7), redox potential, higher water activity (A_W) and antimicrobial substances.

1.4.2. Extrinsic factors

The characteristics of the environment in which the food is maintained such as temperature, atmosphere and relative humidity.

1.4.3. Implicit factors

The properties of the microbial population which are present on the food such as growth rates and interactions (synergistic or antagonistic) between the elements of the population.

1.4.4. Processing factors

The physical or chemical operations involved in food processing such as pasteurization, washing, irradiation, peeling or slicing.

These factors and interactions between them determine the nature of the microbes and their potential to grow and eventually cause spoilage in the food (Curtis and Lawley, 2003; Adams and Moss, 2000).

1.5. Current methods of preservation of fresh produce

Many methods are widely used to prevent the microbial food spoilage. The main aims of the food preservation methods are to increase the shelf-life of the food and to make it safe for the human consumption. The various methods used for the preservation of fresh produce (fruits and vegetables) from the spoilage discussed below:

1.5.1. Refrigeration and Freezing

The rates of growth and reproduction of micro-organisms reduces at low temperature. Food is not preserved at low temperature to kill the microbial cells but to prevent it from spoilage. Some micro-organisms are able to survive in the low temperature (freezing) such as *Cladosporium herbarum* (black spots) and *Sporotrichum carnis* (white spots) (Adams and Moss, 2000). Deep freezing at -60°C reduces the biochemical activities of microbes. Fresh fruits and vegetables are kept at temperature between 10-20°C or may be sometimes lower to reduce their metabolic rate. The highly perishable food products are usually stored at low temperature (below 7°C). Different ways are used to store the food at low temperature to extend their shelf life such as *ice chilling* (0-1°C), *refrigeration* (1-7°C) and *freezing* (-20°C). The microbial growth and enzymatic activity in food at low temperature depends on 3 main factors such as *nature of process, nature of food* and *nature of micro-organisms* (Ray and Bhunia, 2008).

1.5.2. Drying or Reduced water activity

Micro-organisms require water for their growth and reproduction and preservation by dehydration reduces their growth. Drying removes the moisture from the food and slows down the action of enzymes causing ripening. Drying results in the change in weight and original shape of food but by adding water at the time of use it regains it originality. The main objective of reducing the water activity in food is to reduce the growth of vegetative cells and germination and outgrowth of spores of micro-organisms along with the prevention of toxin production by toxigenic moulds and bacteria. There are various methods used for reducing the water activity such as natural dehydration (sun-drying), mechanical drying (involves tunnel drying, roller drying and spray drying) and freeze drying (Ray and Bhunia, 2008; Adams and Moss, 2000).

1.5.3. Treatment with chemicals/fungicides

Chemicals have been used in the preservation of foods for many centuries. The use of chemicals is an important means of preventing microbial spoilage of food. In order to control produce loss, the use of post-harvest fungicides is the most common method (Mattson, 2008). When a micro-organism is surrounded by a concentrated aqueous solution, water passes from its cells to the solution and due to dehydration the micro-organism dies. The commonly used chemicals in Great Britain are sulphur dioxide, chlorine, propionic acid, hydrogen peroxide, chlorine dioxide, hypochlorite washing, organic acids, acidified sodium, chlorite, trisodium phosphate, sorbic acid, sodium or potassium nitrates and nitrites, methyl or propyl p-hydroxybenzoate, tetracylines, biphenyl or o-phenyl phenol and its sodium salt (Ray and Bhunia, 2008; Stewart and Amerine, 1982; Fox and Cameron, 1972; Jongen, 2005) whereas, fungicides such as vinclozolin, pyrimethanil have found much effective in controlling blue mould on strawberries and grapes (Døving and Máge, 2001). The most commonly used chemicals in the preservation of fruits and vegetables are:

Food	Preservative
Fruit- citrus	diphenyl or *o*-phenylphenol
Pears	*o*-phenyl phenol, Copper carbonate
Pineapples, Melons and Peaches, Apple	*o*-phenylphenol
Cabbage, Potato, Tomato, Fruits (dried)	Sulphur dioxide
Tomato, apple cider, salad dressing	Benzoic acid / Benzoates
Apples, Pears, Citrus fruits, Pineapples	Thiabendazole or Sodiumphenylphenate
Citrus fruits	Biphenyl
Grapes	Sulphur dioxide fumigation
Apples, Pears, Bananas, Mangoes, Papayas, Peaches, Cherries, Pineapples	Benomyl

Table 1.2. Various food and preservatives added to preserve them. (Fox and Cameron, 1972; Jay *et al.,* 2005)

1.5.4. *Irradiation*

Food irradiation is a cold process of preserving food. This process can be categorized on the intention of the processing and the dose used. There are various techniques and radiations used in this process such as Ultraviolet light, Beta Rays, Gamma Rays, X-Rays and Microwaves. There are several factors considered while using the effects of irradiation on micro-organisms like type of micro-organisms, number of organisms, composition of food, presence/absence of oxygen, physical state of food and age of organisms (Jay *et al.,* 2005; Jongen, 2005; Adams and Moss, 2000).

1.5.5. Antimicrobial Films or Coatings

The edible films are prepared from edible materials such as proteins, polysaccharides and lipids. These can be used in many different forms such as film wraps, pouches and film coatings for food and food components (Cagri *et al.,* 2004). In fresh fruits and vegetables, carnauba wax coating has been used to reduce moisture loss and to maintain fresh quality by controlling oxygen and carbon dioxide exchange (Cagri *et al.,* 2004). In order to improve the microbial stability of fruits and vegetables, the surface is treated either by spraying or dusting with antimicrobial agents or by dipping them in antimicrobial solution (Labuza and Breene, 1988). Some common antimicrobial agents used for fruits and vegetables are organic acids and their salts, antimycotics, nisin, lysozyme, plant extracts and silver ion antimicrobials (Jongen, 2005).

1.6 Drawbacks of the different storage techniques

The processing of fresh fruits and vegetables at high temperature and reducing water activity decreases the quality and freshness of the fruits and vegetables (Jongen, 2005). Moreover, usage of antimicrobial films or coatings may have intolerance or allergic reactions to the consumer. All these treatments are cost-effective and require different equipments (Jongen, 2005). Several factors have to be kept in mind while doing irradiation as it may produce changes in physical properties such as viscosity and chemical properties like in protein, lipids, carbohydrate, nucleic acids, vitamins as well as histological or morphological characteristics and micro flora of food (Jongen, 2005).

Moreover, several incidents of pesticide poisoning among agricultural workers by the use of toxic organophosphate insecticides have been observed and the Environmental Protection Agency (EPA) has estimated that 0.8% of the 2.25 million agricultural workers suffer from acute pesticide poisoning in the United States annually (Geer *et al.,* 2004). The health effects associated with the use of pesticides to mankind includes acute injury to nervous system, reproductive organs, birth defects, lung damage, dysfunction of endocrine and immune system and cancers (Oleskey *et al.,* 2004). Several pathogens have shown resistance to chemicals due to their widespread use (Palou *et al.,* 2001) and first resistant strain of *Botrytis cinerea* was found to be resistant to dicarboximides on strawberries in 1973 (Bal *et al.,* 1989) whereas, *Penicillium digitatum* and *Penicillium italicum* was found to be resistant to imazalil fungicide commonly used in spain (Cabañas *et al*., 2009) *Candida* sp. have also been reported to be resistant to azole antifungal agents (Schnabel and Jones, 2001).

These reasons concerns about the safety of consumers health. Thus, in order to save fresh fruits and vegetables from the fungal spoilage, these problems associated with the current treatments and methods has led to the search for new and better treatment to prolong the shelf-life of food. Of the variety of methods available ozone treatment has potential to prolong the shelf-life and reduction of fungal spoilage in the fresh produce.

1.7. Ozone

Ozone is a natural gas, generated in the higher layers of the atmosphere i.e., stratosphere. Ozone consists of the three oxygen atoms per molecule (O_3). It is not emitted directly from any man-made source but is produced by the direct action of ultra-violet light on oxygen molecules (Beltrán *et al.,* 1997). Ozone formation in nature takes place by Ultra violet-irradiation from the sun and during lighting discharge (Beltrán *et al.,* 1997). Ozone is a colourless gas with pungent smell and has strong oxidizing power, very reactive, almost unstable in water, leaves no residues in water and naturally decomposes into ordinary oxygen making it the most potent oxidizing agent (Russell and Gould, 2003).

Ozone can be used as a therapeutic agent and is the most powerful oxidant (Tzortzakis *et al.,* 2008) which can act 3000 times faster than chlorine for inactivating viruses, bacteria, fungi and protozoa in drinking water (Ya-Ching *et al.,* 2007; Parish *et al.,* 2003). Ozone can be prepared commercially by two methods which are Corona discharge (CD) and ultraviolet light (UV). Out of these two methods, CD method is more preferred technically and economically as it gives higher ozone concentrations (Jongen, 2005).

1.7.1 Ozone in the control of food spoilage

Ozone has been quoted as "a safe alternative for disinfecting fresh produce" (Russell and Gould, 2003) in more efficient and eco-friendly way of sanitizing than chlorinated methods. In 1997, ozone was granted Generally Recognized As Safe (GRAS) status and received US-FDA approval as food sanitizing agent (Jongen, 2005; Antony-Babu and Singleton, 2009). In June 2001, the use of ozone in gaseous or aqueous phase was approved by the FDA for antimicrobial treatment, storage and processing of food (Jongen, 2005).

Ozone gas is effective against various micro-organisms and leaves no detectable residues and is a powerful antimicrobial agent (Tzortzakis *et al.,* 2007). Ozone kills spoilage or pathogenic micro-organisms by oxidizing key cellular enzymes (Ray and Bhunia, 2008) and reduced effects on spoilage growth in various fruits and vegetables at low ozone concentration has also been observed (Palou *et al.,* 2003; Tzortzakis *et al.,* 2008).

1.7.2. Effect of ozone on fungal growth

Ozone has been used to treat a variety a produce to prevent fungal spoilage of fresh produce. Treatment of inoculated fruits and vegetables such as black pepper, carrot, lettuce and black-berry with ozone resulted in 3-6 log reduction in *Penicillium* and *Aspergillus* species (Ray and Bhunia, 2008) with retardation in fungal spore production and reduced spoilage (Smilanick, 2003). The shelf-life of oranges, strawberries, raspberries, grapes, apples and pears with ozonated water could be extended or doubled with the application of ozone at 2-3ppm for few hours a day (Russell and Gould, 2003). Gaseous ozone has also suppressed fungal development and enhanced shelf life of stored blackberries, table grapes and other refrigerated fruits due to retardation in ripening process by oxidation of ethylene (Palou *et al.,* 2003; Parish *et al.,* 2003; Di Renzo *et al.,* 2005).

In an ozone enriched atmosphere, the lesion development was reduced ($p < 0.01$) significantly in tomato fruit by 50% along with the impacts of ozone enrichment dependent on concentration and duration of exposure (Tzortzakis *et al.,* 2007). According to Ölmez *et al.* (2008), the ozone treatment was found to be better than the chlorine and other organic acid treatments in maintaining the sensory quality of fresh cut green lettuce at (2 ppm) ozone concentration for (0.5-3.5min) exposure time. In a study, ozone treatment in the reduction of post harvest fungal decay of grapes showed better results than sulphur dioxide fumigation (Sarig *et al.,* 1996). In case of strawberries, ozone induced delays in fruit softening and showed beneficial effects on the levels of non-soluble carbohydrates and antioxidants that are known to be key nutritional components (Tzortzakis *et al.,* 2007). Barth *et al.* (1995), observed effectively suppressed fungal development in treatment of blackberries with low ozone concentrations.

Moreover, reduced incidents of spoilage with increased shelf-life in an ozonated atmosphere in apples and grapes (Parish *et al.,* 2003) with reduction in ageing and weight loss in oranges have also been observed (Di Renzo *et al.,* 2005). Beltran (2005), found a 3.3 log reduction in fresh cut potato strips that were 19 vaccum packaged. Whereas, in the case of corn, ozonation (10-12 wt%) reduced aflatoxin levels by 92%, reduces the mutagenic potential and had significant effect on fatty acids of contaminated corn (Prudente and King, 2002). No phototoxic injuries were observed in the ozone treated fruit (dates) when exposed to 0.15 ppm of ozone for 30 days at 15°C and 90% relative humidity, though ozone maintained the firmness and did not affect colour index, ethanol, total soluble solids or pH (Salvador *et al.,* 2006).

However, reduction in spore production and inhibited normal aerial growth of the mycelia in *Penicillium digitatum* and *Penicillium italicum* (Palou *et al.,* 2003) as well as in *Aspergillus niger* (Vijayanandraj *et al.,* 2006), *Aspergillus nidulans* and *Aspergillus ochraceus* were observed under ozone (Antony-Babu and Singleton, 2009). In addition, the sporulation of green mould on lemons, oranges (Harding *et al.,* 1968) and other fruits was found to be reduced under 1.0 ppm ozone level (Palou *et al.,* 2001). Therefore, the reduction in sporulation by ozone could prevent the spread of fungi among fresh produce in storage leading to prevent commercial loss.

Moreover, ozone treatment has shown some changes in surface colour of some fruits and vegetables such as peaches and carrots with reduced ascorbic acid content in broccoli (Kim *et al.,* 1999). A naturally infected Camarosa fruit (strawberry) in the ozone enriched cold storage (1.5 μL/L ozone at 2°C for 3 days) resulted in the decay incidence, weight loss and fruit softening (Nadas *et al.,* 2003). Whereas, ozone treatment showed no reduction in the grapes of grey mould incidence and increased rates of water loss have been reported in ozone treated fruit (Palou *et al.,* 2002). Therefore, some further work is required to reveal the effects of ozone on fresh produce.

1.7.3. Further developments

Despite the fact that ozone has been used to treat a variety of fresh produce, still very less is known about the effect of ozone on the wide variety of different fungal spoilage organisms. Some work has shown that ozone can reduce sporulation of *Penicillium* sp. (Palou *et al.,* 2003) and *Aspergillus* sp. (Vijayanandraj *et al.,* 2006) but for the wider use of ozone it is important to know that ozone is effective against a wide range of spoilage fungi and effective against different strains and isolates. Moreover, variation in resistance to ozone has been observed in two *Aspergillus* species (Antony-Babu and Singleton, 2009). Therefore, it is clear that more work is required to establish if ozone can affect growth and reproduction of different fungal isolates.

1.7.4. Limitations of ozone

The use of ozone also has some limitations such as lack of efficiency, less effective in high pH, temperature and organic solids (Jongen, 2005). The high concentrations of ozone exposure both directly or through food are harmful to humans (Jongen, 2005). Ozone above 0.1 ppm concentrations can cause nose, throat and eye irritation. Being corrosive it requires corrosion-resistant materials (Jongen, 2005). The storage and transportation of ozone is another important drawback as it has a short life and has to be generated on-site which increases treatment costs and require complex equipment (Jongen, 2005). Moreover, it can damage fresh produce and due to toxicity some human safety issues can be raised.

1.8. Aims of this study

In order to evaluate the way in which ozone affects the morphology and spore production of specific species of fungi more work has to be done. Therefore, the specific objectives of this study are:

1. To isolate and identify different fungal species and strains from spoiled produce by molecular techniques.
2. To examine the effect of low-level gaseous ozone (180-200 ppb) on fungal development (radial growth) with spore inoculum.
3. To examine the effect of low-level gaseous ozone (180-200 ppb) on fungal development (radial growth) with mycelial inoculum.
4. To evaluate number of spores produced under low-level ozone (180-200 ppb) with spore and mycelial inoculum.
5. To examine the morphological modifications in various species grown under ozone (180-200 ppb) chambers.