Molecular and Microbiological Methods for the Detection and Measurement of Dry Bubble Disease caused by *Lecanicillium* (*Verticillium*) *fungicola* on Mushroom Farms

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Abstract

Agaricus bisporus (Lange) Imbach – button mushrooms is a commonly cultivated mushroom throughout Europe which has very significant agricultural production. Mushroom cultivation is a monoculture which is exposed to different pathogens and pests. The most economically significant mushroom pathogens is *Lecanicillium fungicola*, the causative agent of Dry Bubble disease. This mycoparasite is responsible for severe losses of cultivated mushrooms and can terminate all mushroom production.

The purpose of this study was to investigate sources of dry bubble disease on mushroom farms using microbiological and molecular approaches. The main focus of the research was to develop a selective medium, to modify the existing selective method and molecular method – Real Time PCR for detection from samples originating from mushroom farms.

The first task of this research was evaluate DNA extractions methods from pure cultures of *L. fungicola* and optimise PCR conditions using a know sets of primers. All tested DNA extraction method gave good genomic DNA useful for PCR.

The next part of this research was identify and detect of *L. fungicola* in samples originating from mushroom farms. A PCR assay was developed and optimised for the detection of *L. fungicola* in casing soil and other mushroom farm debris. Four different methods were evaluated for the isolation of DNA from soil containing different concentrations of conidia of *L. fungicola* including manual extraction and commercially available kits. Only two methods succeeded extracted *L. fungicola* DNA from samples containing soil and casing. The primers for detection of *L. fungicola* designed by Zijlstra *et al.* (2007, 2008 and 2009) gave a 102 bp amplification product and this primer set was tested in PCR reactions for *A. bisporus* and other mushroom pathogens such as *Cladobotryum mycophilum*, *Mycogone perniciosa* and *Trichoderma sp.* and also *Aspergillus fumigatus.*

On this research also was designed a selective primers for *Lecanicillium fungicola* detection from mushroom farms using ITS and MAT1-2-1 region. It was not possible to find truly specific primers for this purpose but some of the sets of primers generated can be used for *in-vitro* test for detection and identification *L. fungicola* from *A. bisporus* tissues.

This study also succeeded in designing selective media for detection of *L. fungicola* from mushroom farm samples. *Lecanicilium fungicola* selective medium

already exists (Rinker *et al.*, 1993), but the growth of *L. fungicola* is very slow due to the inhibitive nature of the ingredients on fungal growth. A modified selective medium and novel selective medium were developed to enable rapid and consistent detection of *L. fungicola* from contaminated soil and casing samples after 6 days of incubation.

Mushroom farms visits were performed for detection of *L. fungicola* from different locations and stages of the crop cycle from spawn running to 3rd flush. *Lecanicillium fungicola* was detected by microbiological tests using novel and modified selective media and molecular method Real Time PCR – TaqMan test using the above mentioned primers.

Key words: *Agaricus bisporus*, detection, selective media, Polymerase Chain Reaction (PCR), Real Time PCR, mushroom farm, *Lecanicillium fungicola*

Presentations and publications

1. **Damięcka (Piasecka) J.** 2007. Mushroom dry bubble caused by *Verticillium fungicola* (Preuss) Hasselbrauk Literatures review. *Postępy Nauk Rolniczych*. 4 :75-83.

2. **Piasecka J.,** Grogan H., Zijlstra C., Baars J.J.P and Kavanagh K. 2008. Detection of *Verticillium fungicola* in samples from mushroom farms using molecular and microbiological methods. 40th Anniversary Meeting Society of Irish Plant Pathologists AFBI Newforge Belfast 11th – 12th September 2008. Poster presentation

3. **Piasecka J.**, Grogan H., Zijlstra C., Baars J.J.P and Kavanagh K. 2008. Detection of *Verticillium fungicola* in samples from mushroom farms using molecular and microbiological methods. 6th International Conference on Mushroom Biology and Mushroom Products. 29th September – 3th October 2008. Poster presentation page 89.

4. **Piasecka J.** 2009. 6th International Conference on Mushroom Biology and Mushroom Product. 29th September - 3th October 2008.*The Newsletter of the British Society for Plant Pathology* No 58 Spring 2009. Meeting Report page 26-27.

5. **Piasecka J.**, Grogan H., Zijlstra C., Baars J.J.P and Kavanagh K. 2009. Detection of *Verticillium fungicola* in samples from mushroom farms using molecular and microbiological methods. Agricultural Research Forum 12th-13th March. Theatre presentation. Page 131.

6. **Piasecka J.**, Grogan H., Zijlstra C., Baars J.J.P and Kavanagh K. 2009. Detecting Dry Bubble on Commercial Mushroom Farms. All Ireland Mushroom Conference and Trade Show, Monaghan 21 May. Poster presentation.

7. **Piasecka J.**, Kavanagh K. and Grogan H. 2009. Identification of *Lecanicillium fungicola* on Irish mushroom farms. Autumn meeting of the Society of Irish Plant Pathologist 11-12 September 2009. Oral presentation

8. **Piasecka J.**, Kavanagh K. and Grogan H. 2009. A selective medium for the detection of *Lecanicillium fungicola* in samples from mushroom farms. Teagasc Walsh Fellowships Seminar 2009. Dublin, Published by: Teagasc, Head Office, Oak Park, Carlow www.teagasc.ie. Poster presentation. Page 56. http://www.teagasc.ie/publications/2009/20091111/Final_Book_Abstracts_WF.pdf.

9. **Piasecka J.**, Kavanagh K. and Grogan H. 2010. Incidence and detection of *Lecanicillium (Verticillium) fungicola* on mushroom farms between 2008 and 2010. Spring meeting of the Society of Irish Plant Pathologist. 09 April 2010. Oral presentation.

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Declaration

This thesis has not been submitted in whole or in part to this or any other university for any degree, and is the original work of the author except where otherwise stated.

Signed: _____

Date: _____

Abbreviations

A	Ampicillin
Abs	Absorbance
bp	Base pare
C	Carbendazim
°C	Degrees celcius
Ch	Chloramphenicol
cm	Centimeter
CP	Crossing point
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5’ - triphosphate
E	Erythromycin
e.g.	For example
EC_{50}	Fungicide concentrations which inhibited mycelial growth by 50 %
EC_{90}	Fungicide concentrations which inhibited mycelial growth by 90 %
FAM	6-carboxyfluorescein
fg	Femtogram
g	Grams
hr	Hours
L	Litre
MEA	Malt extract agar
MG	Malachite green
mg	Milligrams
MGB	3’-minor groove binding probe
min.	Minutes
ml	Millilitre
Mmol	Milimole
MRSM	Modified Rinker’s selective medium
ng	Nanogram
nm	Nanometre
NPDASM	Novel (PDA) selective medium
P	Prochloraz-manganese
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pg	Pictogram
RBM	Base Rinker’s medium
RNase A	Ribonuclease A
rpm	Revolution per minute

RSM	Rinker’s selective medium
RT PCR	Real Time PCR
S	Streptomycin
sec.	Seconds
sp.	Species (singular)
spp.	Species (plural)
T	Tetracycline
TAE	Tris acetate buffer
temp.	Temperature
U	Unit
UV	Ultraviolet
µg	Microgram
µl	Microlitre
µm	Micrometer
µmol	Micromol
V	Volts
V	Volume
v/v	Volume per volume
w/v	Weight per volume

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Chapter 1 Introduction

1.1 Kingdom: Fungi

A fungus is a member of a large group of eukaryotic organisms classified as a separate Kingdom: Fungi (Whittaker, 1969). Kingdom Fungi contains a very large biodiversity of organisms such as yeasts, moulds, rusts, smuts, truffles, morels and mushrooms (Alexopoulos *et al.*, 1996, Stajich *et al.*, 2009). Today more than 69,000 species have been described, but the total number of existing fungi may be more than 1.5 million species (Hawksworth, 1991). Today we can culture artificially only around 5-10 % of fungi (Manoharachary *et al.*, 2005). Molecular studies suggest that fungi are more closely related to animals than plants (Baldauf *et al.*, 1993, Wainright *et al.*, 1993), although early studies and papers suggested that fungi were members of the Plant Kingdom (Scamardella, 1999).

1.1.1 Taxonomy

The revolution in fungal taxonomy began in the early 1990s when molecular methods started to analyse of the nucleotide sequences of ribosomal RNA (rRNA = 18S, 5.8S, 26-28S and 5S) genes (White *et al.*, 1990). Copies of these genes are typically localized within a series of copies of a gene arranged in tandem arrays and thoroughly mixed within a genome (Rooney and Ware, 2005). The ribosomal DNA (codes of ribosomal RNA) (rDNA = 18S, ITS1, 5.8S, ITS2 and 26-28S and 5S) genes are major transcriptional units and are moderately repetitive with 40-240 copies per haploid genome, depending on the species analysed (Garber *et al.*, 1988; Griffin, 1994; Howlett *et al.*, 1997). This region of rDNA is highly similar and it is useful in resolving phylogenetic relationships for closely related taxa due to its relatively rapid evolution rates (James *et al.*, 2006, Hibbett *et al.*, 2007, Richard *et al.*, 2008).

Today, fungal taxonomy is in a state of constant change. One of the most important resources of fungal classification is the on-line databases such as GenBank (http://www.ncbi.nlm.nih.gov/Taxonomy/). Other useful on-line databases are Fungorum (www.indexfungorusm.org), MycoBank (http://www.mycobank.org/) and Tree of Life Web Project (www.tolweb.org.tree). Recent molecular studies of

phylogenetic analyses of Fungi recognise: one kingdom, one subkingdom, seven phyla, ten subphyla, 35 classes, 12 subclasses, and 129 orders (Hibbett *et al.*, 2007). McLaughlin *et al.* (2009) stated that six phyla existed following the AFTOL (Assembling the Fungal Tree of Life–projects) classification (**Figure** 1-1).

The largest group of Kingdom Fungi is the Subkingdom Dikarya. This group includes 98 % of described fungal species. Dikarya includes two Phyla: Ascomycota and Basidiomycota.

The Ascomycota is the largest phylum of Fungi and contains 75 % of all described fungi. It is characterized by the production of meiosporangia (ascospores), which may or may not be produced within a sporocarp (ascoma). Ascomycota is divided into three Subphyla: Pezizomycotina (largest group including the vast majority of filamentous, fruit body producing species), Saccharomycotina (true yeasts including, fungal pathogens of human) and Taphrinomycotina (yeast-like and filamentous fungi plant pathogens) (James *et al.*, 2006; Hibbett *et al.*, 2007). Phylum Basidiomycota includes about 30,000 species of rusts, smuts, yeasts, and mushrooms (Kirk *et al.*, 2001). Most are characterized by meiospores (basidiospores) on the exterior of typically club-shaped meiosporangia (basidia).

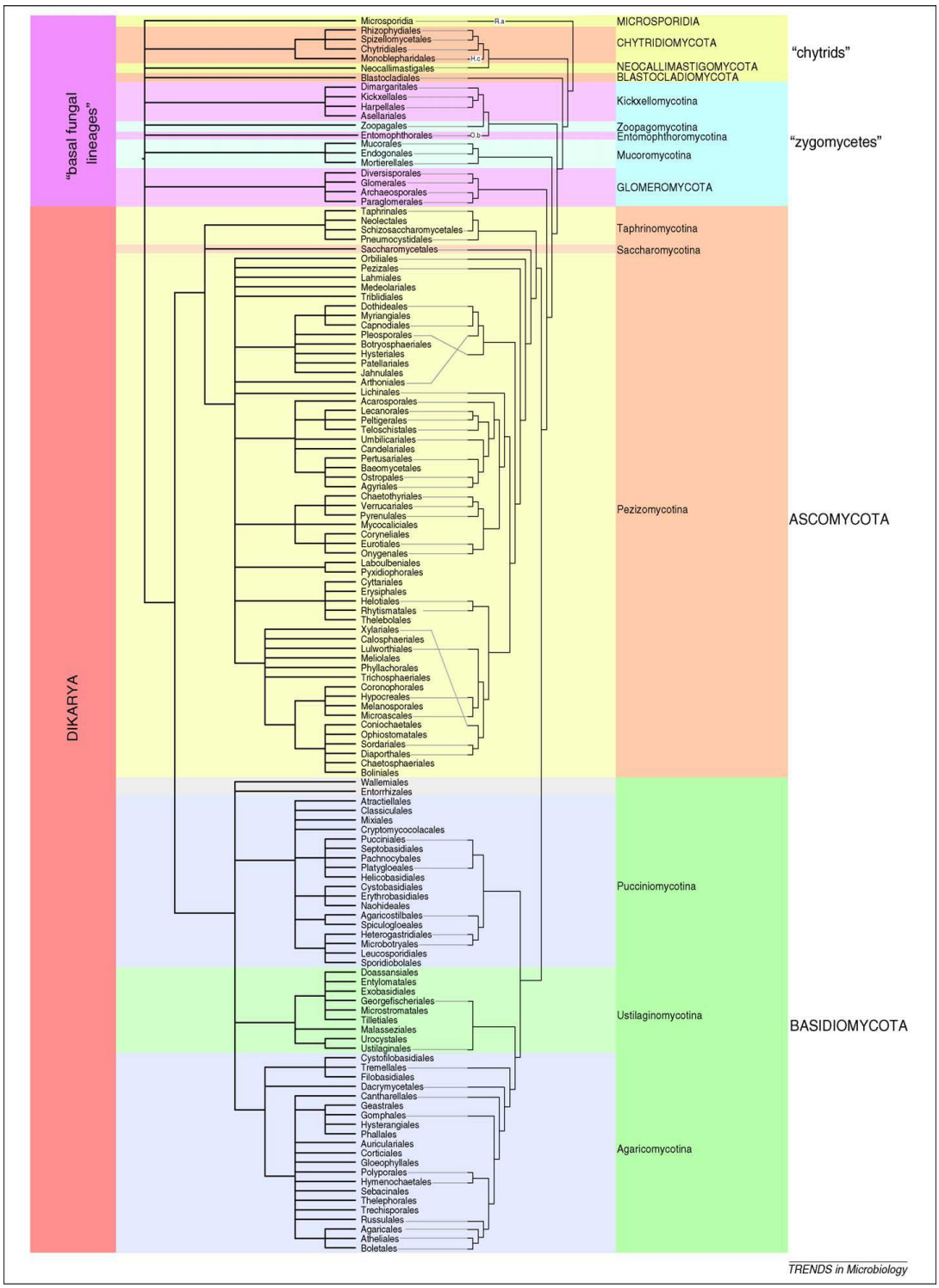

Figure 1-1: Phylogeny and classification of Fungi. The tree on the left represents the AFTOL (Assembling the Fungal Tree of Life-projects) classification. Only nodes corresponding to formally named taxa are resolved. Phyla (suffix -mycota), subphyla (-mycotina) and subkingdom-level taxa (Dikarya) are labelled. Names in quotation marks are informal, non-monophyletic groups. The tree on the right reflects taxon sampling and tree topology from James *et al.* (2006) (the AFTOL classification was developed with reference to many additional studies). Positions of Rozella allomycis, Hyaloraphidium curvatum, and Olpidium brassicae estimated by James and co-workers are indicated by R.a, H.c, and O.b., respectively (McLaughlin *et al.*, 2009).

The Basidiomycota are divided into three Subphyla: Agaricomycotina (68 % of the known Basidiomycota), Pucciniomycotina and Ustilaginomycotina. The subphylum Pucciniomycotina, includes 7,000 species of rust fungi, which are pathogens of land plants. The subphylum Ustilaginomycotina includes 1,500 species of true smut fungi and yeasts. The subphylum Agaricomycotina is the largest group of phylum Basidiomycota and contains around 20,000 described species (James *et al.,* 2006; Hibbett *et al.,* 2007). Almost 98 % of the species contained in Class Agaricomycetes show fruit body formation as mushrooms. The class Agaricomycetes includes around 16,000 described species (James *et al.,* 2006; Hibbett *et al.,* 2007). This class includes many edible species including commercially cultivated mushrooms such as white mushroom – *Agaricus bisporus*, oyster – *Pleurotus osteratus*, shiitake – *Lentinula edodes*, and wild mushrooms such as boletus – *Boletus edulis*, medicinal mushrooms such as lingzhi mushroom – *Ganoderma lucidum*, and many others.

1.1.2 Evolution

The Fungi constitutes a very old, large and diverse group of organisms. The first putative fungi were recorded in Australia dating from 1430 million years ago (Butterfield, 2005). The first fossilized fungal hyphae and spores come from the Ordovician period and are 460 million years old (Redecker *et al.,* 2000; Pidwirny, 2010). The fossil evidence of Ascomycota and Basidiomycota comes from Devonian – Carboniferous, that is 300-400 million years old. At this time, there could be distinguished: Ascomycetes (around 360-330 million years ago) and Basidiomycete (around 300 million years ago) (Tiffney and Barghoorn, 1974).

Recent molecular studies have dated the origin of fungi between 660 million and up to 2.15 billion years ago. Ascomycota and Basidiomycota were dated between 390 million years and up to 1.5 billion years ago. The origin of the Ascomycota was dated to 500-650 million years ago. The fungal organisms evolved 760-1.06 billion years ago (Lucking *et al.,* 2009). The early fungi lived only in water and had only simple aquatic forms with flagellated spores (James *et al.,* 2006). The first information about terrestrial fungi comes from the Cambrian (542-488 million years ago) (Brundrett, 2002) and fungi first colonized the land before land plants. The ability to colonize the land before plants indicates that fungi may have played a crucial role in facilitating the colonization of land by plants (Redecker *et al.,* 2000).

1.1.3 History of Mushrooms in Civilisation

Mushrooms have many different attributes. They are used for food and medicine or may be poisonous, and hallucinogenic. They can also be pathogenic. The first users of mushrooms were the early civilisations of Greeks, Egyptians, Romans, Chinese, and Mexicans. These civilisations used mushrooms for their therapeutic value and very often used them in religious ceremonies as hallucinogenic agents (Chang and Miles, 1989; Stamets, 2000).

The first discovered application of mushrooms was mushroom poisoning discovered by the Greek physician Dioscorides (40-90 AD). In the same century, Pliny the Elder (23-79 AD) gave details to Julia Agrippina, how to use a poisonous fungus to poison her husband Emperor Claudius. The poisonous nature of mushrooms was used to poison many well-known people such as Emperor Jovian in 364AD, Pope Clement VII in 1394, Antipope Urban VI in 1389, French King Charles VI in 1422, and German/Spanish King Joseph Ferdinand in 1699 (Van Griensven, 1988 a; Stamets, 2000).

The first information about wild mushroom consumption and medical use comes from China, where collections of wild mushrooms were recorded e.g. *Auricularia auricula* (600 AD), *Flammulina velutipes* (800 AD), *Lentinula edodes* (ca. 1000 AD) and *Tremella fuciformis* (ca. 1800 AD) (Chang and Miles, 1989).

Linnaeus (1707-1778) named the wild field mushroom as *Agaricus campestris* in his work according to Van Griensven (1988 a).

The first publication of mushroom nomenclature ‘’Systema mycologicum’’ (1815-1818) was written by Swedish mycologist and botanist Elias Fries (1794-1878), providing the fundamental basis for the study of fungi (Fries, 1818).

1.1.4 Fungal Applications

Fungi have played a significant role in human life. Fungi in nature can be classified into three groups: mycorrhizal, parasitic and saprophytic. All these groups have different interactions in nature and have been described in many publications (Stamets, 2000; Chang and Miles, 2004). In nature fungi play a fundamental role in degrading organic material (Wösten *et al.*, 2007).

According to Stamets (2000), humans started to use mushrooms very early in civilisation. The first depiction of using mushroom was in Tassili-n-Ajjer Mountains in Algeria in an image from a cave dating to 5000 years B.C. Mushrooms were used for their hallucinogenic properties and later other cultures such as Mexicans, Greeks, and Egyptians used mushrooms in religious ceremonies. Hallucinogenic properties of mushrooms are still used today. Alexander Fleming accidentally discovered and isolated the first antibiotic – penicillin from *Penicillium notatum* in September 1928 (Fleming, 1980; Sykes, 2001). After that other antibiotics were discovered: streptomycin, chloramphenicol, tetracycline (Singh and Mitchison, 1954, Murphy and Horgan, 2005). Today production of antibiotics is a major branch of the pharmacological industry (Wian and Nielsen; 2007; Dijksterhuis and Samson, 2007).

The oldest written record of basidiomycete mushrooms as a medicinal treatment comes from India and dates to 3000 B.C. (Kaul, 1997). The use of basidiomycete mushrooms as medicine has a long history in Asian countries such as China, Japan and Korea (Daba and Ezeronye, 2003). Basidiomycete mushrooms contain useful medically-active compounds for example as anti-tumour, immunostimulatory agents and anti-cancer, low cholesterol, blood pressure and cardio vascular (Wasser and Weis, 1999a, 1999b).

Fungi have been widely exploited in food production for many years. Yeast is an important microorganism for food production such as bread and alcoholic drinks e.g. beer, wine, vodka, and whiskey (Morais *et al.*, 1996; Ross, 1997; Kavanagh, 2005; Legras *et al.*, 2007). Filamentous fungi do not have many applications in food production compared to yeast. The filamentous fungi are used to produce soft-ripened and blue-vein cheeses. Brie and Camembert from France are the most famous of soft-ripened cheeses made by white mould *Penicillium candida* or *P. camemberti.* The blue cheeses such as Roquefort from France, Stilton from UK, Gorgonzola from Italy and Danish Blue from Denmark are produced by green mould: *Penicillium roqueforti* or *Penicillium glaucum* (Star, 2007). Another filamentous fungal product is a dry-

fermented sausage with a white/creamy coloured appearance. This sausage contains spores of *Penicillium nalgiovense* which give the sausages a characteristic flavour (Stark, 2007). Fruit-body forming fungi such as basidiomycetes can also be human food. The most popular edible mushrooms are *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes*, and many others wild mushrooms such as *Boletus edulis*, *Lactarius deliciosus*, *Armillaria nudle, Agaricus silvicola* and *Tuber melanosporum* etc. (Baar *et al.*, 2007).

Some species of fungi can have a destructive role in human life. Many fungal species cause disease in people, animals and plants (Doohan, 2005, Sullivan *et al.*, 2005). The most important fungal disease in humans is Aspergillosis caused by *Aspergillus fumigatus* (Daly and Kavanagh, 2002). This fungus is widespread in nature and is particularly harmful to people and animals. The most important pathogenic fungus for plants is *Ustilago maydis* which causes smut disease on maize but infected maize called huitlacoche is eaten as a delicacy in Mexico. Another pathogenic fungus is *Magnaporthe grisea* which causes an important disease on rice (Talbot, 2003; Méndez-Morán *et al.*, 2005). Fungi can also cause disease in other fungi. The most important mushroom pathogens of cultivated white mushrooms are *Lecanicillium fungicola*, *Mycogone perniciosa* and *Cladobotryum* spp. These mycoparasites cause a serious loss in mushroom yield and quality (Fletcher and Gaze, 2008).

1.2 White Mushroom – *Agaricus bisporus*

The genus *Agaricus* contains several edible basidiomycete mushrooms occurring widely on grasslands and forests in Europe and North America and others continents. In Europe around 90 species *of Agaricus* occur (Cappelli, 1984). Some of these were isolated and cultivated: *Agaricus arvensis*, *Agaricus bisporus, Agaricus bitorquis, Agaricus macrosporus, Agaricus subfloccosus*, *Agaricus subrufescens* and others (Elliott, 1978; Fritsche 1978; Fermor 1982, Kerrigan 1983; Martinez-Carrera *et al.*, 1995; Noble *et al.*, 1995; Geml and Rimóczi 1999; Kerrigan *et al.*, 1999; Calvo-Bado *et al.*, 2000; Martinez-Carrera *et al.*, 2001). Only *A. bisporus* and *A. bitorquis* are cultivated on an industrial scale (Gea *et al.*, 2003; Van Griensven, 1988 b; Baar *et al.*, 2007). *Agaricus bisporus* is the major species of white mushroom in Europe and North America. *Agaricus bisporus* gives a better quality and yield of fruit bodies than *A. bitorquis*. Some hot countries such as Spain prefer *A. bitorquis,* because this fungus prefers higher temperatures and CO_2 level (Gea *et al.*, 2003).

Cultivated *Agaricus bisporus* mushrooms characteristically have a fruit body which is white and smooth, but some strains have a brown cap and are called brown mushrooms, chestnut, portabella, crimini etc. The size of mushroom depends on strains, time of harvesting and environmental conditions (**Figure** 1-2).

Figure 1-2: *Agaricus bisporus* – white mushroom cultivation.

1.2.1 History of *Agaricus* Cultivation

The earliest information on mushroom cultivation comes from France. The historical sources suggest that during the reign of Louis XIV (1638-1715) mushrooms were grown in the Paris region (Van Griensven, 1988 a). French botanist Tournefort (1707) described for the first time how *Agaricus* mushrooms were cultivated. At this time, cultivation was based on a crop of mushrooms which contained mycelium that was used as the inoculum for freshly prepared manure and this made continuous culture possible (Van Griensven, 1988 a). In 1731, the French method of cultivation was introduced into England by Scottish botanist Philip Miller (1768). The mushroom cultivation technique moved to other European countries such as the Netherlands, German, Italy, Russia and Poland. In the late 19^{th} and early 20^{th} century, mushroom production started in the USA (Van Griensven, 1988 a, b; Szymański 1997; Van Griensven and Roestel, 2004).

The main problem with mushroom cultivation was the preparation of spawn of reasonable quality. The first researchers who achieved germination of the spores of the cultivated mushroom making pure culture spawn were Constantin and Matruchot (Kligman, 1950). In 1902 American researcher, Ferguson, published details of spores germination and the growing of mycelium. After that the first producer of pure culture spawn was the American Spawn Company of St. Paul Minnesota, headed by Louis F. Lambert, a French mycologist (Van Griensven, 1988 a).

Today *Agaricus bisporus* is cultivated in more than 70 countries and is one of the most common and widely consumed mushrooms in the world (Cappelli, 1984) (**Figure** 1-3).

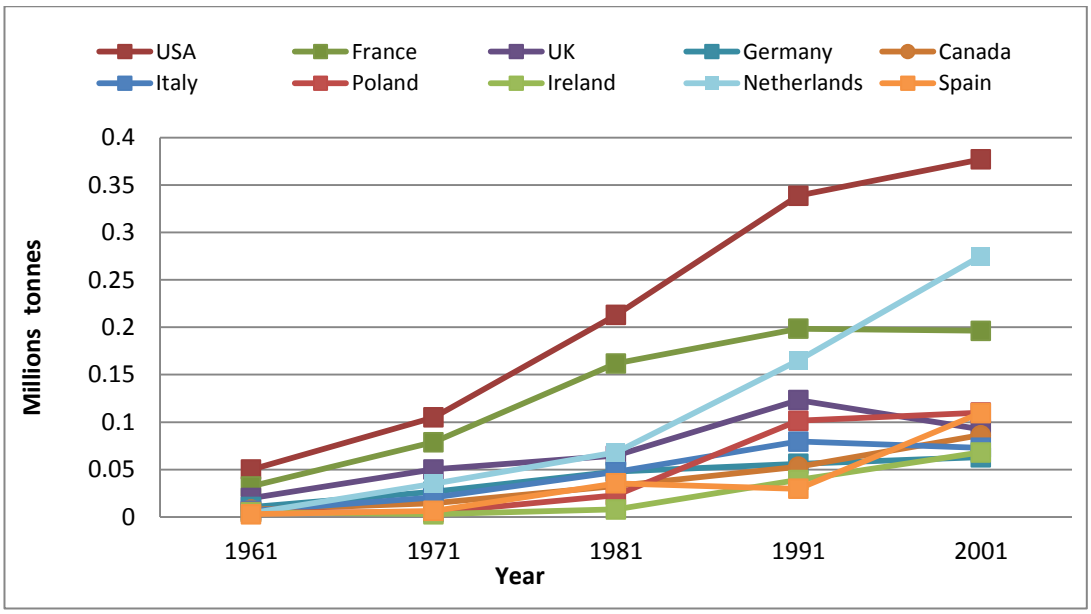

Figure 1-3: Yields of mushroom and truffle production between 1961 and 2001, in the top 10 producing countries (production in tonnes), according to FAO statistics[1].

1.2.2 Cultivation of Other Mushrooms

Chang (1999) lists 10 species of mushroom that are cultivated and make up 92 % of total world mushroom production. However six species account for approximately 87 % of total production: *Agaricus bisporus* (31.8 %), *Lentinula edodes* (25.4 %), *Pleurotus* spp. (14.2 %), *Auricularia auricula* (7.9 %), *Flammulina velutipes* (4.6 %), and *Volvariella volvaceae* (7.9 %). Today, *Agaricus*, *Pleurotus* and *Lentinula edodes* are the basis of worldwide mushroom cultivation. The other three species are grown almost exclusively in Asia according to Chang (1999).

Van Griensven (1988 a) reports that China and Japan were probably the first countries where people cultivated mushrooms. The first information about how to grow shitake comes from 1100 AD (Sung Dynasty) (Chang and Miles 1989). Wang (1987) described a Chinese history of mushroom use and growth. Japanese history of mushrooms dates from the Nara period (710-794 AD) and was described by Yasumasa (2002).

[1] http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor

1.2.3 Global Mushroom Production

Mushroom cultivation is a worldwide practice. Mushroom production has been doubled in Asia during the last decade but in all other continents, there is no significant increase of production (**Figure** 1-4).

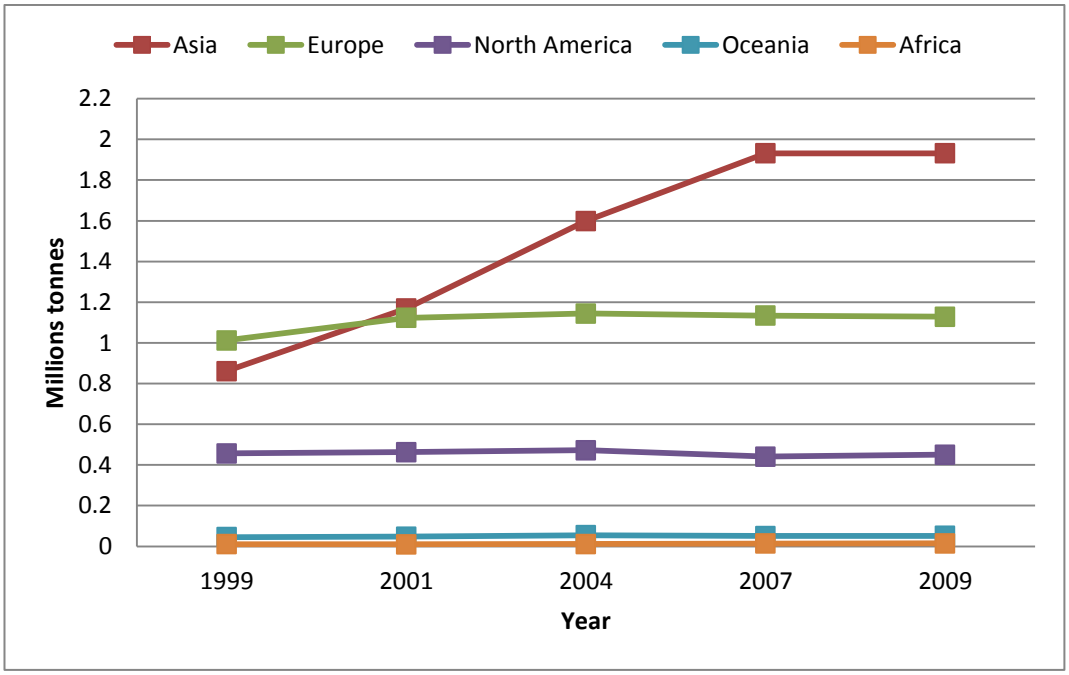

Figure 1-4: World mushroom and truffle production during 1999-2009 (production in tonnes), according to FAO statistics (retrieved October 2010)[2].

The dominant country in the world for mushroom production is China accounting for over 46 % of world production in 2008. China is both a major producer and consumer of a wide variety of edible and medical mushrooms (Chang, 1999). The European and North American countries however produce predominantly *Agaricus bisporus*. The USA is the second largest producer of mushrooms in the world but during recent years production is decreasing. *Lentinula edodes*, *Pleurotus* spp. and truffles are of minor importance in Europe and North America. In the European Union (EU), the Netherlands is the leader for mushroom production and is the third largest producer of mushrooms in the world. Poland is the second largest mushroom producer in Europe.

The third EU country for mushroom production is France where mushroom production is more or less stable. The Irish production of mushrooms in 2008 was 75,000 tonnes putting Ireland in eighth position as a mushroom producer in the world. The European mushrooms production is presented in **Figure** 1-5.

[2] http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor

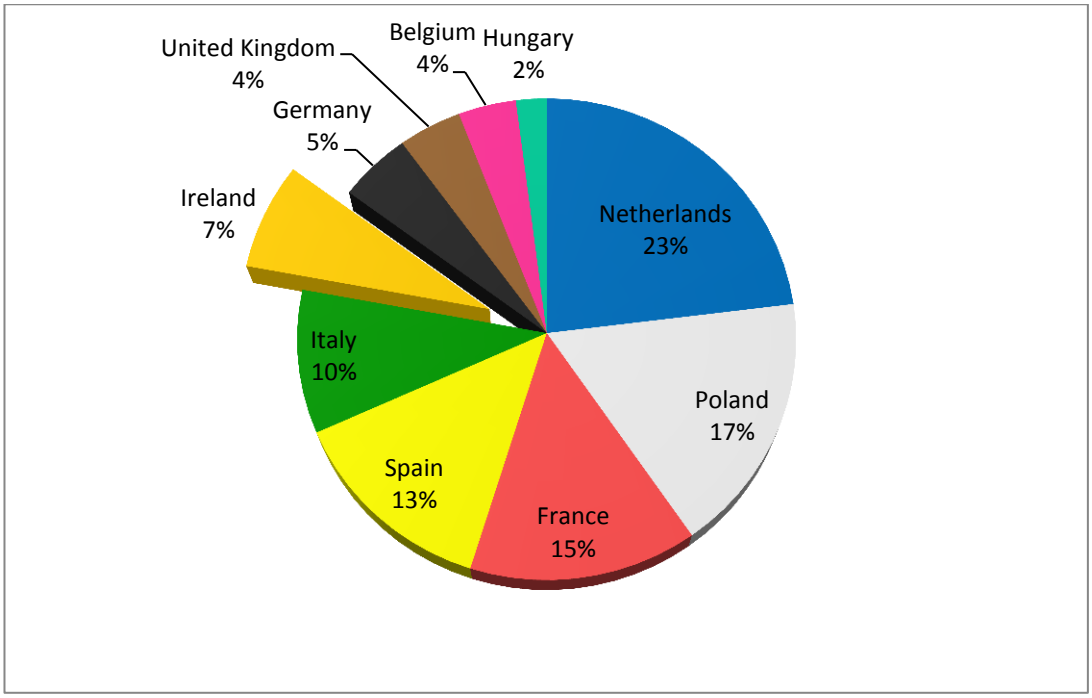

Figure 1-5: European production of mushroom and truffle in 2008, according to FAO statistics[3].

1.2.4 Cultivation of Mushroom

Agaricus bisporus is a heterotrophic, secondary decomposer which utilises a substrate that has already been broken down by other organisms. *Agaricus bisporus* grows well on composted material, which contains other microorganisms such as fungi and bacteria (Fordyce, 1970; Anastasi *et al.*, 2005). *Agaricus bisporus* requires water, carbon sources, nitrogen sources, vitamins and makroelements (P, K etc.) for growth. All these nutrients are provided by two substrates – mushroom compost and mushroom casing soil (Van Gerrits, 1988).

Mushroom compost contains carbon-rich straw, nitrogen-rich manure, gypsum and water. The process of compost preparation is named as composting and has been described by various authors (Fletcher and Gaze, 2008; Van Griensven, 1988 b; Oei, 2003; Chang and Miles, 2004; Szudyga, 2005; Vedder, 1978 and 1986; Baar *et al.*, 2007). This composting process has two stages: fermentation (phase I – 70 °C), pasteurisation and conditioning (phase II – 45 °C), after that the next process of colonisation commences when it is inoculated with *Agaricus bisporus* (mushroom spawn) and incubation for mycelium colonisation to occur (colonisation I), according to Van Gils, (1988) and Baar *et al.*, (2007) (**Figure** 1-7). Today compost productions are

[3] http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor

carried by special compost plants. The compost production is very important process for proper mushroom growth.

Today the most widely cultivated *Agaricus* mushrooms are hybrid strains characterised by a white colour, smooth sporophores and high quality and yield of the fruit body. Spawn is a material used for the commercial inoculation of mushroom compost to produce mushrooms (Oei, 2005). Commercial mushroom ‘’spawn’’ is a pure culture of a particular *Agaricus* strain. It is produced under sterile conditions on some form of grain such as rye, millet, wheat and sorghum (Fritsche 1988).

The essential element for the production of the commercial mushroom is mushroom casing soil. The casing soil induces the formation of sporophores of white mushrooms (Schisler, 1957). Mushroom casing soil generally is a mixture of sphagnum peat which is primary decomposed sphagnum moss, calcium carbonate ($CaCO_3$) (to neutralize pH) and water (about 80 % moisture) (Chikthimmah *et al.,* 2008). The casing soil can be also a mixture of peat with other materials such as marl or spent lime (Visscher, 1988). The pH of casing soil is 7-7.5 and water-holding capacity is 60-80 % depending on type of cultivated mushroom and way of mushroom harvesting. The bacteria contained in casing soil have an important role in fruit body formation of *A. bisporus* (Eger, 1961; Hayes *et al.,* 1969; Flegg and Wood, 1985; Masaphy *et al.,* 1987; Baars and Konings, 2005). Many different bacterial species were observed in casing soil (Hayes *et al.,* 1969; Park and Agnihotri, 1969; Samson, 1986). Baar *et al.,* (2007) reported that thirty two bacterial species were observed in casing soil such as *Pseudomonas* sp., *Bacteriodes* sp. and *Flavobacterium* sp. The most important species involved in basidiome initiation is a *Pseudomonas putida* (Trevisan). Bhatt and Singh (2000) stated that five bacteria out of sixteen isolated from casing soil reduced the growth of the pathogen *L. fungicola* by about 40-60 % in *in vitro* tests. Casing soil also contains a significant population of yeasts, moulds and actinomycetes (Masaphy *et al.,* 1987; Chikthimmah *et al.,* 2008), but casing must be free from competitive moulds and pathogenic organism for *A.bisporus* to grow well.

When compost is fully colonized by mycelia of *A. bisporus* it is covered with around 5 cm of casing soil to cover the compost. Casing soil is at this time very sensitive to contamination by pathogenic fungi and this step must be performed hygienically. When the growing room is filled with the compost and casing the correct growing conditions are provided and mushroom cultivation starts (Van As and Van Dullemen, 1988) (**Figure** 1-6 **A**).

Once the casing is colonised by *Agaricus* mycelium the crop is ‘’aired’’ for about 7 days. This involves a reduction in temperature from 25 °C to 18 °C, a reduction in relative humidity (RH) from 90 % to 80 % and a reduction in CO_2 from 6 % to 0.3 % approximately (**Figure** 1-6 **B**). This is done slowly over 3 days and triggers the formation of mushroom initials. Over the next 3-4 weeks there will be 3 flushes of mushrooms for harvesting (**Figure** 1-6 **C**). This step is very sensitive to contamination by pathogens and pests.

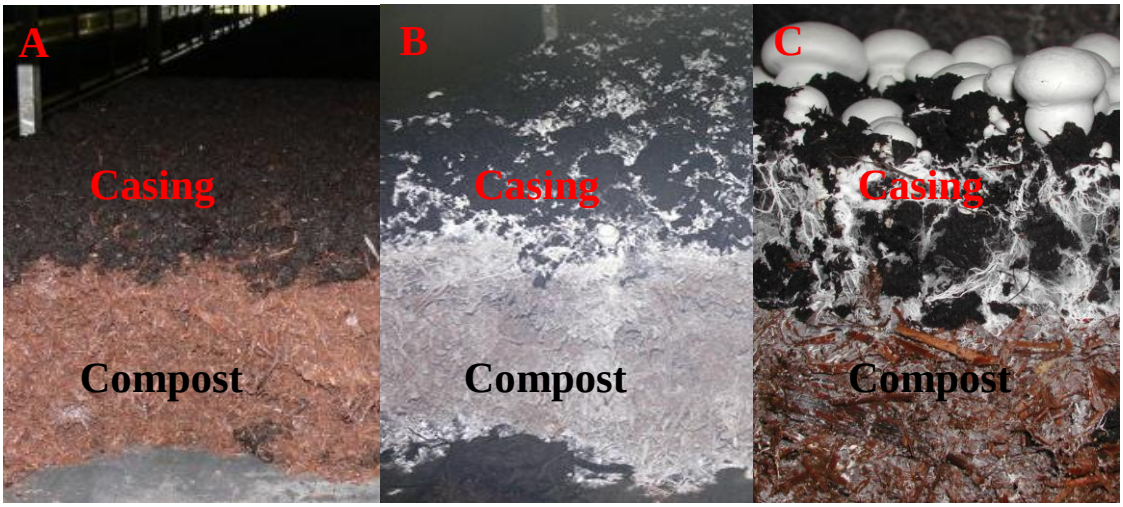

Figure 1-6: Mushroom growth phases: A – Shelf in a growing room after filling with compost (bottom layer – brown) and casing soil (top layer – black); B – compost and casing colonization by *Agaricus bisporus* mycelium; C – The cultivated white mushroom *Agaricus bisporus* – pinning.

Once all the mushrooms have been harvested the crop is terminated, usually by steaming the room and compost. This step consists of increasing temperature in compost to 60-70 °C using steam and maintaining it for 8-12 hours. The aim of this step is to kill all pathogens and pests, which may have developed during cultivation. When the temperature decreases, compost is removed and the mushroom room is cleaned for the next cropping cycle (Van As and Van Dullemen, 1988; Van Gils, 1988; Baar *et al.*, 2007) (**Figure** 1-7).

Today some mushroom growers only harvest two flushes to reduce the risk of pest and disease problems.

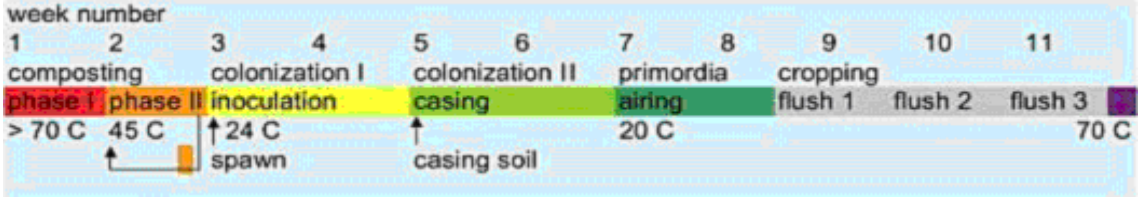

Figure 1-7: Time schedule of *Agaricus bisporus* cultivation (Baar *et al.*, 2007).

1.2.5 Pathogens and Pests of Mushrooms

Mushroom cultivation is a monoculture utilising a pasteurised carbon rich substrate in a clean environment where temperature and relative humidity (RH) are optimal for fungal growth. However other competitive fungi, pathogens and pests grow well under these conditions. The economically significant mushroom pathogens and pests are described in detail in Fletcher and Gaze, (2008). There are 4 main fungi that cause disease of mushrooms: *Lecanicillium fungicola* (dry bubble) *Mycogone perniciosa* (wet bubble), *Cladobotryum* spp. (cobweb) and *Trichoderma* spp. *Trichoderma* spp. which is not a pathogen of *A. bisporus* but it is generally a competitive pathogen of compost (*A. bisporus* substarte). *Lecanicillium fungicola* is the most serious pathogen and causes a disease called dry bubble. This mushroom mycoparasite causes serious loss in yield of mushroom and shows a few symptoms: bubble, spotting and split stipes. The second mushroom disease is *Mycogone perniciosa* which causes wet bubble. The symptoms of wet bubble are very similar to dry bubble symptoms and most times dry and web bubble can be easily mistaken. *Cladobotryum dendroides* and *Cladobotryum mycophilum* cause cobweb disease. This disease is characterized by the growth of coarse mycelium covering affected mushrooms and brown spotting symptoms on the mushrooms. Unlike *Lecanicillium* and *Mycogone*, *Cladobotryum* spp. can also grow over the casing soil (Fletcher and Gaze 2008; Geels *et al.*, 1988, Gams *et al.*, 2004; Baar *et al.*, 2007). *Trichoderma aggressivum* is the most serious pathogen of mushroom compost (Seaby, 1987). Different species of *Trichoderma* spp. have been found associated with mushroom compost that could cause green mould (Maszkiewicz, 1992; Chen *et al.*, 1999; Savoie *et al.*, 2001; Seaby 2006). Green mould diseases are described in many publications as a serious problem of cultivated mushrooms (Sharma *et al.*, 2006; Singh *et al.*, 2006).

Mushrooms are also susceptible to bacterial diseases. Bacterial diseases of mushrooms are caused mainly by *Psudomonas tolaasii*, *P. agarici, P. gingeri, P. aeruginosa, Burkholderia gladioli* pv. *agaricola* and *Janthinobacterium agaricidamnosum* sp. nov. The harvesting "browning" is caused by *P. fluorescens,* which causes brown coloured blotches and spots on the mushrooms (Fletcher and Gaze 2008; Geels *et al.*, 1988; Lincoln *et al.*, 1999).

Other mushroom diseases are caused by viruses. Viral diseases can be extremely infectious and cause great damage. The first information about virus disease was reported in 1950 (Sinden and Hauser, 1950). This virus disease is known as a La France

Virus and it was a new pathogen which affected mushroom cultivation severely, but this virus has not been found in *A. bitorquis* (Fletcher and Gaze 2008). Since then various viral diseases have been described. More recently a new virus disease has emerged and is known as mushroom virus X disease (MVXD) (Gaze *et al.*, 2000; Fletcher and Gaze 2008; Grogan *et al.*, 2003 and 2006).

Agaricus bisporus is affected by other pests such as flies, mites and nematodes. The most serious pests of mushroom crop are scarid and phorid flies. The most common sciarids on mushroom farms are *Lycoriella castanescens* and *L. ingenua*. The phorid flies are *Megaselia halterata* and *M. nigra*. The presence of flies on a mushroom farm is a very significant factor in spreading pathogenic fungi, but they can also damage mycelium and mushroom fruit bodies (Fletcher and Gaze, 2008; Geels *et al.*, 1988).

Prevention and control of pathogens and pests on cultivated mushroom farms is very important if mushroom growers want to have good yields and good quality. The prevention and control of mushroom diseases and pests is described in many publications (Geels *et al.*, 1988; Szudyga, 2005; Sawant *et al.*, 1998; Chang and Miles, 2004; Maszkiewicz, 2006; Fletcher and Gaze, 2008).

1.3 *Lecanicillium fungicola* – Dry Bubble Disease

1.3.1 Taxonomy

Lecanicillium fungicola was described for the first time in 1851 when it was named *Acrostalagmus fungicola* by Preuss (1851). Preuss (1851) isolated *Acrostalagmus fungicola* from unspecified woodland toadstools (Brandy and Gibson, 1969). Forty years later in 1892 two French scientists recognised a species as the cause of the ‘’mole’’ disease of mushrooms and after a morphological diagnosis changed the name to “Verticillium ă petites spores” (Constantin and Dufour, 1892). Later Smith (Smith, 1924), proposed the name *Cephalosporium constatinii*. In 1933, Ware changed the name to *Verticillium malthousei*. In 1936 another researcher renamed it as *Verticillium fungicola* (Hassebrauk, 1936), but in the literature *V. malthousei* existed for a long time.

In the early 1980s two Dutch researchers Gams and Van Zaayen (1982) subdivided *V. fungicola* into three varieties: *Verticillium fungicola* var. *fungicola*, *Verticillium fungicola* var. *aleophilum* and *Verticillium fungicola* var. *flavidum*, but only two varieties *V. fungicola* var. *fungicola* and *Verticillium fungicola* var. *aleophilum* were causal agents of disease in cultivated mushrooms. Differences between these two varieties were based on physiology and morphology but it is difficult to distinguish between them. Zare and Gams (2008) using morphological and molecular techniques assigned *Verticillium fungicola* to the new genus *Lecanicillium* which was described by Zare *et al.*, (2000) and Gams and Zare (2001) and today the current name is *Lecanicillium fungicola* var. *fungicola* (Preuss) Zare & Gams, and *Lecanicillium fungicola* var. *aleophilum* (W. Gams & Zaayen) Zare & Gams comb nov. (Zare and Gams, 2008).

The taxonomic position of *L. fungicola* within the Kingdom Fungi is as follows: National Center for Biotechnology Information and UniProt Taxonomy (www.ncbi.nlm.nih.gov/Taxonomy and www.uniprot.org/taxonomy/170721).

- Kingdom: Fungi,
- Subkingdom: Dikarya,
- Phyla: Ascomycota,
- Subphyla: Pezizomycotina,

- Class: Sordariomycetes,
- Subclass: Hypocreomycetidae,
- Order: Hypocreales,
- Family: Cordycipitaceae,
- Genus: Lecanicillium,
- Species: *Lecanicillium fungicola*

Zare and Gams (2008) assigned *Verticillium fungicola* var. *flavidum* to a genus *Lecanicillium* and refered as a separate species: *Lecanicillium flavidum* (W. Gams & Zaayen) W. Gams & Zare comb.nov.

Lecanicillium fungicola exists commonly in nature (Gams *et al.*, 2004). The fungus was isolated from decaying leaf debris and from the sporophores of other larger Basidiomycete such as *Laccaria laccata*, *Hypholoma capnoides*, *Henningsomyces candidus*, *Thelephora terrestris* and *Marasmiellus ramealis* (Brandy and Gibson, 1969; Zare and Gams, 2008). *Lecanicillium fungicola* is considered to be a mushroom pathogen but Bidochke *et al.* (1999a) isolated it from an insect. Dry bubble disease affects two commonly cultivated white mushrooms – *Agaricus bisporus* (Lange) Sing. and *Agaricus bitorquis* (Quel.) Sacc. (Gea *et al.*, 2003). Dry bubble disease has been also reported in oyster mushrooms (*Pleurotus ostreatus*) (Marlowe and Romaine, 1982; Houdeau and Olivier, 1989).

1.3.2 *Lecanicillium fungicola* as a mushroom pathogen

Dry bubble disease caused by *Lecanicillium fungicola* is prevalent wherever the button mushroom *Agaricus bisporus* is cultivated. *Lecanicillium fungicola* was recorded in many countries such as USA (Lambert, 1938; Forer *et al.*, 1974; Spadafora *et al.*, 1989), Mexico (Largeteau *et al.*, 2004), India (Bhatt and Singh, 2002), China (Chen *et al.*, 1981; Chu, 1982) and Australia (Nair and Macauley, 1987). In Europe dry bubble disease was recorded in France (Chaze and Sarazin, 1936), the Netherlands (Fekete and Kuhn, 1967; Van Zaayen and Gams, 1982), Poland (Maszkiewicz *et al.*, 2006), UK (Smith, 1924; Atkins and Atkins, 1971; Gaze and Fletcher, 1975; Gandy 1972; Wong and Preece, 1987), Ireland (Staunton, 1995), Serbia (Potočnik *et al.*, 2008), Spain (Gea *et al.*, 1995 and 2003) and Denmark (Paludan, 1954). The dry bubble disease is described in numerous publications (Van Griensven, 1988; Fletcher and Gaze, 2008;

Van Zaayen and Gams, 1982; Mamoun and Olivier, 1995; Gea *et al.*, 2005; Maszkiewicz *et al.*, 2006).

In European countries where *A. bisporus* is cultivated *L. fungicola* var. *fungicola* is associated with dry bubble disease but in USA and Canada it is *L. fungicola* var. *aleophilum* (Collopy *et al.*, 2001). *Lecanicillium* (*Verticillium) psalliotae* TRESCHOW has been reported too as a pathogen of cultivated mushrooms causing spotting on the cap (Gandy, 1979; Samuels *et al.*, 1980; Brunett, 1980; Chen *et al.*, 1981; Van Zaayen and Gams, 1982).

1.3.3 Economic Impact

Dry bubble is the most common fungal disease of the commercial mushroom *Agaricus bisporus* (Gandy, 1972). Sinden (1971) reported, that losses caused by *L. fungicola* and the cost of crop protection for this mycoparasite were higher than the cost of control of any other mushroom pest or pathogen, with the exception of the La France viral disease. Russel (1984) reported that the neglected control of *L. fungicola* on mushroom farms can cause yield losses of 10-20 % or higher but sometimes much greater if disease is left uncontrolled. Dry bubble can reduce farm incomes to the point where it is not possible to produce mushrooms economically. In the Netherlands dry bubble was unknown until 1938, but caused losses estimated of 0.5 % of value of all mushroom production in 2001 (Oei, 2003). Other Dutch researchers estimated the yield reduction by *L. fungicola* is 20 million Euro for the Dutch mushroom industry[4]. Bhatt and Singh (2002) reported that *L. fungicola* could decrease yield by 25 %. Forer *et al.*, (1974), calculated that for the period 1971-1972 Pennsylvanian growers lost 9.1 million dollars due to *L. fungicola* induced disease. Spanish researchers estimated losses of 66 million pesos in 1991 (Gea, 1993). Rinker and Wuest (1994) stated that annual losses in Canadian mushroom farms caused by disease were 7 million dollars. At present, in the British mushroom industry *Lecanicillium fungicola* induced dry bubble disease is responsible for losses of approximately £2-3 million (Grogan *et al.*, 2000).

[4] http://www.onderzoekinformatie.nl/en/oi/nod/onderzoek/OND1331230/

1.3.4 Symptoms of dry bubble disease

Various symptoms of dry bubble disease have been observed. The symptoms caused by this fungus depend on time of infection, stage of crop development, genetic variability of the host (Fletcher, 1981; Nair and Macauley, 1987; Sharma and Kumar, 2005, Baar *et al.*, 2007) and quantity of conidia that cause the initial infection. When the contamination occurs early in the crop cycle then symptoms are stronger (Gandy, 1972; Sinden, 1971), and yield is lower (North and Wuest, 1993). Van Zaayen (1981) described experiments using conidia concentrations of 1.5×10^6 and 1.5×10^7 conidia per m^2 inoculated 10 days after casing and all yield was lost. Inoculation at 1.5×10^7 conidia per m^2 caused more severe infection. Mills *et al.* (2000) reported that when a conidia suspension of 10^3 conidia per m^2 was used the symptoms do not always occur in the first two flushes. When conidia concentration of 10^4 conidia per m^2 was used around 15 % of mushrooms were infected during cropping, but when conidia concentrations of 10^6 conidia per m^2 were used, the symptoms were increased 11.8 %, 25 %, 42.1 % and 80 % mushrooms in flush 1, 2, 3 and 4, respectively. However, conidia concentration of 10^8 conidia per m^2 of casing showed total crop loss (Mamoun and Olivier, 1995; Mills *et al.*, 2000). The conidia concentration needed to effectively contaminate cultivation is 10^6 conidia per m^2 of casing and the best time for it is after casing (Mamoun and Olivier, 1995, Mills *et al.*, 2000). Disease symptoms occur after fourteen days from time of inoculation (Holmes, 1971, Damięcka (Piasecka) and Maszkiewicz, 2004). Largeteau and Savoie (2008) stated that more aggressive isolates of *L. fungicola* cause a higher number of bubbles than less aggressive isolates.

For hybrid strains of *Agaricus bisporus* the symptoms can appear 7-10 days after contamination, because hybrid strains generally grow at warmer temperatures and higher relative humidity for better quality (Van Zaayen, 1981; Beyer *et al.*, 2005). Mamoun and Olivier, (1995) reported that disease incidence not only depended on the strain characteristics but also on environmental conditions and inoculum density of pathogen conidia. Dry bubble symptoms are similar whether or not the causal organism is *L. fungicola* var. *fungicola* and *L. f.* var. *aleophilum* although var. *aleophilum* isolates from USA appear to be more aggressive than var. *fungicola* isolates from Europe (Largeteau *et al.*, 2005).

Classical symptoms of dry bubble disease were described by Ware (1933) and later by various authors such as Vedder (1986) and Van Zaayen and Gams, (1982). The general description of *L. fungicola* symptoms was according to Beyer *et al.* (2005). Dry

bubble disease usually present three different symptoms: dry bubble, split stipe and spotting. Dry bubble is the most obvious symptom. It consists of a sphere like mass of mushroom tissue. A single mushroom or a group of mushrooms can develop dry bubbles. Sometimes as the diseased tissue ages, a few small yellowish-brown drops of juice may form. The bubble symptoms usually indicate an early and severe infection of the mushroom pin or even before the pins are visible. The early infection disrupts the growth of the mushroom tissue preventing it from developing into the different tissues of the stem and cap (**Figure** 1-8).

Figure 1-8: White mushroom infected with *L. fungicola*; dry bubble symptom.

Split stipe symptoms develop when infection takes place after the pin begins to develop. If the stipe is infected, the stipe splits as it matures causing a symptom described as split stipe or stipe blowout. The infection disrupts stem elongation on one side of the mushroom, while the healthy side continues growing normally. The tissue on the infected side shatters, splits or ruptures causing this characteristic symptom (**Figure** 1-9).

Figure 1-9: White mushroom infected with *L. fungicola*; split stem symptom.

Spotting symptoms consist of large brown or discoloured spots with a greyish hue in the centre. The greyish hue is the sporulating fungus. This symptom usually develops when infection occurs later, when pins are larger and more developed or when small a spot infection occurs on a pinhead surface (**Figure** 1-10).

Figure 1-10: White mushroom infected with *L. fungicola*; spotting symptom.

Symptomless mushrooms can also show signs of disease after harvest and during storage or on market shelves. Harvesters would unknowingly touch infected mushrooms, move conidia to uninfected areas, and contaminate other places and healthy mushroom sporophores (North and Wuest, 1993; Beyer *et al.*, 2005; Fletcher and Gaze, 2008).

1.3.5 Identification of *Lecanicillium fungicola*

The fungus *Lecanicillium fungicola* grows well on artificial media such as potato dextrose agar (PDA), oat agar (OT), malt extract agar (MEA), nutrient broth (NB), Yeast extract agar (YEA), Southern agar (SA), and mushroom dextrose agar (MDA) (Gams and Van Zaayen, 1982; Brady and Gibson, 1969; Zare and Gams, 2008; Potočnik *et al*., 2008). Gams and Van Zaayen (1982) reported that *L. fungicola* var. *fungicola* and *L. fungicola* var. *aleophilum* are pathogenic to *Agaricus bisporus* and *Agaricus bitorquis*. Morphologically, *L. fungicola* var. *fungicola* and *L. fungicola* var. *aleophilum* have similar conidiophores and are indistinguishable (Zare and Gams, 2008).

1.3.5.1 Morphological Characteristics

The morphology of *L. fungicola* has been described by Brady and Gibson, (1969), Gams and Van Zaayen, (1982) and Zare and Gams (2008).

The general description of *L. fungicola* var. *fungicola* according to Gams and Van Zaayen (1982), states that colonies on MEA reach 1.8-2.8 cm diameter after 10 days at 20 °C. Colonies after this time look white, dirty white, to pale cream-coloured and are dusty to velvety. The reverse is uncoloured, or pale yellowish, pale ochreous to light honey. Octahedral crystals are present and odour is indistinct. The vegetative mycelium hyphae are 0.7-2.5 µm wide. Sporulation is very abundant with conidiophores arising typically from submerged hyphae. Conidiophores are typically erect, 3.5-4 µm wide at the base, thick-walled, with 2-5 (to over 10 in old colonies) whorls of 3-7 phialides. Phialides arise at an oblique angle from the conidiophore and are 14-35 µm long, 1.8-3 µm wide gradually tapering to 0.5-1 µm wide at the tip (**Figure** 1-11 **A, B**). The conidia adhere in mostly globose, slimy heads with the heads of single phialides often coalescing to form large slimy masses (**Figure** 1-11 **C**). The conidia are fusiform, long ellipsoidal to almost cylindrical; the basal end is indistinctly truncated and sometimes curved along the longitudinal axis. Conidia are asymmetrically biconvex to concave-convex to slightly sickle-shaped, smooth-walled and of very unequal size, 3.8-7.2 × 1.2-2.4 µm, length/width ratio 2.5-4.5 with 1-2 or more inconspicuous guttules (**Figure** 1-11 **D**). On mushrooms the ellipsoidal form of conidia may dominate, *in vitro* the fusiform shape is commonly present. Chlamydospores are absent and the teleomorph is unknown.

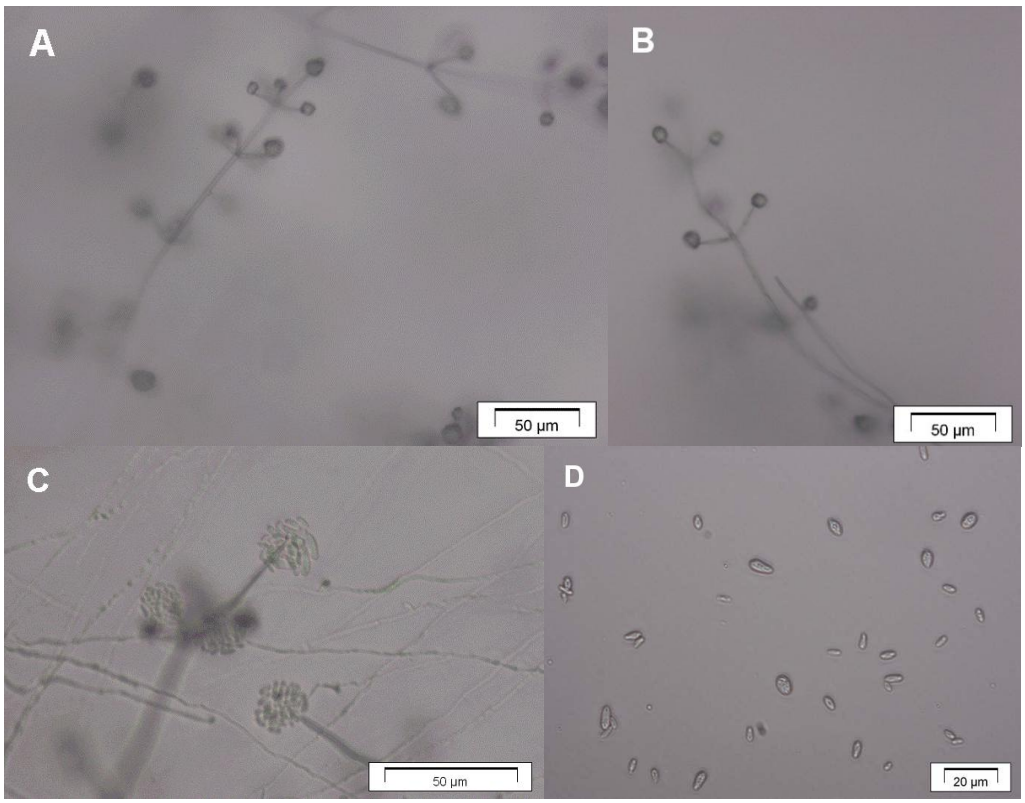

Figure 1-11: *Lecanicillium fungicola* var. *fungicola* wild isolate L.46; A-C – Conidiophores and phialides; D – Conidia

The morphology of *L. fungicola var. aleophilum* according to Gams and Van Zaayen (1982), states that colonies on MEA reach 2.5-3.0 cm diameter after 10 days at 20 °C. Colonies after this time look white, thinly floccose to slightly cottony. The reverse is uncoloured. Octahedral crystals are abundant and odour is indistinct. The vegetative hyphae are 1-3 µm wide. Sporulation is abundant with conidiophores generally arising from submerged hyphae. Conidiophores are erect, up to 400 µm tall about 2.5 (up to 3.5) µm wide at the base, thick-walled, bearing many whorls of 3-10 phialides, which are typically 15-30 µm long, from 1.5-2.2 µm gradually tapering to 0.8-1.2 µm (**Figure** 1-12 **A, B**). Conidia forming mostly globose heads, oblong, fusiform, long ellipsoidal to almost cylindrical but often with conically tapering and ultimately rounded tips, equal at both ends, usually straight, smooth-walled, of very irregular size, 4.5-8 × 1.5-2.5 µm, with two or more inconspicuous guttules (**Figure** 1-12 **C, D**). On mushrooms, the conidia are similar, generally straight, shape and size. Chlamydospores are absent and the teleomorph is unknown.

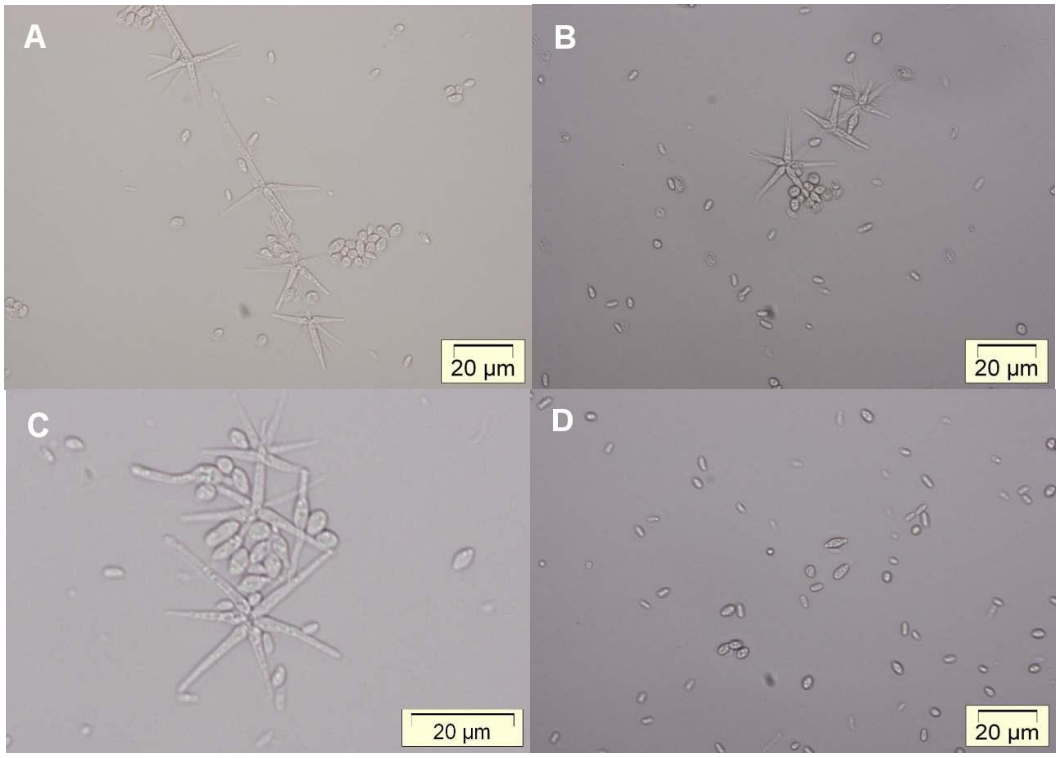

Figure 1-12: *Lecanicillium fungicola* var. *aleophilum* CBS 507.81A. A-C – Conidiophores and phialides; D – Conidia.

These two varieties have only minor differences between them. One of these differences is the speed of growth at 23 °C, but this difference is not very consistent and depends upon the isolate. Another difference is the colour of the colony reverse but *L. f.* var. *fungicola* colonies can also be uncoloured. Another difference is the octahedral crystals which are "present" in *L. f.* var. *fungicola* and "abundant" in *L. f.* var. *aleophilum* but this is a subjective opinion of the researcher. The characteristics of the mycelium, conidiophores and conidia are very similar and difficult to distinguish. Zare and Gams (2008) reported that morphologically these two varieties are indistinguishable.

The only one area of physiological differences is optimal temperature for growth. Fekete, (1967) reported that an optimum temperature for *Lecanicillium fungicola* is 21-24 °C. Van Zaayen and Rutjens, (1981) and Gams and Van Zaayen, (1982) stated the optimal temperature for growth of *L. f.* var. *fungicola* is 20-24 °C, little growth occurring at 27 °C, no growth occurred at 30 °C and thermal death point of conidia is 38-39 °C. Optimal growth for *L. f.* var. *aleophilum* is 24-27 °C, little growth occurring at 30 °C, no growth occurring at 33 °C and thermal death point of conidia is 42 °C. Slightly different optimum temperatures were observed by Zare and Gams (2008), who found *L. fungicola* var. *fungicola* optimum temperature is 18-20 °C, little

growth at 27 °C, and no growth at 30 °C. *L. f.* var. *aleophilum* optimum temperature is 21-27 °C, little growth 30 °C and no growth at 33 °C (Zare and Gams, 2008).

The temperature test is one of the easiest microbiological methods to distinguish these two varieties of *L. fungicola*. This test is used by many researchers to characterise both varieties. *Lecanicillium fungicola* var. *fungicola* and *L. fungicola var. aleophilum* both grow very well at 23 °C, but *Lecanicillium fungicola* var. *fungicola* does not grow at 30 °C, while *L. fungicola var. aleophilum* does (Juarez del Carmen *et al.*, 2002; Largeteau *et al.*, 2004; Largeteau *et al.*, 2006; Potočnik *et al.*, 2008).

1.3.5.2 Molecular Phylogenetics

The development of molecular techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and random amplified of polymorphic DNA (RAPD) has revolutionized fungal systematics (White *et al.*, 1990). Before the creation of the genus "*Lecanicillium*" in 2008 (Zare and Gams, 2008) – dry bubble disease belong to genus "*Verticillium*" and fungi was called – *Verticillium fungicola*.

Bonnen and Hopkins (1997) as a first used a molecular technique – RAPD analysis to examine the intra-species variation of *V. fungicola*. They tested 66 isolates collected over a 45-year period. All isolates were compared by examining colony morphology, fungicide sensitivity, virulence, geographic region, and RAPD grouping. The range of variation in the tested isolates indicated that the population was very homogeneous. Bidochka *et al.*, (1999 b) examined phylogenetic relationships in the genus *Verticillium* using PCR reaction. The authors performed sequence analysis of many *Verticillium* species (including *V. fungicola* isolates) using the internal transcribed space 1 (ITS1) region and a portion of the relatively more conserved nuclear small subunit of ribosomal RNA (rDNA). They reported the phylogenetic data of genus *Verticillium* are polyphyletic (Greek for "of many races") groups based on similar morphological characteristics and have their origin in traditional taxonomy.

Collopy *et al.* (2001 and 2002) examined the molecular phylogenetic variability amongst isolates of *V. fungicola*. Analyses were performed using RAPD analysis of internal transcribed spacers 1 and 2 (ITS1 and ITS2) and 5.8S regions of the nuclear ribosomal DNA (rDNA) transcriptional unit. All 40 isolates collected from different Pennsylvania (USA) mushroom farms in 1998 and 13 isolates collected during last 50 years in North America were identical to ex-type strain of *V. fungicola* var. *aleophilum* and indicating that isolates were part of a clonal population. Sequence analyses of

European isolates were closely related to the ex-type strain *V. fungicola* var. *fungicola*, but Korean isolate was similar to the ex-type strain *V. f.* var. *aleophilum* close to North American group. Only one primer out of six primers for RAPD showed a different banding pattern between both isolates *V. f.* var. *aleophilum* and *V. f.* var. *fungicola*. Collopy *et al.*, (2002), confirmed the results of Bidochka *et al.*, (1999 b) and reported that *V. f.* var. *aleophilum* and *V. f.* var. *fungicola* may not be a species of the existing *Verticillium* genus.

Juarez del Carmen *et al.* (2002) used the same molecular method as Collopy *et al.* (2001) to inspect genetic variation of French isolates. The authors confirmed the results of Collopy *et al.* (2001) and stated that, all French isolates belonged to *V. f.* var. *fungicola*, but in Juarez del Carmen *et al.* (2002) the RAPD patterns of French isolates were not as homogeneous as demonstrated from the data of Collopy *et al.* (2001; 2002).

Largeteau *et al.* (2004) using RAPD and PCR-RFLP techniques reported that Mexican isolates of *V. fungicola* showed the same ITS sequence as a European isolates and were identified as *V. f.* var. *fungicola*. The Mexican isolates of *V. fungicola* var. *fungicola* came to Mexico with materials or machines from Europe (Largeteau *et al*. 2004). On RAPD reaction only one primer out of 5 gave a different profile between *V. f.* var. *fungicola* and *V. f.* var. *aleophilum*. Largeteau *et al.* (2006) using PCR-RFLP, RAPD and AFLP (Amplified Fragment Length Polymorphism PCR) techniques identified the European isolates as a *V. f.* var. *fungicola* and showed them to be as genetically homogeneous as American isolates of *V. f.* var. *aleophilum*.

In 2008 Zare and Gams, (2008), used PCR techniques to examine the ITS region and SSU rDNA (small subunit ribosomal DNA – 18S rDNA, 17S rDNA and 16S like rDNA) to study the *V. fungicola* species complex. They reported that it is very closely related to the genus *Lecanicillium*, and they renamed *Verticillium fungicola* (Preuss) Hassebrauk as *Lecanicillium fungicola* (Preuss) Zare and Gams. They also changed the name of *V. f.* var. *flavidum* Gams & Zaayen to *Lecanicillium flavidum* (Gams & Zaayen) Gams & Zare, comb. nov.

1.3.5.3 Molecular Methods of Fungal Identification

The PCR method has been used to test *V. f.* var. *aleophilum* in North America mushroom farms (Romain *et al.*, 2002). These authors designed specific PCR primers for detection of *V. f.* var. *aleophilum*. This set of primers did not amplify European subspecies *V. f.* var. *fungicola* or *V. f.* var. *flavidum* (not a pathogenic species on mushrooms). This test enabled a confirmed diagnosis of dry bubble disease on fruit body of white mushrooms in less than 3 hours.

Largeteau *et al.* (2007), using Real Time PCR methods, detected association between the amount of *V. fungicola* and the type of symptoms on white mushrooms. More recently TaqMan PCR test has been used for detection of *L. fungicola* from different places on mushroom farm (Zijlstra *et al.*, 2007, Zijlstra *et al.*, 2008, Zijlstra *et al.*, 2009). They designed a probe which detected only *L. fungicola*.

Amey *et al.* (2002) transformed *V. fungicola* using *Agrobacterium tumefaciens* to better understand interactions between pathogen and the host – *Agaricus bisporus*. Foster *et al.* (2004) developed molecular tools for *V. fungicola* that allowed the study of the interaction between *Verticillium* and *Agaricus*. The authors used transformation methods (*Agrobacterium tumefaciens*), marker gene techniques (GUS, GFP) as well as gene-knockout technology.

Collopy *et al.* (2010), using many molecular tools (PCR amplification – Southern analysis, the quantitative RT PCR (Q-PCR), PCR – generated gene fragment, Knockout plasmid construction and *Agrobacterium tumefaciens* – mediated transformation and reported, that the pmk-like mitogen activated protein kinases (MAPK) from *L. fungicola* was not required for virulence of *Agaricus bisporus*.

The popularity and quickness of using PCR method have been employed to study genetic variability, identification and interaction between two fungi (*Agaricus* and *Lecanicillium*). Dry bubble disease interactions have been described by many other mushroom researchers (Mills *et al.*, 2000; Amey *et al.*, 2007; Largeteau *et al.*, 2010; Pantou *et al.*, 2005; Zare and Game 2008; Farrag *et al.*, 2009, Muthumeenakshi and Mills, 2005).

1.3.5.4 Nutrient Requirement of *L. fungicola*

Cross and Jacobs (1969) stated that *L. fungicola* conidia required exogenous supplies of nutrients from mushroom mycelium for germination. Thapa and Jandaik (1987) presented interesting data about conidia germination and germ tube length. The best temperature for conidia germination and length of germ tube was 25 °C followed by 20 and 30 °C. *Lecanicillium fungicola* conidia failed to germinate at 5 and 40 °C. The authors showed the best pH for conidia germination and length of germ tube was 5.5 followed by pH 5; however conidia were able to grow at pH 8 but had only 12 % germination. The best natural extract for germination was extract from mushroom fruit bodies of different stages with almost 98 % of conidia germinating, but sterile water and tap water gave 35 % conidia germination. Thapa and Jandaik (1987) also looked at the effect of different nutrient solutions on conidia germination. The maximum conidia germination and length of germtube was recorded with a sucrose solution (5,000 ppm) followed by sucrose (1,000 ppm) then by glucose (5,000 ppm). Coetzee and Eicker (1991) reported that *L. fungicola* grew very well in medium containing either glucose, mannitol, sucrose, galactose or mannose as a source of carbon. Good growth was recorded on all the nitrogen sources tested. The authors reported that light had a significant effect on sporulation of *L. fungicola* but it had little effect on vegetative growth.

Calonje *et al.* (1997) stated that *L. fungicola* grew very well in media containing sucrose, glucose or fructose as a source of carbon.

1.3.6 Interaction between *Agaricus bisporus* tissue and *Lecanicillium fungicola*

Ware (1933) observed that hyphal strands of *L. fungicola* were present in necrotic mushroom tissue and a short distance beyond the limits of discoloration. Ware (1933) and Matthews (1983) reported that the hyphae of the pathogen penetrated the hyphae of *Agaricus bisporus*, after which the hyphal cells of white mushroom collapsed. Ware (1933) did not observe specialized penetration structures in *Lecanicillium fungicola* using optical microscopy, nor did Matthews (1983) using electron microscopy. North and Wuest (1993) did not observe specialized penetration structures nor did they find evidence of direct penetration by *L. fungicola* using scanning electron microscopy (SEM). They reported that *L. fungicola* mycelia were

closely associated with the surface of the mushroom sporophores and with the internal hyphae of the host. The presence or absence of penetration structures might depend on whether observed tissues are white or brown necrosed.

However, a few years later Dragt *et al.* (1995, 1996) using optical microscopy and transmission electron microscopy (TEM) showed that *L. fungicola* hyphae did in fact penetrate hyphae of *A. bisporus* and this was confirmed by Calonje *et al.* (1997) by TEM and biochemical studies of enzymatic activity of *L. fungicola* in the presence of *A. bisporus* cell wall. These authors, in contrast to the earlier results presented by Ware (1933), Matthews (1983), and North and Wuest (1993) observed evidence of direct penetration and of the presence of specialized penetration structures of *L. fungicola*.

Recently, Shamshad *et al.* (2009) using SEM and TEM failed to confirm the presence of specialized penetration structures or direct penetration by *L. fungicola* in the host tissue but showed that the pathogen mycelium grew very closely alongside the *A. bisporus* mycelium (**Figure** 1-13).

Figure 1-13: Scanning electron microscopy of the surface of an infected mushroom by *L. fungicola*: A – Vegetative hyphae and conidia. (Damięcka (Piasecka) and Maszkiewicz, 2006 a).

Lecanicillium fungicola is able to produce extracellular enzymes such as an amylase, lipase and cellulase (Trigiano and Fergus 1979). Kalberer (1984) reported that *L. fungicola* contained at least one proteolytic enzyme, which is responsible for the attack of the pathogen on *Agaricus bisporus*. Calonje *et al.* (1997) demonstrated that *L. fungicola* produce extracellular enzymes that are required to obtain carbon. Some of the enzyme activities identified includes endopolysacharidases, disacharase,

exopolysaccharidases and proteases. Bidochka *et al.* (1999 a), reported that *L. fungicola* is an opportunistic pathogen and compared to facultative plant pathogens, produced the highest number and range of extracellular proteases which play a significant role during infection.

Mills *et al.* (2000) isolated and identified *beta*-1-6-glucanases, chitinases, serine proteinase, stearase and esterase from culture filtrates of *L. fungicola* grown in the presence of *A. bisporus* cell walls. Later Mills *et al.* (2008) confirmed that *L. fungicola* produced a wide range of hydrolytic enzymes, which play a critical role in the *L. fungicola* infection process, with some of the enzyme activities identified being 1-3-*beta*-glucanase, proteinase, aminopeptidase and chitinase.

Calonje *et al.* (1997) suggested that some lytic enzymes could cause the degradation of the host hyphae, followed by degradation of cytoplasm and death of the cell. Later Calonje *et al.* (2000 a) reported that *L. fungicola* does not seem to exhibit a lytic effect on *A. bisporus* vegetative mycelial wall *in vivo* and suggested that the infection process depends on the chemical composition and structure of the *L. fungicola* cell wall. Bernardo *et al.* (2004 a) and Cabo and Mendoza (2008) confirmed the hypothesis of Calonje *et al.* (2000 a) about *L. fungicola* chemical composition of cell wall and its role in the infection process. One of the components of the *L. fungicola* cell wall is a glucogalactomannan (Calonje *et al.*, 2000 b; Bernardo *et al.*, 2004 a). This polysaccharide is a specific molecule, which binds to sugar-binding protein-lectin, present only in *A. bisporus* fruit body cell walls; this may explain the absence of *Lecanicillium* disease on the *A. bisporus* vegetative mycelia phase as it lacks this lectin (Bernardo *et al.*, 2004 a).

The same mechanism of glucogalactomannan-lectin interaction (pathogen-host recognition and intereaction) also occurs in the oyster mushroom (*P. ostreatus*) fruit body (Bernardo *et al.*, 2006; Cabo and Mendoza, 2008). Amey *et al.* (2003), reported that *beta*-1,6-glucanases showed up-regulation when *L. fungicola* was on a *A. bisporus* cell wall and in the presence of cell wall components including chitin.

More research is needed to understand the interaction between the pathogen and the host and the role of glucogalactomannan and lectin in host colonization and the initiation of *Lecanicillium* disease.

Agaricus bisporus can protect itself from *L. fungicola* invasion by the production of extracellular phenoloxidases, H_2O_2, and antibiotics (Score *et al.*, 1997; Largeteau *et al.*, 2006; Savoie *et al.*, 2004), but the efficiency of self-defence depends on the level of resistance of individual *Agaricus* strains to *L. fungicola*.

The cell wall of *L. fungicola* contains homo- and hetero-polysaccharides, proteins, lipids and minerals with the most common components encountered being glucans, glucogalactomannans, and chitin (Calonje *et al.*, 2000 b). Domenech *et al.* (2002) isolated and identified water soluble polysaccharides contained in three strains of *L. fungicola* var. *fungicola* cell wall using chemical analysis, methylation analysis and nuclear magnetic resonance spectroscopy (NMR). NMR analysis identified these two polysaccharides 1,5-di-O-acetyl-2,3,4,6-*tetra*-O-methyl-galactitol, which correspond to terminal galactopyranose and 1,4,5,6-*tetra*-O-acetyl-1-2-3-*di*-O-methyl mannitol, which correspond to terminal mannopyranose or mannofuranose. This result can be useful for chemotaxonomic characterisation of fungi.

1.3.7 Sources of Infection and Disease Spread

The primary sources of dry bubble disease can be casing ingredients, especially peat containing *L. fungicola* conidia (Wong and Preece, 1987; Fletcher and Gaze, 2008). The infection cannot occur before casing time. If conidia land on the spawned compost, crops do not show disease during the crop cycle (Beyer *et al.*, 2005; Fletcher and Gaze, 2008). The secondary spread vectors of conidia and mycelium of *L. fungicola* can be dust particles and water droplets in the air coming from infected crops (Gandy, 1972; Gaze, 2004; Beyer *et al.*, 2005; Clift and Shamshad, 2008). Grogan (2001) described an experiment in which dust/debris was collected from mushroom farm where dry bubble disease was present. This debris was used to inoculate new crops, which developed symptoms of dry bubble disease. The dust particles can carry sticky conidia and spread to other mushroom houses, land on the casing and infect the pins. The conidia of *L. fungicola* are held in sticky mucilage and they can disperse rapidly when they come in contact with water. Conidia dispersal into water vapour can transport conidia rapidly around a mushroom farm and infect new houses. However, a blast of air does not transport *L. fungicola* conidia when passed over a *Lecanicillium* colony at a speed of 10.75m/s^{-1} (Cross and Jacobs, 1969). Other very important conidia carriers are flies (Cross and Jacobs, 1969).

Mushroom flies are the most important pest of *Agaricus* cultivation all over the world (Bech *et al.*, 1982; Fletcher and Gaze, 2008). Sciarid (Sciaridae) flies are very effective at transmitting *Lecanicillium fungicola* conidia (Gandy, 1972; Finley *et al.*, 1984; Fletcher and Gaze, 2008). White (1981) showed that Phorid flies (Phoridae) were

able to carry *Lecanicillium fungicola* conidia too. Kumar and Sharma, (1998), reported that 100 % sciarids and 76-100 % phorids transmitted *L. fungicola* conidia, *in vitro* experiments using two different media. Recent publications indicate that the sciarid fly *Bradysia ocellaris* (Comstock) is a more competent vector of *L. fungicola* conidia transmitter than *Lycoriella ingenue* (Dufour) (Shamshad *et al.*, 2009).

Humans constitute a very important vector for conidia dispersal because every time they enter a house with dry bubble, conidia can be picked up and be transported to a new crop (Bech *et al.*, 1982; Wong and Preece 1987). Fletcher *et al.* (1986) found viable *L. fungicola* conidia persisted on hands after washing procedure with soap and hot water. Cross and Jacobs (1969) demonstrated that conidia dispersal of the pathogen can be by water splash during some growing manipulations. They showed, that 60 drops of water on infected sporophores from 1m high for 1 minute could spread *Lecanicillium* conidia more than 60 cm. Gandy (1972) confirmed Cross and Jacobs (1969) view that the watering process can spread conidia and significantly reduce yields of healthy crops. Conidia can spread for a long distance with crates and equipment used on mushroom farms (Bech *et al.*, 1982; Griensven, 1988; Jandaik and Sharma 1999, Fletcher and Gaze, 2008). *Lecanicillium fungicola* possesses a sticky mucilage which contains bunches of conidia which can enable them to stick to surfaces.

1.3.8 Detection of *L. fungicola* and other microorganism using classical methods.

Fungi play a significant role as spoilage agents of food. The fungi are responsible for spoiling about 25 % of annual production of plants for humans and animal consumption (Geisen, 2007) and this is why it is so important to identify, detect and monitor contaminated food to find sources of these pathogens. Classical identification methods require specialists or correct identification of fungi. The classical method is time-consuming; the results usually take up to 5 days if the microbes are able to grow on artificial media (Geisen, 2007).

The classical identification method of fungi requires an isolation method. The microbiological method for isolation involves cultivation on media and later sub-cultivation for identification. Various media are considered to be “non-selective” media; on media such as Czapek agar, malt agar, or potato dextrose agar many kinds of fungi can grow. The most important media for isolation and identification of fungi are

selective medium. A selective medium can contain a high amount of sugar which creates favourable conditions for recovery of species with rapid germination times and fast growth. Other selective media are nutritionally rich to allow for and support growth slow-growing fungi while preventing of growth of rapid-growing fungi. The interfering fungi species must be eliminated or constrained by physical or chemical means to allow growth of slow growing species. The most popular fungitoxic agents for suppression of rapid growing fungi are rose bengal, cyclosporine A, cycloheximide, dichloran and malachite green (Bills *et al.*, 2004).

The history of selective media and method for isolation and identification of human and animal pathogenic fungi dates back around 60 years (Georg *et al.*, 1954; Ulrich, 1956). They used antimicrobial agents such as cycloheximide and chloramphenicol to inhibit growth of saprophytic fungi and bacteria. Later on other antibiotics such as streptomycin and penicillin (Hantschke, 1968) were tested. Further knowledge on the selectivity of chemical inhibitors and the physiology of pathogens led to development of selective media for isolation of soil and plant pathogenic fungi (Tsao, 1970).

Today selective media is a common method for isolation and identification of different plant pathogens. The selective media are used for identification of many plant and some mushroom pathogens such as: *Aspergillus carbonarius* and *Aspergillus niger* which are responsible for wine contamination with mycotoxin - ochratoxin A (Pollastro *et al.*, 2006), *Fusarium avenaceum* and *F. verticillioides* (= *F. moniliforme*) – which are common fungal pathogens of wheat, maize and other crops (Thrane, 1996), *Trichoderma* spp. – common plant and compost mushroom pathogen (Elad and Chet 1983) and *Trichoderma harzianum* – common mushroom compost pathogen (Williams *et al.*, 2003).

Wong and Preece (1987) first described a microbiological test for *L. fungicola* detection. The authors used two microbiological media: Agar F (used routinely in bacterial blotch caused by *Pseudomonas tolassi* and *P. gingeri*) and DBR medium (Defined base medium + bromothymol blue + raffinose). They reported that *L. fungicola* grew well on Agar F and produced white fluffy colonies but colonies of *P. tolassi* and *P. gingeri* were found on the same plates. The second medium DBR used by Wong and Preece (1987) was originally developed for testing the bacterium *Erwinia salicis* and its ability to utilize a large number of different carbohydrates, including raffinose. Wong and Preece (1987) tested *L. fungicola* on basal media (DBR) plus indicator and they discovered that *L. fungicola* grew well using raffinose as the sole

carbon source, compared to other fungi common in mushroom farms such as *Penicillium, Mucor, Cladosporium* and *Trichoderma* which grew either poorly or did not grow during 1-7 days of incubation at 25 °C. They used bronopol as a bacterial suppressor to inhibit bacteria growth.

Rinker *et al.* (1993) described a selective medium for *L. fungicola*. Rinker's selective medium for *L. fungicola* contains a basal medium (DBR) presented by Wong and Preece (1987), one antibiotic, two different fungicides and two dyes. On this medium *L. fungicola* grew well and after a few days colonies were present but some species of *Penicillium* also grew. Rinker's selective medium for *L. fungicola* can be used for monitoring sanitation, hygiene levels, and this helps to manage dry bubble disease.

The DBR agar medium contains some reagents which are common ingredients to those used in many different media such as selective medium for *Trichoderma harzianum* (Elad and Chet 1983; Williams *et al.*, 2003).

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1.3.9 Environmental Parameters

Temperature and relative humidity (RH) are important factors for mushroom growing but also for disease development. *Lecanicillium fungicola* likes warm and moist conditions for growth and reproduction (Griensven, 1988). The higher temperature and humidity during summer time may be responsible for the fast development and spread of the fungus on mushroom farms, also dry dusty summer weather may spread disease.

Hybrid strains of button mushroom like to grow at a higher temperature and higher RH for better quality. Consequently, the time of dry bubble disease development is around 7-10 days after contamination. Nair and Macauley (1987) reported that manipulation of the temperature and RH during cropping might reduce the amount of disease development. They showed a decrease in air temperature from 20 °C to 14 °C and relative humidity from 90 % to 80 % for several days could decrease the amount of infected mushrooms (Arrold 1981; Nair and Macauley 1987). Cross and Jacobs (1969) reported that the lowered relative humidity may also reduce the spread of the pathogen, because the presence of water is known to support in the dispersal of *L. fungicola* conidia. Thermal death point for *L. fungicola* is 39 °C after 30 min (Van Zaayen and Rutjens 1981). Sharma and Kumar (2005) recommended 55 °C for *L. fungicola* as a thermal death point under wet heat treatment, but on dry heat, the authors suggested it is 70 °C for one hour to kill *L. fungicola*. Bech *et al.* (1989) reported that dry *L. fungicola* conidia could survive 10 min at 125 °C. Treating casing soil before use at 54.4 °C for 15 min eliminated *Verticillium fungicola* (Wuest and Moore, 1972).

Lecanicillium fungicola conidia can survive for at least one year in moist soil (Cross and Jacobs, 1969). The dried conidia and mycelium in infected mushrooms can survive for seven months (Fekete, 1967). Brady and Gibson (1969) reported that dry conidia could survive a maximum of 7-8 months.

1.3.10 Prevention and Control of Dry Bubble Disease – Management

Control of *L. fungicola* is very difficult because commercial strains of *A. bisporus* do not have any natural resistance to dry bubble disease and they are very highly susceptible to *L. fungicola* (Sharma, 1994; Dragt *et al*., 1995; Jandaik and Sharma, 1999). *Agaricus bitorquis* is less sensitive than *A. bisporus* for *L. fungicola* (Poppe, 1972). Draght *et al.* (1995) reported that several wild isolates of *A. bisporus* out of 100 tested isolates had partial resistance to *L. fungicola* but they do not have complete resistance.

The conidia of *L. fungicola* can easily spread on mushroom farms. The first important thing to do it is to prevent *L. fungicola* conidia entering the mushroom farm. The best method to prevent the entry of this mycoparasite is to use good cultural practices and sanitation (Gandy, 1972; Vedder, 1978; Griensven, 1988; Beyer *et al.*, 2005). Fungicides that are used to control *L. fungicola* must do so without damaging the *Agaricus bisporus* mycelium (Challen and Elliott, 1985). The fungicides control the spread of mycoparasite but they do not prevent entry of *L. fungicola* conidia. The first fungicides used to control dry bubble disease were dithiocarbamates, principally zineb (Yoder *et al.*, 1950), and this was later replaced by mancozeb (Fekete and Kuhn, 1965, 1966; Newman and Savidge, 1969).

Gandy (1971 and 1972) and Holmes *et al.* (1971) proposed fungicides containing carbendazim (Methylbenzimidazole carbamate – MBC). Carbendazim is a systematic benzimidazole fungicide that plays a very important role in plant disease control. Carbendazim works by inhibiting the development of fungi probably by interfering with spindle formation at mitosis (cell devision)[5]. MBC fungicides gave excellent control of *L. fungicola* initially (Wuest and Cole, 1970; Holmes *et al.*, 1971; Ganney and Atkins, 1972), but prolonged use resulted in *L. fungicola* becoming resistant (Wuest *et al.*, 1974; Bollen and Van Zaayen, 1975; Fletcher and Yarham, 1976; Lambert and Wuest, 1976). Recently Potočnik *et al.* (2008) reported that *L. fungicola* Serbian isolates were highly resistant to benomyl and had EC_{50} values higher than 200 mg/l. Carbendazim fungicides are no longer effective in dry bubble disease control. Furthermore they are no longer approved for use on mushrooms in Europe.

[5] http://www.pan-uk.org/pestnews/Actives/Carbenda.htm

The next fungicide used to control dry bubble was a non-systemic fungicide which contained as an active ingredient – chlorothalonil, also known as tetrachloroisophthalonitrile (Gandy and Spencer 1976). This fungicide gave good control of isolates of *L. fungicola* that were resistant to carbendazim and benomyl (Beyer and Kremser, 2004). It also gives a broad spectrum of disease control activity. Chlorothalonil was reported as a multi-site active fungicide with a low risk of resistance development. Van Zaayen (1977), Gandy and Spencer (1981) and Maszkiewicz (2001), demonstrated that the fungicide is efficient in controlling dry bubble disease on mushroom crops. However after 20 years of application *L. fungicola* started to develop resistance and chlorothalonil failed to control dry bubble disease (Fletcher and Hims, 1981; Gea *et al.*, 1996; Bonnen and Hopkins, 1997).

1.3.10.1 DMI fungicides

Van Zaayen (1983) proposed locally systemic fungicides which included prochloraz as an active ingredient. Prochloraz is a member of the sterol demethylation inhibitor (DMI) fungicides and inhibits the sterol C-14 α-demethylation of 24-methylenedihydrolanosterol, a precursor of ergosterol in fungi which is necessary for the development of functional cell walls (Siegel, 1981; Buchenauer, 1987; Brent and Hollomon 2007). The application of DMI results in abnormal fungal growth and death. However, DMI fungicides have no effect on conidia germination because conidia contain enough sterol for the formation of germ tubes. Therefore, DMI fungicides must be applied preventively or at early-stage of infection to be effective. Usually, these fungicides have approximately 14 days of residual activity. DMI fungicides have a very specific site of action so the risk of resistance development is a concern. DMI fungicides are known and used in agriculture since the 1970s (Buchenauer, 1987).

The best result for pathogen control in mushroom cultivation was DMI fungicide which contains complex prochloraz-manganese (prochloraz-Mn) (**Figure** 1-14).

Characteristics of prochloraz-manganese:

Synonyms: Dichlorotetrakis (N-propyl-N-(2-(2,4,6-trichlorophenoxy)ethyl)-1H-imidazole-1-carboxamide)manganese

Molecular Formula: $C_{60}H_{64}Cl_{14}MnN_{12}O_8$

Molecular Weight: 1632.505809

Registry Number: 75747-77-2

Molecular Structure:

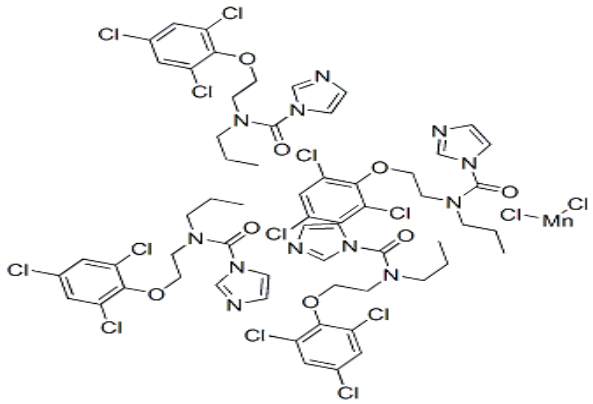


Figure 1-14: Prochloraz-manganese[6].

Prochloraz-manganese complex showed excellent control of *L. fungicola* (Van Zaayen and Adrichem, 1982; Gea *et al.*, 1995 and 1996; Bernardo *et al.* (2004 b). It was more effective than other fungicides containing carbendazim, benomyl, chlorothalonil and formaldehyde, iprodione and the mixture of prochloraz + carbendazim. Today prochloraz-manganese is used in many countries where mushrooms are cultivated (but excluding USA) (Mendoza *et al.*, 2003; Bhatt and Singh 2002; Papadopoulos, 2005; Bernardo *et al.*, 2002; Gea *et al.*, 2003; Grogan *et al.*, 2000; Damięcka (Piasecka) and Maszkiewicz, 2006 b). Fletcher *et al.* (1983) reported that the prochloraz-manganese complex gave best control when it was used as three separate spray applications of 0.3 g/m^2 during cropping. Later Van Zaayen (1983) reported that control of *L. fungicola* was excellent when 3 g/m^2 of prochloraz was applied once as a spray 9 days after casing. Russell (1984) reported the complex prochloraz-manganese achieved good control of *L. fungicola* when it was applied once after 7 days casing run at a rate of 1.5 g/m^2 active ingredient. Prochloraz-manganese killed *L. fungicola* in 83 % of crops while chlorothalonil killed only 1 % (Bhatt and Singh, 2002). However, after thirty years of using prochloraz-manganese on mushroom farms there are concerns regarding a decrease in sensitivity to this fungicide (Desrumaux *et al.*, 1998; Grogan *et al.*, 2000; Gea *et al.*, 2005; Damięcka (Piasecka) and Maszkiewicz, 2004). Moreover, the attitude of many mushroom farm owners to the control of dry bubble is unsatisfactory (Gea *et al.*, 2005). Although Grogan *et al.* (2000) reported that prochloraz-manganese could still control dry bubble disease reasonably well they demonstrated that two isolates

[6] http://www.chemicalbook.com/ChemicalProductProperty_EN_CB1854880.htm

presented different levels of sensitivity to this fungicide. Potočnik *et al.* (2008) reported that prochloraz-manganese is still effective on Serbian isolates of dry bubble disease and *in vitro L. fungicola* isolates showed high sensitivity with EC_{50} (fungicide concentrations which inhibit mycelia growth by 50 %) values of less than 3 ppm.

Kelling *et al.* (2000) reported that the concentration of prochloraz-manganese in casing decreased effectively between 14 and 21 days after treatment and again the concentration of prochloraz-manganese decreased in casing after a second treatment, between 21 and 28 days (Grogan and Juke, 2003). Papadopoulos (2005) stated that decreasing concentrations of fungicide in casing is due to the action of microorganisms.

1.3.10.2 Biological Control and other control methods of *L. fungicola*

Biological control of dry bubble disease is very difficult. First information for biological control of *L. fungicola* comes from French researchers De Trogoff and Ricard, (1976), who used another common pathogen of plants and mushroom compost – *Trichoderma viride*. They sprayed casing soil with 100×10^6 *Trichoderma viride* propagules/litre/m^2 to control *L. fungicola* in several trials on mushroom holdings where dry bubble disease was endemic but this test was not positive. Bhatt and Singh (2000) reported that five bacterial isolates from casing are effective against *L. fungicola*. It is very difficult to use biological control agents in the mushroom cultivation, because bio-control requires a certain amount of the pathogen to be activated so that the control organism can survive. But *Trichoderma viride* is a good biological control of diseases of plants caused by bacteria, fungi and nematodes (Spiegel ad Chet, 1998).

Dimantopoulou *et al.* (2006) and Chrysayi-Tokousbalides *et al.* (2007) demonstrated that is not easy to find new fungicides for the control of *L. fungicola* which will not affect the development of the white mushroom. Tanović *et al.* (2009) tested a few essential oils from aromatic and medicinal plants against *L. fungicola in vitro*. Some essential oils have an ability to suppress growth of the pathogen.

1.3.11 Thermal disinfection

Thermal disinfection (steam cookout) is a very effective method at the end of a crop for eliminating all populations of pathogens and pests in the mushroom house and cleans all the surfaces of the building (Gandy, 1981). The cookout process is effective when the temperature inside a growing room is 65-70 °C for 9-12 hours depending on circumstances (Fletcher and Gaze, 2008). After cookout, the next crop in the growing room should start free from disease, so the main task of the grower then is to keep it clean of disease (Gandy, 1981). If mushroom farm does not have the equipment to produce steam or if the growing rooms cannot be steamed then good practice is to use chemical disinfection (Fletcher and Gaze, 2008).

1.3.12 EU – legislation

Today, there are very few fungicides for the control of fungal pathogens of mushrooms approved for use in EU countries. Chemicals permitted by EU for use on mushroom cultivation are prochloraz and prochloraz-manganese complex (Anonymous 2005, 2009). Details of maximum residue levels permitted in EU on cultivated mushroom are published in EU Commission Directives 2008/17/EC amending certain Annexes to Council Directives 86/362/EEC, 86/363/EEC and 90/642/EEC [7].

The EU restrictions on fungicides are continually under review as new information becomes available on the potentially harmful effects of pesticides on humans.

[7] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:050:0017:0050:EN:PDF

1.4 Aims and Objectives of this study

The overall objectives of this study were to develop detection methods for *Lecanicillium fungicola* which causes dry bubble disease on Irish mushroom farms and that might also be used in other European coutries such as Poland, Serbia, Spain, Netherlands and France etc. Two approaches for detection of *L. fungicola* were designed and tested – selective media which is an easy test for detection of live spores and molecular method – Real Time PCR which is able to detect dead and live material of *L. fungicola*.

The objectives of this work are as follows:

1. To isolate and identify *L. fungicola* isolates from diseased tissue of *A. bisporus* on Irish mushroom farms using morphological test and response to different growth conditions.
2. To analyse the sensitivity of the pathogen to two fungicides: carbendazim and prochloraz-manganese.
3. To evaluate DNA extraction methods and optimise PCR methods and to design selective primers.
4. To develop a sample collection, and preparation of samples for microbiological and molecular test.
5. To develop novel selective medium for *L. fungicola* and modify an existing selective medium for microbiological test for use in mushroom farm samples.
6. To use Real Time PCR method for *L. fungicola* detection from casing samples and from mushroom farm samples.
7. To collect and analyse samples from mushroom farms using novel and modified selective media and molecular test – Real Time PCR.

Chapter 2 Materials and Methods

2.1 Chemicals

In this study the following chemicals were used (**Table** 2-1)

Table 2-1: Chemicals used during experiments.

No.	Chemicals	Supplier	Catalogue No
1	Bromocresol green salt (C21H13O5Br4S Na)	Sigma-Aldrich	B 1256
2	Carbendazim (Kapchem 50 SL)	KapChem-Ireland	
3	2-Propanol (Isopropanol)	Sigma-Aldrich	I 9516
4	3-(N-morpholino)propanesulfonic acid (MOPS)($C_7H_{15}NO_4S$)	Sigma-Aldrich	M 3183
5	Acetic Acid ($C_2H_4O_2$)	Sigma-Aldrich	242853
6	Agar	BD Difco ™	214010
7	Agarose electroforesis	Melford	MB 1200
8	Ammonium dihydrogenphosphat ($NH_4H_2PO_{4)}$	Riedel-de-Maen	30401
9	Ampicillin	Sigma	A9393
10	Bromocresol green inducator	Riedel-De Maen ag Seelze-hannover	32742
11	Bromophenol blue ($C_{19}H_{10}Br_4O_5S$)	Sigma-Aldrich	5525
12	Carbendazim	KapChem	n/a
13	Casing soil	Cooperative Netherlandse Champignonkwekersvereniging	type nat or nat +
14	Casing soil	Irish mushroom farms	n/a
15	Chloramphenicol	Sigma	C0378
16	Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	D 5879
17	DirectLoad ™ Step Ladder, 50bp	Sigma	D3812
18	DNA Ladder 50 bp	BioLabs	N3236S
19	DNA Ladder 100 bp	BioLabs	N0467S
20	Dneasy Plant Mini Kit	Qiagen	69104
21	dNTP mix	Promega	U1511
22	Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	E6758
23	Erythromycin	Sigma-Aldrich	E 5389
24	Ethanol	Sigma-Aldrich	E 7023
25	Ethidium bromide	Sigma-Aldrich	E7637
26	Glacial acetic acid ($C_2H_4O_2$)	Sigma-Aldrich	A9967
27	Glass beads	Sigma	G8772

28	Glycerol	Sigma	G7893
29	GoTaq DNA Polymerase	Promega	M8301
30	Phusion™ High Fidelity DNA polymerase	BioLabs	F-530S
31	Hydrochloric acid (HCl)	Sigma	435570
32	LZ Load Precision Molecular Mass Standard	BioRAD	170-8356
33	Magnesium sulfat heptahydrate ($MgSO_4 \times 7H_2O$)	Merck	5882
34	MagneSphere® Magnetic Separation Stand		Z5342
35	Malachite green salt	Gurr	CI 42000
36	Malt extract	Oxoid	CM0059
37	Malt extract agar (MEA)	Oxoid	CM 0059
38	Molecular Water 1L	Sigma	W4502
39	Nuclease Free water 2 x25 ml	Promega	P1193
40	Nucleon PhytoPure DNA isolation kit	GE (GE Healthcare Life Sciences).	RPN 8510
41	Phenol: Chloroform: Isoamyl alcohol 25:12:1	Flucka	77617
42	Phosphate Buffered Saline (PBS)	Oxoid	BR 0014G
43	Polyadenyli acid potassium (Poly A)	Sigma	P9403
44	Polyethylene glycol (PEG)	Sigma	73034
45	Polymerase Lightcycler 480 Probes Master (5 × 1 ml (2x conc.))	Roche	04 707 494 001
46	Polymerase Maxima Probe qPCR Master mix	Fermentas	K0269
47	Polymerase Maxima Probe/ROX qPCR Master mix	Fermentas	K0232
48	Polymerase Maxima Probe/ROX qPCR Master mix	Fermentas	K0239
49	Potassium Acetate (CH_3CO_2K)	Sigma	P1190
50	Potassium chloride (KCl)	Sigma	P 4504
51	Potato Dextrose Agar (PDA)	Oxoid	CMO139
52	Potato Dextrose Agar (PDA)	Scharlau	01-483
53	Potato Dextrose Agar (PDA)	BD Difco ™	213400
54	Potato dextrose Broth	BD Difco ™	254920
55	Potato Dextrose Broth	Sigma	P 6685
56	Primers for PCR	Genosys Biotechnologies (Europe) Ltd.	n/a
57	Primers for PCR	Integrated DNA technologie, Inc.	n/a
58	Primers for Real Time PCR	Applied Biosystems	4304971
59	Probe TaqMan MGB	Applied Biosystems	4316034
60	Prochloraz-Mn (Sporgon 46 % WP)	BASF	
61	Proteinase K	Sigma	P6556
62	QuickGene Mini 80 device	Fujifilm	
63	QuickGene DNA tissue DT-S DNA	Fujifilm	
64	Raffinose ($C_{18}H_{32}O_{16} \times 5H_2O$)	Sigma	R0250

65	Ribonuclease A	Sigma	R6513
66	Rnase A	Quiagen	1009368
67	Sodium chloride (NaCl)	Sigma	S3014
68	Sodium Dodecyl Sulfate (SDS)	Sigma	L4390
69	Sodum Hydroxide (NaOH)	Sigma-Aldrich	S8045
70	Sporogn 50 WP (a.i. prochloraz-manganese)	BASF	n/a
71	Streptomycin Sulfate Salt	Sigma	S6501
72	Taq DNA polymerase in storage buffer A	Promega	M1860
73	Taq Polymerase DNA	Sigma	D1806
74	Taq Polymerases – GoTaq	Promega	M 830 A
75	Taq Polymerases – High Fidelity DNA	BioLabs	F-530
76	Technical agar	Oxiod	LP0013
77	Tetracycline	Sigma	T3258
78	Tris borate EDTA buffer (TBE) 10x concentrated	Sigma	93290
79	Tris-HCl	Sigma	T5941
80	Trizma base	Sigma	T1503
81	Tween 80	Merck	822187
82	Wizard® Magnetic DNA Purification System for Food	Promega	FF3750
83	ZR Fungal/Bacterial DNA MiniPrep™	Zymo Research Corporation	D6005

n/a – not available

2.2 Consumables

In this study the following consumables were used (**Table** 2-2).

Table 2-2: Consumables used during this study.

No.	Consumables	Supplier	Catalogue No
1	0.2 ml PCR tubes	Sarstedt	72.737
2	1.5 ml vessels	Fisherbrand	FB74031
3	1.5 ml vessels	Sarstedt	72.692
4	15 ml screw-cap tube	Sarstedt	62.554.001
5	2 ml vessels	Eppendorf	0030 120.094
6	50 ml screw-cap tube	Greiner	
7	50ml self-standing centrifuge tube	Sarstedt	62.547.004
8	Aerosol Barrier Pipet Tips 10 ul	Fisher	02-707-439
9	Aerosol Barrier Pipet Tips 1250 ul	Fisher	02-707-404
10	Aerosol Barrier Pipet Tips 200 ul	Fisher	02-707-430
11	Cover slips	Ultima	22221
12	Disposable pipettes	Corniostar	4489
13	Disposable pipettes	Sarstedt	86.1253.001

14	Filter 0.22 µm	Sarstedt	83.1826.001
15	Filter pore size 150 µm pores,	Netherlands	n/a
16	Filter Tips 200 µl	AGB	AXYGTF-200
17	Filter Tips 0.5-10 µl	Lorgarback	825.001
18	Filter Tips 10 µl	AGB	AXYGTF-300
19	Filter Tips 10 µl	Fisher	PMP 326 010 C
20	Filter Tips 1,000 µl	Fisher	PMP 326 030 T
21	Filter Tips 200 µl	Fisher	PMP 326 060 K
22	Filter Tips 30 µl	Fisher	PMP 326 050 N
23	Filtrer Tips 20 µl	AGB	AXYGTF-20
24	Gloves-Disposable Latex	Semperguard	CEO321
25	Lightcycler 480 Multiwell Plate 96 White (includes seating foils)	Roche	04729 692 001
26	Lightcycler 480 Sealing Foil	Roche	4729 757 001
27	Microscope slides	Ramboldi	11120
28	Miracloth	Calbiochem	475855
29	Nonwoven swabs 7.5 × 7.5	Mesoft	
30	Non-woven swabs 10 × 10cm,	Bastos Viegas, s.a.	n/a
31	Parafilm	Manashe	WI 54952
32	Plates Petri dishes		
33	References isolates of *L. fungicola* and *T. aggressivum*	CBS and BCCM	
34	Serological pipettes 25 ml	Costar	4489
35	Sterile Disposable L shaped (Spreaders)	Fisher	LPS-140-041X
36	Sterile Disposable L shaped (Spreaders)	Microspec	PLS5/500
37	Sterile inoculation loops	Fisher	LPS-131-011B
38	Syringe	BD Plastipak	302188
39	Transfer pipettes	Sarstedt	86.1171.010

n/a – not avaliable

2.3 Instruments

In this study the following instruments were used (**Table** 2-3).

Table 2-3: Instruments used during this study.

No.	Instrument	Company	Serial Number
1	Agar cuter		n/a
2	Autoclave	Systec	3170Elv
3	Autoclave	Tomy	SX-500E
4	Balance	Chettler Toledo	Bcollege S502-S
5	Balance	Sartarius	A 200 S
6	Centrifuge	Beckman	GS-6
7	Centrifuge Heraeus Christ	LaboFuge GL	2202
8	Centrifuge – table	Tomy	PHC-0.60
9	Electrophoresis unites	SciePlas	
10	Flow hood	Gelaire	BSB 4
11	Gel visualisation	Manson Technology	G-Box
12	Gel visualisation	Alpha Innotech	
13	Gel visualisation	Alpha DigiDoc TM RT	n/a
14	Haemocytometer	Neubauer	n/a
15	Haemocytometer	Burker Turk	n/a
16	Heating block eppendorf	Stuart	SHT 2
17	Incubater	Status by the Northern Media Supply Ltd.	n/a
18	Incubator	New Brunswick Scientific	Excella E25
19	Incubator	Heraeus-Ihre	25003692
20	Lyophilisation machine	SB8	n/a
21	Magnesphere Technology Rack	Promega	Z5342
22	Magnetic stirrer	Stuart	CB161
23	Microscope	Olympus	CH20
24	Microwave	LG	n/a
25	Microwave	Beaumark	n/a
26	Mortar and pestle	Haldenwager-Berlin	55-0a, 56-00
27	MQ water	Millipore – Synthesis	F7SN96809M
28	MQ water	Millipore – Elix	F7SN08019E
29	PCR machine	Eppendorf – Mastercycler	533300238
30	pH meter	Jenway	3510
31	pH meter	Eutech Instruments	510
32	Pippetors – Socorex	Fisher	PMP-265-0255V
33	Power supply unit	Bio-Rad	041BR64149
34	Power supply unit	Bio-Rad	283BR14245
35	Real Time Machine	Roche	LightCycler 480

36	Refrigerator (+4 and -20 °C)	Whirlpol	n/a
37	Refrigerator (+4 and -20 °C)	Candy	n/a
38	Refrigerator (-70 °C)		n/a
39	Refrigerator (-70 °C)		n/a
40	Reversible 96 well rack	Sigma	R6151-5EA
41	Rotor	Beckman	S/N 1237A
42	Rotor	LaboFuge GL	8159
43	Rotor	Eppendorf	A-2-DNP
44	Safety cabinet	Microflow	n/a
45	Safety cabinet	Bioair	Safeflow 0.9
46	Spectrophotometry	Manson Technology	NanoDrop 1000
47	Ultra Centrifuge	Eppendorf	5417C
48	Ultra Centrifuge	Eppendorf	545221036
49	Ultra Centrifuge	Eppendorf	5804
50	Vortex	Scientific Industries	Vortex-Gene 2

n/a – not avaliable

2.4 Solutions

2.4.1 Solutions for pH Adjustment

2.4.1.1 5 M Hydrochloric Acid (HCl)

Deionised water (40 ml) and hydrochloric acid (43.64 ml) were added slowly to a glass graduated cylinder. The final volume was adjusted to 100 ml. The solution was stored at room temperature.

2.4.1.2 5 M Sodium Hydroxide (NaOH)

NaOH pellets (20 g) were added to deionised water (80 ml) and dissolved using a magnetic stirrer. The final volume was adjusted to 100 ml. The solution was stored at room temperature.

2.4.2 Phosphate buffered saline

One PBS tablet (20 x) was added to 200 ml of distilled water, and dissolved by stirring. The solution was autoclaved and stored at room temperature.

2.4.3 Phosphate buffered saline – Tween 80 (PBST-80)

Tween 80 (0.5 ml) was added to 1 L PBS. The solution was stored at room temperature.

2.4.4 30 % (v/v) Glycerol ($C_3H_5(OH)_3$)

Glycerol (30 ml) was added to 70 ml deionised water. The solution was sterilized by autoclaving and stored at room temperature.

2.4.5 50 % (v/v) Glycerol ($C_3H_5(OH)_3$)

Glycerol (500 µl) was added to 500 µl deionised water. The solution was sterilized by autoclaving and stored at stored at -20 °C.

2.4.6 0.5 M Ethylenediaminetetra acetic acid (EDTA) ($C_{10}H_{16}N_2O_8$)

EDTA (186 mg) was added to 1 L of deionised water. The pH was adjusted to pH 8. The solution was sterilized by autoclaving and stored at room temperature.

2.4.7 1 M Tris ($C_4H_{11}NO_3$)

Tris (121.14 g) was dissolved in 1 L deionised water. The pH was adjusted to pH 7.5. The solution was sterilized by autoclaving and stored at room temperature.

2.4.8 TE buffer

10 ml 1M Tris (pH 7.5) and 2 ml 0.5 M EDTA (pH 8) to 800 ml deionised water. Mixed and adjust to 1 L with deionised water. The buffer was sterilized by autoclaving and stored at room temperature.

2.4.9 20 % Sodium Dodecyl Sulphate (SDS) ($NaC_{12}H_{25}SO_4$)

SDS (20 g) was added to 100 ml deionised water, and dissolved. The solution was stored at room temperature. If SDS precipitated, the solution was incubated at 37 °C until the SDS went back into solution.

2.4.10 20 mg/ml proteinase K (EC 3.4.21.64)

Proteinase K (20 mg) was added to 1 ml of deionised water. The solution was mixed and stored at -20 °C.

2.4.11 6 M Sodium chloride (NaCl) (NaCl saturated H_2O)

NaCl (35 g) was added to 100 ml of deionised water. The solution was mixed, autoclaved and stored at room temperature. NaCl 6 M is saturated salt solution stored at 37 °C.

2.4.12 30 % Polyethylene glycol (PEG) ($C_{2n+2}H_{4n+6}O_{n+2}$)

PEG (30 g) was added to 100 ml deionised water, and dissolved. The solution was stored at room temperature.

2.4.13 1.6 M Sodium chloride (NaCl)

NaCl (9.35 g) was added to 100 ml of deionised water. The solution was mixed, autoclaved and stored at room temperature.

2.4.14 7.5 M Potassium acetate (CH_3CO_2K)

CH_3CO_2K (73.65 g) was added to 100 ml of deionised water. The solution was mixed, autoclaved and stored at -20 °C.

2.4.15 5 µg/µl Polyadenyli acid potassium salt (Poly A) (Carrier RNA)

Poly A (0.5 mg) was added to 100 µl of deionised water. The solution was mixed and stored at -20 °C.

2.4.16 70 % C_2H_6O (Ethanol Absolute, 100 % (200 proof)

Ethanol (15 ml) was added to 35 ml deionised water. The solution was stored at room temperature.

2.4.17 Antibiotics

Antibiotics were filter-sterilised using a filter with a pore size of 0.2 µm (Sarstedt, Nümbrecht, Germany) and were added into cooled ~50 °C medium if required (Table 2-4).

Table 2-4: Common antibiotics and supplements with working concentrations.

Antibiotic	Diluent	Stock Solution	Working concentration	Storage
Streptomycin	water	10,000 mg/l	100 mg/l	-20 °C and at 4 °C
Tetracycline	DMSO	10,000 mg/l	100 mg/l	-20 °C

2.4.18 Aljanabi and Martinez (1997), homogenizing buffer (100 ml of 0.4 M NaCl 10 mM Tris-HCl pH 8.0 and 2 mM EDTA pH 8.0)

Tris-HCl (0.1576 g) and EDTA (0.07448 g) was added to 100 ml deionised water. The pH was adjusted to pH 8 and NaCl (2.337 g) was added. The buffer was sterilized by autoclaving and stored at 4 °C.

2.4.19 Yeates *et al.* (1998), extraction buffer (100 ml of 100 mM Tris-HCl pH 8.0, 100mM sodium EDTA pH 8.0, 1.5 M NaCl)

Tris-HCl (1.58 g) and EDTA (3.72 g) was added to 100 ml deionised water. The pH was adjusted to pH 8 and NaCl (8.77g) was added. The buffer was sterilized by autoclaving and stored at 4 °C.

2.4.20 DNA electrophoresis reagents

2.4.20.1 50x Tris-acetate buffer (TAE) (2M)

Trizma base (242 g) was added to 57.1 ml glacial acetic acid ($C_2H_4O_2$) and 100 ml of 0.5 M EDTA, pH 8.0. The volume was adjusted to 1 L with distilled water. The solution was stored at room temperature.

2.4.20.2 1x Tris-acetate buffer (TAE) (40mM)

50x TAE (20 ml) was added to distilled water (980 ml). The solution was stored at room temperature.

2.4.20.3 Ethidium bromide ($C_{21}H_{20}BrN_3$)

Ethidium bromide was supplied at 10 mg/ml of which 4 µl was used per 100 ml agarose gel.

2.4.20.4 6x DNA loading dye

Bromophenol blue ($C_{19}H_{10}Br_4O_5S$) 250 mg was added to 33 ml glycerol ($C_3H_5(OH)_3$) and 67 ml sterile water. The solution was stored at room temperature.

2.5 Media, agar and casing

2.5.1 Potato dextrose agar

Potato dextrose agar (PDA) was prepared under aseptic conditions. The required amount of PDA (39 g) or PDB (24g) and Agar (15g) or technical agar (15g) was/were added to 1 L distilled water and dissolved. Agar medium was autoclaved and allowed to cool to ~50 °C, the antibiotic (streptomycin) was add when medium was used for fungal isolations from *A. bisporus*. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.2 Potato dextrose broth

Potato dextrose broth (PDB) medium was prepared under aseptic conditions. The 24 g of PDB was added to 1 L distilled water and dissolved. The broth medium (20 or 50 ml) was filled prior to autoclaving into 50 or 250 ml flask closed by cotton and

sealed by tin foil. After autoclaving the medium was cooled to room temperature (RT) and inoculated.

2.5.3 Malt extract agar

Malt extract agar (MEA) medium was prepared under aseptic conditions. The 50 g of MEA was added to 1 L distilled water and dissolved. Agar medium was autoclaved and allowed to cool to ~50 °C. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and stored at 4 °C.

2.5.4 Malt extract broth

Malt extract (ME) broth medium was prepared under aseptic conditions. The 30 g of ME was added to 1 L distilled water and dissolved. The broth medium (20 or 100 ml) was filled prior to autoclaving into 50 or 250 ml flask closed by cotton and sealed by tin foil. After autoclaving the medium was cooled to room temperature and inoculated.

2.5.5 PDA with different amount of malachite green (MG)

Potato dextrose agar (PDA) was prepared under aseptic conditions. The required amount of PDA (39 g) or PDB (24g) and Agar (15g) or technical agar (15g) were added to 1 L distilled water and dissolved. After that, different amounts of malachite green (0, 10, 20 and 30 mg/l) were added depending on experiment. Medium was autoclaved and allowed to cool to ~50 °C. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.6 PDA with different amount of malachite green and one fungicide

Potato dextrose agar (PDA) was prepared under aseptic conditions. The required amount of PDA (39 g) or PDB (24g) and Agar (15g) or technical agar (15g) was/were added to 1 L distilled water and dissolved. After that, different amounts of malachite green (5, 7.5, 10 mg/l) were added depending on experiment. Medium was autoclaved and allowed to cool to ~50 °C and the 1 mg/l prochloraz-manganese (P) or 100 mg/l carbendazim (C) were added. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.7 PDA with different amount of malachite green (MG) and fungicides (P and C)

Potato dextrose agar (PDA) was prepared under aseptic conditions. The required amount of PDA (39 g) or PDB (24g) and Agar (15g) or technical agar (15g) was/were added to 1 L distilled water and dissolved. After that, different amounts of malachite green (0, 5 and 10 mg/l) were added depending on experiment. Medium was autoclaved and allowed to cool to ~50 °C and the 1 mg/l prochloraz-manganese (P) and 100 mg/l or 500 mg/l carbendazim (C) were added. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.8 PDA with different amount of malachite green and two fungicides (PDAPCMG)

Potato dextrose agar (PDA) was prepared under aseptic conditions. The required amount of PDA (39 g) or PDB (24g) and Agar (15g) or technical agar (15g) was/were added to 1 L distilled water and dissolved. After that, different amounts of malachite green (0, 5 and 10 mg/l) were added depending on experiment. The name of amount of malachite green added was indicated as a number in medium name e.g. PDAPCMG0, PDAPCMG5 or PDAPCMG10. Medium was autoclaved and allowed to cool to ~50 °C

and the 1 mg/l prochloraz-manganese and carbendazim 100 mg/l were added. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.9 PDA with different amount of malachite green and two fungicides and two antibiotics (PDAPCMGST)

Potato dextrose agar (PDA) was prepared under aseptic conditions. The required amount of PDA (39 g) or PDB (24g) and Agar (15g) or technical agar (15g) was/were added to 1 L distilled water and dissolved. After that, different amounts of malachite green (0, 5 and 10 mg/l) were added depending on experiment. The name of amount of malachite green added was indicated as a number in medium name e.g. PDAPCMG0, PDAPCMG5 or PDAPCMG10. Medium was autoclaved and allowed to cool to ~50 °C and 100 mg/l streptomycin, 500 mg/l tetracycline and 1 mg/l prochloraz-manganese and carbendazim 100 mg/l were added. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.10 PDA with different concentration of malachite green and two fungicides and two antibiotics and bromoresol green (PDAPCMGSTB)

Potato dextrose agar (PDA) was prepared under aseptic conditions. The required amount of PDA (39 g) or PDB (24g) and Agar (15g) or technical agar (15g) was/were added to 1 L distilled water and dissolved. After that, different amounts of malachite green (0, 10, 20 and 30 mg/l) were added depending on experiment. The bromocresol green was added to and when it was present it media was indicated by letter ‘’B’’. The name of amount of malachite green added was indicated as a number in medium name e.g. PDAPCMG0STB, PDAPCMG10STB, PDAPCMG20STB and PDAPCMG30STB. Medium was autoclaved and allowed to cool to ~50 °C and 100 mg/l streptomycin, 500 mg/l tetracycline and 1 mg/l prochloraz-manganese and carbendazim 100 mg/l were added. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.11 Novel (PDA) selective medium (NPDASM)

The new selective medium contained the following chemicals:

- 24 g/l Potato dextrose
- 20 g/l technical agar
- 10 mg/l malachite green sodium salt
- 30 mg/l bromocresol green sodium salt
- 100 mg/l streptomycin
- 500 mg/l tetracycline
- 1 mg/l prochloraz-manganese (Sporgon 46 % WP)
- 100 mg/l carbendazim (Kapchem 50 SL)

Agar medium was autoclaved and allowed to cool to ~50 °C and the streptomycin, tetracycline, prochloraz-manganese and carbendazim were added. Agar (20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and stored at 4 °C.

2.5.12 Base Rinker' medium (RBM)

Base Rinker's medium contained following chemicals:

- 1 g/l raffinose ($C_{18}H_{32}O_{16}$)
- 30 mg/l bromocresol green sodium salt ($C_{21}H_{13}O_5Br_4S\ Na$)
- 2 g/l Ammonium phosphate ($NH_4H_2PO_4$)
- 0.4 g potassium chloride (KCl)
- 0.4 g/l Magnesium sulphate heptahydrate ($MgSO_4 \times 7H_2O$)
- 20 g/l technical agar

Agar medium was autoclaved and allowed to cool to ~50 °C and other elements were added if experiment required it (malachite green was added before autoclaving). Agar (20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and stored at 4 °C.

2.5.13 Base Rinker medium with different amounts of malachite green

Base Rinker's medium (RBM) was prepared and different amount of malachite green (0, 10, 20 and 30 mg/l) were added depending on experiment. Medium was autoclaved and allowed to cool to ~50 °C. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.14 Modified Rinker's medium with different amounts of malachite green and fungicides

Base Rinker's medium (RBM) was prepared and different amount of malachite green (5 and 10 mg/l) were added depending on experiment. Medium was autoclaved and allowed to cool to ~50 °C and the 1 mg/l prochloraz-manganese and 100 mg/l carbendazim were added. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.15 Modified Rinker's medium with 10 mg/l malachite green and two fungicides (RBMPCMG)

Base Rinker's medium (RBM) was prepared and different amount of malachite green 10 mg/l. Medium was autoclaved and allowed to cool to ~50 °C and the 1 mg/l prochloraz-manganese and 100 mg/l carbendazim were added. The amount of malachite green added was indicated as a number in medium name e.g. RBMPCMG10. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.16 Modified Rinker's medium with different amounts of malachite green and two fungicides and two antibiotics and two fungicides (RBMPCMGST)

Base Rinker's medium (RBM) was prepared and different amount of malachite green (0, 10, 20 and 30 mg/l) were added depend for experiment. The name of amount of malachite green added was indicated as a number in medium name e.g. RBMPCMG0ST, RBMPCMG10ST, RBMPCMG20ST, RBMPCMG30ST. Medium was autoclaved and allowed to cool to ~50 °C and 100 mg/l streptomycin, 500 mg/l tetracycline and 1 mg/l prochloraz-manganese and carbendazim 100 mg/l were added. Agar (15-20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and were stored at 4 °C.

2.5.17 Modified Rinker's selective medium (MRSM)

Modified Rinker's medium contained the following chemicals:

- 1 g/l raffinose ($C_{18}H_{32}O_{16}$)
- 10 mg/l malachite green sodium salt ($C_6H_5C(C_6H_4N(CH_3)_2)_2]Na$
- 30 mg/l bromocresol green sodium salt ($C_{21}H_{13}O_5Br_4S$ Na)
- 2 g/l Ammonium phosphate ($NH_4H_2PO_4$)
- 0.4 g/l potassium chloride (KCl)
- 0.4 g/l Magnesium sulphate heptahydrate ($MgSO_4 \times 7H_2O$)
- 20 g/l technical agar
- 100 mg/l streptomycin
- 500 mg/l tetracycline
- 1 mg/l prochloraz-manganese (Sporgon 46 % WP)
- 100 mg/l carbendazim (Kapchem 50 SL)

Agar medium was autoclaved and allowed to cool to ~50 °C and the streptomycin, tetracycline, prochloraz-manganese and carbendazim were added. Agar (20 ml) was subsequently poured into 90 mm petri dishes, under sterile conditions. The agar plates were allowed to set and stored at 4 °C.

2.5.18 Casing and soil

Clean casing was supplied by Cooperative Netherlandse Champignonkwekersvereniging, (CNC) (type nat or nat +). Irish clean casing soil was collected from an Irish mushroom farm from Carbury, Co.Kildare, Ireland. The soil samples were collected from the NUIM ground.

NUIM ground soil was characterised by Dr. Christopher Williams fromNUIM:

The colour of soil was unform brownish black – Munsell colour: Hue = 2.5 Y, Value = 3, Chroma = 1 (2.5Y 3/1) – this indicates a high organic content. The texture of soil was not gritty to slightly gritty, moderately smooth, moderately sticky and plastic, forms moderately cohesive balls, forms threads which will not bend into rings. Therefore, silty clay loams (i.e. 0-20% sand; 28-40% clay and 50-60% silt). The pH was around 7.45 i.e. circumneutral. The soil characterisation was performed using FitzPatrick (1980).

2.5.19 Casing extract

Casing extract was prepared using 5 g casing soil mixed with 15 ml water in 50 ml screw-cap tube. The mixture was centrifuged for 5 min at 3,000 × g. The top liquid phase was collected and called ‘’casing extract’’.

2.5.20 Dust extract

Dust extract was prepared from dust collected from floor mixed with 15 ml water in 50 ml screw-cap tube. The mixture was centrifuged for 5 min at 3,000 × g. The top liquid phase was collected and called ‘’dust extract’’.

2.6 Procedures

2.6.1 Sterilisation

All growth media and PCR equipment were sterilised prior to use by autoclaving in an autoclave at 121 °C and 15 lb/sq.in. for 15 minutes or 105 °C and 15 lb/sq.in. for 30 minutes. Any chemicals unsuitable for autoclaving were filter-sterilised using a filter with a pore size of 0.2 µm (Sarstedt, Nümbrecht, Germany). All cultures and consumables were autoclaved prior to disposal.

2.6.2 Fungal isolates and bacteria

Isolates of pathogenic fungi of *Agaricus bisporus* were collected from Irish mushroom farms during 2007/2008. *L. fungicola, M. perniciosa* and *C. mycophilum* were isolated from infected sporophores of *A. bisporus* or casing.

All dates of collection, geographic origin and source of isolates used in this study are listed in

Table 2-5**.** The isolates were identified using cultural and microscopy characterisation of hyphae and conidia. Other isolates were used in some experiments (**Table** 2-6).

Table 2-5: Summary of species/strains, date of collection, isolation code, geographic origin, and source of *Lecanicillium fungicola* isolates used in this study. (NUIM – National University of Ireland, Maynooth).

No.	Species	Date	Isolation code	Geographic origin	Courtesy	Source	References
1	*L. fungicola* var. *aleophilum*	1990	MUCL 21766	Tienen, Belgium		MUCL	
2	*L. fungicola* var. *aleophilum*	1986	DC 257	Wentworth Mushroom, Canada	V.L.Wilkinson	PennState	
3	*L. fungicola* var. *aleophilum*	1988	DC 262	Markham Mushroom, Canada	V.L.Wilkinson	PennState	
4	*L. fungicola* var. *fungicola*	1997	VCTC	St Paterne, France	Dr. M.Largeteau	INRA-Bx	Largeteau *et al.*, 2006
5	*L. fungicola* var. *fungicola*	1964	MUCL 8126	Rennes, France		MUCL	
6	*L. fungicola* var. *fungicola*	2007	CR.181	Monaghan, Ireland	Dr. H. Grogan	Teagasc	
7	*L. fungicola* var. *fungicola*	2007	L.2	Connaught Mushrooms, Ireland	In this study	NUIM	
8	*L. fungicola* var. *fungicola*	2007	L.3	Connaught Mushrooms, Ireland	In this study	NUIM	
9	*L. fungicola* var. *fungicola*	2007	L.7	Mullingar, Ireland	In this study	NUIM	
10	*L. fungicola* var. *fungicola*	2007	L.10	Mullingar, Ireland	In this study	NUIM	
11	*L. fungicola* var. *fungicola*	2007	L.15	Connaught Mushrooms, Ireland	In this study	NUIM	

No.	Species	Date	Isolation code	Geographic origin	Courtesy	Source	References
12	*L. fungicola* var. *fungicola*	2007	L.16	Connaught Mushrooms, Ireland	In this study	NUIM	
13	*L. fungicola* var. *fungicola*	2007	L.17	Connaught Mushrooms, Ireland	In this study	NUIM	
14	*L. fungicola* var. *fungicola*	2007	L.18	Cathal Reilly, Ireland	In this study	NUIM	
15	*L. fungicola* var. *fungicola*	2007	L.19	Pat Kierron, Kildorough, Ireland	In this study	NUIM	
16	*L. fungicola* var. *fungicola*	2007	L.20	Monaghan, Ireland	In this study	NUIM	
17	*L. fungicola* var. *fungicola*	2007	L.21	Ballard Mushrooms, Ireland	In this study	NUIM	
18	*L. fungicola* var. *fungicola*	2007	L.22	Monaghan, Ireland	In this study	NUIM	
19	*L. fungicola* var. *fungicola*	2008	L.23	Connaught Mushrooms, Ireland	In this study	NUIM	
20	*L. fungicola* var. *fungicola*	2008	L.29	Cavan, Ireland	In this study	NUIM	
21	*L. fungicola* var. *fungicola*	2008	L.40	Sheelin Mushroom, Ireland	In this study	NUIM	
22	*L. fungicola* var. *fungicola*	2009	L.46	Mullingar, Ireland	In this study	NUIM	
23	*L. fungicola* var. *fungicola*	2008	L.244	Waterford, Ireland	Dr. H. Grogan	Teagasc	
24	*L. fungicola* var. *fungicola*	2009	L.49	Athlone, Ireland	In this study	NUIM	
25	*L. fungicola* var. *fungicola*	2009	L.51	Tipperary, Ireland	In this study	NUIM	

No.	Species	Date	Isolation code	Geographic origin	Courtesy	Source	References
26	*L. fungicola* var. *fungicola*	2009	L.52	Tipperary, Ireland	In this study	NUIM	
27	*L. fungicola* var. *fungicola*	2002	VMX1	District de Veracruz, Mexico	Dr.M.Largeteau	INRA-Bx	Largeteau *et al.*, 2004
28	*L. fungicola* var. *fungicola*	2002	VMX2	District de Veracruz, Mexico	Dr. M.Largeteau	INRA-Bx	Largeteau *et al.*, 2004
29	*L. fungicola* var. *fungicola*	2002	VMX3	District de Veracruz, Mexico	Dr. M.Largeteau	INRA-Bx	Largeteau *et al.*, 2004
30	*L. fungicola* var. *fungicola*	1969	CBS 992.69	Horst, Netherlands		CBS	Bernardo *et al.*, 2004; Gea *et al.*, 2005; Zare and Gams, 2008
31	*L. fungicola* var. *fungicola*	1980	CBS 648.80	Horst, Netherlands	Dr. M.Largeteau	INRA-Bx	Largeteau *et al.*, 2006
32	*L. fungicola* var. *aleophilum*	1981	CBS 507.81A	Helden-panningen, Netherlands		CBS	Gams and Van Zaayen, 1982
33	*L. fungicola* var. *aleophilum*	1981	CBS 357.80	Proefstation Horst, Netherlands		CBS	Van Zaayen and Gams, 1982; Collopy *et al.*, 2001; Zare and Gams, 2008; Rasha *et al.*, 2009
34	*L. fungicola* var. *fungicola*	2008	L.15A	Wielkopolska, Poland	Dr. J. Szumigaj-Tarnowska	IWARZ	
35	*L. fungicola* var. *fungicola*	2008	L.20A	Świetokrzyskie, Poland	Dr. J. Szumigaj-Tarnowska	IWARZ	
36	*L. fungicola* var. *fungicola*	2008	L.25A	Podkarpackie, Poland	Dr. J. Szumigaj-Tarnowska	IWARZ	
37	*L. fungicola* var. *fungicola*	2008	L.29A	Mazowieckie, Poland	Dr. J. Szumigaj-Tarnowska	IWARZ	
38	*L. fungicola* var. *fungicola*	2009	L.30A	Mazowieckie, Poland	Dr. J. Szumigaj-Tarnowska	IWARZ	
39	*L. fungicola* var. *fungicola*	2003	ViV3	Viňca, Serbia	Dr. I.Potočnik	ARI Serbia	Potočnik *et al.*, 2008

No.	Species	Date	Isolation code	Geographic origin	Courtesy	Source	References
40	*L. fungicola* var. *fungicola*	2002	P2V3	Požarevac, Serbia	Dr. I.Potočnik	ARI Serbia	Potočnik *et al.*, 2008
41	*L. fungicola* var. *fungicola*	2003	Be2V	Beograd, Serbia	Dr. I.Potočnik	ARI Serbia	
42	*L. fungicola* var. *fungicola*	2006	NSIV1	Novi Slankamen, Serbia	Dr. I.Potočnik	ARI Serbia	
43	*L. fungicola* var. *fungicola*	2004	ReV4	Resnic Serbia	Dr. I.Potočnik	ARI Serbia	
44	*L. fungicola* var. *fungicola*	2003	RaV1	Rakovica, Serbia	Dr. I.Potočnik	ARI Serbia	Potočnik *et al.*, 2008
45	*L. fungicola* var. *fungicola*	2006	P3V3	Požarevac, Serbia	Dr. I.Potočnik	ARI Serbia	
46	*L. fungicola* var. *fungicola*	unknown	V20	Spain	Dr. J. Szumigaj-Tarnowska	IWARZ	
47	*L. fungicola* var. *fungicola*	unknown	VTPT1	Spain	Dr. J. Szumigaj-Tarnowska	IWARZ	
48	*L. fungicola* var. *aleophilum*	unknown	VTaw	Taiwan	Dr. M.Largeteau	INRA-Bx	Largeteau *et al.*, 2004
49	*L. fungicola* var. *fungicola*	1934	MUCL 9781 (CBS 440.34)	United Kingdom		MUCL/ CBS	Collopy *et al.*, 2001; Ware 1933; Yokoyama 2004 and 2006; Zare and Gams, 2008
50	*L. fungicola* var. *aleophilum*	1979	DC 145	California, USA	V.L.Wilkinson	PennState	
51	*L. fungicola* var. *aleophilum*	1982	DC 167	Pennsylvania, USA	V.L.Wilkinson	PennState	
52	*L. fungicola* var. *aleophilum*	1982	DC 170	Pennsylvania, USA	Dr. I.Potočnik	ARI Serbia	Potočnik *et al.*, 2008
53	*L. fungicola*	2003	V9503-3	Netherlands	Wageningen University	WU	

Table 2-6: Summary of species/strains, date of collection, isolation code, geographic origin, and source of isolates used in this study. (NUIM – National University of Ireland, Maynooth).

No.	Species	Date	Isolation code	Geographic origin	Courtesy	Source
1	*Agaricus bisporus*	2007	Ab.1	Ireland	In this study	Shop
2	*Agaricus bisporus*	2007	Ab.2	Ireland	In this study	Shop
3	*Agaricus bisporus*	2007	Ab.3	Ireland	In this study	Shop
4	*Agaricus bisporus*	2009	21.08.09	Ireland	In this study	Shop
5	*Aspergillius fumigatus*	2007	As.	NUIM, Ireland	Dr. M. Schrettl	NUIM
6	*Bacteria/Yeast*	2008	Bac.1	Casing soil	In this study	NUIM
7	*Cladobotryum mycophilum*	2007	D.1	Connaught Mushrooms, Ireland	In this study	NUIM
8	*Lecanicillium flavidum*	1981	CBS 530.81	Chevaufosse near Malmédy, Belgium	In this study	CBS
9	*Mycogone perniciosa*	2007	M.1	Mullingar, Ireland	In this study	NUIM
10	*Mycogone perniciosa*	2008	M.11	Cavan, Ireland	In this study	NUIM
11	*Mycogone perniciosa*	2008	M.31	Cavan, Ireland	In this study	NUIM
12	*Mucor sp.*	2008	Mucor	Casing soil	In this study	NUIM
13	*Penicillium sp.*	2008	Pen.1	Casing soil	In this study	NUIM
14	*Trichoderma* sp. *Th 2*	2009	Th2 (430)	Ireland	Dr. H. Grogan	Teagasc
15	*Trichoderma aggressivum (Th3)*	2009	Th 3	CBS 433.95		CBS

2.6.3 Fungal isolation

A piece of infected fruit-body of *A. bisporus* was cut by sterile scalpel and put on PDA with streptomycin (100 mg/l) as a bacteriostatic agent for isolation of mycoparasites. After 3-5 days of incubation at 20 °C the agar with clean piece of the clean culture of *L. fungicola*, *C. mycophilum* and *M. perniciosa* was subcultured to new PDA for a cleaning step. If the clean culture was not present the next subculture was performed again.

2.6.4 Bacteria and yeast isolation

The casing extract was mixed with water and 100 µl of casing extract was plated on to PDA. After 3-5 days of incubation at 20 °C the colonies of bacteria and yeast were isolated and subcultured to a new PDA for cleaning step. The clean culture plates were sealed with parafilm, and stored inverted in a sealed plastic bag at 4 °C.

2.6.5 Strain storage and growth

Lecanicullium fungicola strains were maintained on PDA or MEA. A 3 or 6 mm diameter PDA agar plug with active mycelium from clean culture was subcultured and agar plug was inverted, and stabbed into the middle of clean PDA plate and incubated at 20 °C for 10-14 days with periodic checking. Once half-full and full plate growth was observed, plates were sealed with parafilm, and stored inverted in a sealed plastic bag at 4 °C. The half-full and full plate growth was used for future experiments.

Lecanicillium fungicola were grown in 10 or 50 ml of PDB for 4-6 days at room temperature (20-23 °C) with rotary shaking at 100 rpm. After incubation the cultures were harvested using miracloth.

2.6.6 Long term storage

Lecanicillium fungicola, *C. mycophilum* and *M. perniciosa* strains were maintained on PDA. Agar plugs with active mycelium from clean culture were subcultured and agar plug was stabbed into the middle of clean PDA plate and incubated at 23 °C for 10-14 days with periodic checking. Once half-full and full, plate growth was observed. The agar plugs (3-6) were cut from clean culture of fungi and put into 2 ml Eppendof tube and Naftlane tubes and filled by 30 % sterile glycerol. The Eppendorf and Naftlane tubes were left for 30 min at room temperature and put in to -70 °C refrigerator and liquid nitrogen (-196 °C) for long term storage. All isolates were prepared with three repeats for long term storage in -70 °C refrigerator. Only one tube was stored in liquid nitrogen.

2.6.7 Lyophilisation

Lecanicillium fungicola mycelium was harvested from 10-14 days old agar plates using a sterile scalpel and put in to 1.5 ml vessels with a screw cap. Next the tube with mycelium was put in to lyophilisation freeze dryer overnight. Next day the tube was closed and left for long term storage and used for DNA extraction.

2.6.8 Conidia harvesting and counting

Conidia were harvested from clean fungal colonies culture from half or full growth agar plate. To remove conidia, plates were washed with PBS with 0.1 % (v/v) Tween 80, harvested (2,000 × g for 5 minutes) in a Beckmann GS-6 bench centrifuge, washed in PBS and re-suspended in PBS. Conidia were counted using haemocytometer and later diluted to a working concentration.

2.6.9 Temperature test

Fifty three isolates of *L. fungicola* and one *L. flavidum* were allowed to grow at two different temperatures, 23 and 30 °C for 7 days, using three repetitions per isolate and temperature of incubation. Inoculum of agar plug (0.6 cm) with active medium were removed from clean culture from the edge of 21 days old cultures and plated at the

centre of Petri dishes filled with PDA. Data were recorded after 7 days of incubation. Culture and microscopic characteristics were also observed. Mycelium growth was recorded on two perpendicular diameters after incubation.

2.6.10 Sensitivity test for prochloraz-manganese

In this preliminary experiment, different concentrations of fungicide Sporgon 50WP containing 46 % prochloraz-manganese as an active ingredient were tested. Sporgon is the universal fungicide for control of diseases (including dry bubble) in mushroom farms. This test used prochloraz-manganese added with several (0; 0,1; 1; 5; 10; 25; 50; 100 mg/l a.i.) different concentrations to molten potato dextrose agar (PDA).

The medium was autoclaved and when medium had cooled to ca. ~50 °C, the prochloraz-manganese was added and Petri dishes were filled with different medium.

All plates were incubated for 21 days at 23 °C in the dark. Colony size was measured across two diameters after 7, 14, 21 days, using three repetitions per isolate and growth was expressed as a percentage of the control and used to calculate EC_{50} and EC_{90}. The effect of fungicides was studied by analysing means and variance of EC_{50} and EC_{90} (fungicide concentrations which inhibit mycelial growth by 50 and 90 %, respectively) were determined for each isolate by fitting a nonlinear saturation curve. It was b(1-exp{-ct}) where t=logdose using a SAS Software (SAS Institute Inc. 2004. SAS/STAT® 9.1, Cary, NC: SAS Institute Inc.).

2.6.11 Sensitivity test for carbendazim

Kapchem 50 SL containing 50 % carbendazim as an active ingredient is another useful fungicide on mushroom farms to protect button mushrooms. This *in vitro* test used carbendazim as the active ingredient and this was added to a solution of molten sterile PDA at following concentration 0; 0,1; 1; 10; 100 mg/l active ingredient.

The medium was autoclaved and when medium had cooled to ~50 °C, the carbendazim was added and Petri dish was filled with different medium.

For every concentration of prochloraz-manganese and carbendazim three replicates were prepared. Plugs with active mycelium were removed from the clean

culture and plated into the centre of Petri dishes filled with PDA medium and suitable concentrations of fungicide.

All plates were incubated for 21 days at 23 °C in the dark. Colony size was measured across two diameters after 7, 14 and 21 days, using three repetitions per isolate and growth was expressed as a percentage of the control and used to calculate EC_{50} and EC_{90}. The effect of fungicides was studied by analysing means and variance of EC_{50} and EC_{90} (fungicide concentrations which inhibit mycelial growth by 50 and 90 %, respectively) were determined for each isolate by fitting a nonlinear saturation curve. It was b(1-exp{-ct}) where t=logdose using a SAS Software (SAS Institute Inc. 2004. SAS/STAT® 9.1, Cary, NC: SAS Institute Inc.).

2.6.12 Antibiotics

In this experiment, four different antibiotics were tested in different concentrations (**Table** 2-7).

The medium used for antibiotic test contained:

- 24 g/l Potato dextrose
- 20 g/l technical agar or agar
- 5 mg/l malachite green sodium salt
- 30 mg/l bromocresol green sodium salt
- 1 mg/l prochloraz-Mn (Sporgon 46 % WP)
- 100 mg/l carbendazim (Kapchem 50 SL)

The medium was autoclaved and when medium had cooled to ca. ~50 °C, antibiotics, prochloraz-manganese and carbendazim were added and Petri dishes were filled with (15 ml) of different medium. Antibiotics were filter-sterilised using a filter with a pore size of 0.2 µm.

Table 2-7: Antibiotics prepared for test with working concentrations.

Antibiotic	Diluent	Stock Solution	Working concentration	Storage
Ampicillin (*Amp*)	water	10,000 mg/l	100 mg/l	-20 °C
Chloramphenicol (*Cm*)	water	10,000 mg/l	100 mg/l	-20 °C
Erythromycin (*Ery*)	water	10,000 mg/l	100 mg/l 500 mg/l 1 000 mg/l	-20 °C
Streptomycin (*Sm*)	water	10,000 mg/l	100 mg/l 500 mg/l 1 000 mg/l	-20 °C and at 4 °C
Tetracycline (*Tet*)	DMSO	10,000 mg/l	100 mg/l 500 mg/l 1 000 mg/l	-20 °C

2.6.13 Sample preparation for selective medium

Into 50 ml falcon 2 ml or 1 ml of conidial suspension of different concentrations of *L. fungicola* (0 to 10^5 conidia/ml) were added to the 2 g or 1 g of casing soil (autoclaved and not autoclaved depending on the experiment), respectively. Next 2 ml water was added and the sample was vortexed at maximum speed. After that the 50 ml falcon was filled with water and mixed vigorously. Next step was filtration (pore size 150 µm pores, *L. fungicola* conidia are around 5 µm in size). In this experiment was used 1 filter and 1 flask. Every sample was prepared separately starting at sample 1 and finished at sample 6. After every sample filter and flask were cleaned. Filtrate was collected in a 50 ml screw-cap Greiner tube. Volume of the filtrate was reduced by centrifugation for 5 min at 3,000 × g.

Final volume was 1 ml per sample. Each sample 100 µl was added to a filled plate with different medium and spread. The plates were incubated at 20 °C in dark. Results were recorded after 4 to 6 days and checked using optical microscope. Plates were kept for a longer time (14 days) for examination. All samples were prepared in three replicates per medium

2.6.14 Data analysis from agar plates

The colony growth for each isolate was determined on control plate. The two perpendicular diameters of each colony were measured minus the diameter of the agar

plug (3 or 6 mm) or conidial suspension drop (3 mm - 10µl). Results from three replicates for each isolate were calculated as an average, to give average mycelia growth rate was present as a averega radial growth with standard error.

2.6.15 PCR samples preparation

2.6.15.1 Samples containing *A. bisporus*

White mushrooms – Agaricus *bisporus* (Ab) fruit body was bought in a super market. The *A. bisporus* fruit body was cut into small pieces and ground in liquid nitrogen using sterile porcelain pestle and mortar (Section 4.1.1.3).

1) After grinding 80 (Ab1Q), 110 (Ab2Q) and 90 (Ab3Q) mg of *A. bisporus* powder was used for DNA extraction using DNeasy Plant Mini Kit isolation kit.
2) After grinding 150 (Ab1M), 190 (Ab2M) and 150 (Ab3M) mg of *A. bisporus* powder was used for DNA extraction using Aljanabi and Martinez (1997) method.

2.6.15.2 Samples containing *L. fungicola*

The *L. fungicola* (L.2) mycelium was harvested from pure cultures grown on agar plate after 14 days of incubation using sterile scalpel. *Lecanicillium fungicola* mycelium was ground in liquid nitrogen using sterile porcelain pestle and mortar. The 100 mg of *L. fungicola* (V1-V3) powder was used for DNA extraction using DNeasy Plant Mini Kit isolation kit (Section 4.1.1.3).

2.6.15.3 Samples containing *A. bisporus* and *L. fungicola*

White mushrooms – *A. bisporus* (Ab) fruit body was bought in a super market. The *A. bisporus* fruit body was cut into small pieces and ground in liquid nitrogen using sterile porcelain pestle and mortar. The *L. fungicola* (L.2) mycelium was harvested from pure cultures grown on agar plate after 14 days of incubation using sterile scalpel. *L. fungicola* mycelium was ground in liquid nitrogen using sterile porcelain pestle and mortar. The *A. bisporus* and *L. fungicola* powder was mixed (Section 4.1.1.3).

1) When DNA extraction was performed by DNeasy Plant Mini Kit isolation kit the amount of *A. bisporus* was 125 mg and *L. fungicola* 25 mg (AbV1Q), for second sample *A. bisporus* was 47 mg and *L. fungicola* 25 mg (AbV2Q) and *A. bisporus* was 105 mg and *L. fungicola* 35 mg (AbV3Q).
2) When DNA extraction was performed by using Aljanabi and Martinez (1997) method the amount of *A. bisporus* was 100 mg and *L. fungicola* 105 mg

(AbV1M), for second sample *A. bisporus* was 84 mg and *L. fungicola* 106 mg (AbV2M) and *A. bisporus* was 100 mg and *L. fungicola* 140 mg (AbV3M).

2.6.15.4 DNA extraction from other fungi using ZR Fungal/Bacterial DNA kit.

DNA was extracted from agar plate when colonies of fungi had a full growth plate after 3-10 days depending on fungal growth. The mycelium was harvested (around 100 mg) using a sterile scalpel and put into ZR Bead Bashing tube for DNA extraction by ZR Fungal/Bacterial DNA kit. The fungi used in this extraction were *C. mycophilum* (C.1), *M. perniciosa* (M.1 and M.31), *A. fumigatus* (As.) and *L. fungicola* (L.7, CR181 and L.15).

2.6.15.5 Samples collected from mushroom farm and DNA extracted using the method of Yeates *et al.* (1998)

Samples were collected from mushroom farm with problem of dry bubble. The farm was visited at 15.08.2008 in Carbury Mushrooms, Co. Kildare, Ireland. All dates of collection and origin collected and tested are listed in **Table** 2-8**.**

Table 2-8: Summary of collection and origin of collection.

No	No sample	Sample	Room	Flush
1	A (1-3)	Casing	3	3
2	B (4-6)	Casing (repeat)	3	3
3	C (7-9)	Casing	18	3
4	D (10-12)	Casing (repeat)	18	3
5	E (13-15)	Dust from floor	18	3
6	F (16-18)	Dust from floor (repeat)	18	3
7	G (19-21)	Dust from floor	3	3
8	H (22-23)	Dust from floor (repeat)	3	3
9	I (24-26)	Dust from floor	3	3

2.6.15.6 DNA extraction form *Trichoderma* using DNeasy Plant Mini Kit isolation kit

Trichoderma (CBS 433.95) conidia were harvested from 3-5 days old plates (MEA) using PBST (5 ml) and an aliquot of the resulting conidial suspension (100 µl) was used to inoculate 200 ml cultures of Malt extract broth. The cultures were incubated at 20 ℃ for 3 days with constant agitation. The cultures were then filtered through autoclaved miracloth and the mycelia collected. The mycelial mass was frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar. DNA extractions were carried out using the DNeasy Plant Mini Kit isolation kit following the manufacturer's instructions.

2.6.16 Real Time sample preparation

2.6.16.1 Sample preparation of *L. fungicola* conidial suspension mixed with casing for DNA extraction

Into 50 ml falcon 2 ml of conidial suspension of different concentrations of *L. fungicola* (10^1 to 10^7 conidia/ml and 0 to 10^5 conidia/ml) were added to the 2 g of casing soil. Next 2 ml water was added and sample was vortexed at maximum speed. After that 50 ml falcon was filled into water and mixed vigorously. Next step was filtration using filter with pore size 150 µm. The filtration step removed biggest material parts of casing and allowed *L. fungicola* conidia to pass through. Every sample was prepared separately starting from the smallest to the highest conidial suspension concentrations. The same filter and flask, cleaned after every sample was used. The filtrate was collected in a 50 ml screw-cap Greiner tube. Volume of the filtrate was reduced by centrifugation for 5 min at 3,000 × g. The casing pellet was moved to the appropriate tube for isolation of DNA using a Wizard Magnetic DNA Purification System for Food.

2.6.16.2 Sample preparation of proper amounts of *L. fungicola* conidial suspension mixed with casing extract and water.

Into 2 ml Eppendorf tube 0.1 ml of different concentrations of conidial suspension of *L. fungicola* (0 to 10^6 conidia/ml) was added to 0.9 ml of casing extract or sterile water. Samples were mixed using a vortex. DNA was extracted using a commercial kit (Wizard Magnetic DNA Purification System for Food Promega) according to the instructions on the kit.

2.6.17 Molecular biology methods

2.6.17.1 Extraction of genomic DNA from fungi for PCR

Fungal mycelium or conidia were harvested from half and full growth agar plates. For mycelium harvesting a disposable scalpel was used and mycelium was scraped from agar plate containing a clean colony. Conidia were harvested using PBST (5 ml) and conidia number was counted by haemocytometer and diluted to known concentrations. The *A. bisporus* sporofores were cut into small pieces and used for DNA extraction. After extraction DNA was stored at -20 °C. DNA extractions from fungi were performed using four extraction methods.

1) **Aljanabi and Martinez (1997) method.** DNA extraction was performed using:
    a. About 50-100 mg of either fresh mycelium or frieze-dried mycelium was used for DNA extraction.
    b. DNA extraction from soil samples (0.26-0.28g soil) and 200 µl conidial suspension (of fungi of different concentration) was mixed in 2 ml Eppendorf with glass bead with 400 µl of sterile salt homogenizing buffer (0.4 M NaCl 10 mM Tris-HCl pH 8.0 and 2 mM EDTA pH 8.0) (Section 4.1.3.1). The extraction was the same as from clean mycelium.

    The mycelium was collected into 1.5 ml microcentrifuge tube and homogenized in 400 µl of sterile salt homogenizing buffer (0.4 M NaCl 10 mM Tris-HCl pH 8.0 and 2 mM EDTA pH 8.0), using a pestle for 1.5 ml microcentrifuge tubes for 10-15 s. Then 40 µl of 20 % SDS (2 % final concentration) and 8 µl of 20 mg/ml proteinase K (400 mg/ml final concentration) were added and mixed well. The samples were incubated at 55-65 °C for at least 1 h or overnight, after which 300 µl of 6 M NaCl (NaCl saturated H_2O) was added to each sample. Samples were vortexed for 30 sec at maximum speed, and tubes spun down for 30 min at 10,000 × g. The supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample, mixed well, and samples were incubated at -20 °C for 1 h. Samples were then centrifuged for 20 min, 4 °C, at 10,000 × g. The pellet was washed with 70 % ethanol, dried and finally resuspended in 100 µl sterile dH_2O. Genomic DNA was used for PCR amplification of genomic DNA.

2) **Modified Yeates *et al.* (1998) method.** DNA extraction was performed using enzymatic lysis.

a. Soil (0.26 - 0.28 g) was mixed with 200 µl conidial suspension of fungi of different concentrations in 50 ml falcons (Section 4.1.3.1)
b. Soil or casing of 1 g or casing water or dust water of 1 ml was mixed with 1 ml of different concentrations of conidial suspension of *L. fungicola* (10^1-8.95 × 10^7) and (10^6-10^7) in 50 ml falcons (Section 4.1.3.3).
c. The samples collected from mushroom farms contained 10 gram and 5 gram samples in 50 ml falcons (Section 4.1.3.4).

Extraction buffer (1 ml) (100 mM Tris-HCl [pH 8.0], 100mM sodium EDTA [pH 8.0], 1.5 M NaCl) and 20 µl of proteinase K (30 mg/ml) was added in to 50 ml. The sample was incubated in water bath at 37 °C for 30 minutes and mixed 2-3 times during incubation by inverting tube. Next 1 g of glass beads was added and the sample was vortexed vigorously for 2 minutes. Then 100 µl of 20 % SDS was added and the sample was incubated at 65 °C for 90 min. The supernatant was collected after centrifugation at 3,000 × g for 10 min at room temperature. The supernatant was collected, and the soil pellet was re-extracted with further extraction buffer (1 ml), incubation at 65 °C for 10 minutes and centrifugation as above. The supernatants were transferred into 2 ml Eppendorf tube and half-volume of polyethylene glycol (30 %)/sodium chloride (1.6 M) was added. The samples were incubated at room temperature for 2 hr. Samples were centrifuged (10,000 × g for 20 min) and the partially purified nucleic acid pellet was resuspended in 20 µl of TE. Potassium acetate (7.5 M) was added to a final concentration of 0.5 M. Samples were transferred to ice for 5 min then centrifuged (16,000 × g, 30 min) at 4 °C to precipitate proteins and polysaccharides. The aqueous phase (300 µl) was extracted with one volume of phenol/chloroform/isoamyl alcohol 25:12:1 vortex and centrifuged at 14,000 × g for 5 min at 4 °C. After that the upper aqueous phase was collected into new 1.5 ml microcentrifuge tube and one volume of (300 µl) chloroform was added vortex and centrifuged at 14,000 × g for 5 min at 4 °C. Next the aqueous phase was collected into new 1.5 ml microcentrifuge tube and one volume (300 µl) of isopropanol was added. Next the sample was centrifuged at 14,000 × g for 10 min at 4 °C. After centrifugation the ethanol was removed using a pipette. After that sample was left at room temperature for 30 minutes to dry. DNA was pelleted by centrifugation (16,000 × g for 30 min) and resuspended in TE (100 µl).

3) **Nucleon Phytopure Genomic DNA Extraction Kit (GE Healthcare Life Sciences).** All buffers and reagents were supplied with the kit. The mycelia mass was frozen in liquid nitrogen and ground to a fine powder using a porcelain pestle and mortar. In this step the material was mechanically ground. The grinding powder (100 mg) was transferred to 1.5 ml microcentrifuge tube and 600 µl Reagent 1 was added. 4µl RNase (100 µg/ml) was added and the samples were incubated at 37 °C for 30 min. The solution was mixed and after incubation 200 µl of Reagent 2 was added. After that the tubes were inverted several times until a homogenous mixture was obtained. Next the samples were incubated for 10 min at 65 °C mixing 2-3 times during incubation by inverting tube. After incubation samples were placed on ice for 20 min. After incubation on ice samples were removed from ice and 500 µl of cold chloroform (-20 °C) and 100 µl of Nucleon PhytoPure DNA extraction were added. The samples were vortexed vigorously for 10 min at room temperature. After that the samples were centrifuged at 13,000 × g for 10 min. After centrifugation the upper phase containing DNA, was transferred, into a fresh tube. The DNA was precipitated using an equal volume of cold isopropanol (-20 °C). The tube was gently inverted until DNA precipitated. The samples were centrifuged at minimum of 4,000 × g for 5 min to pellet the DNA. DNA pellet was washed with 70 % ethanol and centrifuged again at 4,000 × g for 5 min to pellet the DNA. The supernatant was discarded and the DNA pellet was left to dry for 10 min at room temperature. When DNA pellet was dry 100 µl of TE was added and DNA pellet was suspended.

4) **ZR Fungal/Bacterial DNA kit supplied by Zymo Research (California, U.S.A.).**
    a. Soil (0.26 - 0.28 g) was mixed with 200 µl conidial suspension of fungi of different concentrations in 50 ml falcons (Section 4.1.3.1).
    b. Casing soil (100g) was mixed with 100 µl of difrent concnetratios of *L. fungicola* (10^4-10^7 conidia/g casing) (Section 4.1.3.3).

    All buffers and reagents were supplied with the kit. The samples of mycelia, conidial suspension, conidial suspension mixed with soil and conidial suspension mixed with casing were added to 750 µl DNA buffer in the ZR Bead Bashing tube. The tubes were vortexed vigorously for 5 minutes. The bead bashing tubes were centrifuged at 10,000 × g for 1 minute. Supernatant (400 µl) was transferred to Zymo-Spin IV Spin filters in collection tubes and centrifuged

at 7,000 × g for 1 minute. Fungal/Bacterial DNA binding Buffer (1200 µl) was added to the filtrates in the collection tubes. Filtrate (800 µl) was transferred to Zymo-Spin IIC Columns in collection tubes and centrifuged at 10,000 × g for 1 minute. The filtrate was discarded. The remaining filtrate (800 µl) was added to the Zymo-Spin IIC columns and centrifuged at 10,000 × g for 1 minute. DNA Pre-Wash Buffer (200 µl) was added to the Zymo-Spin Columns in new collection tubes and centrifuged at 10,000 × g for 1 minute. Fungal/Bacterial DNA wash buffer (500 µl) was added to the Zymo-Spin IIC columns and centrifuged at 10,000 × g for 1 minute. The Zymo-Spin IIC columns were transferred to clean 1.5 ml microcentrifuge tubes and DNA Elution Buffer (100 µl) was added to the columns and centrifuged at 10,000 × g for 1 minute to elute the DNA samples.

5) **DNeasy Plant Mini Kit supplied by Qiagen**. All buffers and reagents were supplied with the kit. The mycelia mass and *A. bisporus* tissues were frozen in liquid nitrogen and ground to a fine powder using a porcelain pestle and mortar. (In one experiment with casing and *L. fungicola* conidial suspension the protocol was modified and material was mechanically broken using glass bead (2g) and 400 µl buffer AP1 and 4 µl of RNase A stock solution (100 mg/ml)). The powder (100 mg) was transferred to 1.5 ml microcentrifuge tube and 400 µl buffer AP1 and 4 µl of RNase A stock solution (100 mg/ml). Next the samples were vortexed vigorously for 10 sec and incubated for 10 min at 65 °C mixing 2-3 times during incubation by inverting tube. In this step the cells were lysed. After incubation the 130 µl buffer AP2 was added to the lysate. The tubes were mixed and incubated for 5 min on ice. This step precipitates detergent, proteins and polysaccharides. The lysate was applied to the QIAshredder spin column sitting in a 2 ml collection tube and centrifuged for 2 min at maximum speed. After centrifugation the lysate from collection tube was collected and transferred to a new tube usually 450 µl. After that 1.5 ml volume of buffer AP3/E (675 µl) was added and mixed by pipetting. Next 650 µl of this mixture was applied to the DNeasy mini spin column sitting in a 2 ml collection tube and centrifuged for 1 min at > 6,000 × g. After centrifugation the collection tube was emptied and the rest of mixture was centrifuged. After that the DNeasy column placed in new 2 ml collection tube and 500 µl buffer AW was added to the DNeasy column and centrifuged for 1 min at > 6,000 × g. After centrifugation the collection tube was emptied. Buffer AW 500 µl was added to the DNeasy

column and centrifuged for 2 min at maximum speed to dry the membrane. After centrifugation the DNeasy column was placed to a 1.5 ml microcentrifuge tube and 100 µl of preheated (65 °C) buffer AE was added directly onto the DNeasy membrane and incubated for 5 min at room temperature and then centrifuged for 1 min at > 6,000 × g to elute.

2.6.17.2 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) was used to amplify DNA from clean cultures of fungi, detection of *L. fungicola* from soil, casing, casing extract and dust extract.

PCR conditions for set of primers designed by Largeteau *et al.* (2007), 130 bp amplicon (Section 4.1.1.2 and 4.1.1.3).

Taq Polymerase DNA – Sigma

10x reaction buffer	2 µl
dNTP mix (10 µM)	2 µl
Primer Forward (20 mm/µl)	0.4 µl
Primer Reverse (100 mm/µl)	0.4 µl
Polymerase	0.2 µl
DNA template	1 µl
Sterile water	to a total of 20 µl

The following reaction cycle were used unless otherwise stated:

95 °C (denaturing)	5 min	
95 °C (denaturing)	1 min	x 35 cycles
54 °C (annealing)	1 min	
72 °C (extending)	1 min	
72 °C (extending)	7 min	

PCR conditions for set of primers designed by Largeteau *et al.* (2007), 130 bp amplicon and Zijlstra *et al.* (2007, 2008 and 2009), 102 bp (Section 4.1.2.1) and check sensitivity and specificity of different polymerases (Section 4.1.2.2);

Taq Polymerase DNA – Sigma and High Fidelity DNA polymerase – BioLabs

10x reaction buffer	2 µl
dNTP mix (10 µM)	2 µl
Primer Forward (20 mm/µl)	0.4 µl
Primer Reverse (100 mm/µl)	0.4 µl
Polymerase	0.2 µl
DNA template	5 µl
Sterile water	to a total of 20 µl

The following reaction cycle conditions were used unless otherwise stated:

95 °C (denaturing)	5 min	
95 °C (denaturing)	1 min	x 35 cycles
47 °C (annealing)	1 min	
72 °C (extending)	1 min	
72 °C (extending)	7 min	

The PCR reaction after optimization of PCR assay set of primers designed by Zijlstra *et al.* (2007, 2008 and 2009), 102 bp, polymerase comparison (Section 4.1.2.3);

GoTaq polymerase – Promega and Taq Polymerase DNA – Sigma

5x Reaction Buffer	5 µl
dNTP mix (2.5 mM)	2 µl
Primer Forward (20 mM)	0.65 µl
Primer Reverse (20 mM)	0.65 µl
50 % glycerol	7.5 µl
Polymerase	0.4 µl
DNA template	0.5-3.5 µl (depend of experiment)
Sterile water	to a total of 25 µl

The following reaction cycle conditions were used unless otherwise stated:

95 °C (denaturing)	2 min	
95 °C (denaturing)	30 sec	x 35 cycles
50 °C (annealing)	30 sec	
72 °C (extending)	1 min	
72 °C (extending)	5 min	

The PCR reaction conditions to find selective primers were as follows (Chapter 4);

Taq DNA polymerase in storage buffer A – Promega

10x Reaction Buffer	2.5 µl
MgCl (25mM)	2 µl
dNTP mix (20 mM)	0.5 µl
Primer Forward (20 mM)	0.65 µl
Primer Reverse (20 mM)	0.65 µl
50 % glycerol	8.5 µl
Polymerase	0.4 µl
DNA template	3 µl
Sterile water	to a total of 25 µl

The following reaction cycle conditions were used unless otherwise stated:

95 °C (denaturing)	5 min	
95 °C (denaturing)	15 sec	x 35 cycles
58 °C (annealing)	30 sec	
72 °C (extending)	30 min	
72 °C (extending)	1 min	

2.6.17.3 Visualisation of genomic DNA and PCR and Real Time PCR product by agarose gel electrophoresis

The genomic DNA was visualised by 1 % (w/v) agarose gel electrophoresis. The PCR and Real Time PCR product were visualised by 2 % (w/v) agarose gel electrophoresis. Agarose gel contained 4 µl per 100 ml of ethidium bromide (100 mg/ml).

Genomic DNA samples were prepared for loading 2 µl DNA with 2 µl of 6x loading dye and 6 µl MQ water. Three different molecular weight markers were used throughout this study: LZ Load Precision Molecular Mass Standard, 100 bp ladder and 50 bp ladder BioLabs. Gels were electrophoresed at 50-100 volts for 30-90 min.

The PCR samples were prepared for loading by adding 2 µl PCR product with 2 µl of 6x loading dye and 6 µl MQ water. Two different molecular weight markers were used throughout this study: 100 bp ladder and 50 bp ladder BioLabs. Gels were electrophoresed at 50-100 volts for 30-90 min.

The PCR samples (Chapter 4) were prepared for loading by adding 8 µl PCR product with 2 µl of 6x loading dye. Two different molecular weight markers were used throughout this study: 100 bp ladder and 50 bp ladder BioLabs. Gels were electrophoresed at 50-100 volts for 30-90 min.The gel was prepared in to 1x TAE buffer.

The Real Time PCR samples were prepared for loading by adding 10 µl Real Time PCR product with 2 µl of 6x loading dye. Three different molecular weight markers were used throughout this study: 100 bp ladder, 50 bp ladder and 20 bp ladder. Gels were electrophoresed at 50-100 volts for 30-90 min.

PCR products were prepared for loading by adding 5 volumes of DNA sample to 1 volume of 6x loading dye. DNA fragment size was estimated by running molecular weight markers alongside the unknown samples. Three different molecular weight markers were used throughout this study: 100 bp ladder, 50 bp ladder and 20 bp ladder. Gels were electrophoresed at 50-100 volts for 30-90 min.

2.6.18 Extraction of genomic DNA from casing, casing extract or water for Real Time PCR

The DNA extraction was performed using three different DNA extraction kits. All extractions were performed using protocols attached with kit with small changes. After extraction DNA after extraction was storage at -20 °C.

1) **Wizard® Magnetic DNA Purification System for Food supplied by Promega.**
    a. DNA was extracted from casing extract with different conidial suspension concentrations of *L. fungicola* (0-10^6 conidia/ml) and samples from mushroom farm. Samples were isolated following a producer instructions. All buffers and reagents were supplied with the kit. One ml casing extract mixed with different conidial suspension concentrations of *L. fungicola* was isolated. Samples were prepared into 2 ml Eppendorf tube.
    b. Mushroom farm samples were extracted but not more than 1-1.2 ml. If volume of mushroom farm sample was bigger than 1 ml the samples was centrifuged for 5 min at 10,000 × g and left for 10 min on bench and the excess liquid was removed. Samples were prepared into 2 ml Eppendorf tube.

The 1 ml of sample was vortexed vigorously with 400 µl of lysis buffer A and 4 µl of RNase A. Then sample was incubated for 10 minutes at room temperature with 250 µl of Buffer B. After incubation 750 µl of Precipitation Solution was added. The mixture obtained was centrifuged for 10 min at 13,000 × g. The supernatant was added to 40 µl of resuspended MagneSil™ PMPs and 0.7-1 ml of isopropanol was added. The tube was mixed and incubated at room temperature for 5 min by shaking. Then the tube was placed onto the MagneSphere® Magnetic Separation Stand (Promega) and left in place for 1 min. The liquid phase was discarded leaving the tubes in the stand. The tube was removed from the stand and 250 µl of lysis Buffer B was added to the particles. The tube was mixed and placed on MagneSphere® Magnetic Separation Stand (Promega). After 1 minute incubation at room temperature, the liquid phase was discarded. Then, 1 ml of 70 % ethanol wash solution was added and, after 1 minute in the magnetic stand, the liquid phase was discarded. This step was

repeated three times and in the end the particles were dried at room temperature for 15-30 minute. Nuclease-free water 100 µl was added to particles and the mixture obtained was mixed and incubated at 65 °C for 5 min. The tube was placed onto the MagneSphere® Magnetic Separation Stand (Promega) for 1 min and the DNA was collected by leaving the tube in the stand and carefully transferring the liquid into a clean tube. The final volume was adjusted to 100 µl by adding nuclease-free water. The total volume of DNA samples was 100 µl.

2) **QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA isolation kits supplied by Fujifilm** was used for DNA extraction from casing extract with different conidial suspension concentrations of *L. fungicola* (0-10^5 conidia/ml) and samples from mushroom farm. Samples were isolated following producer instructions for DNA isolation out of liquid samples with low DNA concentrations using the QuickGene tissue kit. All buffers and reagents were supplied with the kit. One ml casing extract mixed with different conidia concentrations of *L. fungicola* was isolated. Mushroom farm sample was extracted but not more than 1 ml. If volume of mushroom farm sample was bigger than 1 ml the samples was centrifuged for 5 min at 10,000 × g left for 10 min on bench and the excess of liquid was from pipetting. Samples were prepared into 2 ml Eppendorf tube. The EDT 30 µl and MDT 180 µl was added to sample. After that tubes were vortexed rigorously for 10 sec. After vortexing the LDT 250 µl and Rnase A 4 µl (100 mg/ml) was added and vortex in maximum speed 10 sec and leave for 2 min. After that 3 µl of Poly A (Carrier RNA) [5 µg/µl] was added and the samples were vortex at maximum speed. Next samples were incubated at 70 °C for 10 min and after that at 95 °C for 2 min. After incubation samples was centrifuged at 6,000 × g for 2 min. Next was added 350 µl of > 99 % ethanol and vortex on maximum speed for 10 sec. After that samples were centrifuged at 6,000 × g for 2 min. The lysate was transferred into the cartridge of QuickGene system and the QuickGene 80 device was used. The air pressure from QuickGene 80 device was used for flow the lysate into collection tube. The DNA was settled on to filter which is on cartridge of QuickGene system. After that the DNA was cleaned 3 times with 750 µl of WDT using pressure. After cleaning step the cartridge of QuickGene was moved to the elution position and 100 µl of CDT was added. The cartridge was left for 90 sec and pressurization was used. Genomic DNA was collected in to 1.5 ml microcentrifuge tube. The total volume of DNA samples was 100 µl.

2.6.18.1 Standard curve for Real Time PCR

The clean genomic DNA of *L. fungicola* (L.15) was isolated using a commercially available ZR Fungal/Bacterial DNA kit supplied by Zymo Research according by protocol.

2.6.18.2 Quantitative Real Time PCR

Primes sets and probes were designed by Zijlstra *et al.* (2007, 2008 and 2009). The DNA hydrolysis probe TaqMan conjugated with 6-FAM dye (6-carboxyfluorescein is a single isomer derivative of fluorescein. Absorbance max 495 nm, Emission max 520 nm. 6-FAM™ is the most commonly used fluorescent dye for attachment to oligonucleotides and is compatible with most fluorescence detection equipment). The Real Time PCR reactions were performed with standard final volume. The quantitative Real Time PCR was carried out on the DNA samples using the Real Time PCR machines. A standard curve was performed using a serial dilution of known amounts of DNA. The dilutions were 10-fold dilutions. Once the optimum conditions were confirmed by the standard curve, the positive control for each reaction was created by using a serial dilution of a DNA sample. The negative control for each reaction was created by using Nuclear free water.

The PCR program used for quantitative Real Time PCR machine (LightCycler 480) following by Zijlstra *et al.* (2007, 2008 and 2009) protocols. The amount of cycles was modified and reaction used 40-50 cycles.

2.6.19 Primer used in this study for PCR and Real Time PCR

Oligonucleotide primers were designed based on available sequences of *L. fungicola* using Integrated DNA technologies Inc. web side for designed and ordering primers and probe (http://eu.idtdna.com/Home/Home.aspx).

The primers used in this study are listed in **Table** 2-9.

Table 2-9: Primers and probe use for PCR and Real Time PCR during this thesis.

Gene	Primer and Probe	Sequence 5'-3'	Product Size (bp)	Reference	Supplier	Attention
rDNA (ITS1)	VFF6F	GTGAACATACCAATCGTTG	130 bp	Largeteau *et al.* (2007)	Genosys Biotechnologies (Europe) Ltd.	Amplified *A. bisporus* also
	VFF8R	CGGATTCAGAAGATACT GGT				
rRNA	f.vff.vfa(r)	Confidential	102 bp	Zijlstra *et al.* (2007, 2008 and 2009).	Genosys Biotechnologies (Europe) Ltd. – for PCR, Applied Biosystems for Real Time PCR	PCR products in casing soil background
	r.vff.vfa(r)					
	Probe					
mRNA (MAT 1-2-1)	Ay 124053 F(116)	AGAACAAGCATGGAGGCAAGTGGT		This study	Integrated DNA technologies Inc.	EMBL Accession number: ACC AB124635; Length: 209
	Ay 124053 F(151)	AAGGACAAGCGCAATGTCGACGTCAA				
	Ay 124053 R (205)	CAGCCATGACAACCTGAAGCCAAA				
mRNA (MAT 1-2-1)	F9	ACAGCATGGTGAAGAAAGCAGACC		This study	Integrated DNA technologies Inc.	EMBL Accession number: ACC AB124635; Length: 209
	F87	ACCTCGCTGACCATTTCAGCGCAAAT				
	R 201	TCTCAAGGAGGGCTGTCTTGATGT				
mRNA (MAT 1-2-1)	F 79	CACATGTGACCTCGCTGACCATTT		This study	Integrated DNA technologies Inc.	EMBL Accession number: ACC AB124635; Length: 209
	F 142	AGGAGGTGCGACAACGCTACAAGAAA				
	R 167	TTTCTTGTAGCGTTGTCGCACCTC				
rDNA	Af 324874 F (57)	AACATACCAATCGTTGCTTCGGCG		This study	Integrated DNA technologies Inc.	EMBL Accession number: ACC AB124635; Length: 209
	Af 324874 F (138)	CTTGCGGCGGATTCAGAAGATACT				
	Af 324874 R (191)	GCCGGAGGCCATCAAACTCTTTGTA				
rDNA	F 1958	TCGATGAAGAACGCAGCGAAATGC		This study	Integrated DNA technologies Inc.	EMBL Accession number: AB107135; Length: 2294
	F 2017	TCGAATCTTTGAACGCACATTGCGCC				
	R 2100	AAGGGAGCTCGAGGGTTGAAATGA				
rDNA	F 1540	TCAGCTTGCGTTGATTACGTCCCT		This study	Integrated DNA technologies Inc.	EMBL Accession nuber: AB107135; Length 2294
	F 1659	CGGAAAGCTCTCCAAACTCGGTCATT				
	R 1723	TCACCAACGGAGACCTTGTTACGA				
rDNA	F 1934	ACAACGGATCTCTTGGTTCTGGCA	109 bp	This study	Integrated DNA technologies Inc.	EMBL Accession number: AB107135; Length: 2294
	R 2042	GGCGCAATGTGCGTTCAAAGATTC				

2.7 Mushroom farms visits

From 2008 to 2010 samples were collected during 18 visits to 9 Irish mushroom farms with different levels of dry bubble disease (**Table** 2-10). In total 438 samples were collected from different locations and stages of the crop cycle from spawn running to 3rd flush. Samples were examined using microbiological (selective medium) and molecular (Real Time PCR) methods (Chapter 7).

Table 2-10: Summary of mushroom farms visits number visits, orgin, county, number of samples collected and tested on selective media and on Real Time PCR.

No.	Number of visit	Date	Origin and mushroom farm code	County	Number of samples collected and tested on Selective media	Number of samples tested on Real Time PCR
1	1	22.10.2008	EQ, Hillcrest Cornanagh, Ballybay	Monagham	24	24
2	2	28.10.2008	EQ, Hillcrest Cornanagh, Ballybay	Monagham	18	15
3	3	04.12.2008	EQ, Hillcrest Cornanagh, Ballybay	Monagham	36	31
4	4	18.12.2008	EQ, Hillcrest Cornanagh, Ballybay	Monagham	29	18
5	1	04.03.2009	MMcG, Corglas, Carrigallen	Leitrim	24	24
6	1	04.03.2009	MC, Carrickacroy, Kilnaleck	Cavan	11	11
7	1	06.04.2009	EK– Sheeling Mushrooms, Kilnakeck	Cavan	38	36
8	1	06.04.2009	DG, Ballinarry, Kilnaleck	Cavan	7	7
9	1	16.06.2009	JK, Ballard, Slanemore, Mullingar	Westmeath	20	18
10	1	16.06.2009	GR, Walderstown, Athlone	Westmeath	23	21
11	1	14.07.2009	JH, Outroth, Cahir	Tipperary	31	11
12	1	14.07.2009	JQ, Clonmore south Cahir	Tipperary	33	19
13	5	09.02.2010	EQ, Hillcrest Cornanagh, Ballybay	Monagham	26	24
14	2	09.02.2010	EK– Sheeling Mushrooms, Kilnakeck	Cavan	26	24
15	2	15.02.2010	JH, Outroth, Cahir	Tipperary	17	17
16	2	15.02.2010	JQ, Clonmore south Cahir	Tipperary	33	33
17	2	22.02.2010	JK, Ballard, Slanemore, Mullingar	Westmeath	24	24
18	2	22.02.2010	GR, Walderstown, Athlone	Westmeath	18	18
SUMARY					**438**	**375**

2.7.1 Samples categorised by crop stage and other locations on mushroom farm.

Samples were collected from different stages of crop and from other locations on the mushroom farm:

1. **Ready to use** (casing equipment, flies, growing room floor inside, old fashion mushroom farm, picker's equipment, shelves, structure inside growing room);
2. **Spawn running** (flies, growing room floor inside, machine, old fashion mushroom farm, shelves, structure inside growing room);
3. **Casing/at airing** (casing equipment, door handle, flies, growing room floor inside, machine, old fashion mushroom farm, outside samples, picker's equipment, shelves, structure inside growing room, water equipment);
4. **1st flush** (Crates, door handle, flies, growing room floor inside, machine, outside samples, picker's equipment, picker's accessories, shelves, structure inside growing room, water equipment);
5. **2nd flush** (crates, door handle, flies, growing room floor inside, outside samples, picker's accessories, shelves, structure inside growing room);
6. **3rd flush** (crates, door handle, flies, growing room floor inside, old fashion mushroom farm, outside samples, picker's equipment, picker's accessories, shelves, structure inside growing room);
7. **Canteen** (crates, growing room floor inside, outside samples, picker's accessories);
8. **Outside samples** (Crates, machines, outside samples, picker's accessories, water equipment);
9. **Worker's sleeves^** (only selective media).

2.7.2 Sample categorised by location

Samples were collected from different locations of mushroom farms.

1. **Canteen samples** – floor, knife blades, kettle, canteen scales, door knob, green crates for old gloves, canteen toilet door;
2. **Casing equipment** – casing bag, cac buckets, nets, casing;
3. **Crates**
4. **Door handle**
5. **Flies**
6. **Growing room floor inside** – floor inside front and back, crack in concrete, casing debris
7. **Machines** – ruffling, filling, empting, tractor
8. **Old fashion mushroom farm** – table legs, steel racks and plastic cover, rack
9. **Outside samples** – floor close to the growing room, floor around canteen, water
10. **Picker's equipment** – picker's trolleys, platforms and step
11. **Picker's accessories** – hair net and gloves, scales and number rolls
12. **Shelves** – middle and bottom
13. **Structure inside growing rooms** – air duct, lights, radiator, steel frame, ventilation unit, control panel
14. **Water equipment** – hose, tree, water tank
15. **Workers gloves/fingers, cloths and sleeves^** (only selective media)

2.7.3 Materials used for sample collection

The equipment used for sample collection from mushroom farms was: 3 x 100 ml bottles with sterile water, sterile swabs, and 50 ml self-standing centrifuge tubes, permanent marker and gloves. Sterile swabs were prepared by placing in a 50 ml falcon, filled with small amount of water and autoclaved for 30 min in 105 °C into autoclave bag.

2.7.4 Sample preparation

Samples from mushroom farm were collected by passing a sterile wet swab over the selected surface. After that the used swab was put into a self-standing 50 ml centrifuge tube. Swabbing of each location was repeated. The samples were stored at 4 °C overnight and sample preparation started on the next day after mushroom farm visit.

Sample tubes were filled with sterile water up to 50 ml and mixed vigorously for 30 min at 120 rpm. After that the samples were filtered with gravity using a square piece of UV sterilised Miracloth and sterile plastic funnel. Miracloth traps big parts of debris and allow *L. fungicola* conidia (3-6 µm) to pass through. Every sample was prepared separately. Samples were prepared from the youngest growing room to the oldest one. Sample filtrates were concentrated by centrifugation (GS-6 Centrifuge, Beckman) for 10 min at 3,000 × g. After centrifugation samples were gently moved to rack and left overnight to sediment. Next day upper layer was removed using disposable transfer pipettes and debris pellet and a small amount of water was left in the bottom of the 50 ml falcon. Final volume of the sample was around 3 ml. That sample was used to detect *L. fungicola* using microbiological tests and Real Time PCR. For microbiological test 600 µl of samples was used and the rest of the sample was transferred into 2 ml Eppendorf and DNA was extracted by Wizard® Magnetic DNA Purification System for Food supplied by Promega and QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA isolation kits supplied by Fujifilm.

2.7.5 Selective media

For this experiment 100 µl was spread onto a modified Rinker's medium (MRSM) and Novel PDA selective medium (NPDASM). All samples were repeated 3 times per selective medium. All samples were inoculated 6-7 days at 20 °C and after this time the results were recorded using optical microscopy.

2.7.6 DNA extraction

DNA extraction was performed using Wizard Magnetic DNA Purification System for Food (Promega) and QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA (Fujifilm) isolation kit followed by extraction protocol.

2.7.7 Real Time PCR

A result of Real Time PCR was recorded as positive when 6-FAM signal was present after 45 cycles. If 6-FAM signal was present after 45 cycles and/or was negative the sample was recorded as negative. Primers and Probe used in this test were designed by Zijlstra *et al.,* (2007, 2008 and 2009).

2.7.8 Data analysis

The measurement methods were compared using McNemar's test for comparison of proportions from paired binary outcomes. This is a nonparametric test for a 2 × 2 contingency table with matched subjects where the outcomes are not independent. McNemar's Test was calculated using SAS Software (SAS Institute Inc. 2004. SAS/STAT® 9.1, Cary, NC: SAS Institute Inc.).

McNemar's test was first published in a *Psychometrika* article in 1947 – by Quinn McNemar, who was a professor in the Psychology and Statistics department at Stanford University (McNemar, 1947). The McNemar's test is also called the McNemar test for symmetry, or the McNemar symmetry chi square test with one degree of freedom (DF) – non-parametric test[8]. McNemar's test is a non-parametric-test used to compare two population proportions that are related or correlated to each other. Percentages or proportions of events resulting from 2 observations made on the same or matched experimental units under 2 different conditions may be tested for equality using this procedure (Lehr, 2006). McNemar's test is a test on 2 × 2 classification tables with matched pairs of data, which tabulates the outcomes of two tests on a sample of *n* subjects (on paired dichotomous observations to test the significance of the difference between proportions) (Lu, 2010), as follows (**Table** 2-11).

Table 2-11: Example of table calculation of McNemar's test.

Table of Result Medium A by Medium B				
		Result Medium B		
		0 (No Growth)	1 (Growth)	Total
Results Medium A	0 (No Growth)	A	B	A + B
	1 (Growth)	C	D	C + D
	Total	A + C	B + D	N=A+B+C+D
P value				

The statistic of the McNemar's test has a chisquare distribution with 1 degree of freedom (DF) (rows – 1) (columns – 1) = 1). The statistic for the test is:

$$x^2 = \frac{(B-C)^2}{B+C} \qquad (1)$$

Following Lu (2010), "The McNemar's test should be used when B + C is 10 or greater (McNemar, 1947). The exact binomial distribution can be used without resorting to the asymptotic chi-square distribution.

Null hypothesis (H_0): For the null hypothesis, McNemar's test assumes that the totals for the rows are equal to the totals for the columns. In this application this indicates that both media works equally well.

[8] *Nonparametric* methods were developed to be used in cases when the researcher does not know the parameters of the distribution of the variable of interest in the population (hence the name *nonparametric*) and observation are independent – http://www.statsoft.com/textbook/statistics-glossary/n/button/n/

Alternative Hypothesis (H_1): The alternative hypothesis assumes that these totals are not equal. In this application rejecting the null hypothesis would be evidence that the media performed differently.

Significance testing: In McNemar's test, significance is tested by using the chi-square table with one degree of freedom for the statistic above. If the calculated value for McNemar's test value is greater than the table value, we will reject the null hypothesis. If, however, the calculated value is less than the table value, we will accept the null hypothesis (http://www.statisticssolutions.com/methods-chapter/statistical-tests/mcnemar-test/).

Chapter 3 The medium to detect *Lecanicillium fungicola* on mushroom farms

The easiest and cheapest method of identification of *L. fungicola* sources on mushroom farms is a microbiological test. Such a test would detect only living conidia and mycelium of the pathogen. Living material can spread to every new cycle of mushroom cultivation, leading to disease outbreaks. Live pathogenic material can also be spread to some sheltered places such as soil or grass and wait for activation for a long time (Cross and Jacobs, 1969; Fekete, 1967; Brady and Gibson, 1969).

Wong and Preece (1987) first used microbiological tests for detection of *L. fungicola* in samples gathered on mushroom farms. They used two different media: Pseudomonas Agar F [9] and DBR medium agar (Defined base medium – $NH_4H_2PO_4$, KCl, $MgSO_4 \times 7H_2O$ + Bromothymol blue + Raffinose) and Bronopol (sometimes added as bacteria suppresser). The pH was adjusted to pH 7. However, this medium was not very selective for *Lecanicillium fungicola*. On this medium other fungi also grew such as *Penicillium*, *Mucor*, *Cladosporium*, *Trichoderma* and also bacteria (Wong and Preece, 1987).

Rinker *et al.* (1993) first described a selective medium (RSM) for *Lecanicillium fungicola* detection. Rinker's selective medium (RSM) contained DBR agar medium without bromothymol blue described by Wong and Preece (1987). The DBR agar medium contains some reagents which are common ingredients to those used in many different media such as selective medium for *Trichoderma harzianum* (Williams *et al.*, 2003). Rinker's selective medium also contains two dyes (malachite green sodium salt, bromocresol green sodium salt), one antibiotic as a bacteria suppresser (ampicillin anhydrous) and two fungicides used in mushroom farms to inhibit growth of other fungi, benomyl (Benlate 50 WP) and chlorothalonil (Bravo 500). The pH was adjusted to pH 4.

One of the ingredients used in RSM is malachite green which has strong anti-fungal activity and also inhibits *L. fungicola* growth to a certain extent. The raffinose (polysaccharides) used in this medium is a good source of carbon for *L. fungicola*. On

[9] http://www.bd.com/ds/technicalCenter/inserts/Pseudomonas_Agars.pdf

Rinker's selective medium *L. fungicola* grows very slowly and colonies are small and difficult to find without a light microscope after 4-6 days.

The differentification of isolates of *L. fungicola* into varieties *fungicola* and *aleophilum* is performed by physiological differences in them is response to temperature. The temperature test has been performed by many researchers for identification of wild isolates of *L. fungicola* (Largeteau *et al.*, 2004, Gea *et al.*, 2005, Potočnik *et al.*, 2008) and it is one of the easiest methods but not fast for identification of wild isolates of *L. fungicola*. According to Zare and Games (2008) optimal growth temperature of *L. fungicola* var. *fungicola* is 18-24 °C, but it is not able to grow at 30 °C. The *L. fungicola* var. *aleophilum* optimal growth temperature is 21-27 °C, but these isolates are also able to grow at 30 °C. This difference is very useful and can identify a variety of *L. fungicola* as the morphological differences between both varieties are indistinguishable.

The sensitivity of fungal isolates to prochloraz-manganese and carbendazim was examined to check the range of resistance of Irish and other isolates of *L. fungicola*. However, the widespread use of prochloraz-manganese in Europe has decreased the sensitivity to this fungicide. Grogan *et al.* (2000) demonstrated that in *in vitro* test the *L. fungicola* isolates showed decreased sensitivity to prochloraz-manganese. However, Grogan *et al.* (2000) demonstrated that prochloraz-manganese can still achieve a reasonable level of control of dry bubble caused by two isolates showing different degrees of sensitivity to this fungicide.

The objective of this study was to develop a new medium and modify existing medium (Rinker *et al.*, 1993) for the better and faster detection of *Lecanicillium fungicola* in samples originating from mushroom farms. The objective was to find a minimum level of conidia of *L. fungicola* using developed and modified Rinker's selective medium. Another objective was to design a method for sample preparation collected on mushroom farms. That selective medium would be helpful to mushroom growers in order to detect *L. fungicola* on mushroom farms and find possible sources of *L. fungicola* and so help keep disease on mushroom farms under control. The second objective of this study was to examine and identify wild Irish, Polish, Serbian and Spanish isolates of *L. fungicola* using temperature test and to determine their sensitivity to prochloraz-manganese and carbendazim use. Tests of isolates of *L. fungicola* from other European countries could answer the question what kind of varieties of *L. fungicola* are present in Europe and it also might be possible to use the microbiological

approach – selective media for detection *L. fungicola* in other European contries such as Poland, Serbia and Spain.

3.1 Results

3.1.1 Isolation, identification and characterisation of wild isolates *L. fungicola* and other fungi, bacteria and yeast

3.1.1.1 Fungus, bacteria and yeast isolate

Cladobotryum mycophilum and *Mycogone perniciosa* were also isolated and microscopy characterisation was performed using identification provided by Brady and Gibson, (1976) and Gams & Hooz., (1970) respectively. *Penicillium* sp., *Mucor* sp. identification was performed by observation of phenotypic characterisation of colony and by microscopic studies of the conidia. A bacterial/yeast suspension was obtained from casing extract but organisms were not identified. Details of all organisms are given in **Table 2.6**. Additional cultures were obtained from NUIM (*A. fumigatus*) and Dr. H. Grogan (*Trichoderma* sp.) (**Table 2.6**).

3.1.1.2 Microscopic and temperature identification of wild isolates of *L. fungicola*

All wild Irish isolates of *L. fungicola* had the cultural and microscopic characteristic of *L. fungicola* using identification key provided by Gams and Van Zaayen (1982). The morphological characteristics were examined and the Irish wild isolates had dense white aerial mycelia, the reverse of plates was white and during incubation started to be white-grey. The conidiophores were erect and groups with divergent phialides with slightly inflated base. The conidia were produced in gelatinous heads (**Figure** 3-1). The isolates of *L. fungicola* var. *aleophilum* did not show differences in growth compared with *L. fungicola* var. *fungicola* at 23 °C, but *L. fungicola* var. *aleophilum* was able to grow at 30 °C. This physiological difference helps to identify Irish, Polish, Serbian and Spanish wild isolates as *L. fungicola* var. *fungicola* and Canadian and USA wild isolates as a *L. fungicola* var. *aleophilum*. Other tested isolates were already identified and described in publications or classified in data

bases (Gams and Van Zaayen, 1982 and Zare and Gams, 2008) (**Figure** 3-2). Details of all organisms are presented in **Table 2.5**.

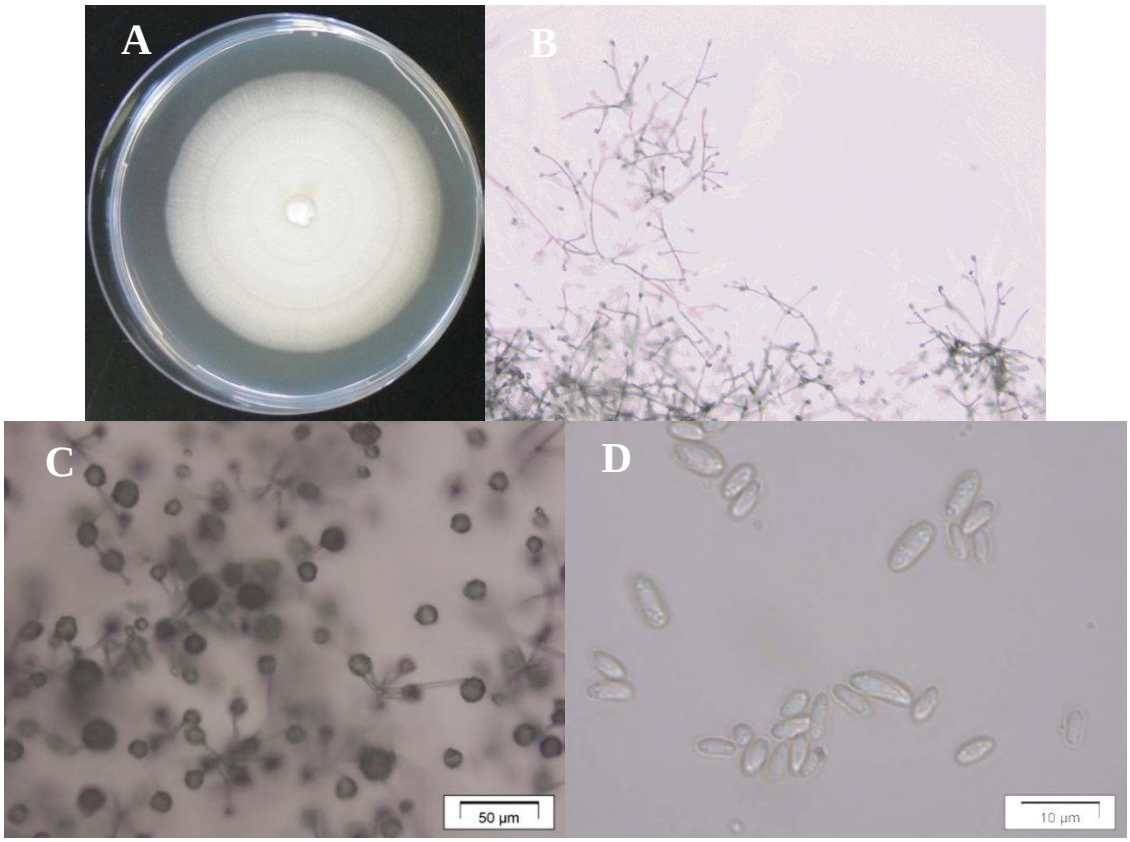


Figure 3-1: *Lecanicillium fungicola* var. *fungicola* wild Irish isolate L.46. A – Agar plate colony after 21 days, B – mycelium, C – Conidiophores and gelatinous heads with conidia, D – conidia.

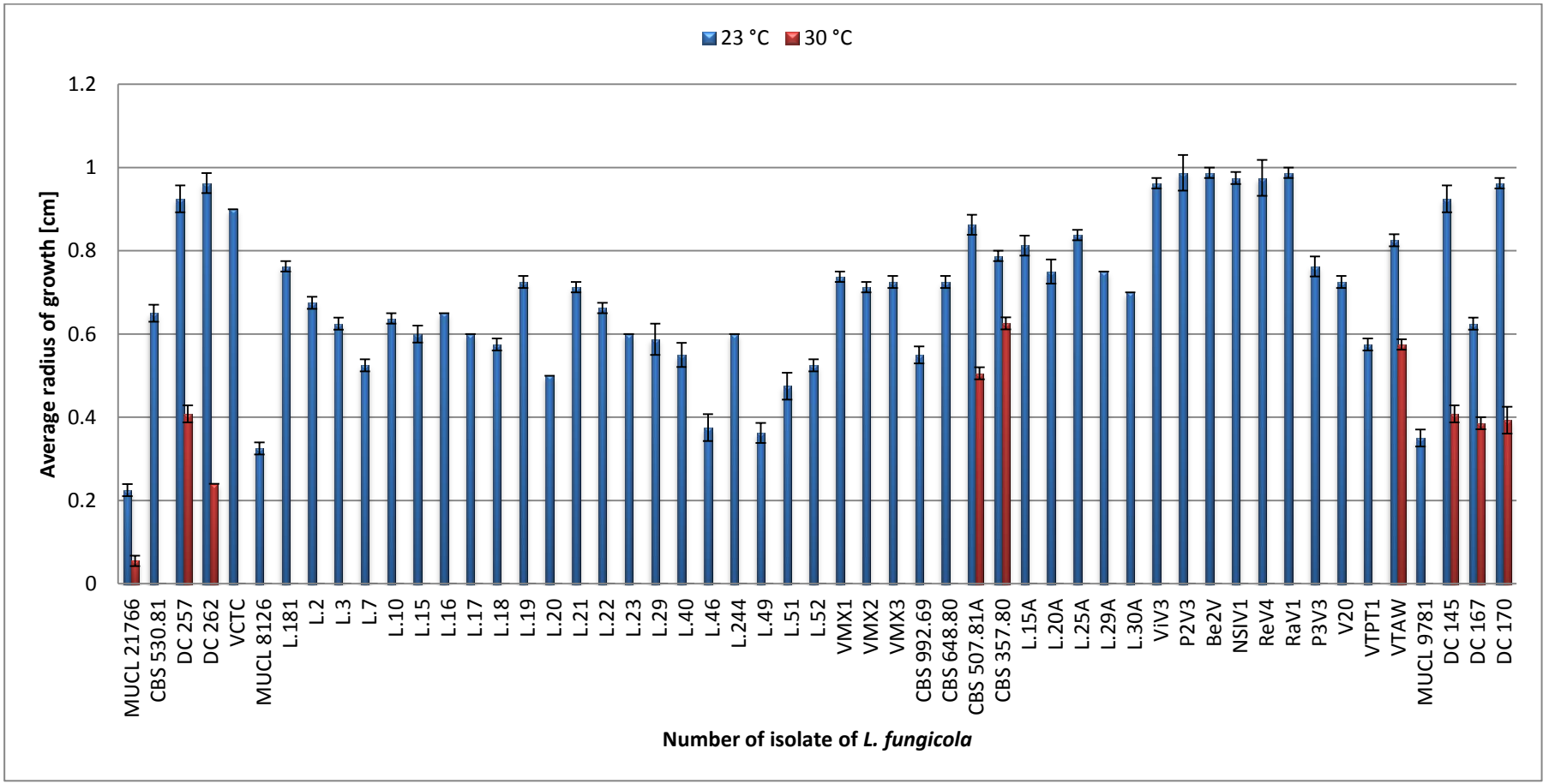


Figure 3-2: Isolates of *L. fungicola* by mycelia growth at 23°C and 30°C, after 7 days of incubation in dark. Standard error is shown.

3.1.1.3 Prochloraz-manganese sensitivity of *L. fungicola*

Fifty two isolates were tested with different concentration of prochloraz-manganese listed in **Table 2-5**. Isolates from Canada, France, Netherlands, UK and USA were very sensitive to prochloraz-Mn and the EC_{50} (fungicide concentrations which inhibited mycelial growth by 50 %) values were between 0.54-1.06 mg/l, The Belgium and Taiwan isolates of *L. fungicola* var. *aleophilum* showed EC_{50} values between 1.47-1.62 mg/l. The Irish, Polish, Serbian and Spanish wild isolates were more resistant to prochloraz-manganese and the EC_{50} value were between 1.86-3.88 mg/l. The EC_{50} values of Mexican isolates were between 2.08-3.18 (**Table** 3-1).

Table 3-1: *In vitro* sensitivity of *L. fungicola* isolates to prochloraz-manganese after 21 days incubation at 23 °C. (EC_{50} and EC_{90} are fungicide concentrations which inhibit mycelial growth by 50 and 90 %, respectively).

No.	Origin	Number of isolate	Isolates	Toxicity parameters			
				EC_{50}	CI (EC_{50})	EC_{90}	CI (EC_{90})
1	Belgium	1	MUCL 21766	1.47	1.24-1.75	13.61	10.68-17.65
2	Canada	1	DC (257)	0.59	0.46-0.76	4.37	2.92-6,57
3	Canada	1	DC (262)	0.82	0.75-0.90	5.61	4.88-6.46
4	France	1	VCTC	0.54	0.51-0.57	3.03	2.77-3.30
5	France	1	MUCL 8126	1.06	0.99-1.12	8.41	7.66-9.23
6	Ireland	21	*	3.88	1.72-6.28	45.77	27.55-86.97
7	Mexico	1	VMX1,	1.86	1.71-2.03	18.22	16.18-20.65
8	Mexico	1	VMX2	1.89	1.77-2.02	18.93	17.30-20.78
9	Mexico	1	VMX3	3.86	2.75-5.49	30.00	21.04-45.81
10	Netherlands	1	CBS 992.69	0.79	0.73-0.85	5.37	4.78-6.0
11	Netherlands	1	CBS 648.80	0.66	0.62-0.68	4.08	3.79-4.38
12	Netherlands	1	CBS 507.81A	0.73	0.54-1.01	6.40	3.78-12.0
13	Netherlands	1	CBS 357.80	0.72	0.66-0.77	4.95	4.36-5.6
14	Poland	1	L.15A	4.07	3.67-4.51	83.35	66.76
15	Poland	1	L.20A	2.82	2.50-3.19	72.63	52.68
16	Poland	1	L.25A	1.38	1.28-1.49	19.45	16.62-23.43
17	Poland	1	L.29A	1.72	1.51-1.97	17.21	14.32-21.04
18	Poland	1	L.30A	2.08	1.88-2.29	47.38	36.71-69.53
19	Serbia	1	ViV3	1.54	1.35-1.76	26.41	19.84-40.97
20	Serbia	1	P2V3	2.17	2.02-2.33	32.65	28.7637.82
21	Serbia	1	Be2V	1.38	1.27-1.50	20.50	17.21-25.36
22	Serbia	1	NSIV1	2.18	1.95-2.44	35.78	28.93-47.42
23	Serbia	1	ReV4	2.48	2.24-2.75	50.21	40.02-69.04
24	Serbia	1	RaV1	1.36	1.17-1.58	19.39	14.41-29.75
25	Serbia	1	P3V3	1.93	1.74-2.15	39.65	30.94-57.09
26	Spain	1	V20	2.52	2.14-2.97	78.04	48.11
27	Spain	1	VTPT1	4.65	3.67-5.89	56.05	41.86-88.84
28	Taiwan	1	VTAW	1.62	1.22-2.18	19.86	12.63-42.55
29	UK	1	MUCL.978	0.68	0.60-0.77	4.03	3.34-4,87
30	USA	1	DC.145	0.42	0.39-0.46	2.42	2.14-2.74
31	USA	1	DC.167	0.78	0.72-0.84	5.13	4.57-5.76
32	USA	1	DC.170	0.89	0.79-0.99	7.62	6.34-9.25

* mean value – see Figure 3.3; CI – 95 % confidence intervals; EC_{50} and EC_{90} expressed in mg/l

The twenty one wild Irish isolates showed high resistance to prochloraz-manganese, the EC_{50} values were between 2.16-5.35 mg/l (**Figure** 3-3). The EC_{90} values were between 19.11-86.97 mg/l.

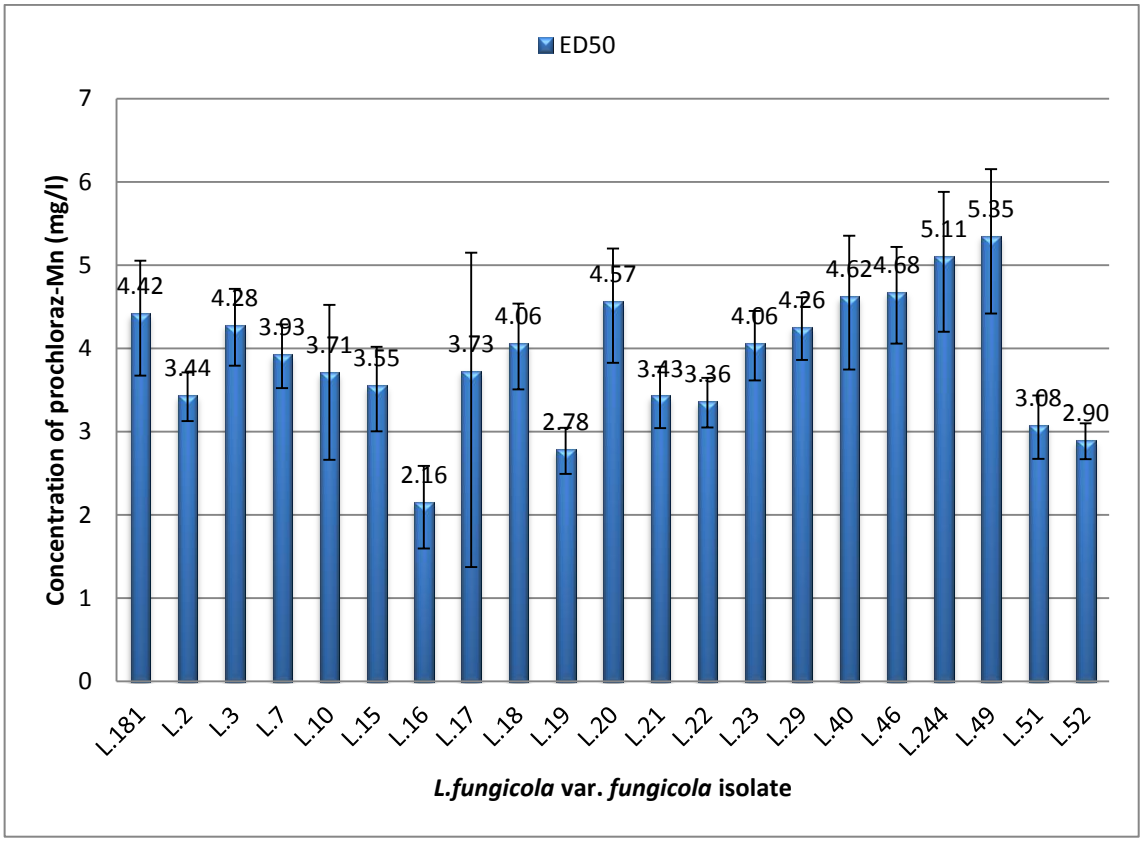


Figure 3-3: *In vitro* sensitivity (EC_{50}) of Irish isolates of *L. fungicola* var. *fungicola* to prochloraz-manganese after 21 days incubation at 23 °C. (EC_{50} is fungicide concentration which inhibits mycelial growth by 50 %). The error bars indicate the 95 % confidence interval of EC_{50}.

3.1.1.4 Carbendazim sensitivity of *L. fungicola*

Fifty two isolates were tested for sensitivity to different concentrations of carbendazim listed in **Table 2-5**. Only six isolates were very sensitive for carbendazim and they showed the EC_{50} values between 7.11-13.70 mg/l. Only one isolate from USA showed EC_{50} and had a range 79.31-108.61 mg/l (**Table** 3-2).

The wild isolates from Ireland, Poland, Serbia and Spain and some isolated from culture collections were resistant to carbendazim and the results did not cover a range that included the EC_{50} and EC_{90}.

Table 3-2: *In vitro* sensitivity of *L. fungicola* isolates to carbendazim after 21 days incubation at 23 °C. (EC_{50} and EC_{90} are fungicide concentrations which inhibit mycelial growth by 50 and 90 %, respectively).

No.	Origin	Amount of isol.	Isolates	Toxicity parameters			
				EC_{50}	CI (EC_{50})	EC_{90}	CI (EC_{90})
1	Belgium	1	MUCL 21766	9.10	7.41-11.03	63.14	51.66-78.59
2	France	1	MUCL 8126	9.40	7.63-11.42	69.18	56.22-86.88
3	Netherlands	1	CBS 992.69	11.04	8.78-13.70	88.93	69.59-n/a
4	Netherlands	1	CBS 507.81A	10.57	7.82-13.95	85.31	62.62-0
5	Netherlands	1	CBS 357.80	9.17	7.47-11.11	65.95	53.92-82.17
6	UK	1	MUCL 9781	7.93	6.27-9.81	52.36	42.42-65.81
7	USA	1	DC170	92.32	79.31-108.61	n/a	n/a

CI – 95 % confidence intervals; EC_{50} and EC_{90} expressed in mg/l, n/a – the actual limit was out of the data range.

The Irish wild isolates showed 62 % to 96 % of control growth when concentration of carbendazim was 50 mg/l, but when concentration of carbendazim was increased to 100 mg/l the growth of *L. fungicola* var. *fungicola* had a range 47 % to 103 % of control growth (**Figure** 3-4).

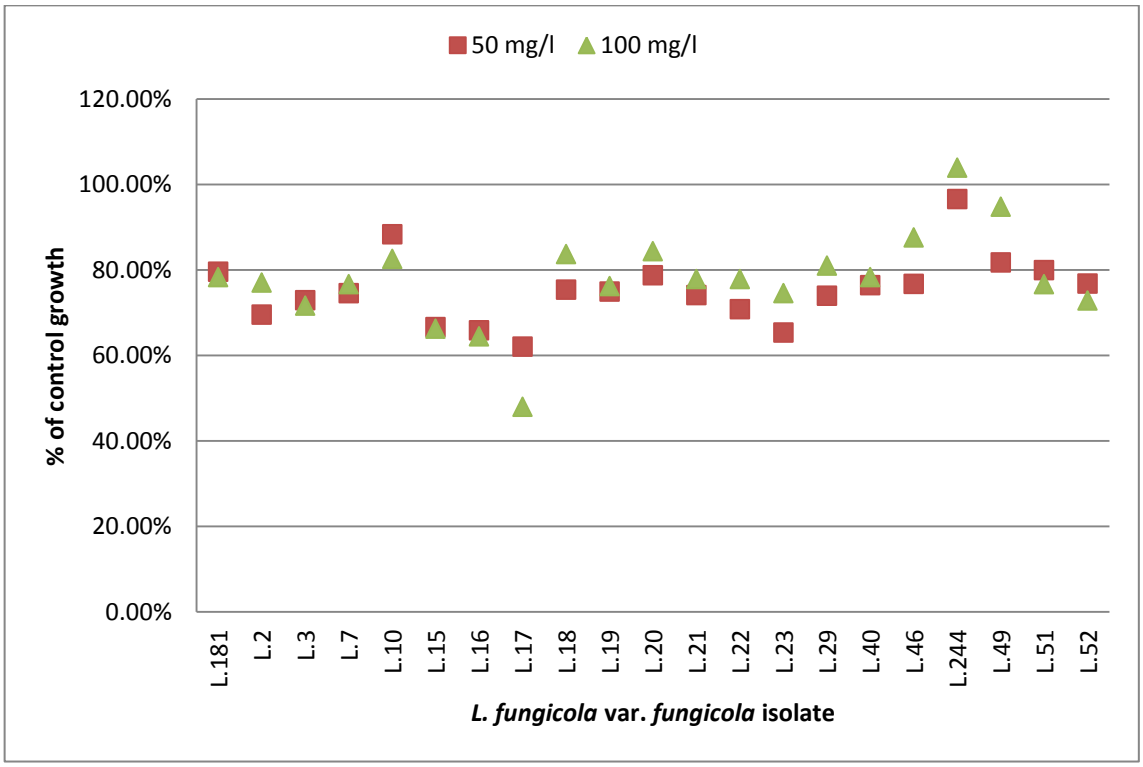


Figure 3-4: In vitro response of Irish isolates of L. f. var. fungicola for two concentrations of carbendazim 50 and 100 mg/l active ingredient. After 21 days at 23 °C.

3.1.1.5 Summary

All tested wild Irish, Polish, Serbian and Spanish isolates were identified as *L. fungicola* var. *fungicola*. The Canadian and USA isolates were identified as *L. fungicola* var. *aleophilum*. Other tested isolates were already identified and described in publications or classified in data bases (**Table 2-5**).

The wild Irish, Polish, Serbian and Spanish isolates of *L. f.* var. *fungicola* were moderately sensitive to prochloraz-manganese with EC_{50} values ranging from (1.16-6.28 mg/l). The Irish wild isolates were more resistant to prochloraz-manganese compared to Polish, Serbian and Spanish isolates and EC_{50} values range was 4.56 mg/l between isolates (EC_{50} = 1.72 to 6.28 mg/l). The Serbian isolates were more sensitive than Irish, Polish and Spanish isolates. The EC_{50} values for Serbian isolates were between 1.16 to 2.74 mg/l. Polish and Spanish isolates showed very similar EC_{50} values and the range was 1.51-4.51 mg/l and 2.13-3.18 mg/l, respectively. The Mexican isolates showed EC_{50} values between 2.08-3.18 mg/l and it was similar to response for wild European isolates.

French isolates of *L. f.* var. *fungicola* showed a high sensitivity to prochloraz-manganese and EC_{50} values was 0.51-0.57 mg/l. Other French isolates MUCL 8219 was isolated from wheat seed (*Triticum sp.*) and EC_{50} values were between 0.99 to 1.12 mg/l. That hight sensitive for prochloraz-manganese may be explain by use prochloraz as a fungicide in wheat cropping (Leroux and Marchegay, 1991).

The Canadian and USA isolates of *L. f.* var. *aleophilum* were sensitive to prochloraz-managnese which were isolated before this fungicide was used.

The Belgian isolate of *L. fungicola*, isolated from watercress (*Nasturtium aquaticum*) showed moderate sensitivity to prochloraz-managnese and the EC50 value was 9.10 mg/l. That moderate sensitivity to prochloraz-managnese could be explained by the presence of prochloraz in hydroponic cultivation (groundwater).

Netherlands isolates were sensitive to prochloraz-manganese but this can be explained by time of collection of isolates before prochloraz-manganese was used and the varieties of *L. f.* var. *aleophilum*.

The wild Irish, Polish, Serbian and Mexican isolates did show resistance to carbendazim. Only six isolates were very sensitive to carbendazim. One isolates from USA showed EC_{50} but this value was very high. Other tested isolates were resistant to carbendazim.

Nair and Macauley (1987) reported that in *in vitro* test the *A. bisporus* was little affected by prochloraz-manganese and EC_{50} value was about 25 mg/l.

3.1.2 Preliminary evaluation of chemicals for their effect on *L. fungicola* (CR.181 and L.2) growth and conidial germination.

3.1.2.1 Effect of different concentrations of malachite green on colony growth

Two Irish isolates CR181 and L.2 were tested using two media: PDA and Rinker's base medium (RBM) medium, containing different concentrations of malachite green (0, 10, 20 and 30 mg/l).

The conidia of *L. fungicola* grew very well in PDA and RBM medium without malachite green. When the concentration of malachite green was 10 mg/l in both media, conidial germination was 80-100 % of control. When the concentration of malachite green was 20 mg/l or higher conidia of both isolates failed to grow (**Figure** 3-5).

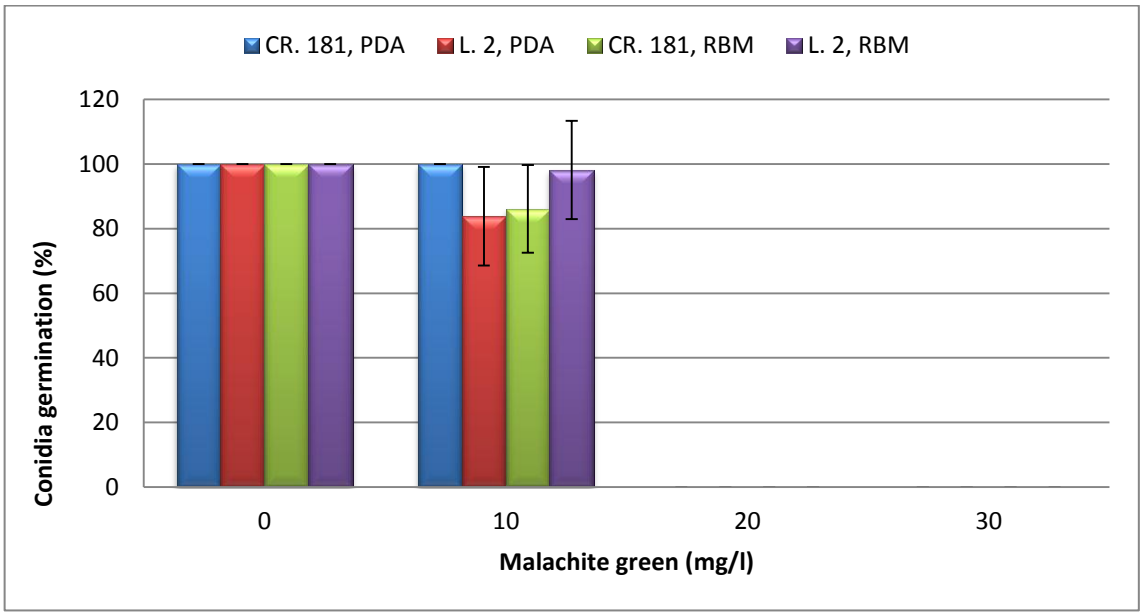


Figure 3-5: Comparison of conidia germination in PDA and RBM with different concentrations of malachite green (MG) after 7 days incubation. Standard error is shown. All presence colonies were measured.

The radius of growth from individual colonies of *L. fungicola* after 7 days at 23 °C in PDA control medium without malachite green had a range 0.7-0.8 cm for CR 181 isolate and 0.75-0.8 cm for L. 2 isolate. When concentration of malachite green was 10 mg/l, the radius of growth decreased to 0.2-0.3 cm for isolate CR 181 and 0.2-0.25 cm for L. 2. Increasing the concentration malachite green to 20 mg/l or higher prevented the growth of isolates (**Figure** 3-6).

On Rinker's base medium (RBM) without malachite green radius of growth after 7 days was 0.1 - 0.15 cm for CR 181 isolate and 0.15 - 0.2 cm for L. 2 isolate. When the concentration of malachite green was 10 mg/l, the radius of growth decreased to 0.05-0.1 for both tested isolates. Higher concentrations of malachite green prevented the growth of *L. fungicola* isolates (**Figure** 3-6). After 14 days of incubation small colonies of both isolates were visible when the concentration of malachite green was 20 mg/l (results not presented).

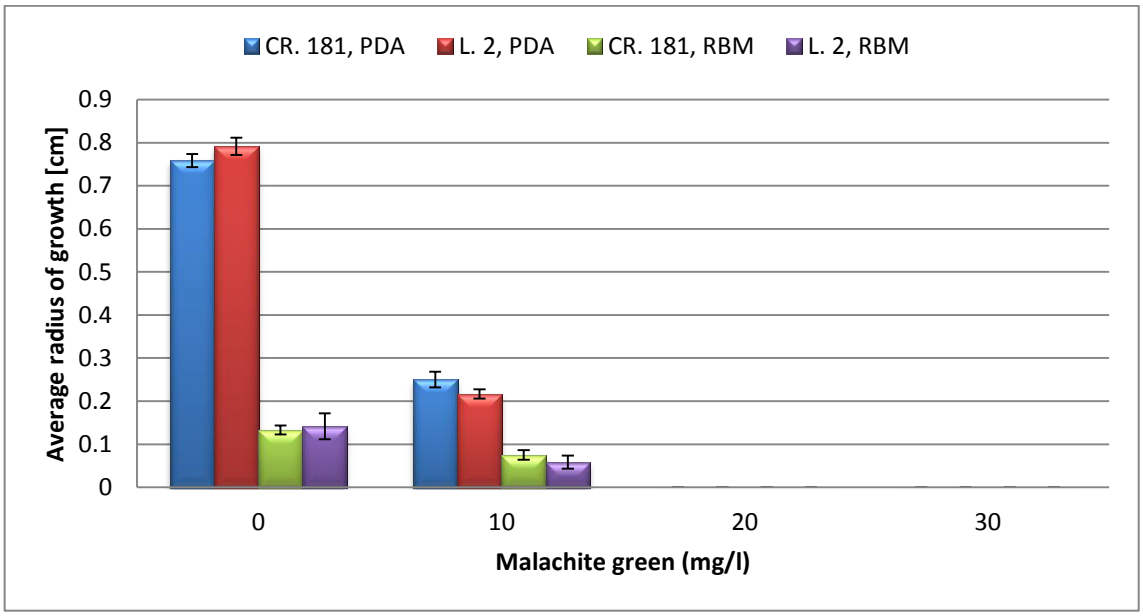


Figure 3-6: Average radius of *L. fungicola* colony growth in PDA and RBM medium with different concentrations of malachite green (MG), after 7 days of growth. Standard error is shown. All presence colonies were measured.

Colonies of *L. fungicola* on Rinker's base medium (RBM) with 0 mg/l malachite green were very small compared to those on PDA medium with 0 mg/l malachite green. Only on PDA medium with 10 mg/l of malachite green *L. fungicola* had a white colour and was easy to count. On Rinker's base medium with 10 mg/l malachite green colonies of *L. fungicola* were difficult to find as they were small and transparent (**Figure** 3-7).

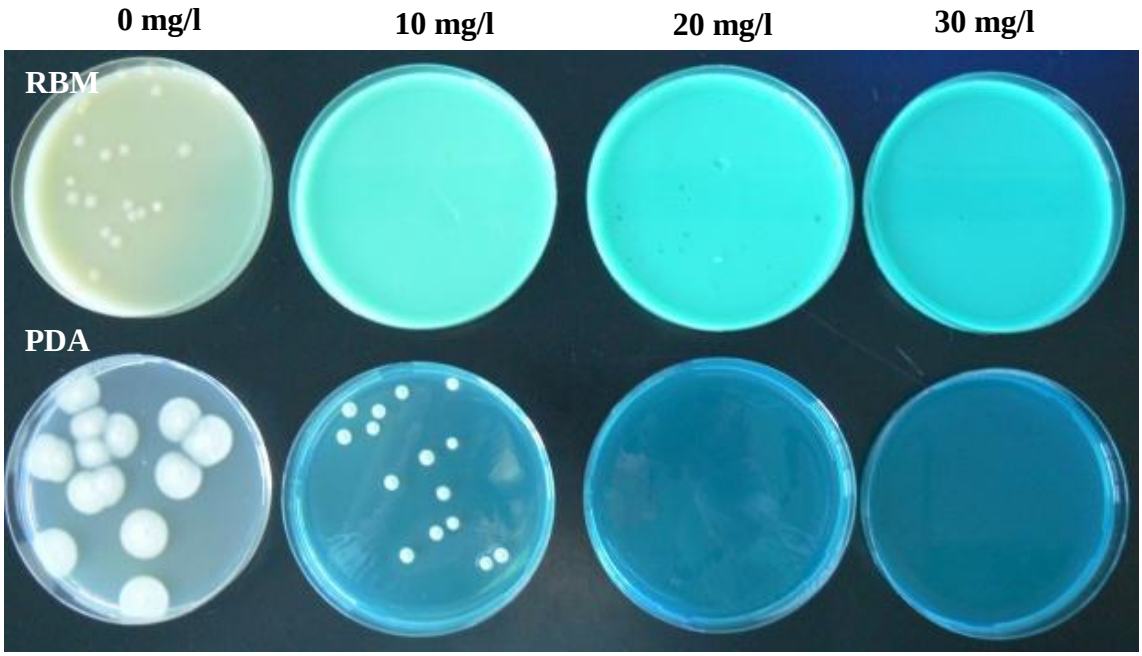


Figure 3-7: Size of *L. fungicola* colonies in PDA and RBM medium with different concentrations of malachite green (MG) (0, 10, 20, 30 mg/l) after 7 days of growth. Isolate CR 181.

When media were inoculated by agar plug with active mycelium of *L. fungicola*, *L. fungicola* showed growth at all concentrations of malachite green.

In PDA, control medium without malachite green the radius of fungal growth was mean value 0.7-1 cm for both isolates. When the concentration of malachite green was 10 mg/l, the radius of growth decreased and for isolate CR 181 it was 0.3-0.4 cm and for isolate it L.2 it was 0.3-0.5 cm. When the concentration of malachite green was increased to 20 mg/l, *L. fungicola* did not show growth inhibition and the radius of growth was 0.2-0.4 cm for isolate CR 181 and 0.4-0.5 cm for isolate L.2. When concentration of malachite green was increased to 30 mg/l the radius of growth of *L. fungicola* was much smaller and was 0.2-0.4 cm for isolate CR 181 and 0.3-0.4 cm for isolate L.2 (**Figure** 3-8).

On Rinker's base medium (RBM) without malachite green the radius of growth was between 0.2-0.4 cm for both isolates. When concentration of malachite green was increased to 10 mg/l, the radius of mycelia growth decreased and was 0.2-0.3 cm for both isolates. When the concentration of malachite green was increased to 20 mg/l *L. fungicola* isolates showed considerable growth inhibition and radius of growth was 0.1-0.2 cm for both isolates. When concentration of malachite green was increased to 30 mg/l the radius of growth of *L. fungicola* was much smaller and was 0-0.2 cm for isolate CR 181 and 0.1-0.2 cm for isolate L. 2 (**Figure** 3-8).

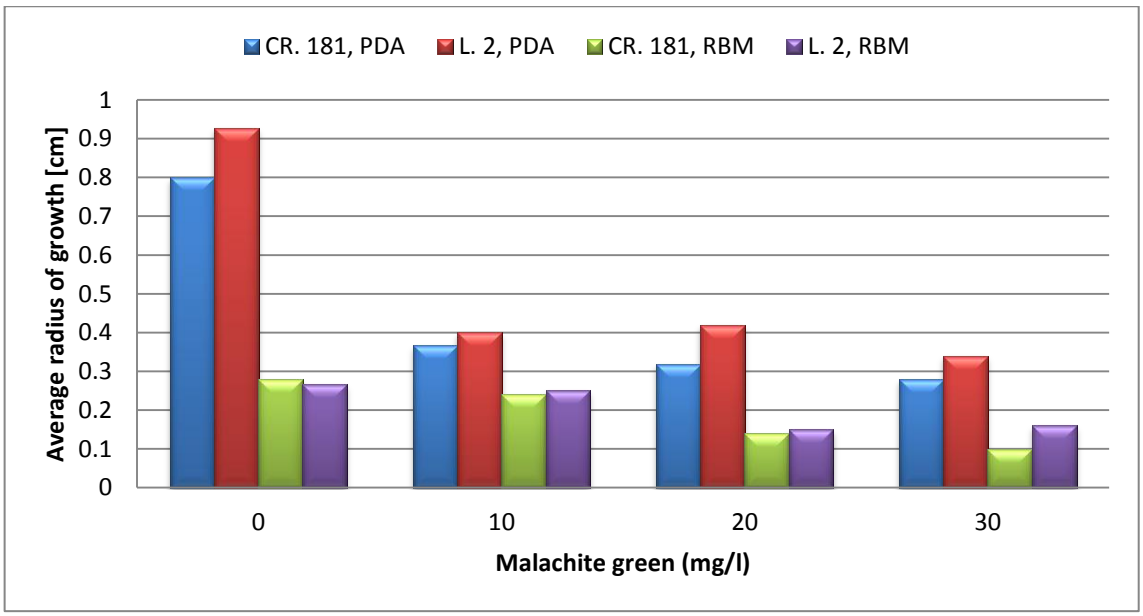


Figure 3-8: Average radius of growth of two isolates of *L. fungicola* in PDA and RBM medium with different concentrations of malachite green (MG) after 7 days of incubation. One colonie of agar plug was measured.

The growth of agar plugs with active mycelium of *L. fungicola* in PDA medium was inhibited when concentration of malachite was 10 mg/l. The growth of mycelium was 33-57 % of control growth. When the concentration of malachite green was increased to 20 mg/l growth of *L. fungicola* was 25-57 % of control growth. However when the concentration of malachite green was 30 mg/l growth of *L. fungicola* was inhibited by 22-50 % of control growth.

On Rinker's base medium *L. fungicola* growth was 50-100 % of control growth when concentration of malachite green was 10 mg/l. When the concentration of malachite green was increased to 20 mg/l the growth of *L. fungicola* was 33-66 % of control growth. When concentration of malachite green was increased to 30 mg/l the mycelium growth was by 34-100 % of control.

When media were inoculated with agar plugs of active mycelium of *L. fungicola* growth on all concentrations of malachite green was observed. In PDA medium *L. fungicola* grew much faster than on Rinker's base medium. When concentraion of malachite green was increased in PDA medium mycelium of *L. fungicola* had a white colour and was easy to measure. In contrast to Rinker's base medium (RBM) where *L. fungicola* mycelium growth was difficult to measure and growth was much slower (**Figure** 3-9).

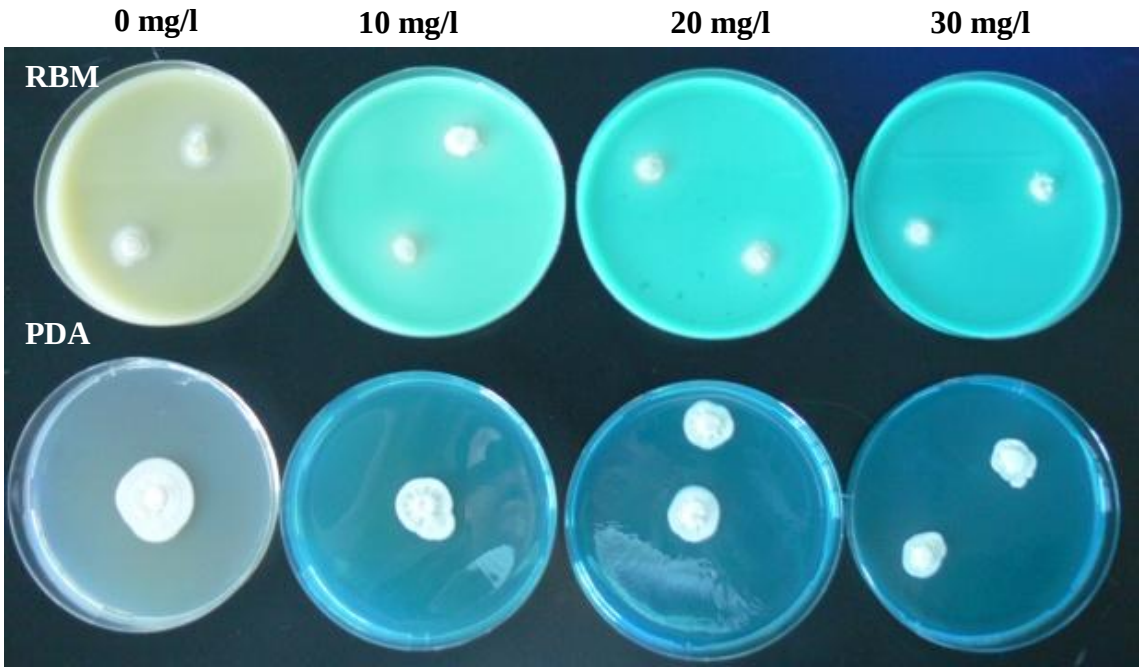


Figure 3-9: Size of *L. fungicola* colonies (agar plugs) in PDA and Rinker's base medium (RBM) with different concentrations of malachite green (0, 10, 20, 30 mg/l) after 7 days of growth. Isolate CR 181.

3.1.2.2 Fungicide sensitivity

Two isolates CR181 and L.2 were tested using two media: Rinker's base medium and PDA medium containing different concentrations of the fungicides: prochloraz-manganese and carbendazim.

- **Sensitivity test for prochloraz-Mn**

Both isolates of *L. fungicola* showed the same response to different concentrations of prochloraz-Mn. When the concentration of prochloraz-manganese was 0.1 mg/l the radius of growth was 0.8-0.9 cm. When the concentration of prochloraz-manganese was increased to 1 mg/l, *L. fungicola* grew very well and the radius of growth was 0.6-0.7 cm. Increasing the concentration to 5 mg/l prochloraz-manganese the radius of growth decreased to 0.3 cm in both isolates and the radius of growth was four times smaller than control radius. *Lecanicillium fungicola* growth was nearly halted when the concentration of prochloraz-manganese was 25, 50 and 100 mg/l after 7 days at 23 °C (**Figure** 3-10).

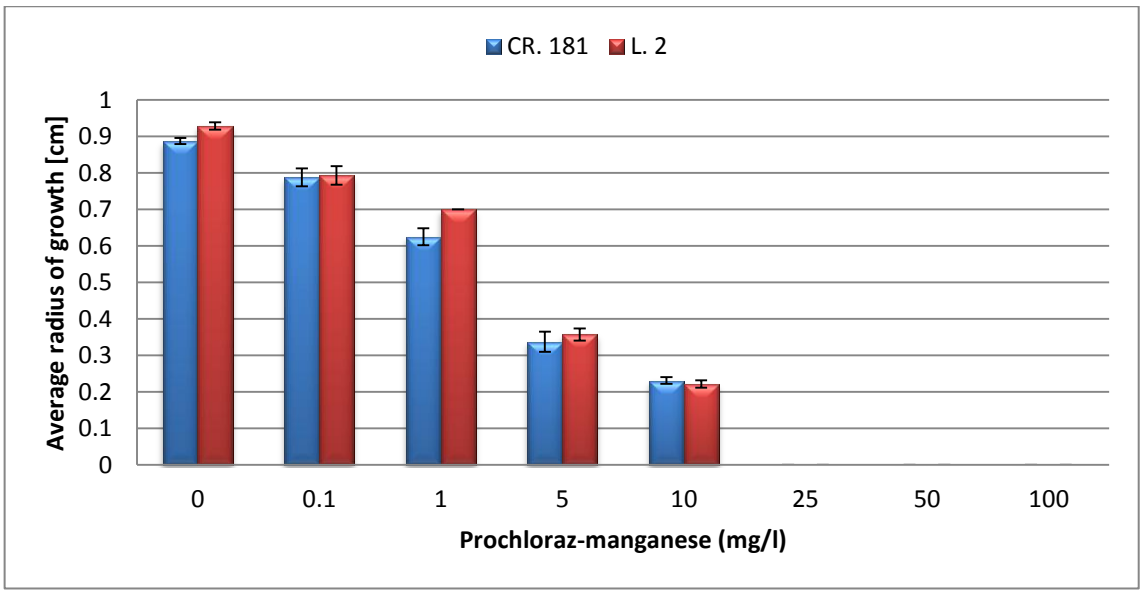


Figure 3-10: Average radius of growth of *L. fungicola* on PDA medium with different concentrations of prochloraz-manganese of two isolates of *L. fungicola*. Results after 7 days. Standard error is shown.

When the concentration of prochloraz-manganese was 0.1 and 1 mg/l *L. fungicola* showed more than 80 % of control growth. Increasing the concentration of prochloraz-manganese to 5 mg/l *L. fungicola* showed 40 % of control growth.

- **Sensitivity test for carbendazim**

The second tested fungicide was carbendazim. There was no significant difference between the two isolates of *L. fungicola* in response to carbendazim. When the concentration of carbendazim was 0.1 mg/l to 100 mg/l, both isolates had this same radius of growth as control and had a colony radius of 0.82-0.96 cm (**Figure** 3-11).

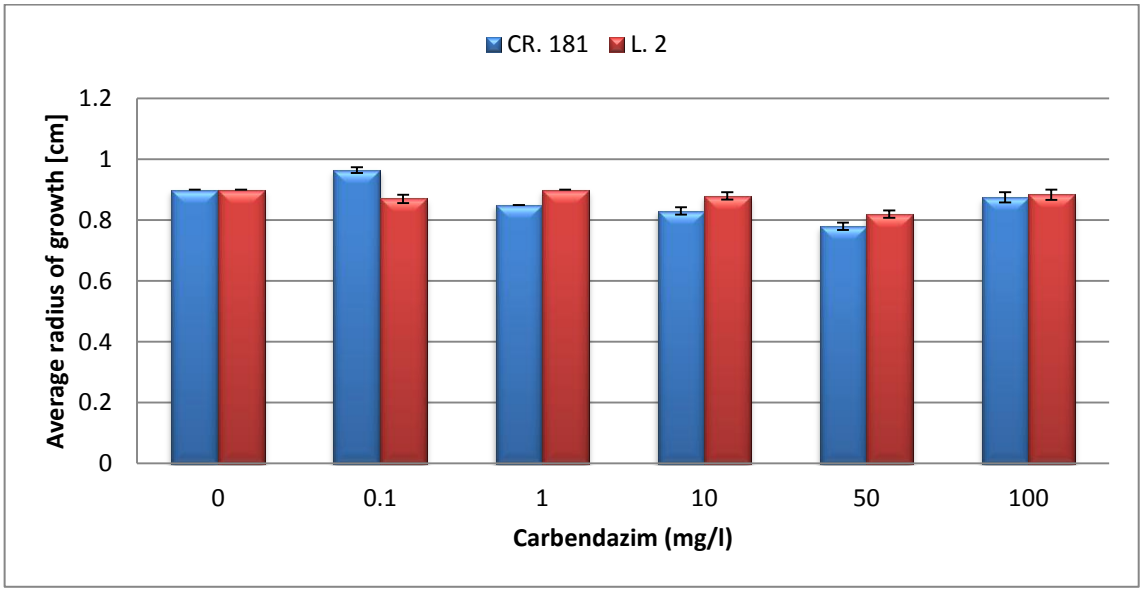


Figure 3-11: Average radius of growth of *L. fungicola* in PDA medium with different concentrations of carbendazim. Results after 7 days. Standard error is shown.

The concentration of carbendazim between 0.1 to 100 mg/l did not show any significant inhibition of growth of *L. fungicola* and at highest concentration of carbendazim (100 mg/l), *L. fungicola* growth was as good as without any fungicide. Both tested isolates showed resistance to carbendazim.

3.1.2.3 Summary

Preliminary tests with two wild isolates of *L. fungicola* showed the response of these fungi to different concentrations of malachite green and two fungicides containing prochloraz-manganese and carbendazim after 7 days of incubation.

Lecanicillium fungicola grew very well when concentration of malachite green was 10 mg/l. PDA medium with 10 mg/l malachite green gave much better growth of *L. fungicola* conidia than Rinker's base medium with 10 mg/l malachite green. When medium was inoculated by agar plug *L. fungicola* grew in all malachite green concentrations. The best mycelial growth was in PDA medium containing 10 and 20 mg/l malachite green. However, Rinker's base medium containing 10 mg/l malachite green *L. fungicola* showed growth too.

In the next experiments concentrations of malachite green would be between 5 to 10 mg/l as this is best to allow growth of *L. fungicola*.

Lecanicillium fungicola was sensitive to the fungicide prochloraz-Mn. The concentration of prochloraz-manganese was 1 mg/l *L. fungicola* growth showed 80 % of growth. Carbendazim did not affect growth of *L. fungicola* and at the highest tested concentration *L. fungicola* showed 90 % of control growth.

3.1.3 Effect of malachite green (anti-fungal drug) on fungal growth

3.1.3.1 Different concentration of malachite green: *L. fungicola*

Malachite green is an important reagent in selective medium. This reagent eliminated the growth of competitive fungi (Rinker 1993) but also inhibited growth of *L. fungicola*. The objective of this experiment was to compare the effect of different concentrations of malachite green in PDA medium with different fungicides. The PDA medium containing 100 mg/l carbendazim or 1 mg/l prochloraz-Mn. When PDA contained 100 mg/l carbendazim and 5, 7.5 or 10 mg/l malachite green the radius of growth of *L. fungicola* was 0.75 cm with higher conidia concentration 4.90×10^5 conidia per 5 µl. When conidia concentration was 4.90×10^3 conidia per 5 µl the radius

of growth was decreased to 0.5-0.6 cm. However in PDA medium containing 1 mg/l prochloraz-manganese and 5 mg/l malachite green the radius of growth of *L. fungicola* was 0.7 cm with higher conidia concentration (4.90×10^5 conidia per 5 µl) but when conidia concentration was 4.90×10^3 conidia per 5 µl the radius of growth was 0.5 cm. The growth of *L. fungicola* decreased when the concentration of malachite green increased to 7.5 or 10 mg/l and the radius of colony growth was 0.55-0.6 cm when the concentration of conidia was 4.90×10^5 conidia per 5 µl and 0.35-0.4 cm concentration of conidia was 4.90×10^3 conidia per 5 µl. When the concentration of malachite green increased to 7.5 or 10 mg/l, radius of colony growth decreased to 0.05 cm for both conidia concentrations of *L. fungicola* (**Figure** 3-12 and **Figure** 3-14).

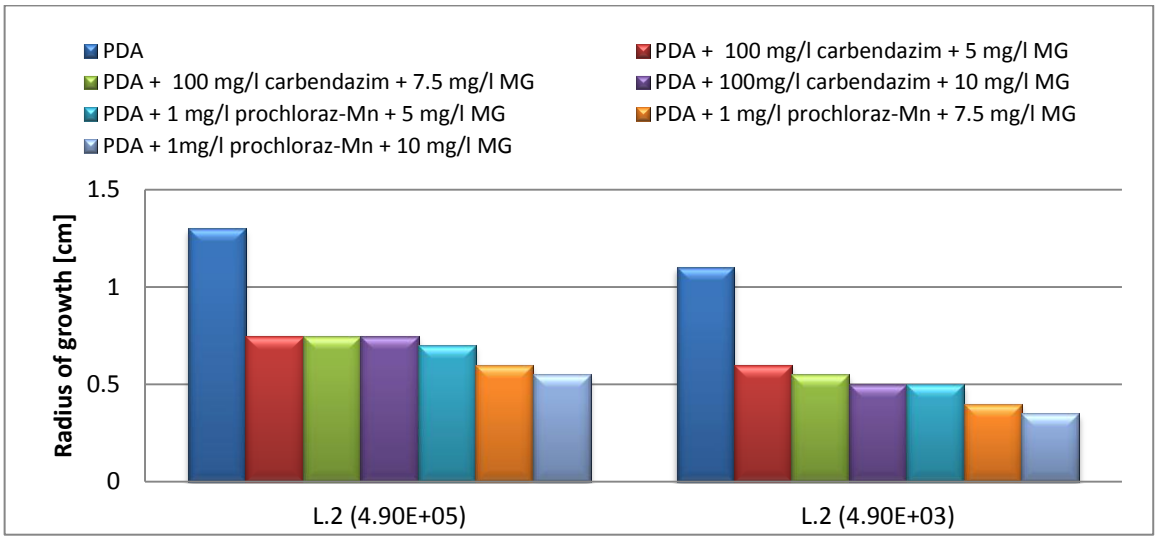


Figure 3-12: *In vitro* response of *L. fungicola* in PDA medium with different concentrations of malachite green and with different fungicides. Conidia concentration applied per drop. After 7 days at 23 °C. Conidia concentration per 5 µl, one drop.

The percentage growth of *L. fungicola* in PDA medium containing 100 mg/l carbendazim and 5, 7.5 or 10 mg/l malachite green was 58 %, when conidia concentration was 4.90×10^5 conidia per 5 µl but when conidia concentration was 4.90×10^3 conidia per 5 µl growth of *L. fungicola* was 45-55 %. In PDA medium, containing 1 mg/l prochloraz-manganese and 5 mg/l malachite green growth of *L. fungicola* was 54 % of control growth and decreased when concentration of malachite green increased to 7.5 or 10 mg/l. Colony growth was 46 and 42 % of control growth when conidia concentration was 4.90×10^5 conidia per 5 µl. Lower conidia concentration of 4.90×10^3 conidia per 5 µl the growth of *L. fungicola* was 31-36 %. *Lecanicillium fungicola* showed 57 to 31 % of growth when concentration of malachite green was between 5-10 mg/l.

3.1.3.2 Other common fungi and their response to different concentrations of malachite green

The next series of experiments was to test susceptibility of other fungi to different concentrations of malachite green and two fungicides. Two different media were tested: PDA with 100mg/l carbendazim and PDA with 1 mg/l prochloraz-manganese with different concentrations of malachite green 5, 7.5 and 10 mg/l. Two different conidia concentrations were tested for each fungus (**Figure** 3-13). *Cladobotryum mycophilum* (D.1) grew well on PDA control medium without malachite green and fungicides in both conidia concentration but the growth was not present in any conidia concentration and in media containing a malachite green and one of the tested fungicides (**Figure** 3-13). *Mycogone perniciosa* (M.1) grew well on PDA control medium without malachite green and fungicides in higer conidia concentrations, the lower conidia concentrations did not show growth. This fungus showed some growth in all tested media when conidia concentration was 5×10^3 and 5×10 per 5 µl. The radius of growth of *M. perniciosa* was 0.15-0.6 cm depending on conidia concentration and concentration of malachite green. *Aspergilus fumigatus* (As.) grew well on PDA control medium without malachite green and fungicides. Small growth was present in PDA medium containing 100 mg/l carbendazim or 1 mg/l prochloraz-manganese and concentrations of malachite green was 5 and 7.5 mg/l malachite green and conidia concentration was 2.05×10^5. When conidia concentration was 2.05×10^3 the *A. fumigatus* did not grow. The radius of growth of *A. fumigatus* was very small and growth of this fungus was nearly stopped in higher conidia concentrations (**Figure** 3-13 and **Figure** 3-14).

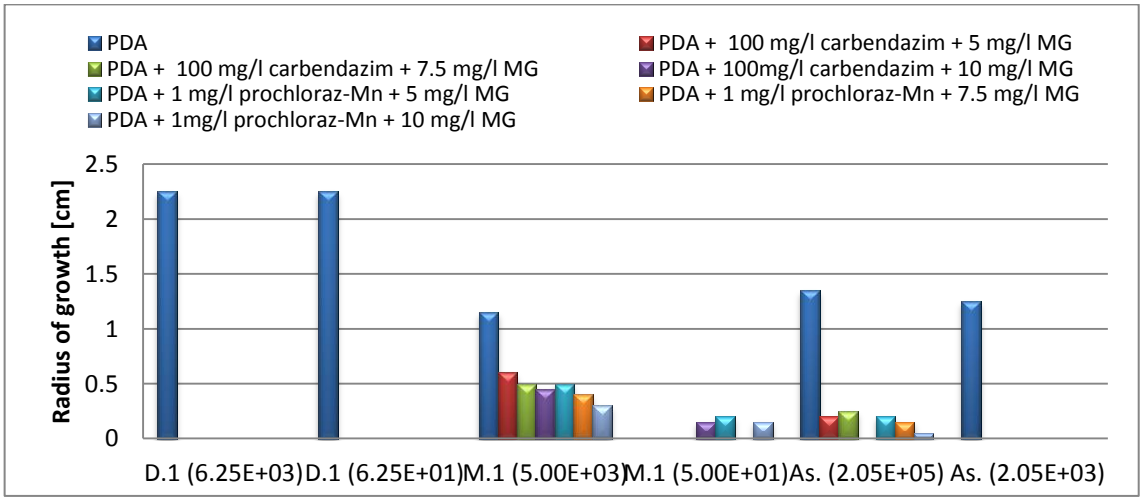


Figure 3-13: *In vitro* response of *L. fungicola* and other fungi at different concentrations in PDA medium with different concentrations of malachite green (MG) and two fungicides after 7 days incubation at 23 °C. *Cladobotryum mycophilum* (D.1), *Mycogone perniciosa* (M.1), *Aspergilus fumigatus* (As.), conidia concentration per 5 µl.

Isolate	Conidia concentration per plate	PDA (control)	Carbendazim 100 mg/l			Prochloraz-manganese 1 mg/l		
			5 mg/l MG	7.5 mg/l MG	10 mg/l MG	5 mg/l MG	7.5 mg/l MG	10 mg/l MG
L.2	4.90×10^5							
L.2	4.90×10^3							
D.1	6.25×10^3							
D.1	6.25×10^1							
M.1	5.0×10^3							
M.1	5.0×10^1							
As.	2.05×10^5							
As.	2.05×10^3							

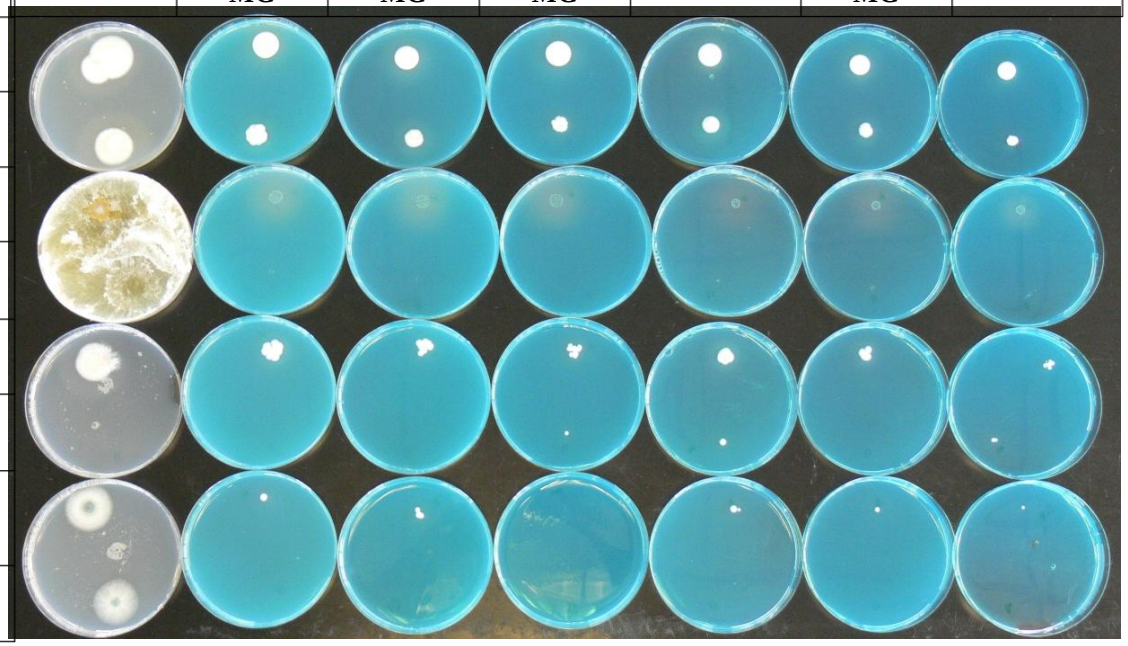


Figure 3-14: *In vitro* response of *L. fungicola* (L.2) and other fungi in PDA medium with different concentration malachite green (MG) and two fungicides after 7 days incubation at 23 °C. *Cladobotryum mycophilum* (D.1), *Mycogone perniciosa* (M.1), *Aspergilus fumigatus* (As.).

3.1.3.3 Inhibition of other fungi without malachite green

The next study of novel and modified medium was to check if it was possible to design a medium without malachite green. Malachite green is a good anti-fungal salt but inhibits the growth of *L. fungicola* as well. In this experiment different fungi at different conidia concentrations were tested. In PDA control medium all tested fungi grew very well (**Figure** 3-15).

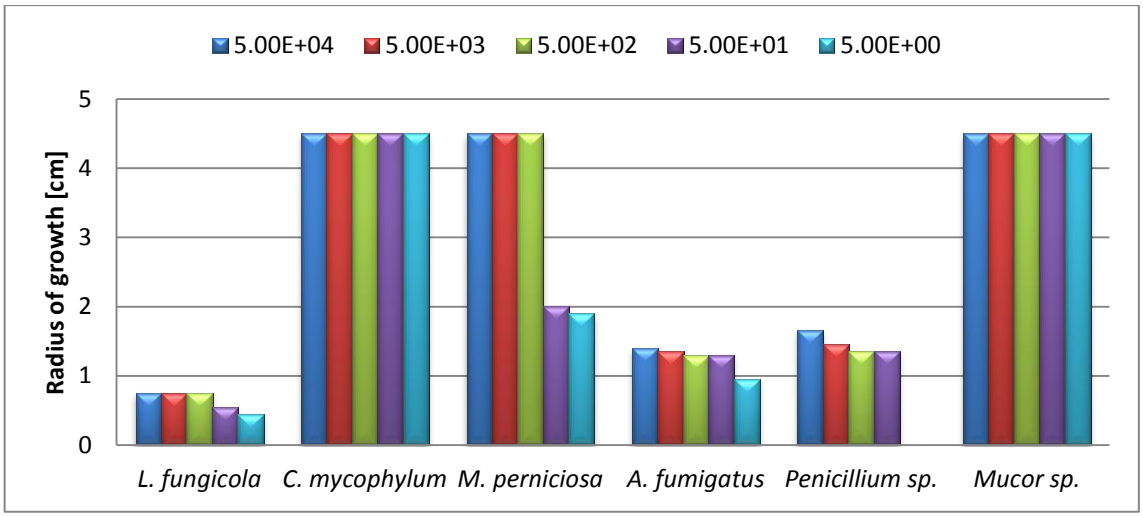


Figure 3-15: *In vitro* response of different conidia concentrations of *L. fungicola* and other fungi on PDA medium condia concentration per 5 µl.

When PDA medium contained two fungicides: prochloraz-manganese – 1 mg/l and carbendazim – 100 mg/l, *L. fungicola* showed growth when conidia concentration was 50 conidia per 5 µl drop and growth was 72 % of control growth in PDA control medium. However in this medium *C. mycophilum* also grew. *Mucor* sp. as a fast growing fungus covered all the plate after 7 days (**Figure** 3-16).

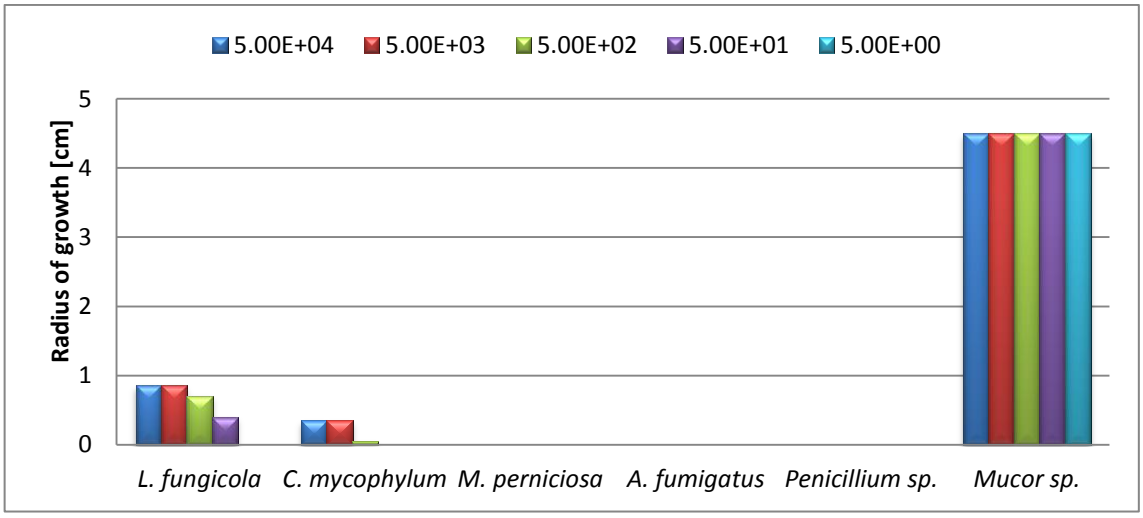


Figure 3-16: *In vitro* response of other fungi with different conidia concentrations on PDA with 1 mg/l prochloraz-Mn and 100 mg/l carbendazim after 7 days at 23 °C, condia concentration per 5 µl.

When concentration of carbendazim was increased to 500 mg/l, *L. fungicola* showed growth and growth of colonies was the same when concentration of carbendazim was 100 mg/l. Higher concentrations of carbendazim did not significantly inhibit growth of *C. mycophilum* and *Mucor* sp. Both of these fungi showed same growth when concentration of carbendazim was 100 mg/l (**Figure** 3-17). Media without malachite green inhibited growth of *C. mycophilum* but did not stop the growth of fast growing fungi such as *Mucor sp.*

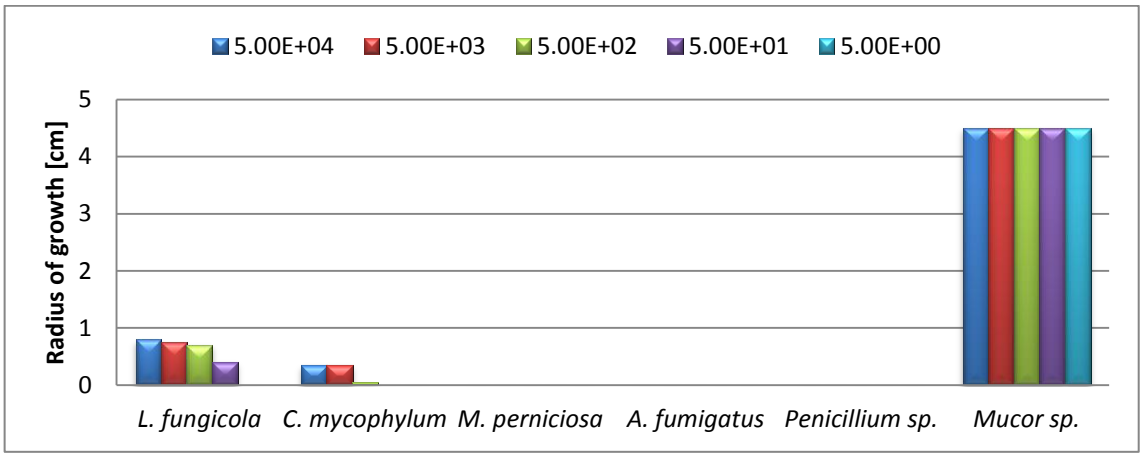


Figure 3-17: *In vitro* response of other fungi with different conidia concentrations on PDA with 1 mg/l prochloraz-Mn and 500 mg/l carbendazim after 7 days at 23 °C, condia concentration per 5 µl.

The selective medium must contain malachite green to stop growth of other fast growing fungi such as *Mucor* sp. The next part of this experiment was to test PDA medium and Rinker's base medium with malachite green. The tested medium consisted of PDA, 5 mg/l malachite green, 1mg/l prochloraz-manganese and 100 mg/l carbendazim. On this medium only *L. fungicola* grew. Other tested fungi were inhibited (**Figure** 3-18).

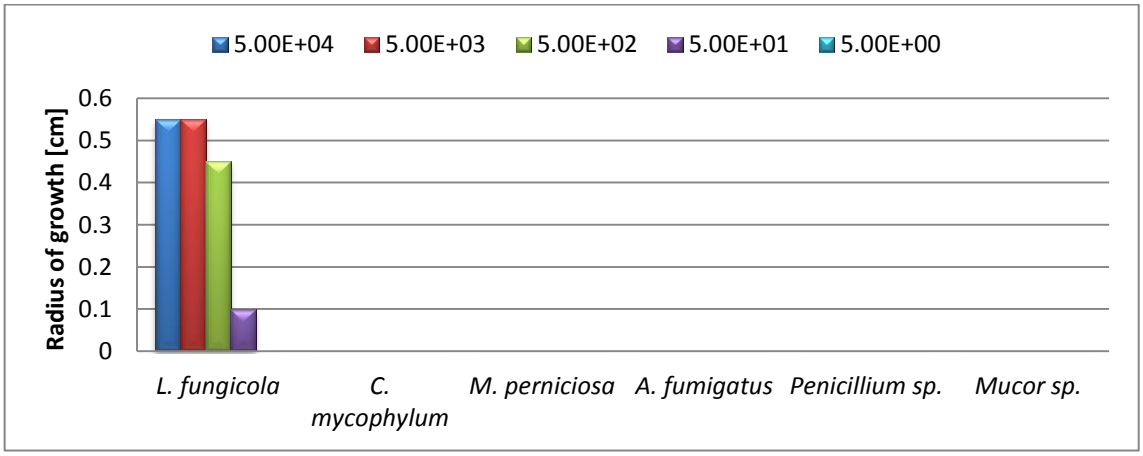


Figure 3-18: *In vitro* response of *L. fungicola* and other fungi with different conidia concentrations on PDA with 5 mg/l malachite green and 1 mg/l prochloraz-Mn and 100 mg/l carbendazim after 7 days at 23 °C, condia concentration per 5 µl.

When concentration of carbendazim was increased 5 times *L. fungicola* showed the same response when concentration of carbendazim was 100 mg/l. Other tested fungi were also inhibited (**Figure** 3-19).

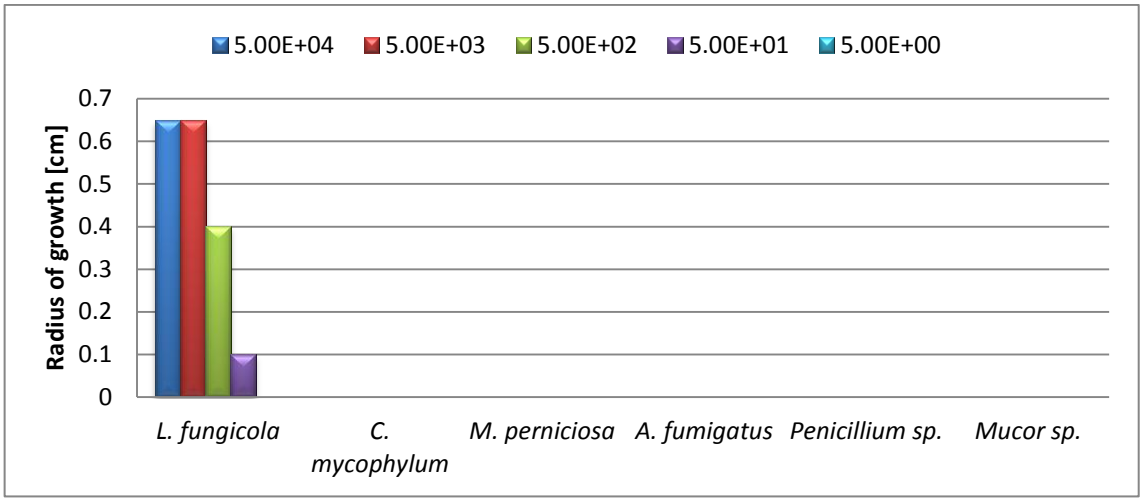


Figure 3-19: *In vitro* response of *L. fungicola* and other fungi with different conidia concnetrations on PDA with 5 mg/l malachite green and 1 mg/l prochloraz-Mn and 500 mg/l carbendazim after 7 days at 23 °C, condia concentration per 5 µl.

The Rinker's base medium consisted of 5 mg/l malachite green, 1 mg/l prochloraz and 100 mg/l carbendazim. In this medium *L. fungicola* colonies grew as well but the size of colonies was smaller than in PDA medium with 5 mg/l malachite green, 1 mg/l prochloraz and 100 mg/l carbendazim (**Figure** 3-20).

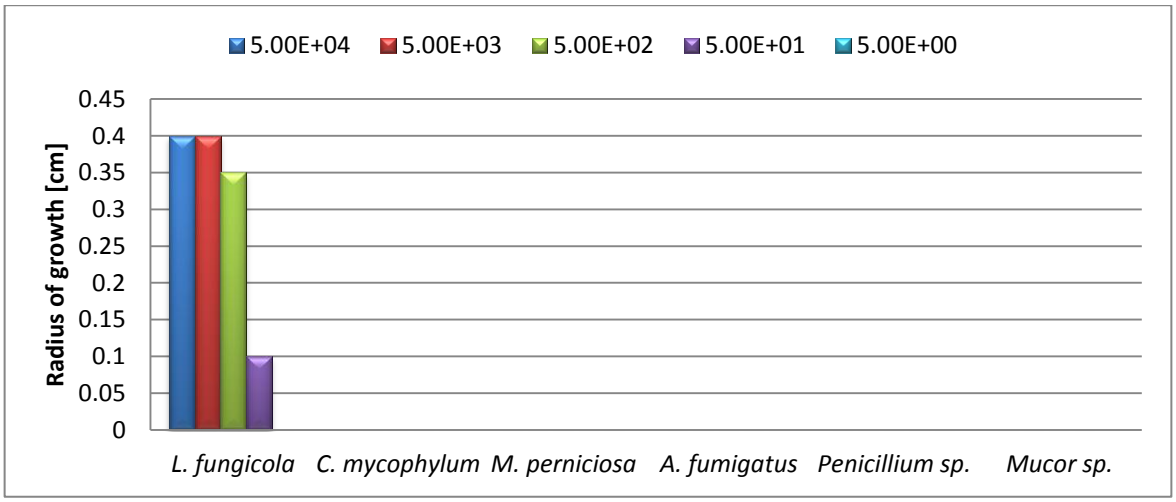


Figure 3-20: *In vitro* response of *L. fungicola* and other fungi with different conidia concentrations on RBM with 5 mg/l malachite green and 1 mg/l prochloraz-Mn and 500 mg/l carbendazim after 7 days at 23 °C, condia concentration per 5 µl.

All data is presented in **Figure** 3-21 and **Figure** 3-22.

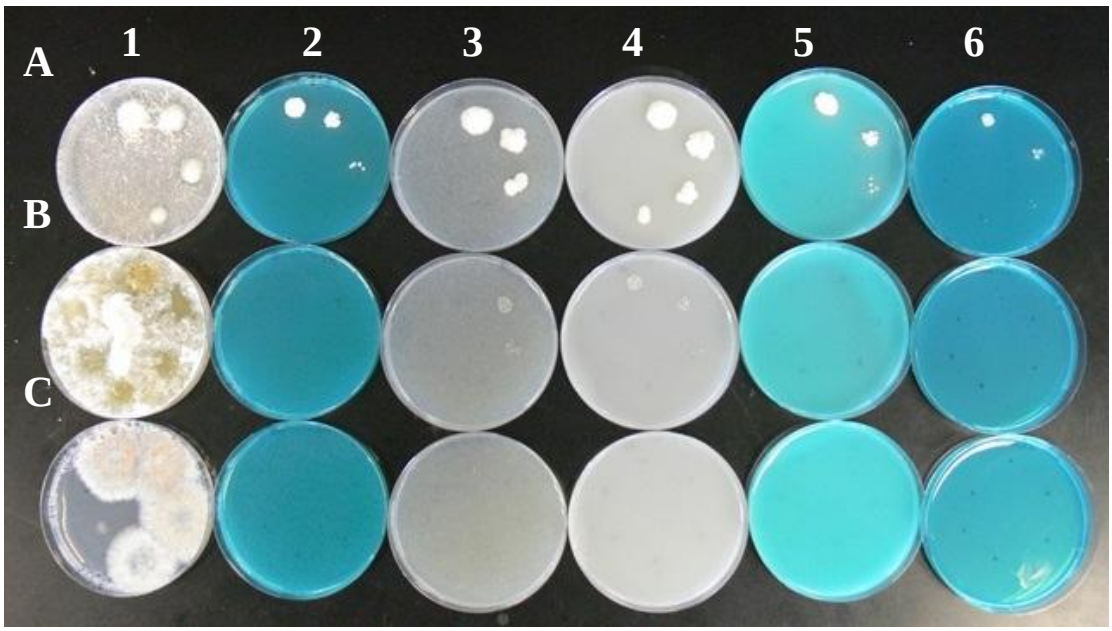


Figure 3-21: *In vitro* response of A – *L. fungicola;* B – *C. mycophilum*; C – *M. perniciosa* on different media. After 7 days.

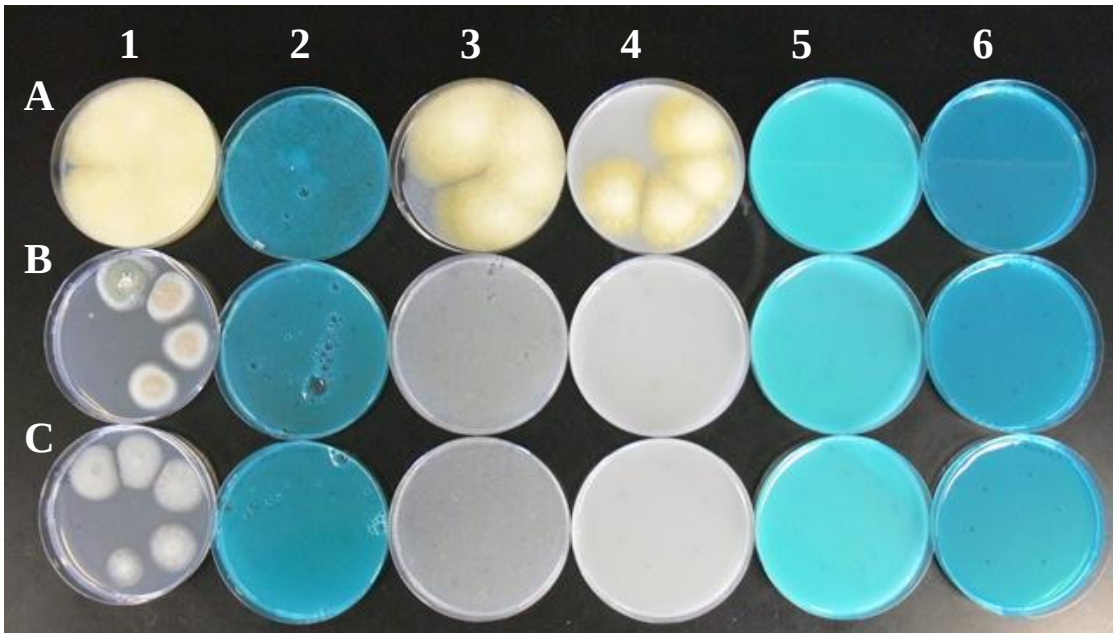


Figure 3-22: *In vitro* response of A – *Mucor* sp.; B – *Penicillium* sp.; C – *A. fumigatus* on different media. After 7 days.

Legend (**Figure** 3-21 and **Figure** 3-22)

1. PDA
2. PDA + 5mg/l MG + 1mg/l Prochloraz-Mn + 100mg/l carbendazim
3. PDA + 1mg/l Prochloraz-Mn + 100mg/l carbendazim
4. PDA + 1mg/l Prochloraz-Mn + 500mg/l carbendazim
5. PDA + 5mg/l MG + 1mg/l Prochloraz-Mn + 500 mg/l carbendazim
6. RBM + 5mg/l MG + 1mg/l Prochloraz-Mn + 100 mg/l carbendazim

The selective medium was also tested for common pathogens of mushroom compost *Trichoderma aggressivum* type 2 and *Trichoderma atroviride* type 3 which are

fast growing fungi and inhibition of their growth is very important. The *T. aggressivum* and *T. atroviride* after 3 days incubation at 23 °C covered all plates when the medium was PDA without malachite green and fungicides (data not shown). Growth was measured on PDAPCMG10 and RBMPCMG10 medium. On each medium only *L. fungicola* grew and growth of *Trichoderma* was inhibited (**Figure** 3-23). In PDAPCMG10 medium colonies of *L. fungicola* had a white colour but on RBMPCMG10 colonies were transparent.

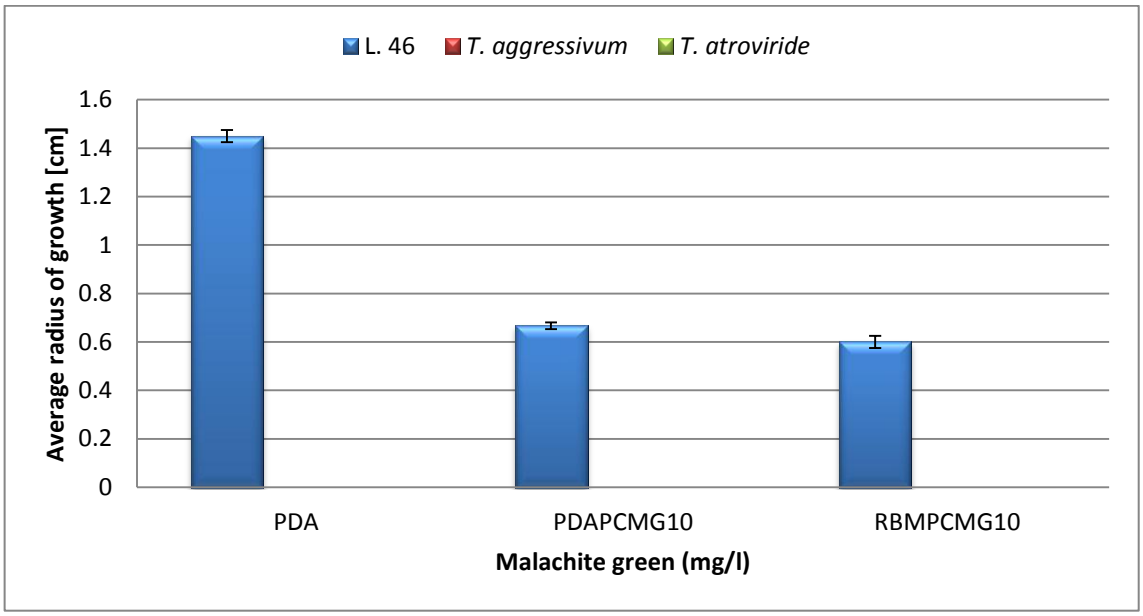


Figure 3-23: *In vitro* response of *L. fungicola* (L. 46) and two isolates of *Trichoderm*a Th2 and Th3 on PDAPCMG and RBMPCMG after 7 days. Agar plug. Standard error is shown.

3.1.4 Examination of effects of different antibiotics on growth of *L. fungicola*

3.1.4.1 Different antibiotics and concentration of antibiotics

Bacterial and yeast populations are very common in casing and soil. The bacterial population in casing is between 8.2 and 8.5 log CFU per gram casing and 6.7 log CFU (colony formation unite) of yeast per gram casing (Chikthimmah *et al.*, 2008). It is important to eliminate any possible nutrient competition between bacteria and yeast and *L. fungicola* (Rinker *et al.*, 1993). The control medium contained: PDA with 5 mg/l malachite green 100 mg/l carbendazim, 1 mg/l prochloraz-manganese (PDAPCMG5) without antibiotics. The tested medium contained PDAPCMG5 and different antibiotics: ampicillin (A), chloramphenicol (Ch) (only 100 mg/l), erythromycin (E) streptomycin (S) and tetracycline (T) with different concentrations 100, 500 and 1,000

mg/l. *L. fungicola* was not sensitive to any antibiotic that was tested. At all tested concentrations of antibiotics *L. fungicola* grew well and colonies had a radius 0.35-0.45 cm (77 to 112 % of control growth) for agar plug and 0.45-0.6 cm (81 to 120 % of control growth) for 5×10^3 conidia per 5 µl drop.

The next part of the study was to check sensitivity of bacteria/yeast isolated from casing soil and bacteria/yeast contained in fresh casing soil extract. The streptomycin salt (1,000 mg/l) showed some inhibition of bacteria/yeast growth. The highest concentration of tetracycline (1,000 mg/l) repressed growth of bacteria contained in casing extract soil. Only tetracycline showed some positive inhibition of bacteria/yeast from casing soil extract, but bacteria/yeast isolated from casing still grew (**Table** 3-3).

Table 3-3: Effectiveness of different concentrations of different antibiotics (100, 500 and 1,000 mg/l) against bacteria/yeast and fresh casing extract solution in PDAPCMG5 media. The radius of growth came from 5 µl drop for one replication is shown (mm).

Media (PDAPCMG5) and antibiotics	**Solutions tested**	**without antibiotics**	**100 mg/l**	**500 mg/l**	**1,000 mg/l**
PDAPCMG5	Bacteria/yeast	9.5	n/a	n/a	n/a
	Fresh casing extract	5	n/a	n/a	n/a
Ampicillin	Bacteria/yeast	n/a	10.5	0.92	6.7
	Fresh casing extract	n/a	5	0.5	5
Chloramphenicol	Bacteria/yeast	n/a	8.8	nt	nt
	Fresh casing extract	n/a	5	nt	nt
Erythromycin	Bacteria/yeast	n/a	13.3	10.8	7.7
	Fresh casing extract	n/a	4.7	5	5
Streptomycin	Bacteria/yeast	n/a	8.3	7.8	4.7
	Fresh casing extract	n/a	4.7	5	4.3
Tetracycline	Bacteria/yeast	n/a	13.2	13.3	11.7
	Fresh casing extract	n/a	3.2	1.3	0

The values are indicated by average radius of growth (mm); n/a – not available for test, nt – not tested.

3.1.4.2 Effect of two different antibiotics on growth of *L. fungicola*

The next part of the study was to combine two antibiotics and examine bacteria/yeast from fresh casing solution. The control medium contained: PDA with 5 mg/l malachite green 100 mg/l carbendazim and 1 mg/l prochloraz-manganese (PDAPCMG5). The test medium contained PDAPCMG5 and two different antibiotics and different concentrations: ampicillin (A), chloramphenicol (Ch) (only 100 mg/l), erythromycin (E) streptomycin (S) and tetracycline (T) at 100, 500, 1,000 mg/l. Lecanicillium fungicola did not show resistance to any tested antibiotics and grew well in all concentrations of both antibiotics. At all tested concentrations of antibiotics L. fungicola grew well and growth had a radius of 0.35 to 0.55 cm (77 to 122 % of control growth) for agar plug and 0.4 to 0.6 cm (72 to 120 % of control growth) for 5×10^3 conidia 5 µl drop.

The bacteria and yeast isolated from casing soil and bacteria and yeast contained in casing soil extract were prevented from growing when the concentration of antibiotics was: streptomycin 500mg/l and ampicillin 1,000 mg/l, streptomycin 1,000 mg/l and ampicillin 1,000 mg/l, streptomycin 100 mg/l and tetracycline 100 mg/l, streptomycin 100 mg/l and tetracycline 500 mg/l, streptomycin 100 mg/l and tetracycline 1,000 mg/l, streptomycin 500 mg/l and tetracycline 100 mg/l, streptomycin 500 mg/l and tetracycline 500 mg/l, streptomycin 1,000 mg/l and tetracycline 500 mg/l, streptomycin 1,000 mg/l and tetracycline 1,000 mg/l. All concentrations of streptomycin and tetracycline stopped growth of isolated bacteria/yeast and bacteria/yeast from casing soil extract (**Table** 3-4 and **Figure** 3-24).

Table 3-4: Effectiveness of different concentrations of two different antibiotics (100, 500 and 1,000 mg/l) against bacteria/yeast and fresh casing extract solution in PDAPCMG5 media. The radius of growth came from 5 µl drop for one replication is shown (mm).

		Streptomycin (mg/l)							
Antibiotic added to PDAPCMG5	Antibiotic con. (mg/l) /Solutions tested	0		100		500		1 000	
		Bacteria /yeast	Fresh casing extract	Bacteria/ yeast	Fresh casing extract	Bacteria/ yeast	Fresh casing extract	Bacteria/ yeast	Fresh casing extract
Ampicillin	0	9.5	5	n/a	n/a	n/a	n/a	n/a	n/a
	100	n/a	n/a	9	4.5	7.7	4.7	4.5	4
	500	n/a	n/a	9	4.5	6.5	4.5	4.5	1.2
	1000	n/a	n/a	4.5	1	4.5	0	4.5	0
Chloramphenicol	0	9.5	5	n/a	n/a	n/a	n/a	n/a	n/a
	100	n/a	n/a	7.3	5.2	4.8	4.8	4.3	4.3
Tetracycline	0	9.5	5	n/a	n/a	n/a	n/a	n/a	n/a
	100	n/a	n/a	0*	0	0*	0*	0*	0
	500	n/a	n/a	0*	0	0*	0	0*	0
	1,000	n/a	n/a	0*	0	0*	0	0*	0

* – fungal growth was observed the radius was 0.5-1 mm. The values are indicated by average radius of growth (mm); n/a – not available for test, nt – not tested.

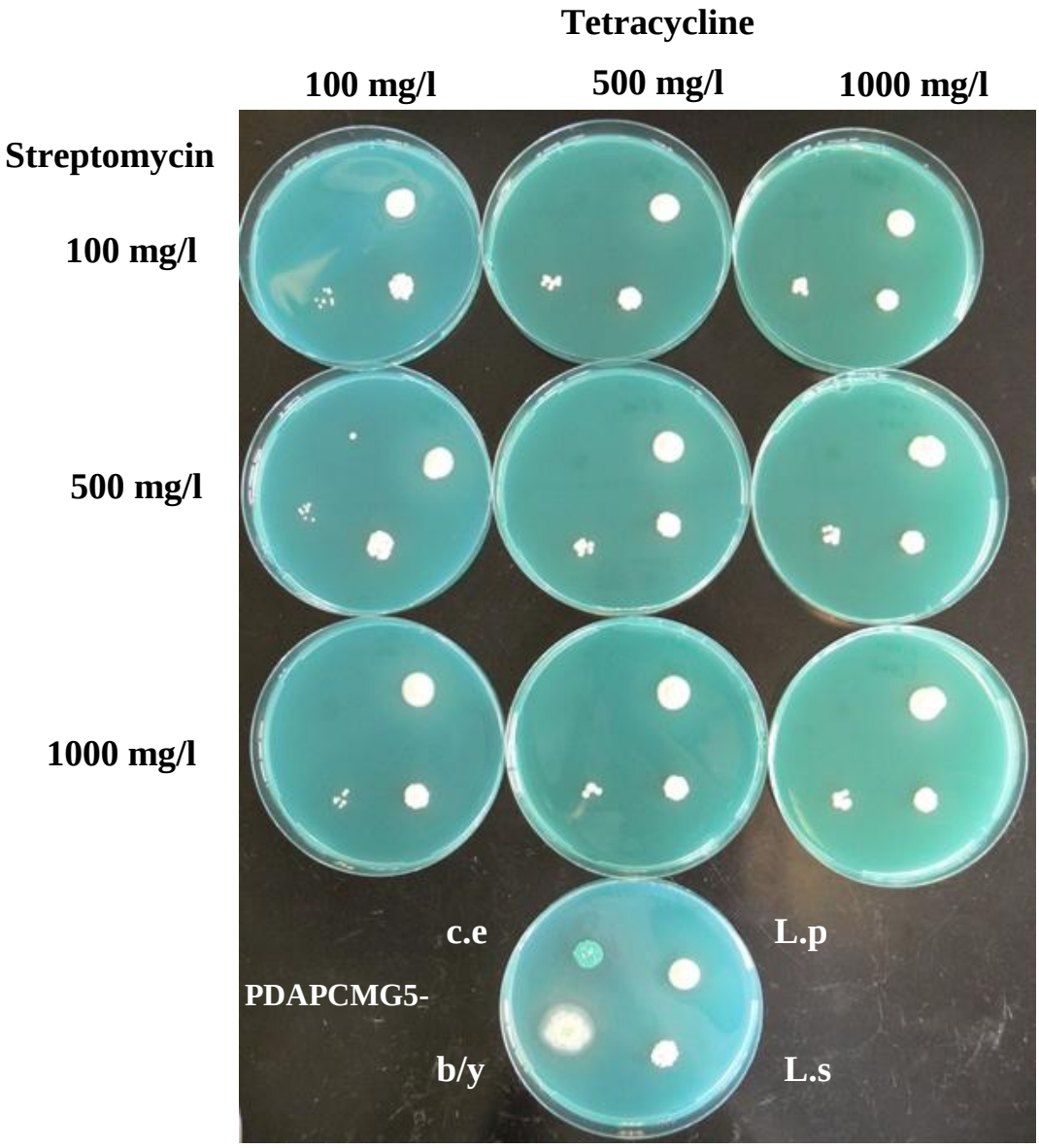


Figure 3-24: *In vitro* response of *L. fungicola* (L.2) on agar plug (L.p) and 5×10^3 conidia per 5 µl drop (L.s), bacteria/yeast (b/y) and casing extract (c.e). PDA control medium contain PDAPCMG5 as a control and with different concentrations of two antibiotics: tetracycline and streptomycin (100, 500 and 10,000 mg/l). In some samples with b/y solution fungal growth was observed. After 6 days on 23°C in dark.

The two antibiotics streptomycin and tetracycline which gave satisfactory results were used in the next part of the experiment. In this experiment *L. fungicola* conidia was mixed with extract casing soil. The control medium contained: PDA with 5 mg/l malachite green 100 mg/l carbendazim, 1 mg/l prochloraz-manganese (PDAPCMG5). The tested medium contained PDAPCMG5 and different concentrations of streptomycin and teteracycline (100, 500 and 1,000 mg/l). On PDAPCMG5 – control medium there was growth of lots of bacteria/yeast (around 200 colonies). When PDAPCMG5 medium contained 100 mg/l of both antibiotics some yeast grew on medium also. Few colonies of yeast grew when concentration of antibiotics was 500 mg/l streptomycin and 100 mg/l tetracycine and 1,000 mg/l streptomycin and 100 mg/l tetracycline. When concentration of teteraycyline was increased to 500 mg/l and concentration of streptomycin was 100 mg/l yeast did not grow and this antibiotic combination gave best result for inhibition of growth of competition organisms (**Figure** 3-25**).**

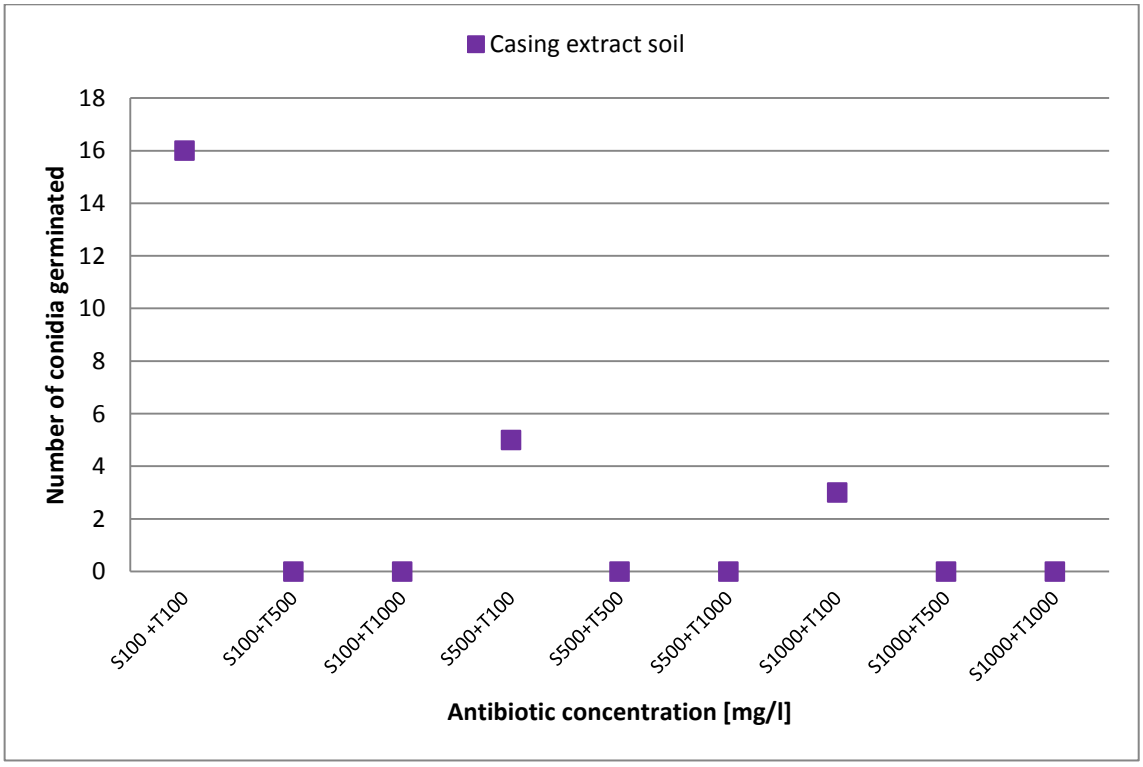


Figure 3-25: Effectiveness of different concentrations of 100, 500, 1,000 mg/l of two antibiotics: Streptomycin (S) and Tetracycline (T) against casing extract soil in PDA medium. PDAPCMG5 – contain PDA + 5mg/l MG + 100mg/l carbendazim + 1mg/l prochloraz-Mn, after 6 days.

The *L. fungicola* (1×10^5 conidia per 100 µl) conidia were mixed with fresh casing extract and 100 µl was spread. The amount of colonies growing on PDAPCMG5 – control medium had 49 colonies and 60 to 112 colonies were present on other media combinations and was very similar. The size of *L. fungicola* colonies was 0.3 to 0.4 cm (75 to 84 % of control growth) when concentration of steptomycin and tetracycline were 100 mg/l of each antibiotic These antibiotic concentrations inhibited growth of almost all yeast but still a few colonies of yeast were able to grow (**Figure** 3-27).

When concentration of antibiotics was 100 mg/l streptomycin and 500 mg/l tetacycline, yeast stopped growing and this combination of antibiotics gave very good results (**Figure** 3-25) and the radius of growth of *L. fungicola* colonies was 0.25 to 0.3 cm (55-68 % of control). In other tested concentrations of antibiotics *L. fungicola* radius of colonies was 0.2 to 0.3 cm (44-68 % of control growth) (**Figure** 3-26).

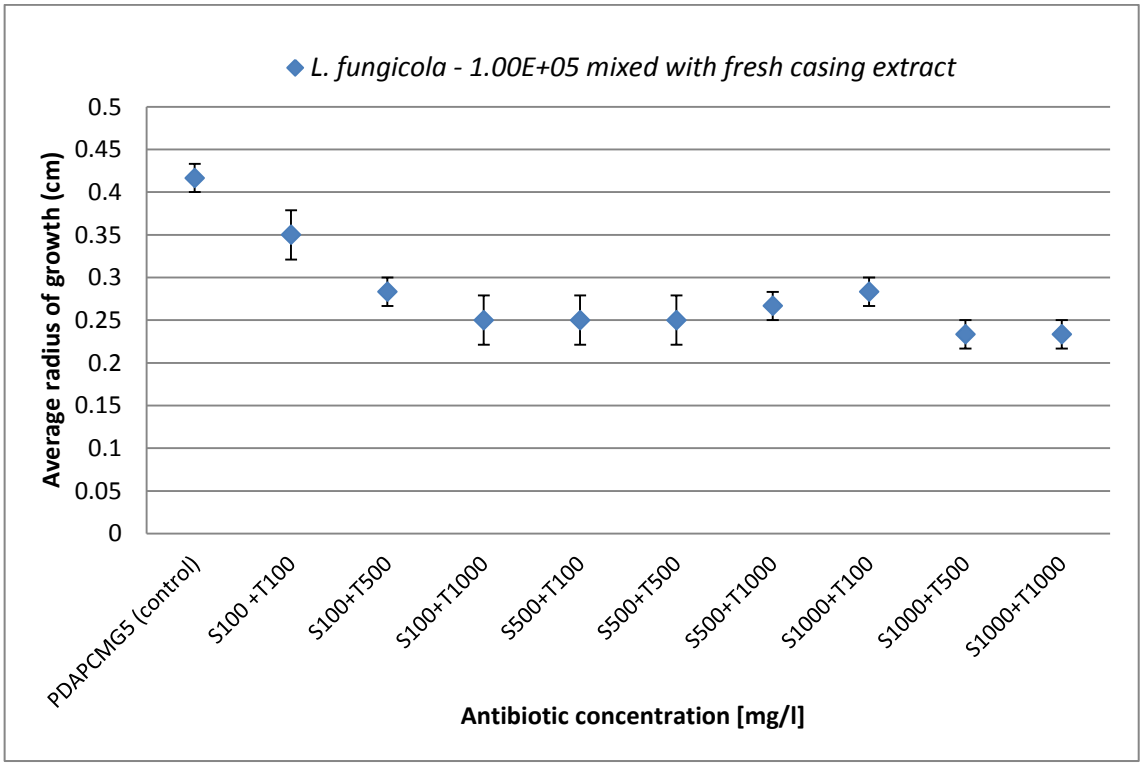


Figure 3-26: *In vitro* response of *L. fungicola* to two antibiotics: Streptomycin (S) and Tetracycline (T) on different concentration 100, 500, 1,000 mg/l in PDAPCMG5 medium contain PDA + 5mg/l MG + 100mg/l carbendazim + 1mg/l prochloraz-Mn, after 6 days. Standard error is shown.

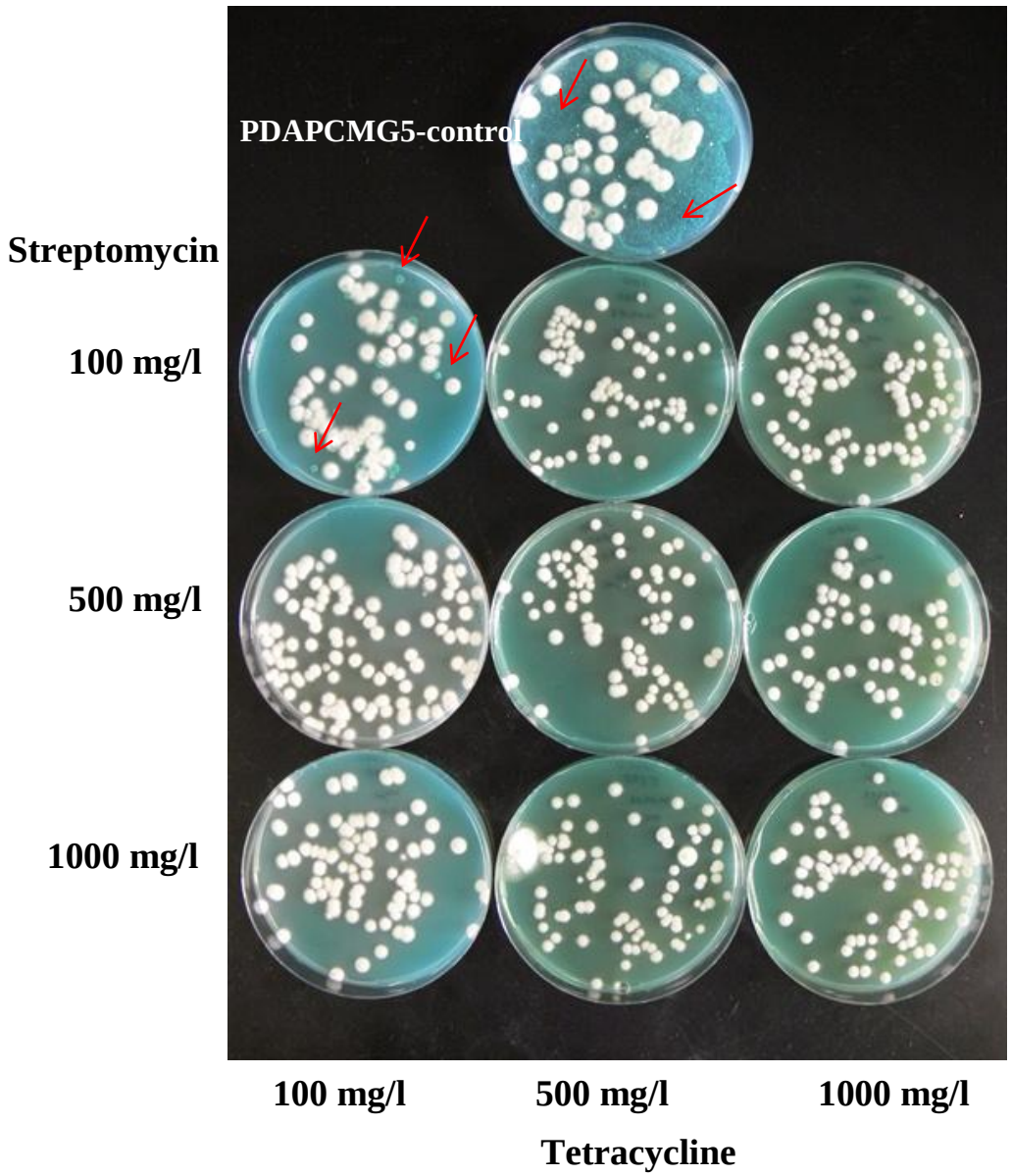


Figure 3-27: *In vitro* response of *L. fungicola* to two antibiotics: Streptomycin (S) and Tetracycline (T) on different concentration 100, 500, 1,000 mg/l PDAPCMG5 medium. PDAPCMG5 – control medium contain PDA + 5mg/l MG + 100mg/l carbendazim + 1mg/l prochloraz-Mn, after 6 days incubation. (Red arrows indicate bacteria/yeast colonies. The plate contained PDAMGCP-contol medium was covered for bacteria/yeasts). After 6 days.

3.1.5 The PDAPCMG5ST selective medium test for different conidia concentration of *L. fungicola*

On PDAPCMG5ST medium *L. fungicola* grew well when conidia concentration per 5 µl was 4.48×10^5, 5×10^4, 5×10^3 and 5×10^2. Smaller concentrations of *L. fungicola* 5 and 5×10^1 conidia per 5 µl drop did not grow (**Figure** 3-28).

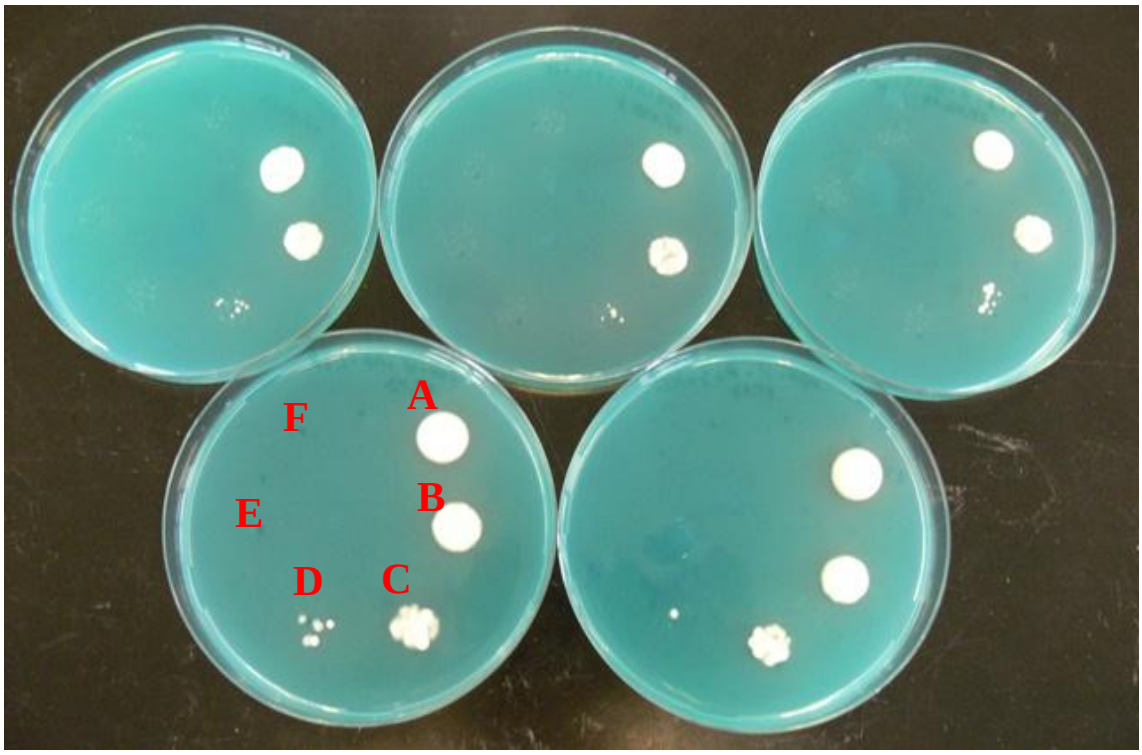


Figure 3-28: Response *of L. fungicola* in PDAPCMG5ST medium contain PDA + 5 mg/l malachite green and 100 mg/l streptomycin and 500 mg/l tetracycline, 1 mg/l prochloraz-manganese and 100 mg/l carbendazim after 6 days.

A. 4.48×10^5 conidia per 5 µl (8.95×10^7 conidia/ml)
B. 5×10^4 conidia per 5 µl (1×10^7 conidia/ml)
C. 5×10^3 conidia per 5 µl (1×10^6 conidia/ml)
D. 5×10^2 conidia per 5 µl (1×10^5 conidia/ml)
E. 5×10^1 conidia per 5 µl (1×10^4 conidia/ml)
F. 5 conidia per 5 µl (1×10^3 conidia/ml)

3.1.5.1 Response of *L. fungicola* and other fungi to modified Rinkers' selective medium (RBMPCMGST)with different concentrations of malachite green compared to novel selective medium

Rinker's orginal medium was modified and tested for *L. fungicola*, *Mucor*, *Penicillium*, casing soil extract and an unknown fungus isolated from casing soil extract. (It was difficult to identify this fungus because conidia were not present). Modified Rinker's medium contained: RBM + 1 mg/l prochloraz-Mn, 100 mg/l carbendazim, 100 mg/l streptomycin, 500 mg/l tetracycline, (RBMPCMGST) and different concentrations of malachite green (10, 20 and 30 mg/l). *Lecanicillium fungicola* grew very well in all tested concentrations of malachite green. The radius of growth of agar plug of *L. fungicola* was 0.23 cm on RBMPCMG10ST and RBMPCMG20ST and a little decreased when concentration of malachite green was 30 mg/l and was 0.2 cm. The colonies radius of growth of *L. fungicola* was 0.5 cm when concentration of malachite green was 10 and 30 mg/l. The radius of growth was smaller when concentrations of malachite green was 20mg/l and was 0.45 cm. Other fungi such as *Penicillium* sp. and the unknown fungus were inhibited when concentration of malachite green was 30 mg/l. Compared to the growth of *L. fungicola* on designed PDAPCMG10STB medium (PDA + 1 mg/l prochloraz-Mn, 100 mg/l carbendazim, 100mg/l streptomycin, 500 mg/l tetracycline and 10 mg/l malachite green and 30 mg/l bromocresol green) the radius was much larger than in RBMPCMG10ST. However other fungi such as *Penicillium* sp. and the unknown fungus grew as well in PDAPCMG10STB and RBMPCMGST with all tested concentrations of malachite green (10, 20 and 30 mg/l). *Penicillium* sp. showed growth in PDAPCMG10STB as well as RBMPCMG10ST and RBMPCMG20ST. Unknown fungus showed the highest growth when medium was PDAPCMG10STB, but on RBMPCMG10ST, RBMPCMG20ST growth was high too, only on RBMPCMG30ST in medium the growth was inhibited by half (**Figure** 3-29).

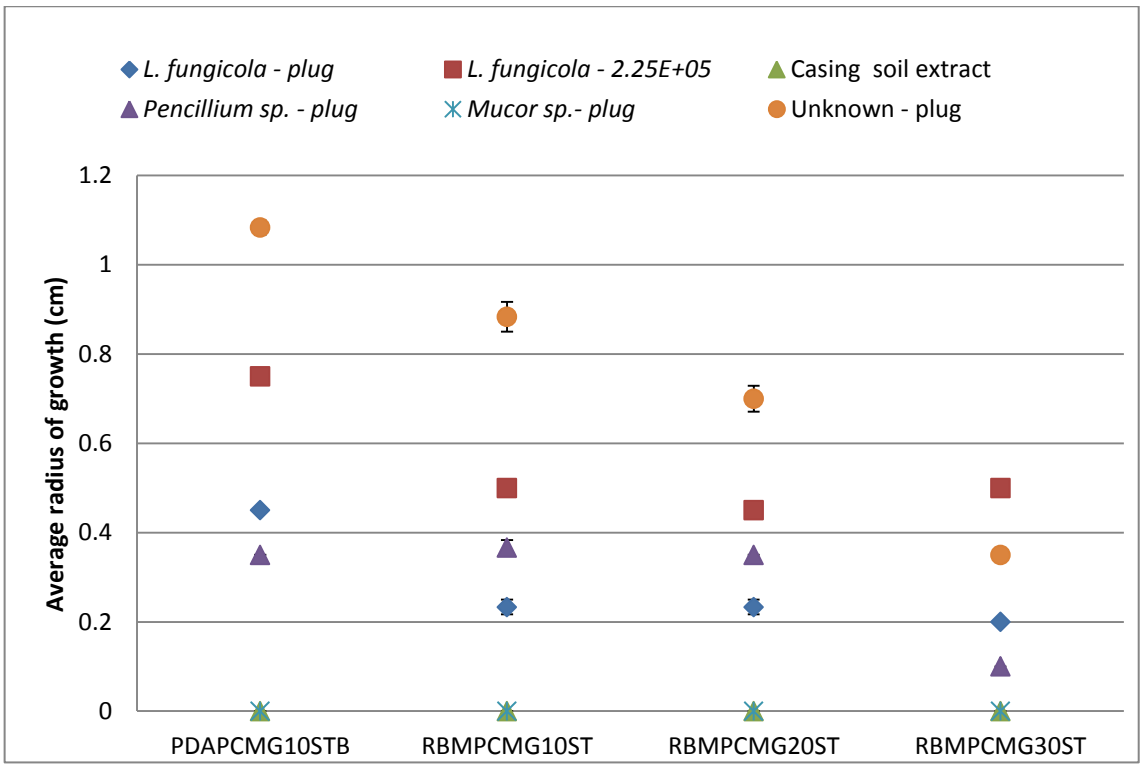


Figure 3-29: *In vitro* response *of L. fungicola* and different fungi present in casing soil on PDAPCMG10STB and RBMPCMGST with different concentrations of malachite green (10, 20 and 30 mg/l) after 7 days. Standard error is shown.

3.1.5.2 Summary

All wild isolates of *L. fungicola* from mushroom farms were identified as *L. fungicola* var. *fungicola* and showed similar response to prochloraz-manganese and carbendazim.

Malachite green is a significant reagent in selective medium. When the concentration of malachite green was too high, growth of *L. fungicola* was inhibited. Lower concentrations of malachite green showed slight inhibition of growth of *L. fungicola*, but decreasing the malachite concentration could allow growth of other fungi. *Lecanicillium fungicola* grew very well when the concentration of malachite green was between 5-10 mg/l and colonies were easy to spot after 7 days of incubation. When medium contained only PDA other tested fungi such as *C. mycophilum*, *M. perniciosa*, *A. fumigatus* and *Trichoderma* sp. grew well, but when PDA contained some amount of malachite green and fungicides, growth of these tested fungi was inhibited. *M. perniciosa* showed slight growth in all tested concentrations of malachite green when in media. Next tested combination of reagent used on selective medium was made of two fungicides prochloraz-manganese (P) and carbendazim (C) in one medium and varied malachite green concentration. The aim of this test was to stop growth of other fungi and allow growth of only *L. fungicola*. In PDA, medium all tested fungi grew very well

and some covered all the plate after 7 days of incubation. When medium contained only PDAPC and two fungicides without malachite green *Mucor* sp. showed growth too. When media were PDAPCMG5 or RBMPCMG5 *C. mycophilum*, *M. perniciosa*, *Mucor* sp., *A. fumigatus* and *Penicillium* sp. were inhibited. *L. fungicola* grew very well on this medium and colonies had white colour. Malachite green is an essential ingredient of selective medium. The higher concentration of malachite green inhibited growth of *L. fungicola* too, but smaller concentrations still inhibited other fungi and allowed growth of *L. fungicola*.

Next part of the evaluation of designed selective medium was to find an appropriate antibiotic to inhibit growth of bacteria and yeast. *Lecanicillium fungicola* is a fungus and fungi are resistant to antibiotic activity therefore anti-bacterial activity of antibiotics will have little effect on fungal growth. Four different antibiotics were tested and only higher tested concentrations of tetracycline inhibited growth of microorganism contained in casing soil. Antibiotics were tested in combination and good results were obtained when streptomycin and tetracycline were combined. The microorganisms present in casing soil were prevented from growing in almost all tested concentrations of streptomycin and tetracycline. For the selective medium the concentration of antibiotics was chosen as 100 mg/l streptomycin (S) and 500 mg/l tetracycline (T).

On RBMPCST and 10, 20 and 30 mg/l malachite green the *L. fungiola* grew very weakly compared to PDAPCMG10STB medium. In both tested media other fungi present in casing, *Penicillium* sp. and unknown fungus also grew. Bromocresol green (B) was used to increase visibility of *L. fungicola* colonies and bromocresol green did not have a negative effect on *L. fungicola* growth (Rinker *et al.*, 1993).

On PDAPCMG10STB *L. fungicola* grew well but other fungi, such as *C. mycophilum*, *M. perniciosa*, *A. fumigatus*, *Penicillium* sp. and *Mucor* sp. did not show growth. On RBMPCMG5ST only *L. fungicola* grew. Other fungi such as *C. mycophilum*, *M. perniciosa*, *A. fumigatus*, *Penicillium* sp. and *Mucor* sp. did not show growth. When concentration of malachite green was 10 mg/l in both media *Trichoderma* sp. did not growth.

3.1.6 Novel (PDA) selective medium and modified Rinker's medium tested for *L. fungicola* (V9503-3) detection on casing

3.1.6.1 *Lecanicillium fungicola* growth on novel and modified Rinker's media

Different concentrations of conidia of *L. fungicola* (0-10^5 conidia per gram casing) were tested on two media – PDAPCMGSTB and RBMPCMGST with different concentrations of malachite green (0, 10, 20 and 30 mg/l). In PDA and MEA media *L. fungicola* grew well when conidia concentration was 10^3-10^6 per ml. On RBMPCMGST medium *L. fungicola* radius of growth were much smaller than in PDAPCMGSTB medium. When the concentration of malachite green was 10 mg/l or higher the radius of growth of *L. fungicola* was decreased on both media PDAPCMGSTB and RBMPCMGST (**Figure** 3-30). In PDAPCMG10STB medium *L. fungicola* grew when conidia concentration was 1×10^4 and 1×10^6 conidia/ ml. On RBMPCMG10ST, RBMPCMG20ST and RBMPCMG30ST *L. fungicola* grew only when conidia concentration was $1 \times 10^3 - 1 \times 10^6$ conidia/ml.

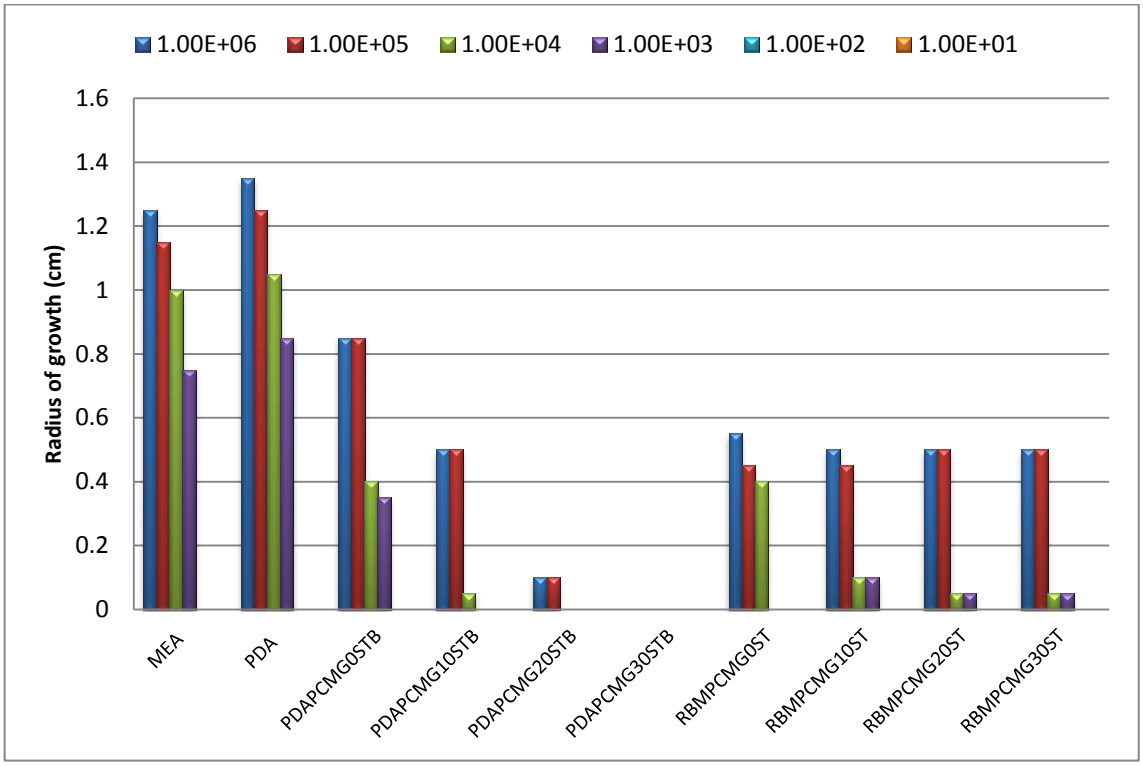


Figure 3-30: *In vitro* response of different conidia concentration of *L. fungicola* for different concentrations of malachite green (10, 20 and 30 mg/l) tested for tow media PDAPCMGSTB and RBMPCMGST after 7 days.

3.1.6.2 Detection of *L. fungicola* in casing soil using novel and modified Rinker's selective medium

Different conidia concentrations of *L. fungicola* (0-10^5 conidia/g casing) were mixed with 2 g clean casing and 2 ml water and 100 µl was spread on novel (PDAPCMGST) and modified Rinker's media (RBMPCMGST) with different concentrations of malachite green (0, 10, 20 and 30 mg/l). Casing soil contains lots of different fungi, bacteria and yeast. On both tested media *Penicillium* sp., yeast and an unknown fungus also grew. *Lecanicillium fungicola* detection level was very poor and there was correlation with conidia concentration. However some colonies of *L. fungicola* grew in PDAPCMGST with 10, 20 and 30 mg/l malachite green and RBMPCMGST medium with 20 and 30 mg/l malachite green (**Figure** 3-31). *Lecanicillium fungicola* conidia grew on both media, when concentration of conidia was 1 × 10^5 conidia/gram casing. On PDAPCMGST and RBMPCMGST medium *Penicillium* sp. and unidentified fungus grew also. However, *L. fungicola* was very easy to identify after 7 days and other fungi did not overgrow *L. fungicola* colonies (**Figure** 3-32).

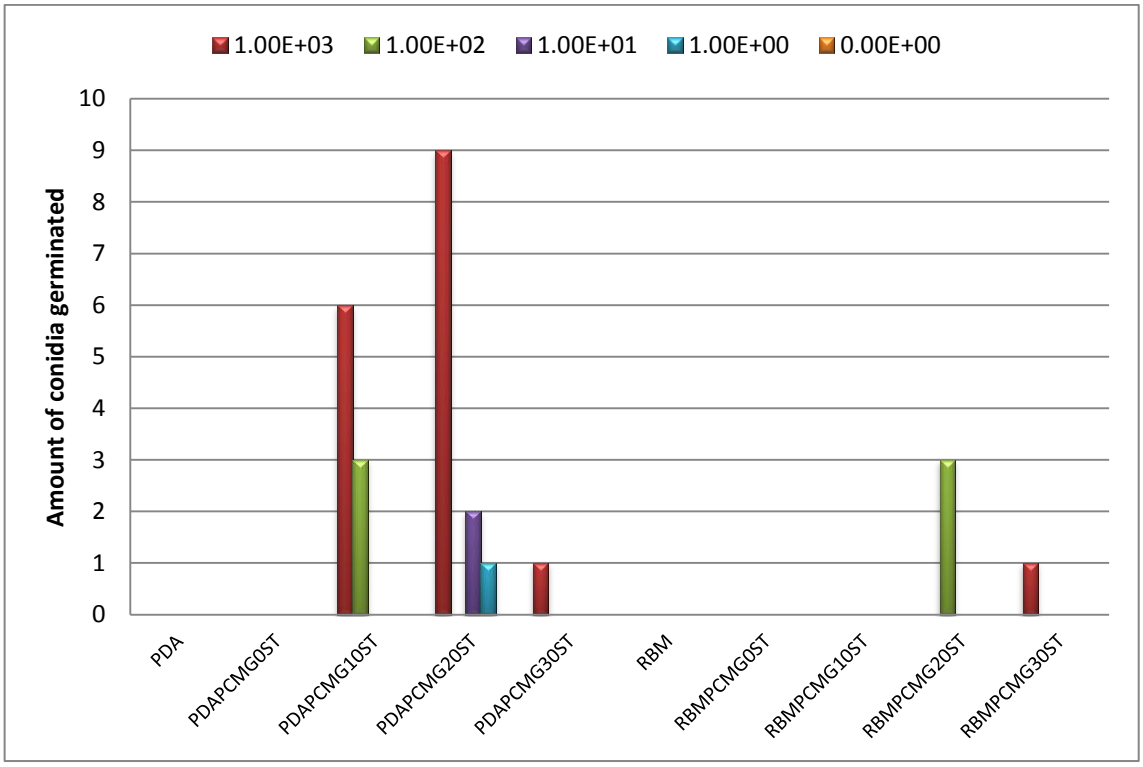
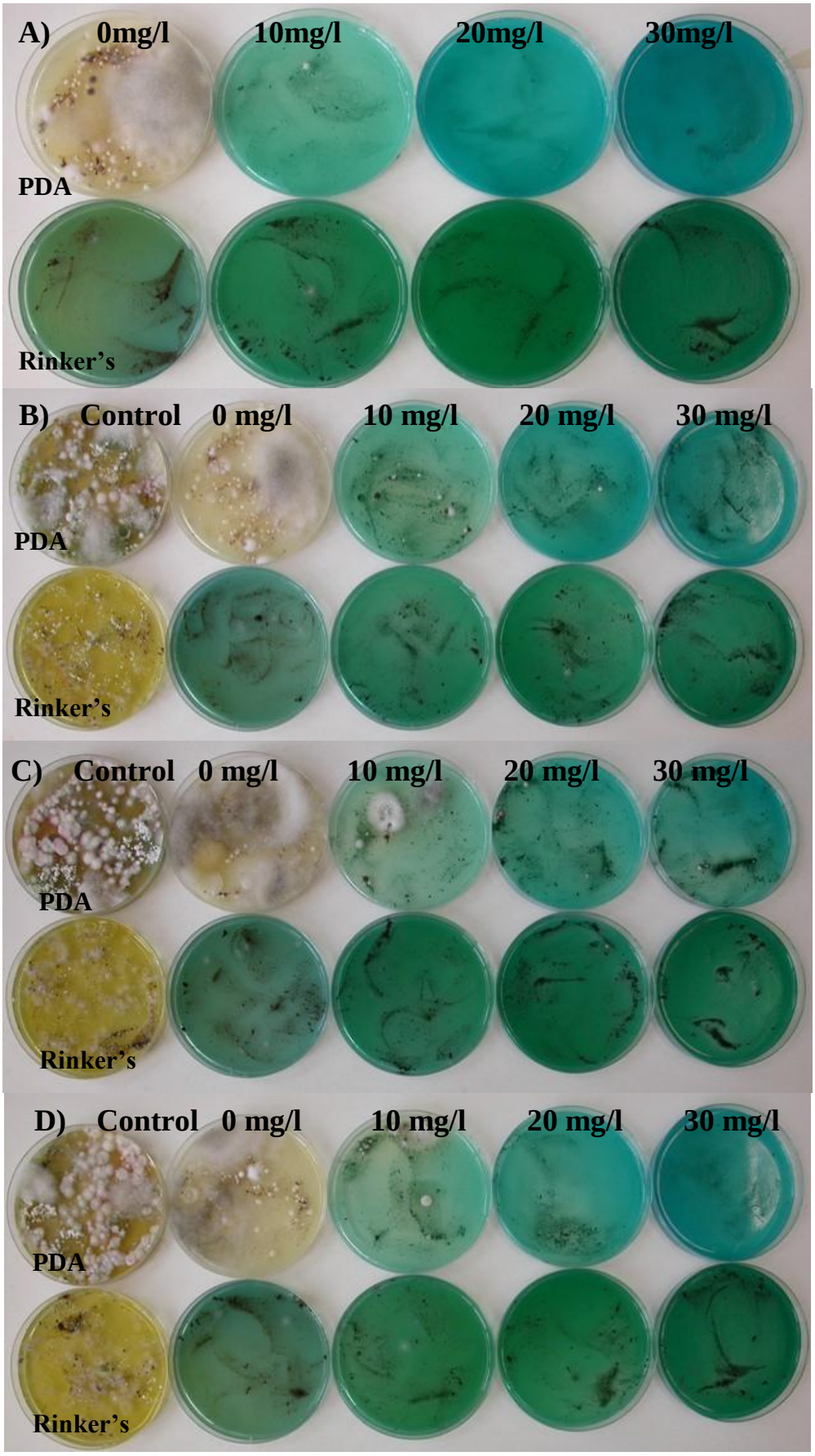


Figure 3-31: In *in vitro* response of different conidia concentrations of *L. fungicola* (10^1-10^4 conidia/ml) PDAPCMGST and RBMPCMGST with different concentration of malachite green (10, 20 and 30 mg/l). After 7 days.


A) 0mg/l 10mg/l 20mg/l 30mg/l
PDA
Rinker's
B) Control 0 mg/l 10 mg/l 20 mg/l 30 mg/l
PDA
Rinker's
C) Control 0 mg/l 10 mg/l 20 mg/l 30 mg/l
PDA
Rinker's
D) Control 0 mg/l 10 mg/l 20 mg/l 30 mg/l
PDA
Rinker's

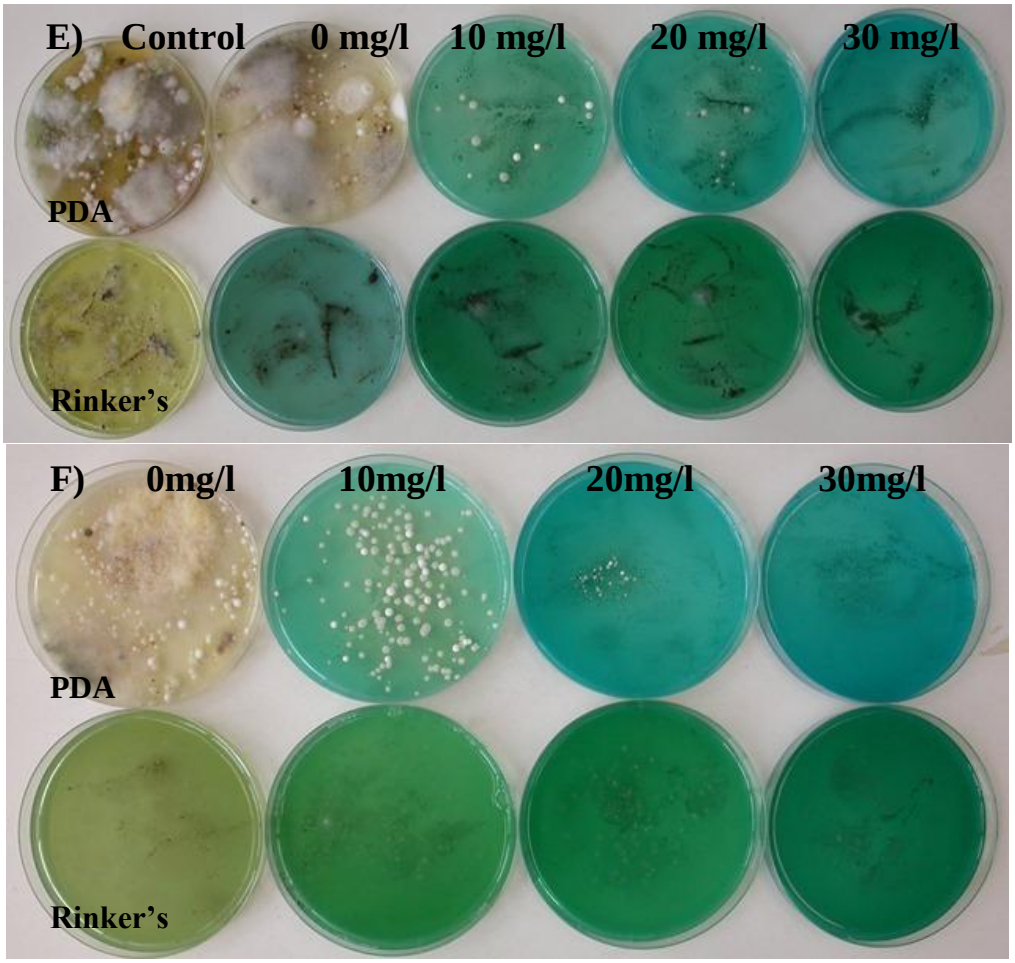


Figure 3-32: *Lecanicillium fungicola* detection level (0-10^4 conidia per plate 100 µl) on PDAPCMGST and Rinker's modified (RBMPCMGST) medium contained different concentration of malachite green (10, 20 and 30 mg/l). Control media – contain PDA and RBM. After 7 days incubation for 20 °C in the dark.

A. Control casing sample (without *L. fungicola* (10^0 conidia/g casing)
B. Casing sample with 1 conidia of *L. fungicola* per plate (10^1 conidia/g casing)
C. Casing sample with 1 × 10 conidia of *L. fungicola* per plate (10^2 conidia/g casing)
D. Casing sample with 1 × 10^2 conidia of *L. fungicola* per plate (10^3 conidia/g casing)
E. Casing sample with 1 × 10^3 conidia of *L. fungicola* per plate (10^4 conidia/g casing)
F. Casing sample with 1 × 10^4 conidia of *L. fungicola* per plate (10^5 conidia/g casing)

3.1.6.3 Method of detection of *L. fungicola* from sterile casing

Different concentrations of *L. fungicola* conidia (1 ml) were mixed with 1 g sterile casing and sterile water at concentrations from 0-10^5conidia/ml. After that the suspension was filtered, centrifuged and concentrated it was spread (100 µl) on plate filled with appropriate medium. After 6 days conidia of *L. fungicola* grew very well on test media (**Figure** 3-35). *L. fungicola* was detected on all media when conidia concentration was 1×10^5 conidia/g casing and amount of germinated conidia was determined to be high (uncounted). On the novel medium and RBMPCMGST medium *L. fungicola* conidia were detected more reliably at 1×10^4 conidia/g casing. The detection level on novel medium PDAPCMGSTB with 10 mg/l and 20 mg/l malachite green was 10 conidia/g casing (**Figure** 3-33). The conidia germination ability of *L. fungicola* was tested in PDA medium. When the concentration of *L. fungicola* conidia was 10^3 per plate (10^4 conidia/ml) the amount of germinated conidia was 30-36 but when conidia concentration was 10^2 per plate (10^3 conidia/ml) amount of germinated conidia was 5-8 (**Figure** 3-34). Very similar conidia germination rates were present on PDAPCMG10STB medium when conidia concentration was 10^3 per plate (10^4 conidia/ml) (**Figure** 3-33).

After 14 days of incubation colonies of *L. fungicola* became more visible and easier to identify, but 14 days is too long to wait for results and this amount of time is not satisfactory for *L. fungicola* conidia detection on samples from mushroom farm.

Lecanicillium fungicola colonies were very easy to find on PDAPCMGSTB after 6 days. On RBMPCMGST *L. fungicola* colonies were transparent and very difficult to find and count (**Figure** 3-35), but on PDAPCMGSTB some *Penicillium* sp. colonies also grew.

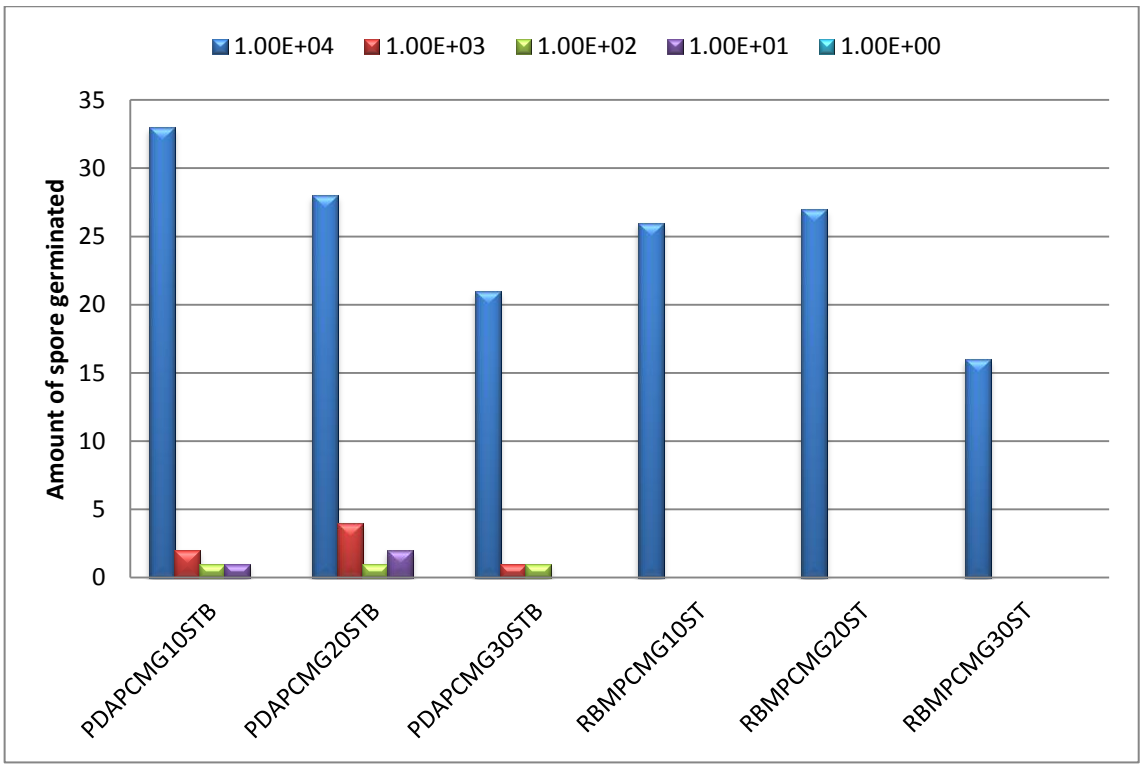


Figure 3-33: In *in vitro* response of different conidia concentrations of *L. fungicola* (1-10^4conidia/ml) on PDAPCMGSTB and RBMPCMGST with different concentration of malachite green (10, 20 and 30 mg/l). After 6 days.

Figure 3-34: Amount of germinated conidia of *L. fungicola* in PDA medium. A) 10^3 conidia of *L. fungicola* per plate (10^4 conidia/ml) – average 33 conidia. B) 10^2conidia of *L. fungicola* per plate (10^3 conidia/ml) – average 7 conidia. After 7 days.

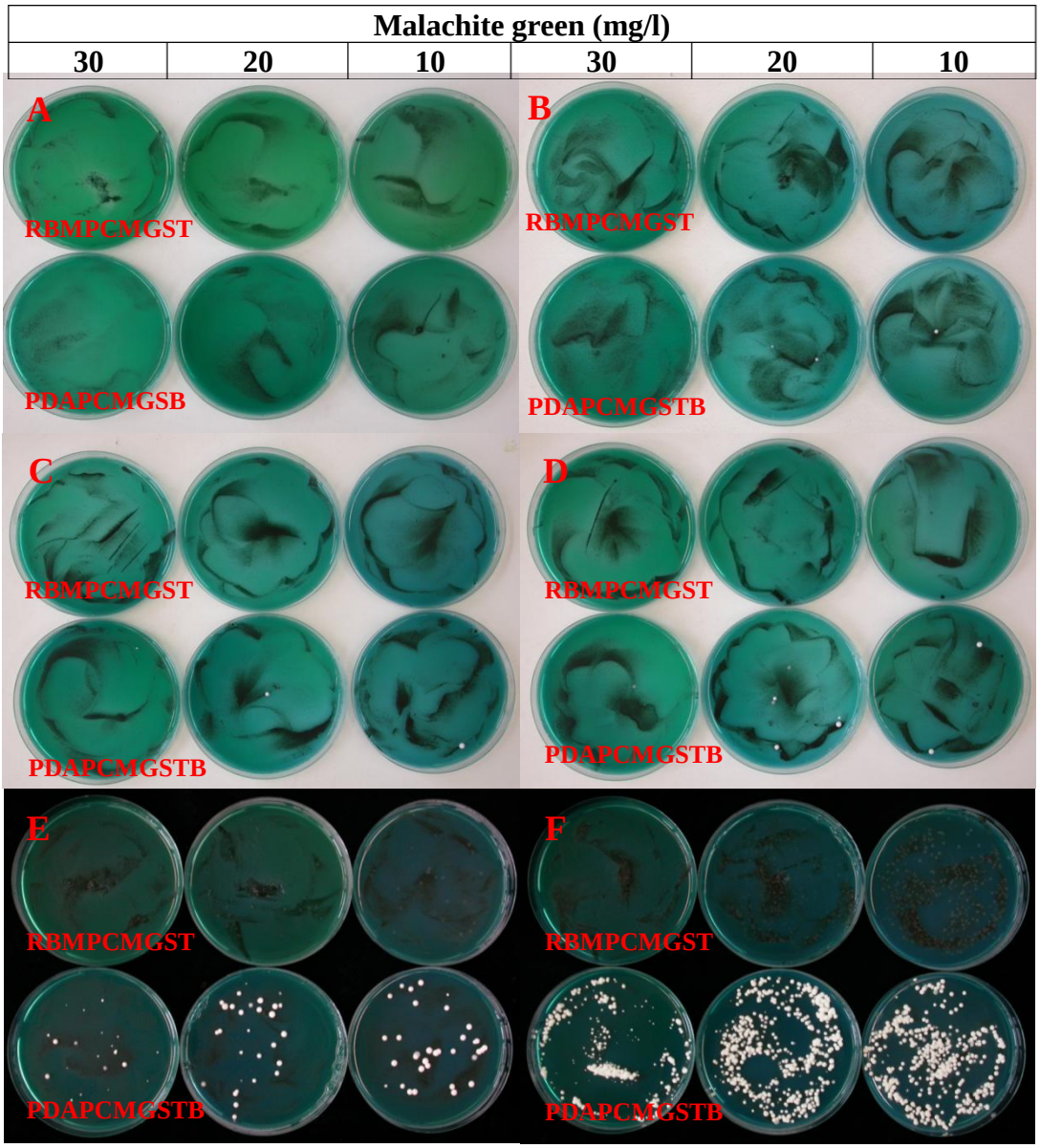


Figure 3-35: *Lecanicillium fungicola* detection level (0-10^4 conidia per plate 100 µl) on PDAPCMGSTB and RBMPCMGST contained different concentrations of malachite green (10, 20 and 30 mg/l). (Dark streaks are casing material. White dots *L. fungicola* colonies). After 7 days incubation for 20 °C in the dark.

A. Control casing sample without *L. fungicola* (10^0 conidia/g casing)
B. Casing sample with 1 conidia of *L. fungicola* per plate (10^1 conidia/g casing)
C. Casing sample with 1 × 10 conidia of *L. fungicola* per plate (10^2 conidia/g casing)
D. Casing sample with 1 × 10^2 conidia of *L. fungicola* per plate (10^3 conidia/g casing)
E. Casing sample with 1 × 10^3 conidia of *L. fungicola* per plate (10^4 conidia/g casing)
F. Casing sample with 1 × 10^4 conidia of *L. fungicola* per plate (10^5 conidia/g casing)

3.1.6.4 *Lecanicillium fungicola* detection in non-sterile casing tested on two media

Lecanicillium fungicola conidia at different concentrations were mixed with non-sterile casing and sterile water. The suspension was filtered, centrifuged and concentrated and 100 µl suspensions was spread on plate filled with appropriate medium. After 4 days *L. fungicola* growth was visible at highest concentration of conidia on PDAPCMG10STB and PDAPCMG20STB medium. However *L. fungicola* conidia grew very well after 6 days of incubation. After this time colonies of *L. fungicola* had a bright white colour and were easy to see. *Lecanicillium fungicola* colonies were numerous when conidia concentration was 1×10^4 and 1×10^5 per ml. When conidia concentration of *L. fungicola* was lower (10^3 conidia/ml) the amount of germination was very similar. The amount of colonies *L. fungicola* growth on 10-10^3 conidia/ml conidia concentration was to high and it could be a human error for conidia counting or dilution procedure.

The detection level of *L. fungicola* in autoclaved casing soil was 10 conidia/g casing (**Figure** 3-36 and **Figure** 3-37).

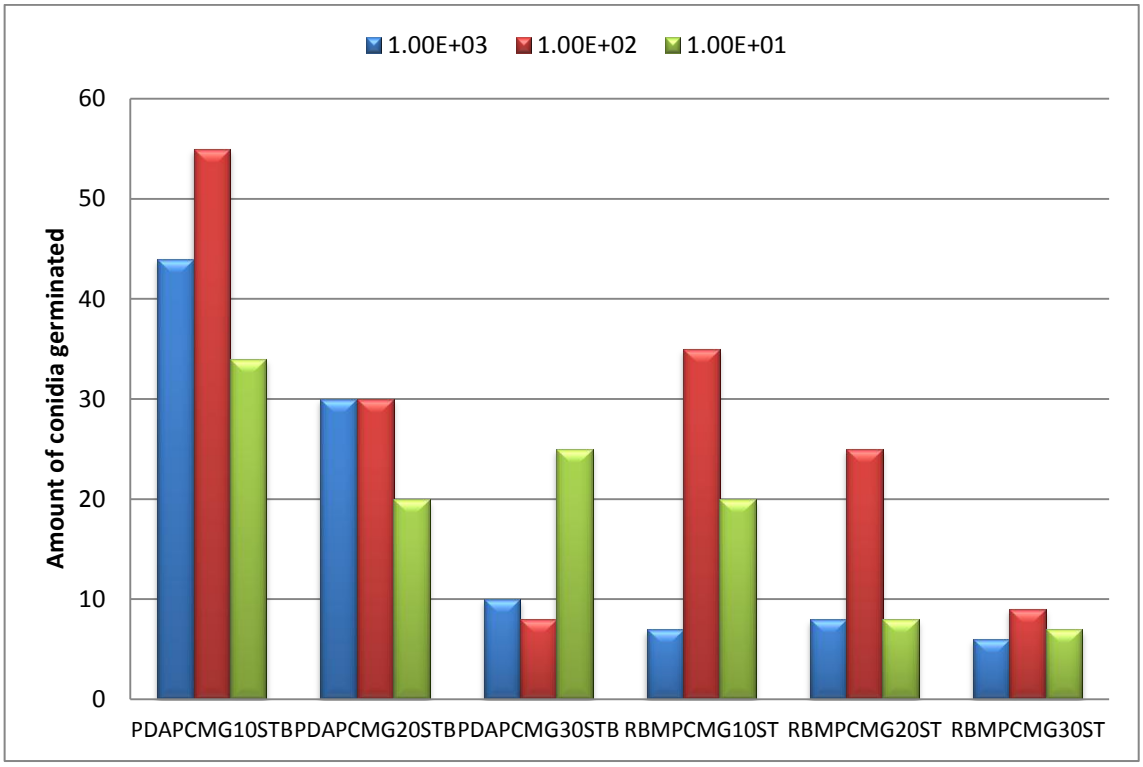


Figure 3-36: *In vitro* response of different conidia concentrations of *L. fungicola* (10^1-10^3conidia/ml) PDAPCMGSTB and RBMPCMGST with different concentration of malachite green (10, 20 and 30 mg/l). After 6 days.

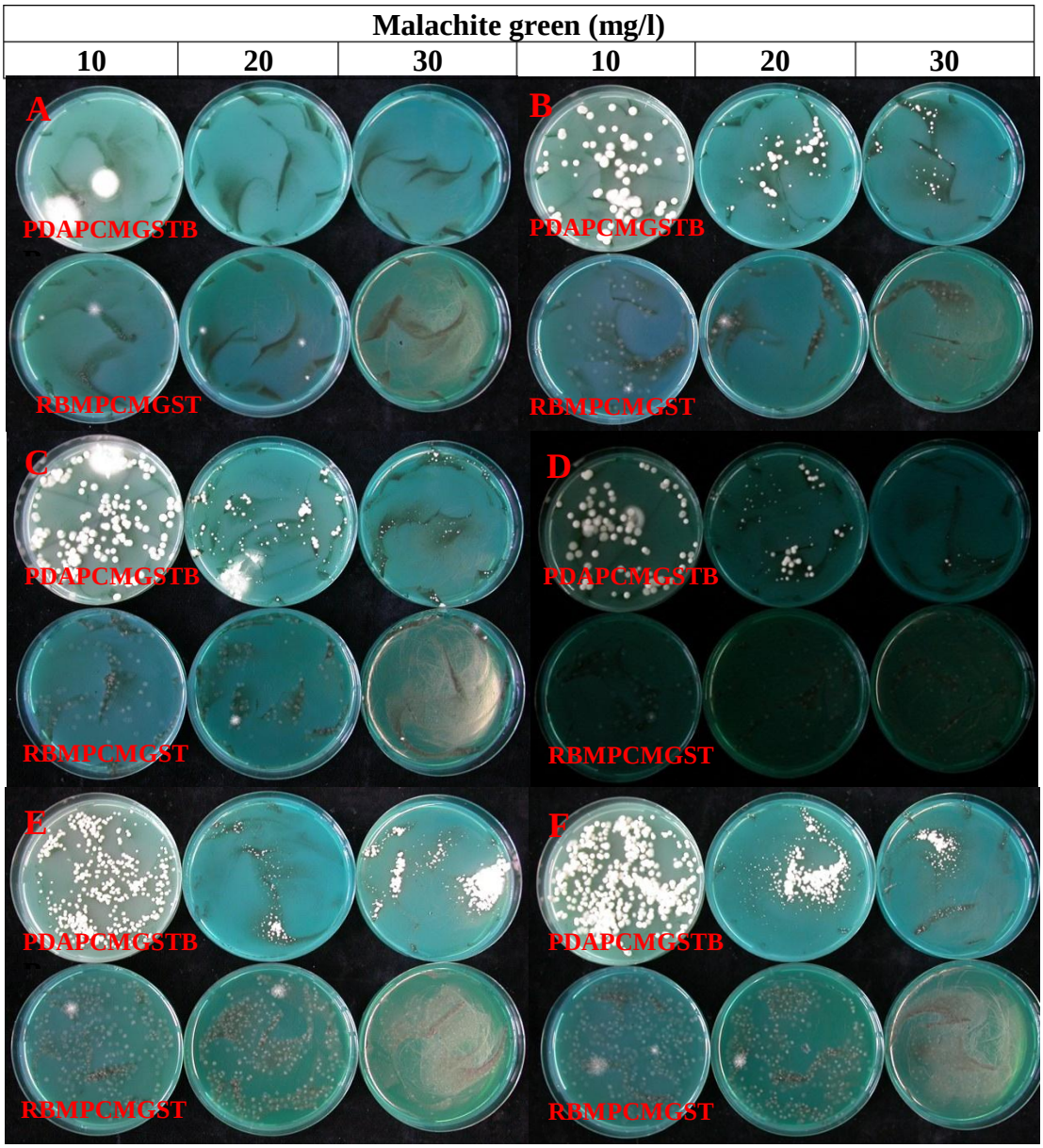


Figure 3-37: *Lecanicillium fungicola* detection level (0-10^4 conidia per plate 100 µl) on PDAPCMGSTB and RBMPCMGST with different concentrations of malachite green (10, 20 and 30 mg/l). (Dark streaks are casing material. White dots *L. fungicola* colonies). After 7 days incubation for 20 °C in the dark.

A. Control casing sample without *L. fungicola* (10^0 conidia/g casing)
B. Casing sample with 1 conidia of *L. fungicola* per plate (10 conidia/g casing)
C. Casing sample with 1 × 10^1 conidia of *L. fungicola* per plate (10^2 conidia/g casing)
D. Casing sample with 1 × 10^2 conidia of *L. fungicola* per plate (10^3 conidia/g casing)
E. Casing sample with 1 × 10^3 conidia of *L. fungicola* per plate (10^4 conidia/g casing)
F. Casing sample with 1 × 10^4 conidia of *L. fungicola* per plate (10^5 conidia/g casing)

3.1.6.5 *Lecanicillium fungicola* detection on PDAPCMGSTB and RBMPCMGST medium in non-sterile casing

Previous experiment 3.1.6.4 was repeated but this time amount of casing soil was 2 g and conidia amount was 2 ml with different concentrations of conidia (0-10^5 conidia/ml). Different conidia concentrations of *L. fungicola* were mixed with casing and sterile water and after this solution was filtered, centrifuged and concentrated it was spread on plate (100 µl) filled with the appropriate medium. After 4 days, conidia of *L. fungicola* grew on novel medium PDAPCMG10STB and PDAPCMG20STB and RBMPCMG10ST. In PDA medium *L. fungicola* growth was covered with other fungi (**Figure** 3-38). *Lecanicillium fungicola* grew on medium when conidia concentration per plate was 20 or higher. After 6 days colonies of *L. fungicola* were much easier to find. The colonies of *L. fungicola* had a bright white colour on PDAPCMGSTB. On RBMPCMGST *L. fungicola* colonies were transparent and difficult to find (**Figure** 3-39). The detection threshold was 20 conidia per gram casing but *L. fungicola* conidia were detected more reliably at 2×10^4 conidia per gram casing (2×10^3 conidia per plate). When conidia concentration was 2×10^5 conidia/g casing colonies of *L. fungicola* were too numerous to count (**Figure** 3-40). On PDAPCMGSTB in all amount of malachite green an unidentified fungus also grew but after 4 or 6 days this fungus did not overgrow *L. fungicola* colonies (**Figure** 3-40).

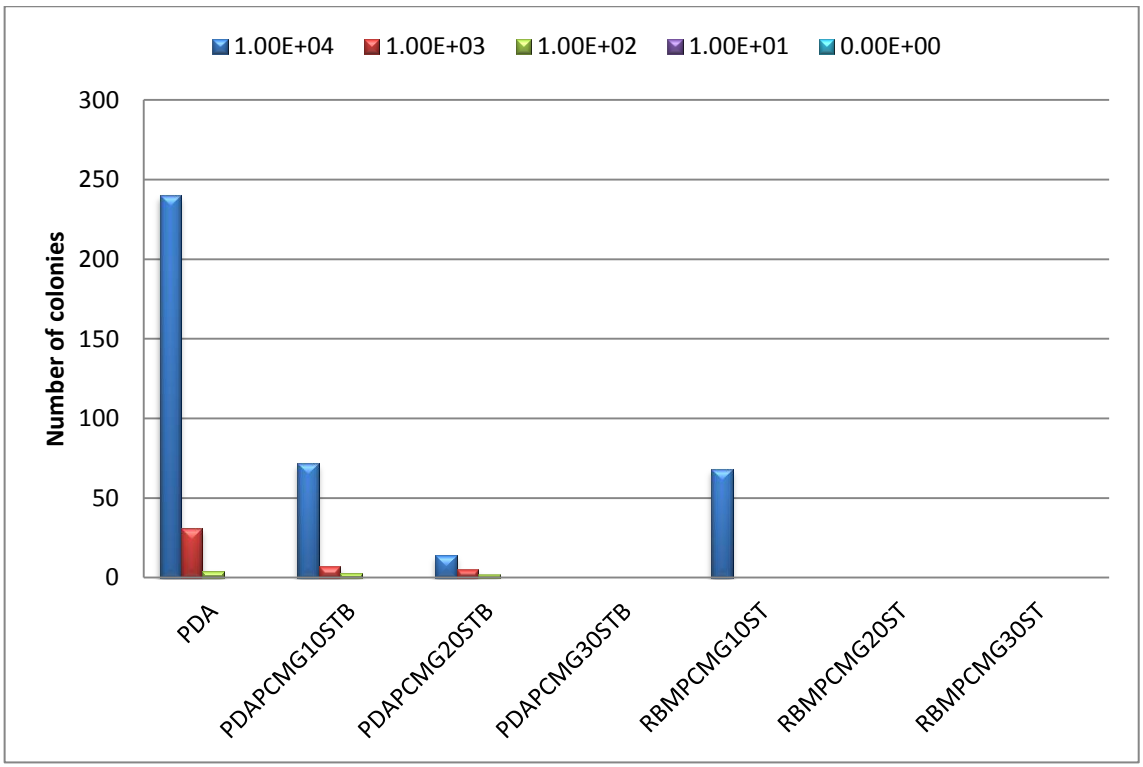


Figure 3-38: In *in vitro* response of different conidia concentrations of *L. fungicola* (0-10^4 conidia/ml) PDAPCMGSTB and RBMPCMGST with different concentration of malachite green (10, 20 and 30 mg/l). After 4 days.

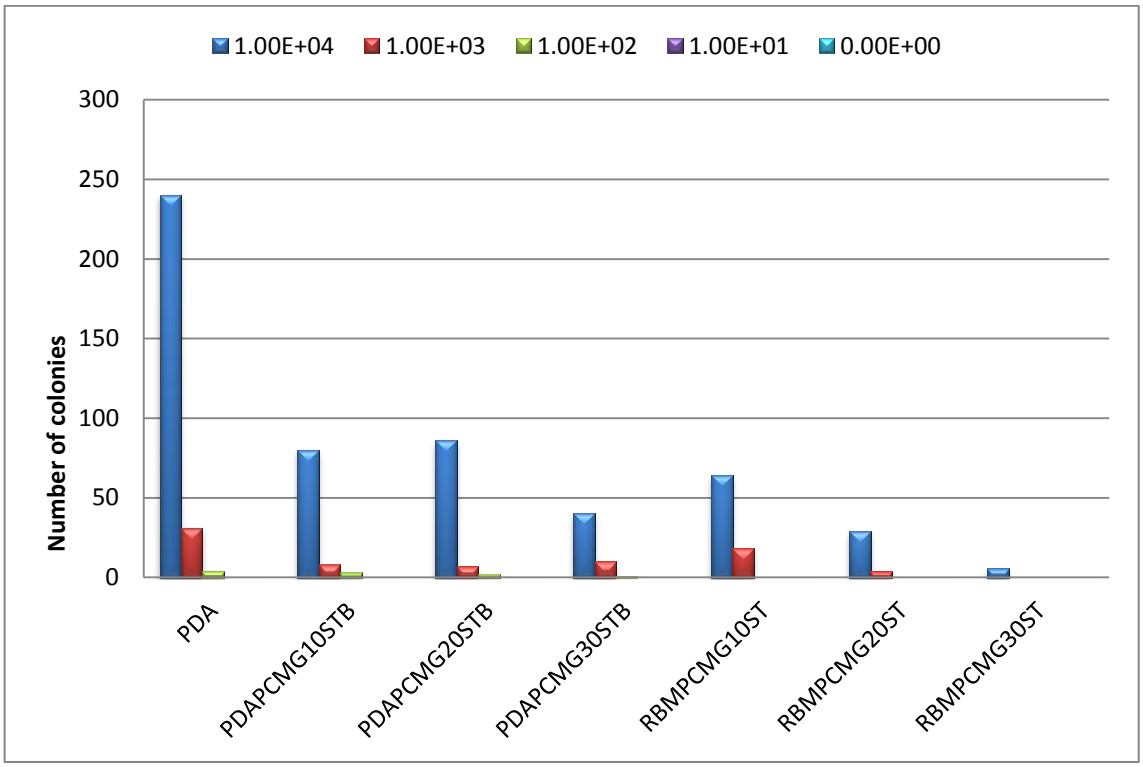
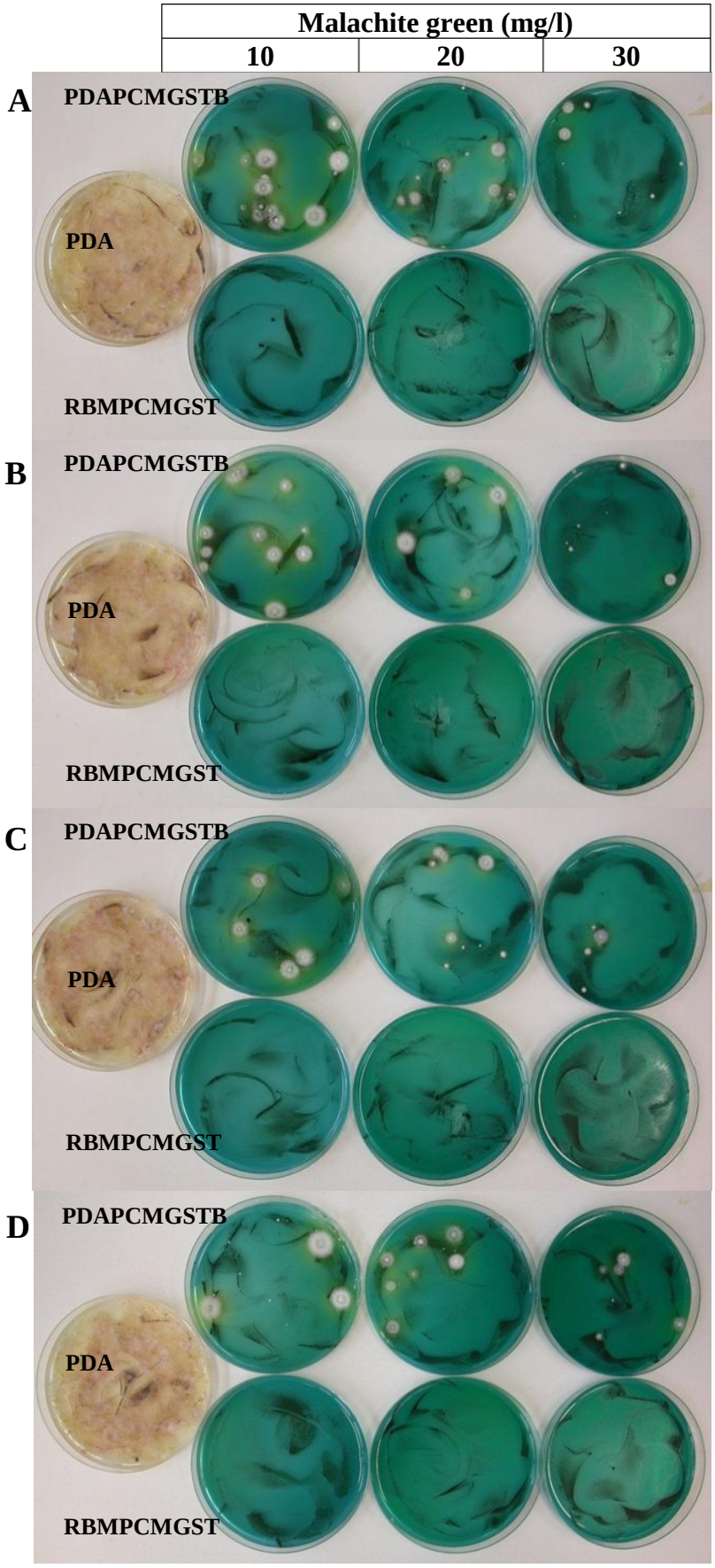


Figure 3-39: In *in vitro* response of different conidia concentrations of *L. fungicola* (0 - 10^4 conidia/ml) PDAPCMGSTB and RBMPCMGST with different concentration of malachite green (10, 20 and 30 mg/l). After 6 days.


Malachite green (mg/l)
10
20
30
A
PDAPCMGSTB
PDA
RBMPCMGST
B
PDAPCMGSTB
PDA
RBMPCMGST
C
PDAPCMGSTB
PDA
RBMPCMGST
D
PDAPCMGSTB
PDA
RBMPCMGST

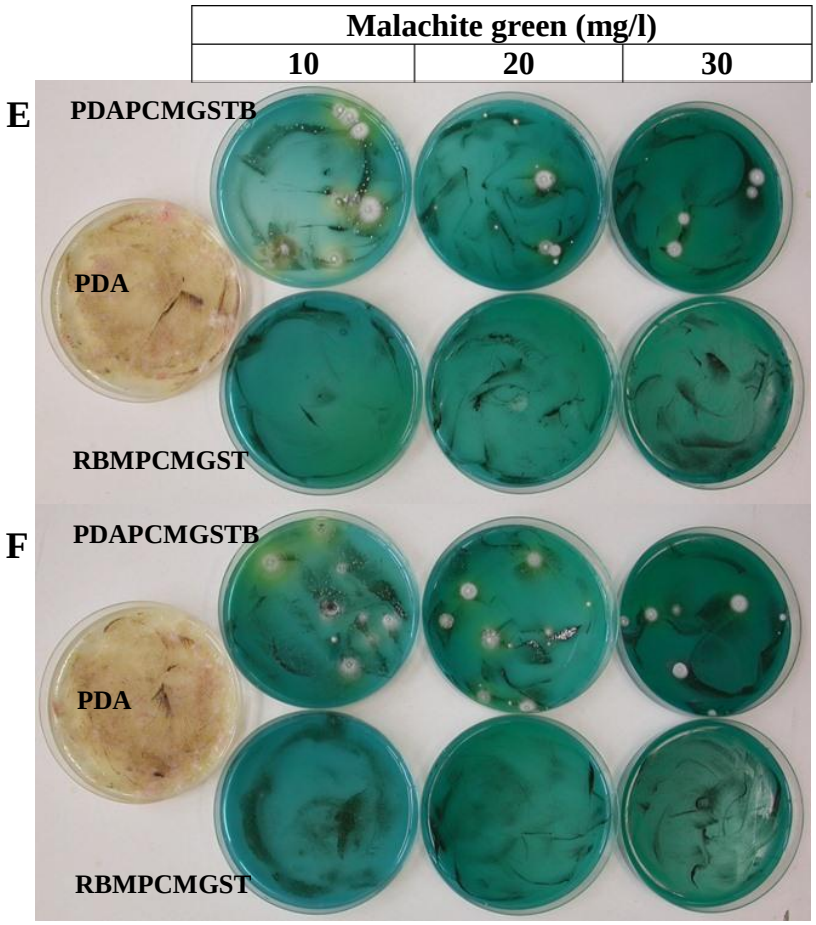


Figure 3-40: *Lecanicillium fungicola* detection level (0-10^4 conidia per plate 100 µl) on PDA without malachite green and PDAPCMGSTB and RBMPCMGST with different concentration of malachite green (10, 20 and 30 mg/l). (Dark streaks are casing material. White dots *L. fungicola* colonies). After 4 days incubation for 20 °C in the dark.

A. Control casing sample without *L. fungicola* (10^0 conidia/g casing)
B. Casing sample with 2 conidia of *L. fungicola* per plate (2 x 10^1 conidia/g casing)
C. Casing sample with 2 × 10 conidia of *L. fungicola* per plate (2 × 10^2 conidia/g casing)
D. Casing sample with 2 × 10^2 conidia of *L. fungicola* per plate (2 × 10^3 conidia/g casing)
E. Casing sample with 2 × 10^3 conidia of *L. fungicola* per plate (2 × 10^4 conidia/g casing)
F. Casing sample with 2 × 10^4 conidia of *L. fungicola* per plate (2 × 10^5 conidia/g casing)

3.1.6.6 Conidia germination in PDA medium

The different conidia concentrations were spread on PDA medium. When the amount of conidia was 10 conidia/ml (1 conidium per plate) the conidia germination rate was 100 %, but when the conidia concentration was 10^2 conidia/ml (10 conidia per plate) the percentage of germinated conidia was 40 and decreased with higher conidia concentrations.

When conidia concentration was 10^4 conidia/ml (10^3 conidia per plate) germination was only 25 %. In higher conidia concentration 10^5-10^7 conidia/ml the amount of colonies were very high (**Figure** 3-41).

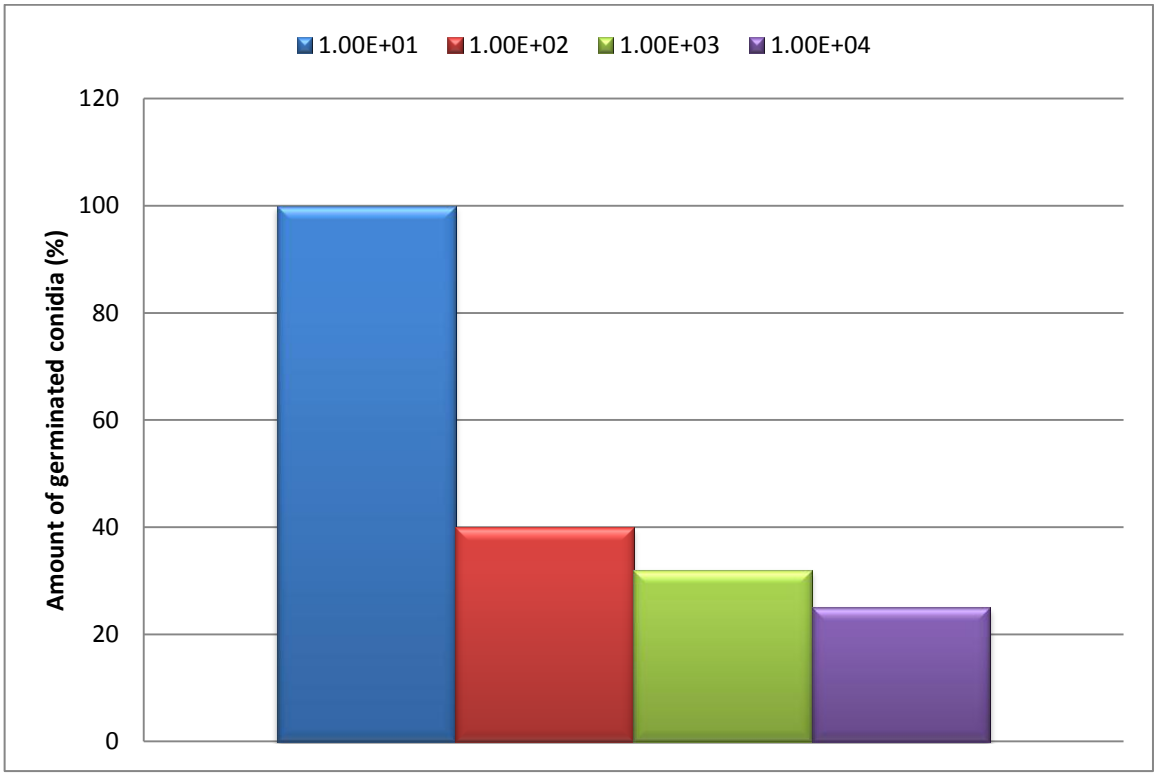


Figure 3-41: *In vitro* response of *L. fungicola* conidia germination for different conidia concentrations. After 6 days in PDA medium. Conidia amount given per plate.

3.1.6.7 Summary

Casing soil consists of high amounts of peat which contains a lot of bacteria, yeast and fungi (Masaphy *et al.*, 1987, Chikthimmah *et al.*, 2008). The button mushrooms require a casing layer for growth. Casing particles are spread around the growing room and outside of mushrooms growing room. The selective medium must be able to allow growth of *L. fungicola* but prevent growth of other microorganisms. *Lecanicillium fungicola* grows very slowly so it is important to inhibit the growth of other fast growing fungi. After a previous part of experiments the PDA selective medium contained PDA, 5 mg/l malachite green, 100 mg/l carbendazim and 1 mg/l prochloraz-Mn (PDAPCMG5). On this (PDAPCMG5) medium *L. fungicola* and some fungus contained on casing extract – an unidentified fungus grew and some isolates of *Penicillium* sp. *Cladobotryum mycophilum*, *M. perniciosa*, *A. fumigatus*, *Penicillium* sp. and *Mucor* sp. did not show growth.

The method for sample preparation is an original method of this procedure and it was filtered, centrifuged and concentrated. The biggest part of casing would stay on filter but small and loose conidia of *L. fungicola* could pass easily through the filter. After filtration the solution was concentrated using a centrifuge for volume decreasing.

Lecanicillium fungicola conidia were found only when conidia were added. The amount of *L. fungicola* conidia growing on PDAPCMGSTB and RBMPCMGST medium did not show any correlation with conidia concentrations. The detection threshold was 10 conidia/g casing (1 conidia per plate) but *L. fungicola* conidia were detected more reliably at 10^4 conidia/g casing (10^3 conidia per plate).

PDA and RBM allowed all microorganisms contained in casing soil to grow. When medium contained: PDA with 0 mg/l malachite green, 100 mg/l carbendazim and 1 mg/l prochloraz-manganese and 100 mg/l streptomycin and 500 mg/l tetracycline (PDAPCST) fungi contained in casing soil also grew. On Rinker's medium with RBM, 0 mg/l malachite green, 100 mg/l carbendazim and 1 mg/l prochloraz-manganese and 100mg/l streptomycin and 500 mg/l tetracycline (RBMPCMG0ST) competitive fungi were inhibited but *L. fungicola* was very difficult to see. When PDAPCST medium contained different concentration of malachite green (10, 20, 30 mg/l) unidentified fungus also grew. This fungus was not present on modified Rinker's medium (RBMPCMGST) with different concentrations of malachite green (10, 20, 30 mg/l) but colonies of *L. fungicola* were difficult to find after 6-7 days of incubation. The detection level of *L. fungicola* was higher on PDAPCMG10ST and RBMPCMG10ST than on these media with 20 or 30 mg/l malachite green. The colonies of *L. fungicola* were very

easy to notice on PDAPCMG10ST medium after 7 days, in contrast to RBMPCMG10ST, when colonies of *L. fungicola* were transparent and difficult to find and count. The weak point of PDAPCSTB with different amount of malachite green was growth of an unidentified fungus (it did not produce conidia) and isolates of *Penicillium* sp. The unidentified fungus also grew on RBMPCMGST with different amount of malachite green, but colonies and amount of an unidentified fungus was much smaller.

3.2 Discussion

All tested wild Irish, Polish, Serbian and Spanish isolates were identified as *L. fungicola* var. *fungicola*. The Canadian and USA isolates were identified as *L. fungicola* var. *aleophilum*. Other tested isolates were already identified and described in publications or classified in data bases.

The Irish wild isolates of *L. fungicola* var. *fungicola* were not very sensitive to prochloraz-manganese (EC_{50} = 1.72 to 6.28 mg/l) compared to other wild isolates collected from Poland (EC_{50} = 1.51 to 4.51 mg/l), Serbia (EC_{50} = 1.16 to 2.74) and Mexico (EC_{50} = 2.08 to 3.18). These results suggest that the *L. fungicola* of Irish populations of isolates showed a similar sensitivity for tested fungicide and could suggest a slight tolerance to prochloraz-manganese. The *L. fungicola* var. *aleophilum* isolates from Canada (EC_{50} = 0.46 to 0.89) and USA (EC_{50} = 0.39 to 0.99) showed high sensitivity to prochloraz-manganese what can be explained by the date of isolate collection and Canadian and USA restrictions on use of this fungicide. The Netherlands (*L. f.* var. *fungicola* EC_{50} = 0.62 to 0.85 and *L. f.* var. *aleophilum* EC_{50} = 0.54 to 1.01) and UK (EC_{50} = 0.59 to 0.76) references isolates were very sensitive to prochloraz-manganese because they were collected before this fungicide was introduce in to mushroom farms. Gea *et al.* (2003) stated that *L. fungicola* var. *fungicola* isolated form *A. bisporus* tissue in Spanish showed a EC_{50} values between 1.2-8.1 mg/l and mean was 3.14 mg/l, he also isolated a *L. fungicola* var. *aleophilum* from *A. bitorquis* tissues in Spain and found the sensitivity for prochloraz-manganese the EC_{50} for var. *aleophium* EC_{50} values were between 0.7-5.6 mg/l and mean was 2.68 mg/l. That result can suggest a slightly lower resistance of *L. f.* var. *aleophilum*. Gea *et al.* (2005) reported that some mushroom farms reported unsatisfactory levels of control of dry bubble disease by prochloraz-manganese which may be explained by development of a resistance by *L. fungicola* with 30 years of use of this fungicide. Grogan *et al.* (2000) and Gea *et al.* (2003) reported that 70 % of *L. fungicola* isolates from United Kingdom and Spanish were moderately sensitive to prochloraz-manganese and EC_{50} values had a range 5 to 8 mg/l prochloraz-manganese. The Belgian and one French isolate were sensitive to prochloraz-manganese but it is difficult to explain because these isolates were isolated from other organism not from *Agaricus* sp. tissue. Other French isolate was very sensitive to prochloraz-manganese and EC_{50} values were between (0.51 to 0.57 mg/l).

All Irish, Polish, Serbian and Mexican isolates of *L. fungicola* var. *fungicola* were resistant to 50 and 100 mg/l carbendazim. Some Netherlands and UK (old) isolates

were sensitive to carbendazim. One Belgian and one French isolate were sensitive too but they were not isolated from *A. bisporus* tissue but from different organisms what may explain sensitivity. The Canadian and USA isolates were resistant to carbendazim also. Only one isolate from USA showed EC_{50} value.

Selective medium is a cheap and easy method to monitor the efficacy of sanitation in mushroom farms. This medium could help mushroom producers and researchers detect *L. fungicola*. The first information about selective medium was published by Rinker *et al.* (1993). The fungicides used in Rinker's selective medium are no longer available. On this medium *C. mycophilum* strains and *Penicillium* strains also grew. It is the only publication about selective medium for *L. fungicola*.

The purpose of this work was to develop a novel medium and to modify an existing selective medium to detect *L. fungicola* in samples coming from mushroom farms. The novel medium contains nutrients, dyes, fungicides and antibiotics. The composition of novel PDA selective medium was tested by many *in vitro* experiments. The best composition of the novel (PDA) selective medium contains: potato dextrose agar – 39 g/L or potato dextrose broth 24g/L and agar 15-20 mg/L, malachite green sodium salt – 10 mg/l, bromocresol green sodium salt – 30 mg/l, tetracycline 500 mg/l, streptomycin – 100 mg/l, prochloraz-manganese (Sporogn) – 1mg/l and carbendazim (KapChem) – 100 mg/l (NPDASM). This composition of novel PDA selective medium gave a higher number of growing colonies of *L. fungicola* and colonies are easy to see after 6 days of incubation at 20-23 °C. In *in vitro* experiments other tested fungi such as *C. mycophilum*, *M. perniciosa*, *A. fumigatus*, *Mucor* sp., *Penicillium* sp. and *Trichoderma* sp. did not show growth. *C. mycophilum*, *A. fumigatus*, *Mucor* sp. and *Trichoderma* sp. can very easily cover all the plate after 3-7 days of incubation and out-grow *L. fungicola* colonies. This is why it was was so important to inhibit these fungi on selective medium and allow growth of *L. fungicola*. *M. peniciosa* is the other mushroom pathogen present in casing. This fungus is many times incorrectly identified as *L. fungicola*. This is why it was so important to eliminate growth of this fungus.

In vitro experiments with casing soil however showed different results. Casing soil contains lots of bacteria, yeast and fungi. The high concentration of two antibiotics did not permit growth of any bacteria and eliminated growth of some sensitive yeast. The two fungicides used on novel selective medium did not permit growth of other fungi. Malachite green as an anti-fungal salt inhibited growth of competitive fungi such as *Trichoderma* sp. and *Mucor* sp. But some wild strains of *Penicillium* sp. and unidentified fungus contained in casing were resistant to all the fungicides and

malachite green. Identification of growing fungus was difficult because it did not produce conidia. *Penicillium* sp. strains had a different morphology and after 6 days *Penicillium* sp. colonies started to be white-yellow and jagged which is easy to differentiate. The unidentified fungus colonies were bigger than *L. fungicola* colonies and colour of unidentified fungus was white-grey. However, despite these problems with growth of *Penicillium* sp. and unidentified fungus, *L. fungicola* colonies were easy to find on novel PDA selective medium. Colonies of *L. fungicola* on this medium had a bright white colour and smooth texture, contrary to *Penicillium* sp. colonies.

The novel medium was compared to a modified version of Rinker's selective medium (Rinker *et al.,* 1993). Modified Rinker's selective medium contained base Rinker's medium ($NH_4H_2PO_4$ – 2g/l, KCl – 0.4g/l, $MgSO_4 \times 7H_2O$ – 0.4g/l, Raffinose – 1 g/l, Bromocresol green sodium salt – 30 mg/l = RBM) and malachite green sodium salt – 10 mg/l, tetracycline 500 mg/l, streptomycin – 100 mg/l, prochloraz-manganese (Sporgon) – 1mg/l and carbendazim (KapChem) – 100 mg/l (MRM). The composition of modified Rinker's selective medium gave a higher number of growing colonies of *L. fungicola* but colonies were not easy to see after 6-7 days of incubation at 20-23 °C. Other tested fungi such as *C. mycophilum*, *M. perniciosa*, *A. fumigatus*, *Mucor* sp. and *Penicillium* sp. did not show growth. On Rinker's modified medium an unidentified fungus originating from casing also grew. Colonies of *L. fungicola* on this medium were transparent and difficult to observe. However, on this medium a smaller amout of unknown fungus and some *Penicillium* strains also grew, but size of colonies of these fungi was much smaller than on novel (PDA) selective medium (NPDASM). The reliable detection level of *L. fungicola* conidia on casing experiment was 10^4 conidia per gram casing, but threshold was 10 conidia per gram casing.

Very interesting results were observed for *L. fungicola* conidia germination. The amount of germinated conidia on control medium was always much smaller than the determined conidia concentrations. The percent of germinated conidia decreased when conidia concentration increased. Similar occurrence was present in casing experiment. Bhatt and Singh (2000), examined a bacteria isolated from casing soil. Five of isolated bacteria reduced growth of *L. fungicola*. Later Berendsen *et al.* (2008) observed this same mechanism of *L. fungicola* conidia germination in casing soil. They suggested the presence of a self-inhibitor mechanism of *L. fungicola*. They also observed an inhibition of small amount of conidia germination of *L. fungicola* in casing soil by microbial activity.

In *in vitro* experiments the best medium for *L. fungicola* detection was novel (PDA) medium which contained PDA, 10 mg/l malachite green, 30 mg/l bromocresol green, 100 mg/l carbendazim and 1 mg/l prochloraz-manganese and 100mg/l streptomycin and 500 mg/l tetracycline (NPDASM) and modified Rinker's medium contained Rinker's base medium and 10 mg/l malachite green, 30 mg/l bromocresol green, 100 mg/l carbendazim and 1 mg/l prochloraz-manganese and 100 mg/l streptomycin and 500 mg/l tetracycline (MRM).

Experiments in Chapter 6 will use novel selective medium and modified Rinker's medium for detection of *L. fungicola* on mushroom farms. That work will support commercial mushroom producers in detecting possible sources of *L. fungicola*. It will help to measure and manage dry bubble disease and also monitor the efficiency level of sanitation and hygiene in mushroom farms.

Chapter 4 Evaluation of DNA extraction methods and use of PCR for the detection of *Lecanicillium fungicola* and designing selective primers for the identification and detection of *Lecanicillium fungicola* from casing soil

The fundamental tool for molecular biology research is the polymerase chain reaction (PCR) invented by Cetus Corporation (Mullis *et al.*, 1986). PCR reactions have changed and developed making it easier, simpler and faster to diagnose. In 1993 Kary Mullis received the Nobel Prize for chemistry for develop PCR. PCR has become an ideal method for the systematics, detection and identification of different microorganisms especially pathogens but not only. The crucial goal of this technique is to improve the sensitivity of detection as well as limit the time it takes to prepare the samples for PCR analysis (Dean *et al.*, 2004).

PCR has become a crucial and universal tool for biological research and laboratory diagnostic applications. PCR offers a simple technique for the amplification and analysis of nucleic acids. However, the first stage of any experiment containing PCR assay is the provision of the pure suspension of nucleic acids, either DNA or RNA. The extraction of nucleic acids is an essential precursor to practically all PCR assays. Isolation of DNA and RNA from various starting materials can be performed using a variety of techniques (Bartlett, 2003 a). The sensitivity of PCR techniques is dependent for the quality of extraction of nucleic acids and primer design.

The designing of specific primers for PCR is a crucial task for efficiency and specificity of the PCR. The effective primers have to do only one task: amplify the desired amplicon. The primer should hybridize only with the intended target and not to other, non-specific, targets, and the efficiency must be high. When primers give a non-specific amplification product aberrant amplicons are generated and these will very

rapidly consume the primers and remove them from the reaction for the intended target (Hyndman and Mitsuhashi, 2003).

The most important element for PCR assay is the optimization of conditions for a particular PCR. Optimization can be time consuming and complicated because of the various parameters that are engaged. According to Grunenwald (2003), these parameters are: quality and concentration of DNA template, design and concentration of primers, concentration of magnesium ions, concentration of the four deoxynucleotides (dNTPs), PCR buffer systems, selection and concentration of DNA polymerase, PCR thermal cycling conditions and concentrations of PCR additives/co-solvents.

The analysis of PCR product is done by electrophoresis gel using pulsed electric fields. The electrophoresis gel is a highly flexible approach that provides information about the size of the DNA amplification product. For DNA electrophoresis the most common gel is an agarose or polyacrylamide gel. The agarose electrophoresis gels are stronger and easy to make. However the resolution of this gel is poor but it can separate DNAs from 200 to 50,000 bp which is more than adequate for PCR-based system. The polyacrylamide gels are more useful for separation of smaller fragments of DNA under 300-500 bp. However, this kind of gel contains acrylamide which it is a neurotoxin. These types of gels are also more difficult to pour and handle than agarose gels (Bartlett, 2003 b).

The agarose electrophoresis gel of DNA requires a buffering system. The most common buffering systems are Tris acetate EDTA buffer (TAE) and Tris borate EDTA buffer (TBE). The TAE has a relatively low buffering capacity, but it is widely accepted because it facilitates recovery of material from agarose gel. The TBE has a higher buffering capacity and it is preferred for small molecules and longer electrophoresis. Electrophoresis is performed using a special tank connected to the power supply unit. The visualisation of PCR assay is performed by DNA dyes (ethidium bromide, SyBR green, etc.) which intercalate into the DNA sequence and are visible under UV light (Bartlett, 2003 b).

Existing practices in identification and detection of fungi from different sources rely primarily on conventional cultivation and microscopic techniques (Deak, 1994). In particular, identification of *L. fungicola* from mushroom farm samples using morphological characteristics and physiological criteria is time consuming. The use of molecular techniques as powerful tools for detecting and identifying *L. fungicola* from *A. bisporus* tissue and samples from mushroom farms would have many advantages.

Detection techniques using nucleic acids are based on the use of unique oligonucleotide sequences either as probes in hybridization assay, or as primers for enzymatic amplification of DNA fragments using the polymerase chain reaction (PCR) (Pedersen *et al.*, 1997).

Designing selective primers for *L. fungicola* var. *fungicola* is very difficult as was shown by Largeteau *et al.* (2007). Their primers for detection of *L. fungicola* var. *fungicola* were designed from ITS1 region of rDNA, but these primers also amplified *A. bisporus* DNA giving the same size amplification product as *L. fungicola* var. *fungicola*. Other researchers (Romaine *et al.*, 2002), designed primers for *L. fungicola* var. *aleophilum* using the sequence of a product based on the method described by Chen *et al.* (1999). This set of primers is able to detect *L. fungicola* var. *aleophilum* from affected *A. bisporus* tissue giving a 162 bp amplicon. This primer set is able to amplify only *L. fungicola* var. *aleophilum* and does not amplify *L. fungicola* var. *fungicola*.

In this study selective primers for *L. fungicola* identification and detection were designed for the mating type (MAT) locus. The MAT locus is a region in genomic DNA responsible for sexual reproduction of fungi (Fraser and Heitman, 2004). Most self-sterile (heterothallic) filamentous ascomycetes have a dimictic mating system with two alleles (idiomorphs) located in a single locus. One idiomorph is MAT1-1 and the second is MAT1-2, such mating type genes have been identified in a number of filamentous ascomycetes belonging to fungal groups that are widely separated in evolutionary terms (Varga, 2003). The hallmark of the MAT1-2 locus is the MAT1-2-1 gene, encoding a protein with a high mobility group (HMG) domain habouring the three invariant residues histidine, proline, and glycine. In addition to the MAT1-2-1 gene, other genes may also be present at the MAT1-2 locus (Coppin *et al.*, 1997). In contrast to the genomes of heterothallic species, the genomes of self-fertile (homothallic) filamentous ascomycetes contain genes indicative of both mating types that can be either linked or unlinked (Galagan *et al.*, 2005; Pöggeler *et al.*, 1997; Rydholm *et al.*, 2007; Yun *et al.*, 1999). The mating-type genes MAT1-1-1 and MAT1-2-1 are the most conserved genes in the mating-type loci MAT1-1 and MAT1-2, respectively (Turgeon and Yoder, 2000). The sequence of MAT1-2-1 region of *L. fungicola* var. *fungicola* was described by Yokoyama and Hara and submitted to the EMBL/GenBank/DDBJ (2003) databases and published by Yokoyama *et al.* (2004 and 2006) (**Figure** 4-1).

Sequence information

Length: **209** BP, A Count: **66**, C Count: **55**, T Count: **40**, G Count: **48**

```
ggatagacac agcatggtga agaaagcaga ccccaagttg acaaacaatg aagtttgtaa      60
gttcatatca ccggtcttca catgtgacct cgctgaccat ttcagcgcaa atcctcggac     120
ggtcctggaa tgccgaacct caggaggtgc gacaacgcta caagaaaatg tctgaagaca     180
tcaagacagc cctccttgag agacatccc                                       209
```

Figure 4-1: MAT1-2-1 sequence information of *L. fungicola*. (http://tanga.vub.ac.be:8080/mrs/query.do?db=embl&query=AB124635).

Another useful source of selective primer sequences is based on ribosomal or internal transcribed space (ITS) sequences (rDNA), despite the fact that variability in these regions is not very high between different fungi (Geisen, 2007). The fungal ribosomal genes are organized in a tandem repeat and inside the rDNA repeat, two variable non-coding ITS regions are nested between the highly conserved nuclear small subunit rRNA (SrDNA), 5.8S and large subunit rRNA genes (**Figure** 4-2). The ribosomal region spanning ITS1-5.8S-ITS2 is often between 600-800 bp long and is found in multiple copies which make it practicable to amplify DNA fragments from samples containing target DNA (Gardes and Bruns, 1993). The fungal ribosomal genes are highly conserved at the genus level or even higher (Bruns *et al.*, 1991). The internally transcribed spacers (ITS1 and ITS2) and the intergenic spacer (IGS) have evolved faster than the ribosomal genes and may therefore be more useful for the development of specific oligonucleotide primers and/or probes, aimed at differentiating at the genus, species or subspecies level (White *et al.*, 1990).

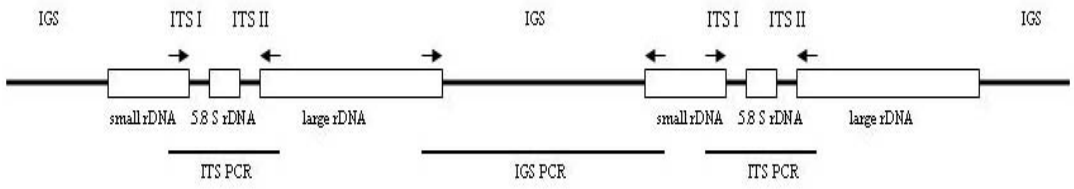


Figure 4-2: Schematic presentation of the organisation of fungal rDNA genes with arrows indicating possible target sequences (Geisen, 2007).

Several studies have shown that ITS regions are highly variable among and within different fungal species (Chen *at al.*, 2000; O'Donnell 1992; Muthumeenakshi *et al.*, 1992). The *L. fungicola* var. *fungicola* ITS1-5.8S-ITS2 region was described by Collopy *et al.* and submitted (29-NOV-2000) to the EMBL/GenBank/DDBJ databases; the publication about this sequence was published by Collopy *et al.* (2001) (**Figure** 4-3).

Sequence information

Length: **559** BP, A Count: **133**, C Count: **172**, T Count: **112**, G Count: **142**

```
aaggtgaacc tgcggaggga tcattacaga gtttacaact cccaaaccca aatgtgaaca        60
taccaatcgt tgcttcggcg gactcgtccc ggcgtccggg tggccttgcg ctgcccgcgg       120
cccggatcca ggcggccgcc ggaggccatc aaactctttg tattaccagt atctttctgaa       180
tccgccgcaa ggcaaaacaa atgaatcaaa actttcaaca acggatctct tggttctggc       240
atcgatgaag aacgcagcga aatgcgataa gtaatgtgaa ttgcagaatt cagtgaatca       300
tcgaatcttt gaacgcacat tgcgcccgcc agcattctgg cgggcatgcc tgttcgagcg       360
tcatttcaac cctcgagctc cctttgggga gcccggcgtt ggggaccggc ctctaccgcc       420
gcccccgaaa tacagtggcg gccccgtcac ggcgacctct gcgtagtaac tcaacctcgc       480
accggaaacc cgacgtggcc acgccgtaaa acaccccact tctgaacgtt gacctcggat       540
caggtaggaa tacccgctg                                                    559
```

Figure 4-3: ITS1-5.8S-ITS2 sequence information of *L. fungicola*. (http://tanga.vub.ac.be:8080/mrs/query.do?db=embl&query=AF324874).

The *L. fungicola* var. *fungicola* ITS1-5.8S-ITS2 and 28S rRNA region was described by Yokoyama and Hara and submitted to the EMBL/GenBank/DDBJ (2003) databases; the publication about this sequence was published by Yokoyama *et al.* (2004 and 2006) (**Figure** 4-4).

Sequence information

Length: **2294** BP, A Count: **585**, C Count: **546**, T Count: **565**, G Count: **598**

```
catgtctaag tataagcaat tatacagcga aactgcgaat ggctcattat ataagttatc        60
gtttatttga tagtaccttа ctacttggat aaccgtggta attctagagc taatacatgc       120
taaaaatccc gacttcggaa gggatgtatt tattagatta aaaaccaatg ccctctgggc       180
tccttggtga ttcataatаа cttttcgaat cgcatggcct tgcgccggcg atggttcatt       240
caaatttctt ccctatcaac tttcgatgtt tgggtattgg ccaaacatgg tcgcaacggg       300
taacggaggg ttagggctcg accccggaga aggagcctga gaaacggcta ctacatccaa       360
ggaaggcagc aggcgcgcaa attacccaat cccgattcgg ggaggtagtg acaataaata       420
ctgatacagg gctcttttgg gtcttgtaat tggaatgagt acaatttaaa tctcttaacg       480
aggaacaatt ggagggcaag tctggtgcca gcagccgcgg taattccagc tccaatagcg       540
tatattaaag ttgttgtggt taaaaagctc gtagttgaac cttgggcctg gctggccggt       600
ccgcctcacc gcgtgtactg gtccggccgg gcctttccct ctgtggaacc tcatgccctt       660
cactgggtgt ggcgggggaaa caggactttt actttgaaaa aattagagtg ctccaggcag       720
gcctatgctc gaatacatta gcatggaata ataaaatagg acgtgtggtt ctattttgtt       780
ggtttctagg accgccgtaa tgattaatag ggacagtcgg gggcatcagt attcaattgt       840
cagaggtgaa attcttggat ttattgaaga ctaactactg cgaaagcatt tgccaaggat       900
gttttcatta atcaggaacg aaagttaggg gatcgaagac gatcagatac cgtcgtagtc       960
ttaaccataa actatgccga ctagggatcg gacgatgtta ttttttgacg cgttcggcac      1020
cttacgagaa atcaaagtgc ttgggctcca gggggagtat ggtcgcaagg ctgaaacttа      1080
aagaaattga cggaagggca ccaccagggg tggagcctgc ggcttaattt gactcaacac      1140
ggggaaactc accaggtcca gacacaatga ggattgacag attgagagct ctttcttgat      1200
tttgtgggtg gtggtgcatg gccgttctta gttggtggag tgatttgtct gcttaattgc      1260
gataacgaac gagaccttaa cctgctaaat agcccgtatt gctttggcag tacgccggct      1320
tcttagaggg actatcggct caagccgatg gaagtttgag gcaataacag gtctgtgatg      1380
cccttagatg ttctgggccg cacgcgcgct acactgacgg agccagcgag tacttccttg      1440
gccgaaaggc ccgggtaatc ttgttaaact ccgtcgtgct ggggatagag cattgcaatt      1500
attgctcttc aacgaggaat ccctagtaag cgcaagtcat cagcttgcgt tgattacgtc      1560
cctgcccttt gtacacaccg cccgtcgcta ctaccgattg aatggctcag tgaggcgtcc      1620
ggactggccc agggaggtgg gcaactacca cccagggccg gaaagctctc caaactcggt      1680
catttagagg aagtaaaagt cgtaacaagg tctccgttgg tgaaccagcg gagggatcat      1740
tacagagttt acaactccca aacccaaatg tgaacatacc aatcgttgct tcggcggact      1800
cgtcccggcg tccgggtggc cttgcgctgc ccgcggcccg gatccaggcg gccgccggag      1860
gccatcaaac tctttgtatt accagtatct tctgaatccg ccgcaaggca aaacaaatga      1920
atcaaaactt tcaacaacgg atctcttggt tctggcatcg atgaagaacg cagcgaaatg      1980
cgataagtaa tgtgaattgc agaattcagt gaatcatcga atctttgaac gcacattgcg      2040
cccgccagca ttctggcggg catgcctgtt cgagcgtcat ttcaaccctc gagctccctt      2100
tggggagccc ggcgttgggg accggcctct accgccgccc ccgaaataca gtggcggccc      2160
cgtcacggcg acctctgcgt agtaactcaa cctcgcaccg gaaacccgac gtggccacgc      2220
cgtaaaacac cccacttctg aacgttgacc tcggatcagg taggaatacc cgctgaactt      2280
aagcatatca ataa                                                        2294
```

Figure 4-4: ITS1-5.8S-ITS2 and 28S rRNA sequence information of *L. fungicola*. (http://tanga.vub.ac.be:8080/mrs/query.do?db=embl&query=AB107135).

The aim of this study was to evaluate different DNA extraction methods and PCR assays to find the best method for DNA extraction from pure culture of *L. fungicola.* The second aim of this study was to test primers described by Largeteau *et al.* (2007) for the detection of *L. fungicola* and the optimization of PCR assay with selective primers from *L. fungicola* designed by Zijlstra *et al.* (2007, 2008 and 2009) for Real Time PCR (TaqMan) and test these primers for different mushroom pathogens and *Agaricus bisporus* DNA. The last task of this study was to find a good DNA extraction method for the detection of *L. fungicola* DNA from soil and casing samples. The DNA extraction procedures can eliminate many interfering substances contained in soil and casing soil. The objective of this study also was designed selective primers for PCR assay for detection of *L. fungicola* from mushroom farm samples using mating type (MAT1-2-1) locus and rDNA (ITS1-5.8S-ITS2 and 28S rRNA) sequences from *L. fungicola*.

4.1 Results

The DNA extraction method and PCR methods are described in Chapter 2 – Section 2.6.15 and Section 2.6.17 and Section 2.6.19.

4.1.1 Evaluation of DNA extraction methods for pure cultures of *L. fungicola* and *Agaricus bisporus*

4.1.1.1 Comparison of DNA extraction methods for pure cultures of *L. fungicola*

Lecanicillium fungicola (CR.181) DNA was isolated using 4 different methods – one manual procedure according to Aljanabi and Martinez (1997) and three commercially available DNA isolation kits: Nucleon Phytopure Genomic DNA Extraction Kit, ZR Fungal/Bacterial DNA kit and DNeasy Plant Mini Kit. Genomic DNA was extracted according to the protocols.

Aljanabi and Martinez (1997) – genomic DNA was extracted from pure cultures of *L. fungicola* grown in liquid media (ME). The genomic DNA showed poor molecular weight quality genomic DNA with a high amount of RNA which is undesirable. When genomic DNA was isolated from pure cultures grown on agar plates the samples showed high-quality genomic DNA with a greater proportion of higher

molecular weight DNA and less shearing. In this manual method genomic DNA contained a high amount of RNA which is undesirable (**Figure** 4-5).

Nucleon Phytopure Genomic DNA Extraction Kit – genomic DNA was isolated from pure cultures of *L. fungicola* grown in liquid media (ME). The genomic DNA showed high-quality genomic DNA with a greater proportion of higher molecular weight DNA and less shearing. When isolated from cultures grown on agar plates the genomic DNA was weak. Using this DNA isolation kit genomic DNA contained a high amount of RNA which is undesirable (**Figure** 4-5).

ZR Fungal/Bacterial DNA – genomic DNA was isolated from pure cultures of *L. fungicola* grown in liquid media (ME). The genomic DNA was weak. When DNA was extracted from pure cultures grown on agar plates the genomic DNA had high-quality genomic DNA with a greater proportion of higher molecular weight DNA and less shearing. The electrophoresis gel did not show contamination with RNA (**Figure** 4-6).

DNeasy Plant Mini Kit genomic DNA was isolated from pure cultures of *L. fungicola* grown in liquid media (ME) and from pure cultures grown on agar plate. The genomic DNA showed high-quality genomic DNA with a greater proportion of higher molecular weight DNA and less shearing. The electrophoresis gel did not show contamination with RNA (**Figure** 4-6).

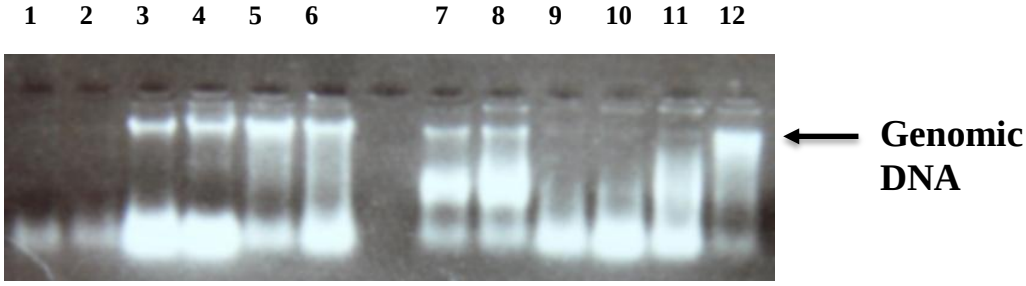


Figure 4-5: Electrophoresis profile of genomic DNA of *L. fungicola* isolated by methods of Aljanabi and Martinez (1997) (Lines 1-6), Nucleon Phytopure Genomic DNA Extraction Kit (Lines 7-12). Where 2 µl of genomic DNA was loaded for each sample. *Lecanicillium fungicola* grown in liquid media (Lines 1-2, 7-8) and grown on agar plates after 14 days (Lines 3-6, 9-12).

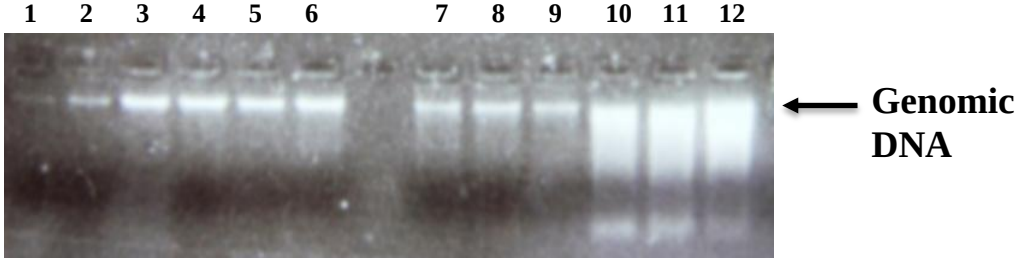


Figure 4-6: Electrophoresis profile of genomic DNA of *L. fungicola* isolated by ZR Fungal/Bacterial DNA kit (Lines 1-6), DNeasy Plant Mini Kit (Lines 7-12) isolation kit where 2 µl of genomic DNA was loaded for each sample. *Lecanicillium fungicola* grown in liquid media (Lines 1-2, 7-9) and grown on agar plate after 14 days (Lines 3-6, 10-12).

The extraction of genomic DNA was repeated using the same methods as previously described and with freeze-dried material of *L. fungicola*.

Aljanabi and Martinez (1997) – DNA extraction method showed similar results to the previous results. The genomic DNA showed high-quality genomic DNA with a greater proportion of higher molecular weight DNA and less shearing, but DNA contained a high amount of RNA. This method required modification with added RNase A to eliminate RNA contamination (**Figure** 4-7).

Nucleon Phytopure Genomic DNA Extraction Kit – the genomic DNA showed high-quality genomic DNA with a greater proportion of higher molecular weight DNA and less shearing. The RNA was eliminated by adding RNase A following by producer optional recommendations (**Figure** 4-7).

ZR Fungal/Bacterial DNA – DNA extraction method showed similar results to the previous results. The genomic DNA showed high-quality genomic DNA with a greater proportion of higher molecular weight DNA and less shearing in all samples (freeze-dried and agar plate samples) (**Figure** 4-8).

DNeasy Plant Mini Kit – DNA extraction method showed similar results to the previous results. The genomic DNA high-quality genomic DNA with a greater proportion of higher molecular weight DNA and less shearing in freeze-dried, agar plate samples. When genomic DNA was isolated from pure cultures grown in liquid media the amount was very low (**Figure** 4-8).

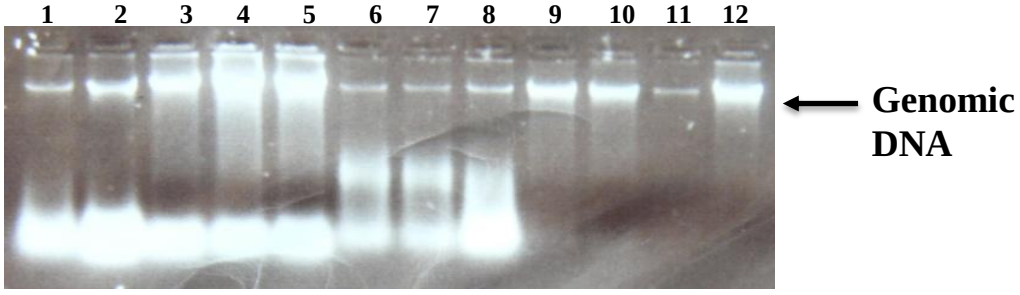


Figure 4-7: Electrophoresis profile of genomic DNA of *Lecanicillium fungicola* isolated by methods of Aljanabi and Martinez (1997) (Lines 1-8), Nucleon Phytopure Genomic DNA Extraction Kit (Lines 9-12) where 2 µl of genomic DNA was loaded for each sample. *Lecanicillium fungicola* was isolated from lyophilised mycelium 1-2 and 9-10 and grown on agar plate after 14 days (Lines 3-5, 11-12) and liquid media (Lines 6-8).

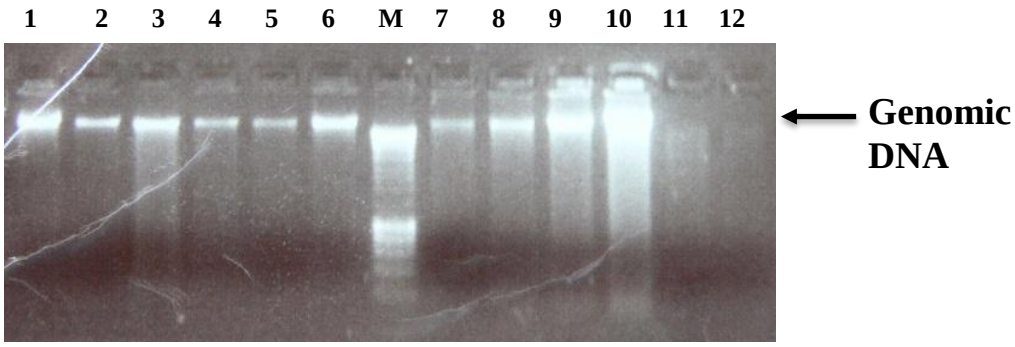


Figure 4-8: Electrophoresis profile of genomic DNA of *Lecanicillium fungicola* isolated by ZR Fungal/Bacterial DNA kit (Lines 1-6), DNeasy Plant Mini Kit (Lines 7-12) isolation kit where 2 µl of genomic DNA was loaded for each sample. Genomic DNA of *L. fungicola* was isolated from lyophilised mycelium (Lines 1-3, 7-8) and grown on agar plates after 14 days (Lines 4-6, 9-10) and liquid media (Lines 11-12). M – Marker 100 bp DNA.

4.1.1.2 PCR assays

The DNA extracted using Nucleon Phytopure Genomic DNA Extraction Kit, ZR Fungal/Bacterial DNA kit and DNeasy Plant Mini Kit were analysed by PCR for evaluation of suitability for amplification. The primers used for PCR were described by Largeteau *et al.* (2007) and this primer set amplified a 130 bp sequence of the ITS1 region of *L. fungicola*. The amplification products were present in all tested samples (**Figure** 4-9).

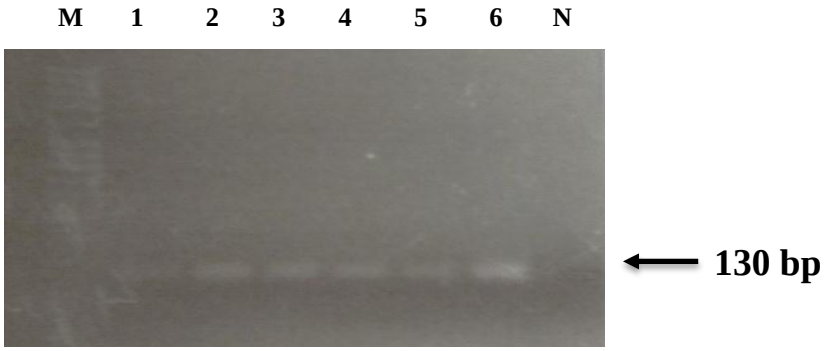


Figure 4-9: Electrophoretic profiles of PCR products from *L. fungicola* using DNA extracted using ZR Fungal/Bacterial DNA kit (Line 1 and 5-6), DNeasy Plant Mini Kit (Line 2-3) and Nucleon Phytopure Genomic DNA Extraction Kit (Line 4). For PCR reaction 1 µl of genomic DNA was used. The diagnostic 130 bp amplicon appears in line 1-6. The Tm (midpoint temperature in degrees Celsius) in PCR reaction was 54 °C. M – Marker 50 bp DNA, N – water control.

Different amounts of DNA (0.25-10 ng/µl) of *L. fungicola* (CR. 181) genomic DNA extracted using DNeasy Plant Mini Kit for PCR reactions were used. The amplification product was present in all samples with *L. fungicola* DNA. The 130 bp amplicon was present in all concentrations of DNA of *L. fungicola* (**Figure** 4-10).

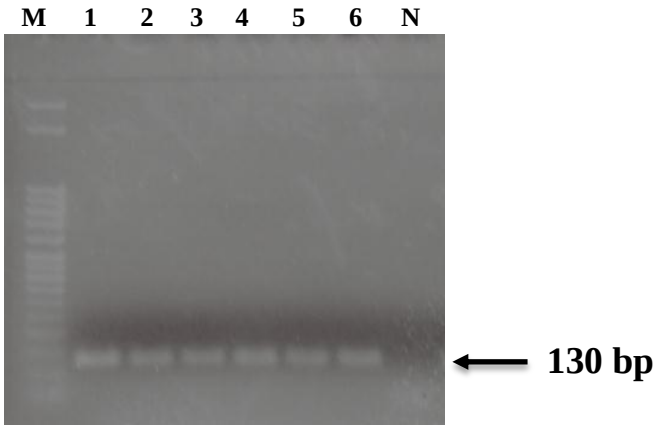


Figure 4-10: Electrophoretic profile of PCR products from *A. bisporus* and *Lecanicillium fungicola* using DNA extracted using DNeasy Plant Mini Kit isolation kit (Line 1-6). For PCR reaction 1 µl of genomic DNA was used. Line 1 – 10 ng/µl, 2 – 5ng/µl, 3 – 2.5 ng/µl, 4 – 1 ng/µl, 5 – 0.5 ng/µl, 6 – 0.25 ng/µl of *L. fungicola* genomic DNA. The Tm in PCR reaction was 54 °C. M – Marker 50bp DNA, N – water control.

4.1.1.3 DNA extraction and PCR assay from mixed samples containing *L. fungicola* and *A. bisporus*

Genomic DNA was extracted from pure culture of *L. fungicola* (L.2) and tissue of *A. bisporus* (Ab) and from mixed samples of *L. fungicola* and *A. bisporus*. The DNA extraction was performed using two commercially available DNA extraction kits: ZR Fungal/Bacterial DNA kit and DNeasy Plant Mini Kit and one manual method Aljanabi and Martinez (1997). The high molecular weight quality genomic DNA of *A. bisporus* and *A. bisporus* mixed with *L. fungicola* was present when DNA extraction was performed by DNeasy Plant Mini Kit. The Aljanabi and Martinez (1997) DNA extraction method was modified and RNase A was added. High-quality genomic DNA with a greater proportion of higher molecular weight DNA and less shearing was present in only one sample of *A. bisporus* and all samples of *A. bisporus* mixed with *L. fungicola*. Good genomic DNA of *L. fungicola* was present when DNA was extracted using ZR Fungal/Bacterial DNA kit (**Figure** 4-11).

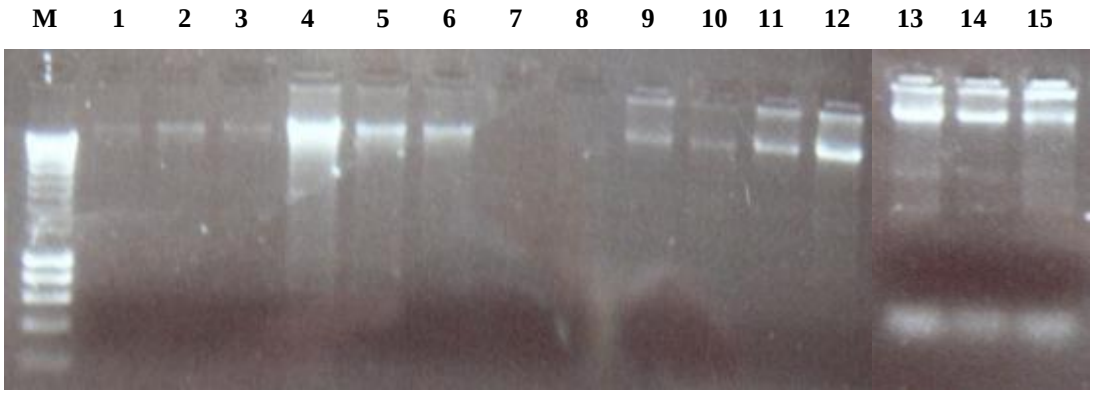


Figure 4-11: Electrophoresis profile of genomic DNA of *A. bisporus* and *Lecanicillium fungicola* isolated by DNeasy Plant Mini Kit isolation kit (Line 1-6) and Aljanabi and Martinez (1997) (Line 7-12), ZR Fungal/Bacterial DNA kit (Line 13-15), where 2 µl of genomic DNA was loaded for each sample. Genomic DNA of *A. bisporus* (Line 1-3 and 7-9) (Ab1Q to Ab2Q and Ab1M to Ab3M), *A. bisporus* and *L. fungicola* (Line 4-6 and 10-12) (AbV1Q to AbV3Q and AbV1M to AbV3M), *L. fungicola* (Line 13-15) (V1 to V3). M – Marker 100 bp DNA.

The extracted DNA was anlysed by PCR assay using the primer set described by Largeteau *et al.* (2007). The amplification product was present in all tested samples containing *A. bisporus*, *A. bisporus* mixed with *L. fungicola* and *L. fungicola* DNA. The amount of amplified product in samples containing *A. bisporus* DNA only was very small with DNeasy Plant Mini Kit, but quite strong with the DNA isolated by the Aljanabi and Martinez (1997) DNA extraction method. These primers are able to amplify *A. bisporus*. Largeteau *et al.* (2007) set of primers are not specific for *L. fungicola* detection only (**Figure** 4-12).

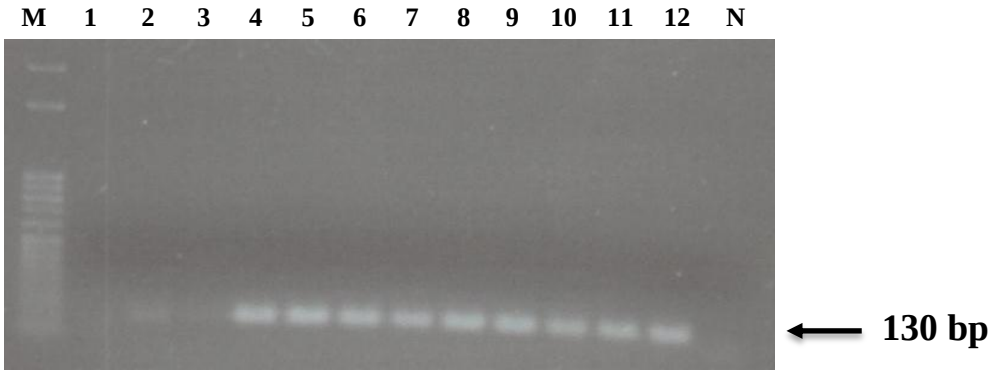


Figure 4-12: Electrophoretic profile of PCR products from *A. bisporus* and *Lecanicillium fungicola* using DNA extracted using DNeasy Plant Mini Kit isolation kit (Line 1-6) and Aljanabi and Martinez (1997) manual method (Line 7-12). For PCR reaction 1 µl of genomic DNA was used. The amplification product of *A. bisporus* (Line 1-3 and 7-9) (Ab1Q to Ab3Q and Ab1M to Ab3M), *A. bisporus* and *L. fungicola* (Line 4-6 and 10-12) (AbV1Q to AbV3Q and AbV1M to AbV3M). The Tm (midpoint temperature in degrees Celsius) in PCR reaction was 54 °C. M – Marker 50 bp DNA, N – water control.

The PCR assay was repeated for the DNeasy Plant Mini Kit. The results confirmed that the primers amplified DNA from both fungi *A. bisporus* and *L. fungicola* giving the same size 130 bp amplicon (gel not shown).

The DNA extractions were performed using the DNeasy Plant Mini Kit. Different concentrations of *A. bisporus* genomic DNA (0.25-10 ng/µl) were used for PCR reaction. The amplification product (130 bp) was present in samples containing 10 and 5 ng/µl DNA of *A. bisporus*. The amplicon was not present in lower DNA concentrations. The efficiency level for amplification of *A. bisporus* was low (**Figure** 4-13).

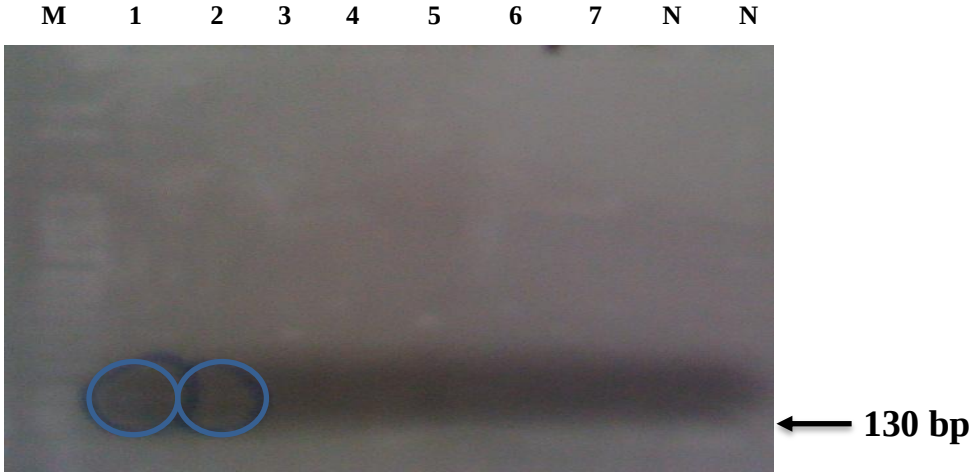


Figure 4-13: Electrophoretic profile of PCR products from *A. bisporus* (Ab1Q) using DNeasy Plant Mini Kit DNA extraction kit (Line 1-7). For PCR reaction 1 µl of genomic DNA was used. The amplification product of *A. bisporus*. Line 1 – 10 ng/µl, 2 – 5ng/µl, 3 – 2.5 ng/µl, 4 – 1 ng/µl, 5 – 0.5 ng/µl, 6 – 0.25 ng/µl of *L. fungicola* genomic DNA. The Tm in PCR reaction was 54 °C. M – Marker 50 bp DNA, N – water control.

4.1.1.4 Summary

The comparison of four DNA extraction methods in this study highlighted differences in the quality and quantity of genomic DNA of *L. fungicola* depending on the isolation method. The **ZR Fungal/Bacterial** DNA kit was considered the best because it always gave high molecular weight quality genomic DNA without RNA for all types of samples. This system is less time-consuming and less technically demanding than the other DNA extraction methods. The ZR Fungal/Bacterial DNA kit used Lysis Tube containing a BeatingBead^{TM} to mechanically disrupt the fungal cells, after which the DNA was extracted and purified using a column system which is supplied by the producer. The manual DNA extraction method described by **Aljanabi and Martinez (1997)**, gave high quantity genomic DNA but it contained a large amount of RNA which is undesirable. An improvement of this method would be the addition of RNase A during DNA extraction to eliminate RNA contamination. This method works well for

all types of samples giving lots of DNA. The quality of DNA was between A280/A260 ratio= 1.3 to 1.8. The manual method is relatively inexpensive but it is time consuming and uses toxic reagents. The **Nucleon Phytopure Genomic DNA Extraction Kit** and **DNeasy Plant Mini Kit** gave high molecular weight quality genomic DNA in almost all type of tested samples. The **Nucleon Phytopure Genomic DNA Extraction Kit** required an RNase A during DNA extraction in order to obtain DNA without RNA. These two methods of DNA extraction require grinding of samples in liquid nitrogen using a mortar and pestle to disrupt the fungal cells. The **Nucleon Phytopure Genomic DNA Extraction Kit** is a chloroform DNA extraction method. The **DNeasy Plant Mini Kit** is a typical column DNA extraction method. Both these kits are time consuming and equipment demanding. The quality of DNA extracted using **Nucleon Phytopure Genomic DNA Extraction Kit** and **DNeasy Plant Mini Kit** was between A280/A260 ratio = 1.3 to 1.85.

Primers described by Largeteau *et al.* (2007), amplified a 130 bp amplicon from the ITS1 region of rDNA of *L. fungicola* var. *fungicola* and *A. bisporus*. These experiments confirmed the conclusion of Largeteau *et al.* (2007) that the primers were non-specific for detection of *L. fungicola* from *A. bisporus* tissues. When *A. bisporus* DNA was present in PCR assays the amplification product was the same size (130 bp) as *L. fungicola* DNA. The amount of amplification product of *A. bisporus* was very low when the extraction method was DNeasy Plant Mini Kit. The amount of *A. bisporus* amplification product was much higher when the Aljanabi and Martinez (1997) DNA extraction method was used, which was not expected but it could be a human error. When the amount of *A. bisporus* DNA was 5 ng per reaction or higher the amplification product was present. These primers were unsuccessful for detection of *L. fungicola* from samples containing *A. bisporus* material.

4.1.2 Optimisation of primers for *L. fungicola* detection

4.1.2.1 Comparison of the different efficiency of primers

Two set of primers were compared, those designed by Largeteau *et al.* (2007), and Zijlstra *et al.* (2007, 2008 and 2009) (Zijlstra primers). The DNA was extracted from samples of *L. fungicola* (L.2), *A. bisporus* (Ab) and *L. fungicola* and *A. bisporus* (mixed) by DNeasy Plant Mini Kit and tested by PCR assay using two sets of primers. The *L. fungicola* DNA gave amplification product of 130 bp using Largeteau *et al.*

(2007) primers and 102 bp when primers designed by Zijlstra primers were used. The DNA extracted from *A. bisporus* gave an amplification product of 130 bp when the primers designed by Largeteau *et al.* (2007) were used but did not give a 102 bp amplificon with the Zijlstra primers. The Zijlstra primers did give amplification products of *A. bisporus* between 916 bp and 1350 bp. The larger amplicons were not present when *L. fungicola* DNA was mixed with *A. bisporus* DNA. The Zijlstra primers are considered to be the most suitable for work with the mixed DNA, but they need to be optimised for PCR assay (**Figure** 4-14).

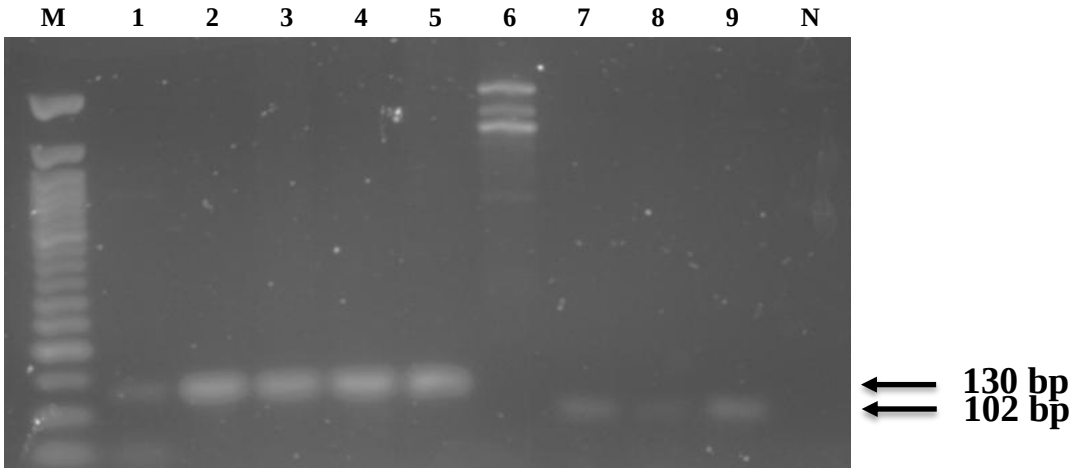


Figure 4-14: Electrophoretic profile of PCR products from *A. bisporus* using DNA extracted using DNeasy Plant Mini Kit isolation kit (Line 1-9), where 5 µl of genomic DNA was used for PCR reaction. The amplification product of *A. bisporus* (Line 1, 6) (Ab2Q), *A. bisporus* mixed with *L. fungicola* (Line 2, 7) (AbV2Q), and *L. fungicola* (Line 3-5, 8-9) (V1). Largeteau *et al.* (2007) set of primers (Line 1-5) amplification product 130 bp and Zijlstra *et al.* (2007, 2008 and 2009) set of primers amplified a product of 102 bp. The Tm in PCR reaction was 47 °C. M-marker 50 bp DNA, N – Water control.

4.1.2.2 Sensitivity and specificity of different polymerases for *L. fungicola* detection.

Two polymerases were tested in order to improve the amplification product. In addition other fungi were included to test the specificity of Zijlstra primers. DNA was extracted using ZR Fungal/Bacterial DNA kit from different fungi: *C. mycophilum* (C.1), *M. perniciosa* (M.1), *A. fumigatus* (As), *L. fungicola* (L.1), and *A. bisporus* (As). Zijlstra primers were used. Two different polymerases, Taq polymerase DNA supplied by Sigma and High Fidelity DNA polymerase supplied by BioLabs, were tested for sensitivity and specificity. The 102 bp *L. fungicola* DNA amplification product was present with both tested polymerases but the signal was very weak and the PCR assay needed to be optimised. Other fungi did not give a 102 bp amplicon but some gave other non-specific products. The High Fidelity DNA polymerase gave more non-specific amplicons than Taq polymerase DNA (**Figure** 4-15).

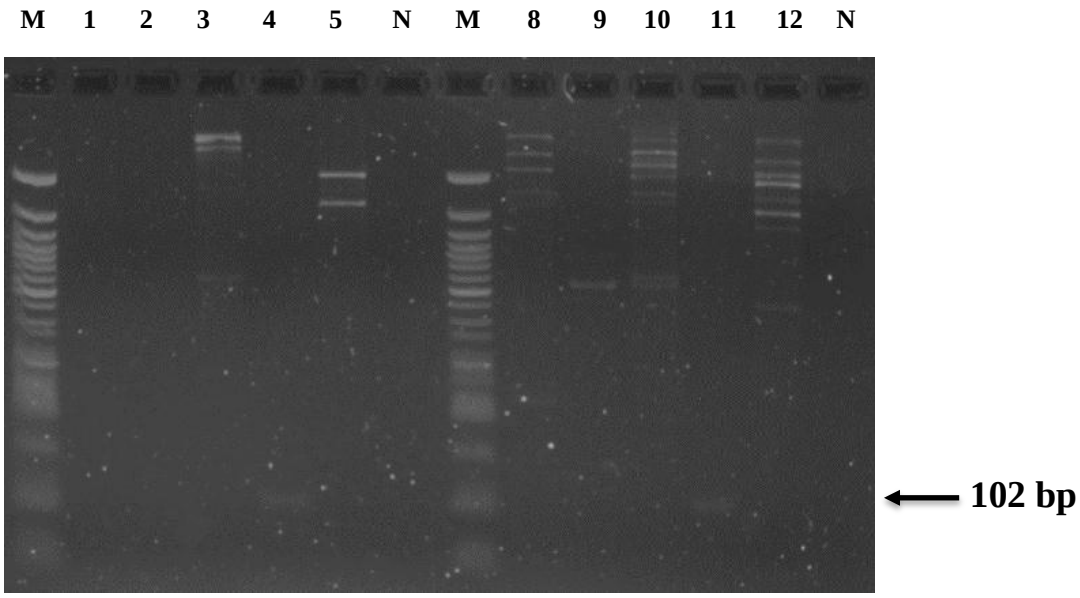


Figure 4-15: Electrophoretic profile of PCR products. DNA was extracted using ZR Fungal/Bacterial DNA kit, where 5 µl of genomic DNA was used for PCR reaction. The amplification product of *C. mycophilum* (Line 1, 8), *M. perniciosa* (Line 2, 9), *A. fumigatus* (Line 3, 10), *L. fungicola* (Line 4, 11) and *A. bisporus* (Line 5, 12) (Ab2Q). Taq polymerase DNA – Sigma (Line 1-5), High Fidelity DNA polymerase – BioLabs (Line 8-12). Zijlstra primers amplified a product of 102 bp. The Tm in PCR reaction was 47 °C. M – Marker 50 bp DNA, N – Water control.

4.1.2.3 Development and optimization of PCR assay

Experiments were performed to search for the amplification conditions that gave strong amplification of the specific products of *L. fungicola*. The optimized PCR assay reaction contained a total of 25 µl and can be summarized as follows: 1x PCR buffer, 2 mmol/L $MgCl_2$, 2 mmol/L dNTPs, 7.5 µl of 50 % glycerol, 0.52 µmol/L each primers, 2 Units Taq and 3.5 µl DNA template, H_2O was added to fill to 25 µl. The optimized annealing temperature was 50 °C.

The PCR reaction was: 2 min 95 °C for template denaturation and enzyme activation, amplification was obtained with 35 cycles of denaturation at 95 °C for 30 sec., annealing at 50 °C for 30 sec. and extension at 72 °C for 1 min followed to 72 °C for 5 min.

The polymerase experiment was repeated using the new optimised PCR conditions. DNA was extracted using ZR Fungal/Bacterial DNA kit from different fungi: *C. mycophilum* (C.1), *M. perniciosa* (M.1) and *L. fungicola* (L.7 and L.1). Primers used for this test were designed by Zijlstra *et al.* (2007, 2008 and 2009) (Zijlstra primers). In this experiment two different polymerases, GoTaq polymerase supplied by Promega and Taq polymerase DNA supplied by Sigma and were tested for sensitivity and specificity. The *L. fungicola* DNA amplification product was present with both

tested polymerases and had a size of 102bp. The sensitivity and specificity were very good for both tested polymerases. Other fungi gave non-specific amplicons which were different to the *L. fungicola* amplicon. The Zijlstra primers gave a positive signal only for *L. fungicola* DNA, but also some gave non-specific amplicons for mushroom pathogens. Both tested polymerases gave good results so it was decided to select only one for future work and GoTaq polymerase was selected (**Figure** 4-16).

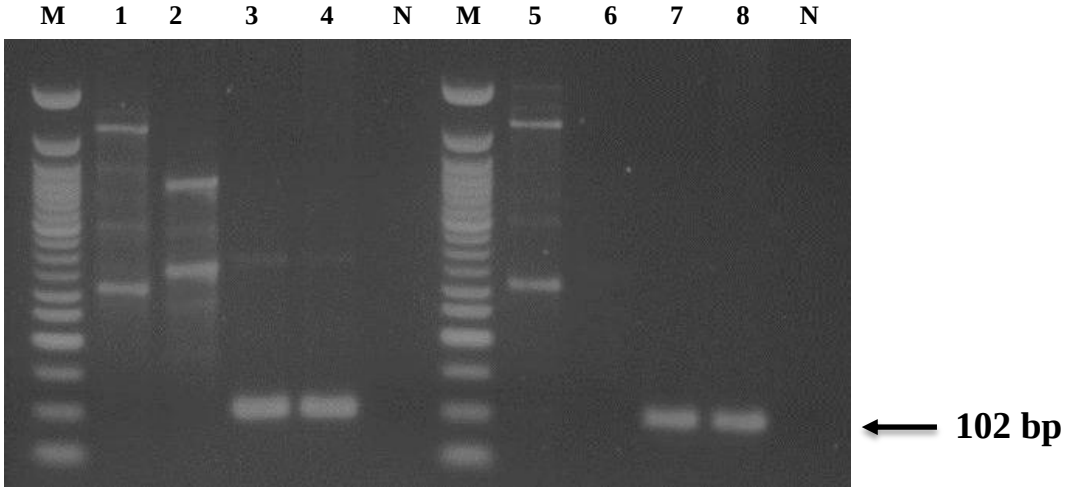


Figure 4-16: Electrophoretic profile of PCR products. DNA was extracted using ZR Fungal/Bacterial DNA kit, where 3.5 µl of genomic DNA was used for PCR reaction. The amplification product of *C. mycophilum* (Line 1, 5), *M. perniciosa* (Line 2, 6), *L. fungicola* (Line 3-4, 7-8). GoTaq polymerase – Promega (Line 1-4), Taq polymerase DNA – Sigma (Line 5-8), Zijlstra *et al.* (2007, 2008 and 2009) set of primers amplified a product of 102 bp. The Tm in PCR reaction was 50°C. M – marker 50 bp DNA, N – water control.

The PCR assay was repeated using only GoTaq polymerase to include *A. bisporus* along with *C. mycophilum* (C.1), *M. perniciosa* (M.1), *L. fungicola* (L.7 and CR181), and *A. bisporus* (Ab). The *L. fungicola* gave the expected size of amplicon at 102 bp. Other tested fungi gave non-specific amplification products. *A. bisporus* DNA in one sample gave a positive amplicon (**Figure** 4-17**)** but when samples were repeated the *A. bisporus* did not show this size of amplification product (**Figure** 4-18). This sample may have been contaminated during the preparation of PCR reaction.

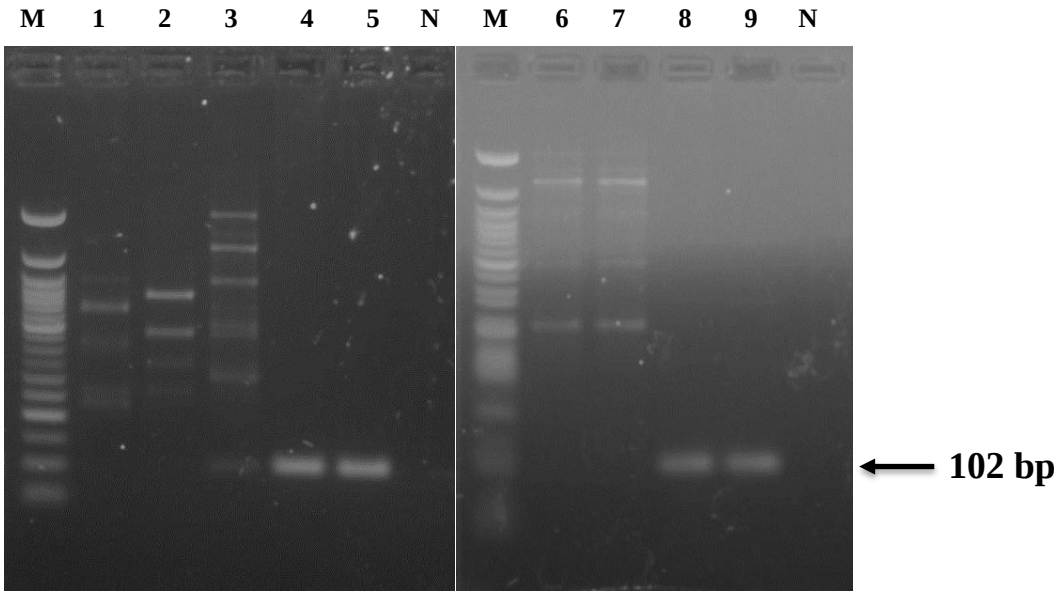


Figure 4-17: Electrophoretic profile of PCR products. DNA was extracted using ZR Fungal/Bacterial DNA kit, where 3.5 µl of genomic DNA was used for PCR reaction. The amplification product of *C. mycophilum* (Line 1), *M. perniciosa* (Line 2), *A. bisporus* (Line 3 (possibly contaminated by *L. fungicola* DNA), 6-7), *L. fungicola* (Line 4-5, 8-9). GoTaq polymerase – Promega and primers designed by Zijlstra *et al.* (2007, 2008 and 2009). The Tm in PCR reaction was 50 °C. M – marker 50 bp DNA, N – water control.

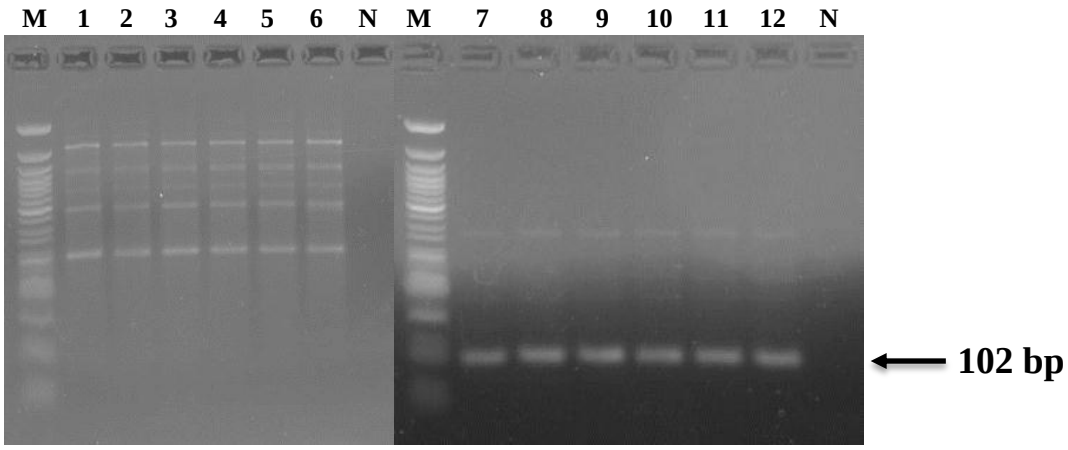


Figure 4-18: Electrophoretic profile of PCR products. DNA was extracted using ZR Fungal/Bacterial DNA kit, where 3.5 µl of genomic DNA was used for PCR reaction. Line 1-6 – *A. bisporus* (Ab2Q) and *L. fungicola* (Line 7-12) (L.2). GoTaq polymerase – Promega and Zijlstra primers. The Tm in PCR reaction was 50 °C. M – marker 50 bp DNA, N – water control.

4.1.2.4 PCR identification of *L. fungicola* isolates from mushroom farms using PCR assay

Ten isolates of *L. fungicola* (L.7, L.10, L.15, L.16, L.17, L.18, L.19, L.20, L.21, and L.22) were collected from mushroom farms and were subjected to PCR assay using the Zijlstra primers. DNA was extracted using ZR Fungal/Bacterial DNA kit and had high molecular weight quality genomic DNA (**Figure** 4-19).

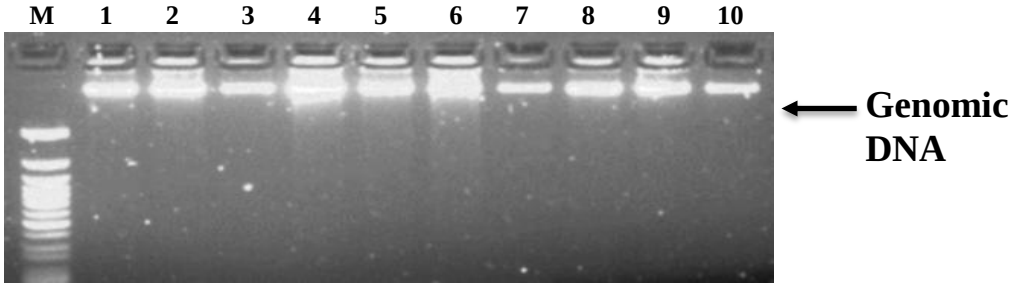


Figure 4-19: Electrophoresis profile of genomic DNA of *Lecanicillium fungicola* isolated by ZR Fungal/Bacterial DNA kit (Line 1-10). Line 1 – L.7, 2- L.10, 3 – L.15, 4 – L.16, 5 – L.17, 6 – L.18, 7 – L.19, 8 – L.20, 9 – L. 21, 10 – L. 22, where 2 µl of genomic DNA was loaded for each sample. Genomic DNA of *L. fungicola* grown on agar plates after 14 days.

The 102 bp PCR product was present in all isolates of *L. fungicola*. Some isolates of *L. fungicola* (L.15, L.18, and L.19, L.21) presented non-specific amplification. The non-specific amplicon was 400 bp (**Figure** 4-20).

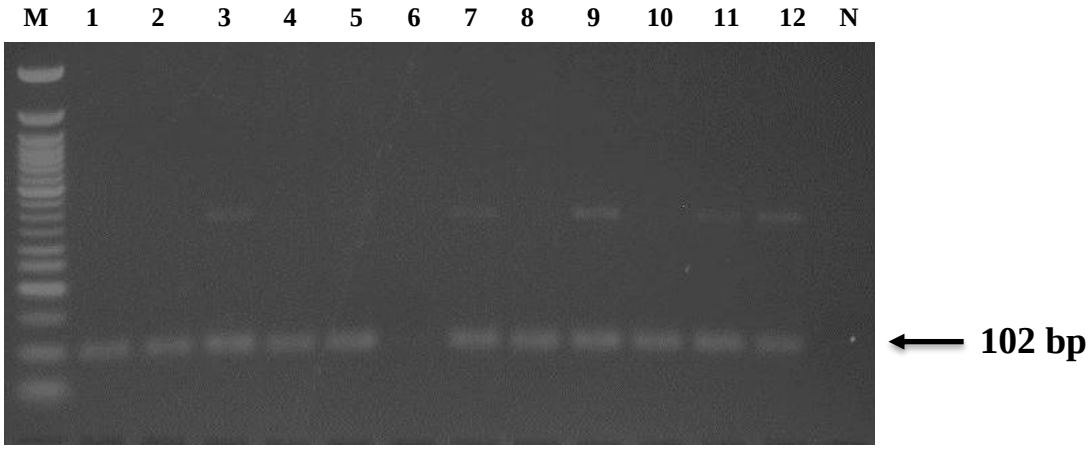


Figure 4-20: Electrophoretic profile of PCR products. DNA was extracted using ZR Fungal/Bacterial DNA kit, where 3.5 µl of genomic DNA was used for PCR reaction. The amplification product of wild isolates of *L. fungicola* (L.) was tested. Line 1 – L.7, 2 – L.10, 3 and 11 – L.15, 4 – L.16, 5 – L.17, 6 and 12 – L.18, 7 – L.19, 8 – L.20, 9 – L. 21, 10 – L. 22, where 2 µl of genomic DNA was loaded for each sample. GoTaq polymerase – Promega and Zijlstra primers. The Tm in PCR reaction was 50°C. M – Marker 50 bp DNA, N – water control.

4.1.2.5 PCR testing of live and dead *L. fungicola* material

Six replicates of 1 ml samples of *L. fungicola* (L.7) conidia (9.8×10^7 conidia/ml) were prepared. Three samples of conidia of *L. fungicola* were autoclaved before extraction to kill them and three samples containing viable conidia material were used. DNA extraction was performed using the ZR Fungal/Bacterial DNA kit. High molecular weight quality genomic DNA was present only in living material of *L. fungicola*. The autoclaved *L. fungicola* conidia did not show genomic DNA (**Figure** 4-21).

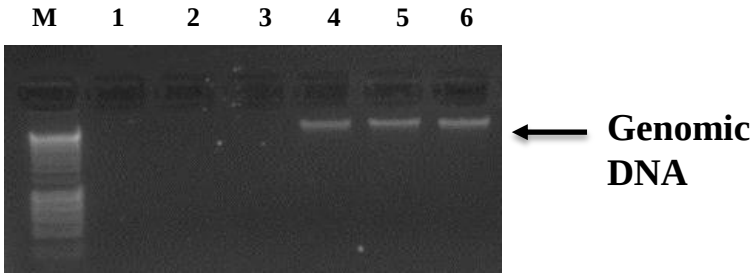


Figure 4-21: Electrophoresis profiles of genomic DNA of *Lecanicillium fungicola* isolated by ZR Fungal/Bacterial DNA kit (Line 1-6). Autoclaved conidia (Line 1-3) and live conidia (Line 4-5) where 2 µl of genomic DNA was loaded for each sample. Genomic DNA of *L. fungicola* was isolated from conidia concentration 9.8×10^7 conidia/ml.

The extracted DNA was analysed by PCR. The amplification product was present in all tested samples extracted from autoclaved and live conidia of *L. fungicola*. The amount of amplified product in samples containing autoclaved (dead) and viable conidia of *L. fungicola* DNA were the same (**Figure** 4-22).

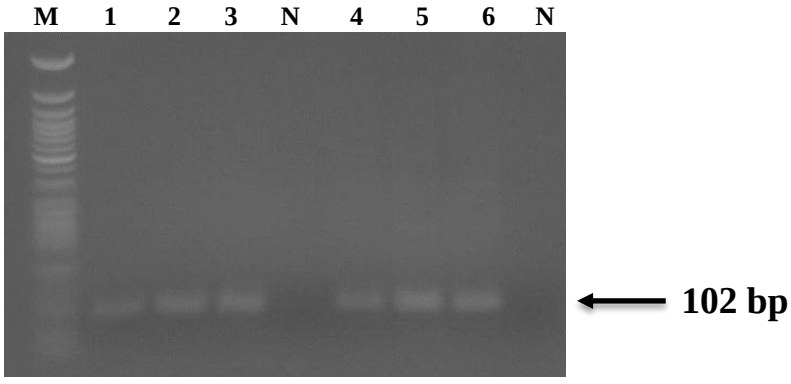


Figure 4-22: Electrophoretic profiles of PCR product, where 3.5 µl of genomic DNA was used for PCR reaction. The amplification product of wild isolates of *L. fungicola* autoclaved conidia (Line 1-3) and living conidia (Line 4-5) where 2 µl of genomic DNA was loaded for each sample. GoTaq polymerase – Promega and Zijlstra primers. The Tm in PCR reaction was 50 °C. M – Marker 50 bp DNA, N – water control.

4.1.2.6 Summary

Primers described by Largeteau *et al.* (2007), amplified an ITS1 region of rDNA of *L. fungicola* and *A. bisporus* giving 130 bp amplification products for both fungi. This set of primers was not really used by Largeteau *et al.* (2007) to analyse *L. fungicola*. Largeteau *et al.* (2010) designed selective primers for detection *L. fungicola* form *A. bisporus* tissue. Primers using by Largeteau *et al.* (2010) amplifying the *L. fungicola* eIF4E gene encoding the cap binding protein eIF4E. They qualified residual host DNA. The primer set described by Zijlstra *et al.* (2007, 2008 and 2009), amplified an rRNA region of *L. fungicola* giving a 102 bp amplification product. Zijlstra *et al.* (2007, 2008 and 2009) primers were designed for Real Time PCR reaction with TaqMan probe. DNA extraction using ZR Fungal/Bacterial DNA kit gave the best quality DNA and was a less time-consuming and technically demanding DNA extraction method. The PCR assay was optimised for efficiency, sensitivity and specify of *L. fungicola* PCR product. The highest efficiency was achieved with GoTaq polymerase supplied by Promega. The primer sets were tested with different fungi such as *C. mycophilum, M. perniciosa, A. bisporus* and *A. fumigatus*. These fungi gave many non-specific amplification products, but none gave a 102 bp amplicon. These results suggest that Zijlstra *et al.* (2007, 2008 and 2009) set of primers are selective for *L. fungicola* detection.

The autoclaved and not autoclaved samples of *L. fungictola* were tested. The autoclaved material did not show genomic DNA but extraction from autoclaved material gave less DNA and it is present in the suspension. Maybe if I will use a larger amount of genomic DNA for electrophoresis the genomic DNA will be show in autoclaved material. The PCR products form autoclaved and not autoclaved material gave a similar intensity but DNA quantity must be diluted to about 10 times to give products of different intensity. However inhibitions might be less abundant in autoclaved material.

4.1.3 Evaluation of DNA extraction methods from soil and casing samples of *L. fungicola*

4.1.3.1 Comparison of methods to extract DNA from soil and casing samples

The soil was collected from the NUIM grounds (0.26-0.28 g soil) and was mixed with 200 µl of conidia suspension and tissue of different fungal: *L. fungicola*, *C. mycophilum*, *M. perniciosa*, *A. fumigatus* and *A. bisporus*. The DNA was extracted by three different extraction methods.

Aljanabi and Martinez (1997) – the DNA was extracted using glass beads. Only one sample of soil mixed with *L. fungicola*, *C. mycophilum*, *M. perniciosa*, *A. fumigatus* and *A. bisporus* showed high molecular weight quality genomic DNA. Other samples did not show good quality genomic DNA. The genomic DNA was poor quality or not existing (**Figure** 4-23).

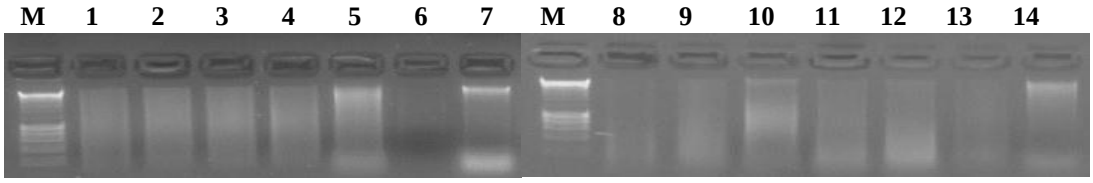


Figure 4-23: Electrophoresis profile of genomic DNA extracted using the method of Aljanabi and Martinez (1997). Genomic DNA of *L. fungicola* 9.8×10^7 conidia/ml (Line 1 and 8), *C. mycophilum* 1.25×10^6 conidia/ml (Line 2 and 9), *M. perniciosa* 1.0×10^6 conidia/ml (Line 3 and 10), 4.1×10^7 conidia/ml *A. fumigatus* (Line 4 and 11), *A. bisporus* (Line 5 and 6), Soil (Line 6 and 13) and mixture of fungi (*L. fungicola*, *C. mycophilum*, *M. perniciosa*, *A. fumigatus* and *A. bisporus*) (Line 7 and 14). M – Marker 100 bp DNA.

ZR Fungal/Bacterial DNA kit – the DNA was extracted and high molecular weight quality genomic DNA was present in all samples (**Figure** 4-24).

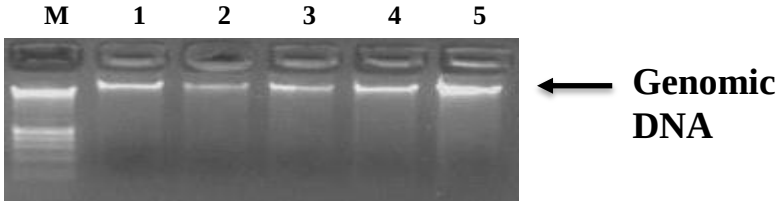


Figure 4-24: Electrophoresis profile of genomic DNA extracted using ZR Fungal/Bacterial DNA kit. Genomic DNA of *L. fungicola* 9.8×10^7 conidia/ml (Line 1), *C. mycophilum* 1.25×10^6 conidia/ml (Line 2), *M. perniciosa* 1.0×10^6 conidia/ml (Line 3), *A. bisporus* (Line 4) and mixture of fungi (*L. fungicola*, *C. mycophilum*, *M. perniciosa*, *A. fumigatus* and *A. bisporus*) (Line 5). M – Marker 100 bp DNA.

Yeates *et al.* (1998) – the DNA was extracted and high molecular weight quality genomic DNA was present in almost all samples (**Figure** 4-25).

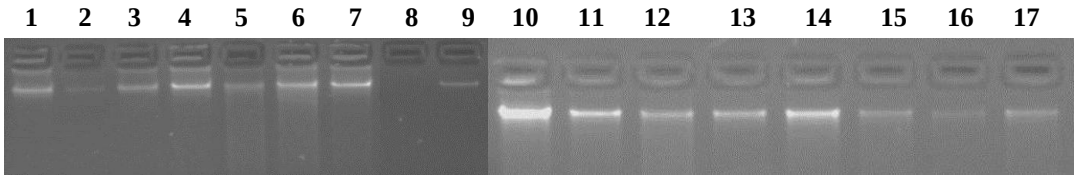


Figure 4-25: Electrophoresis profile of genomic DNA extracted using Yeates *et. al.* (1998). Genomic DNA of *L. fungicola* 9.8×10^7 conidia/ml (Line 1 and 10), *C. mycophilum* 1.25×10^6 conidia/ml (Line 2 and 11), *M. perniciosa* 1.0×10^6 conidia/ml (Line 3 and 12), 4.1×10^7 conidia/ml *A. fumigatus* (Line 4 and 13), *A. bisporus* (Line 5 and 14), mixture of fungi (*L. fungicola*, *C. mycophilum*, *M. perniciosa*, *A. fumigatus* and *A. bisporus*) (Line 6, 15 and 17) and soil (Line 7 and 16). *L. fungicola* conidia without soil (Line 8 and 9).

4.1.3.2 PCR assay of soil extractions

Aljanabi and Martinez (1997) – the extracted DNA was analysed by PCR assay. The PCR product was present on electrophoresis gel. Amplification product was not present. The *L. fungicola* DNA did not show a 102 bp amplicon in any sample (**Figure** 4-26).

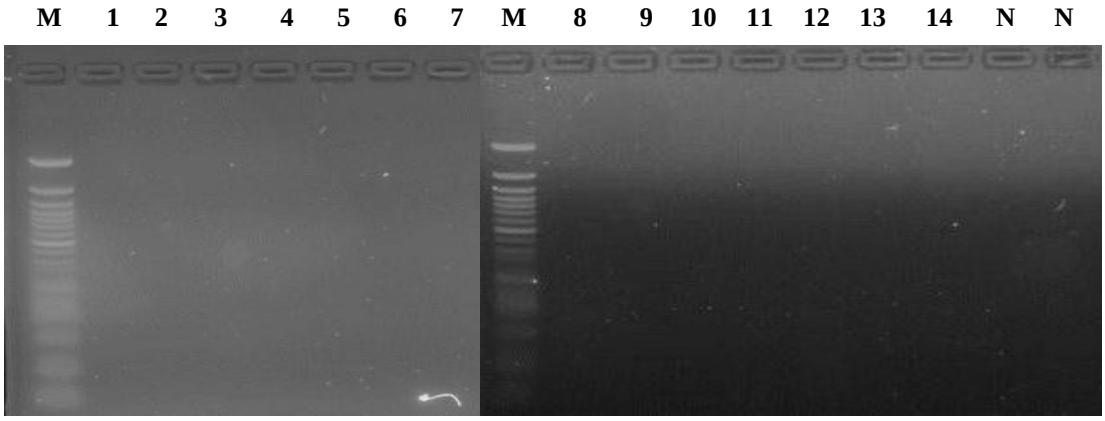


Figure 4-26: Electrophoretic profile of PCR products. DNA was extracted using the method of Aljanabi and Martinez (1997). For PCR reaction 3.5 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola* (Line 1 and 8), *C. mycophilum* (Line 2 and 9), *M. perniciosa* (Line 3 and 10), *A. fumigatus* (Line 4 and 11), *A. bisporus* (Line 5 and 6), Soil (Line 6 and 13) and mixture of fungi (*L. fungicola*, *C. mycophilum*, *M. perniciosa*, *A. fumigatus*, *A. bisporus*) (Line 7 and 14). The Tm in PCR reaction was 50°C. M – Marker 50bp DNA, N – water control.

ZR Fungal/Bacterial DNA kit – the extracted DNA was analysed by PCR assay using 3.5 µl of template. The PCR product visualised. No samples presented an amplification product (102 bp) samples (**Figure** 4-27).

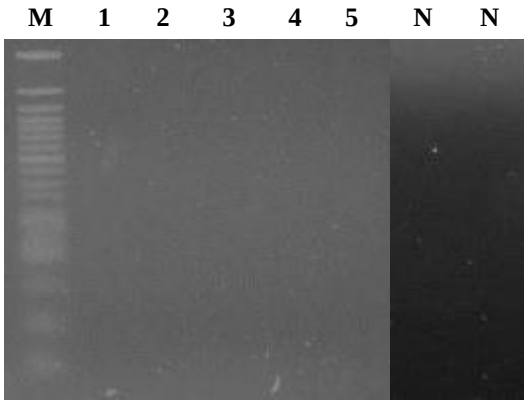


Figure 4-27: Electrophoretic profile of PCR products. For PCR reaction 3.5 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola* 9.8×10^7 conidia/ml (Line 1), *C. mycophilum* 1.25×10^6 conidia/ml (Line 2), *M. perniciosa* 1.0×10^6 conidia/ml (Line 3), *A. bisporus* (Line 4) and mix of fungi (*L. fungicola, C. mycophilum, M. perniciosa, A. fumigatus* and *A. bisporus*) (Line 5). The Tm in PCR reaction was 50 °C. M – Marker 50 bp DNA, N – water control.

ZR Fungal/Bacterial DNA kit - the PCR assay was repeated with different amount of template. When the amount of *L. fungicola* template was 1 µl the 102 bp PCR products was present but when amount of *L. fungicola* DNA template was 10 µl the PCR product was absent (**Figure** 4-28).

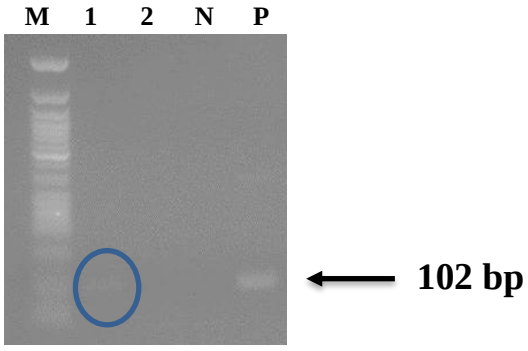


Figure 4-28: Electrophoretic profile of PCR products. DNA was extracted using ZR Fungal/Bacterial DNA kit. For PCR reaction 1 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola* 9.8×10^7 conidia/ml (Line 1 and 2). Line 1 – contain a 1 µl of template, Line 2 – contain 10 µl of template. The Tm in PCR reaction was 50 °C. M – Marker 50bp DNA, N – water control, P – positive control DNA of *L. fungicola*.

ZR Fungal/Bacterial DNA kit – the PCR assay was repeated with 1µl of template. The PCR product was present on electrophoresis gel. In some samples containing *L. fungicola* the amplification product was present and showed a 102 bp amplicon. Other samples from other fungi did not show an amplicon the same size as *L. fungicola*. Samples containing *A. bisporus* DNA showed some non-specific amplicon around 1,000 bp. The samples containing soil mixed with *L. fungicola* conidia (9.8×10^7 conidia/ml) samples also showed an amplification product of size 102bp (**Figure** 4-29).

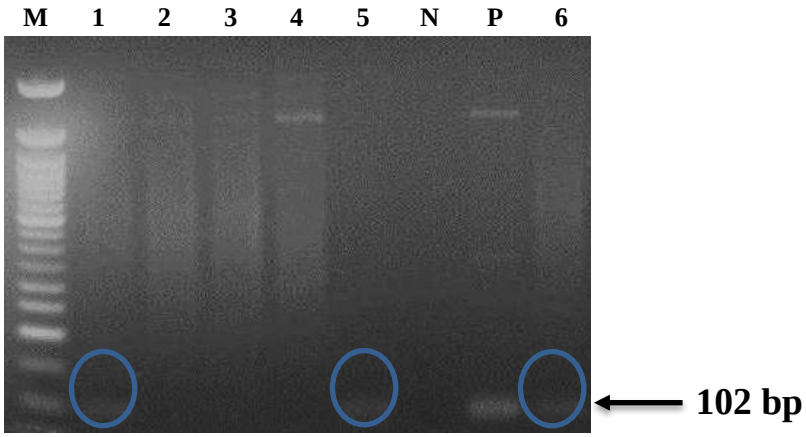


Figure 4-29: Electrophoretic profile of PCR products. DNA was extracted using ZR Fungal/Bacterial DNA kit. For PCR reaction 1 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola* 9.8×10^7 conidia/ml (Line 1), *C. mycophilum* 1.25×10^6 conidia/ml (Line 2), *M. perniciosa* 1.0×10^6 conidia/ml (Line 3), *A. bisporus* (Line 4) and mix of fungi (*L. fungicola*, *C. mycophilum*, *M. perniciosa*, *A. fumigatus* and *A. bisporus*) (Line 5). For PCR reaction 0.5 µl of *L. fungicola* 9.8×10^7 conidia/ml (Line 6). The Tm in PCR reaction was 50 °C. M – Marker 50bp DNA, N – water control.

Yeates *et al.* (1998) – the extracted DNA was analysed by PCR assay. The PCR product was present on electrophoresis gel. Samples containing soil and soil mixed with fungi did not show an amplification product. Only samples containing clean *L. fungicola* DNA showed an amplification product at 102 bp size (**Figure** 4-30).

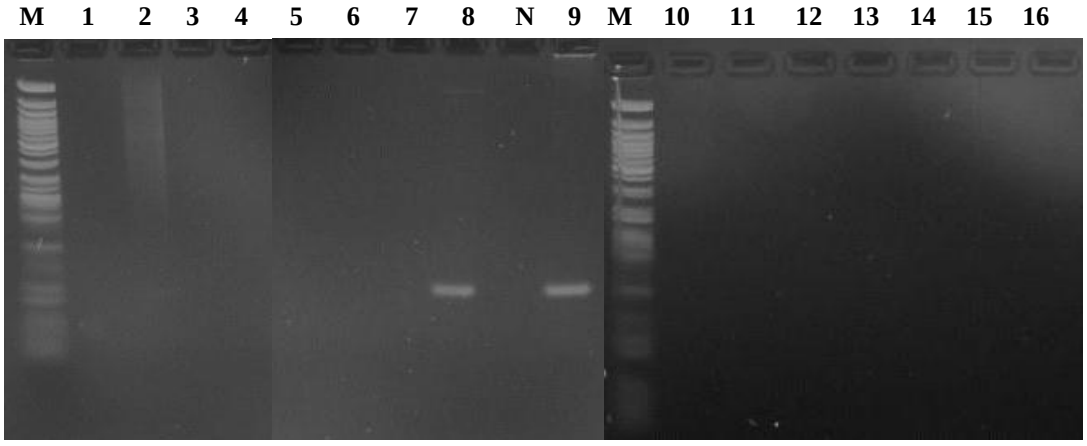


Figure 4-30: Electrophoretic profile of PCR products. DNA was extracted using the method of Yeates *et al.* (1998). For PCR reaction 3.5 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola* 9.8×10^7 conidia/ml (Line 1 and 10), *C. mycophilum* 1.25×10^6 conidia/ml (Line 2 and 11), *M. perniciosa* 1.0×10^6 conidia/ml (Line 3 and 12), 4.1×10^7 conidia/ml *A. fumigatus* (Line 4 and 13), *A. bisporus* (Line 5 and 14), mix of fungi (*L. fungicola*, *C. mycophilum*, *M. perniciosa*, *A. fumigatus* and *A. bisporus*) (Line 6, 15) and soil (Line 7 and 16). *L. fungicola* conidia without soil (Line 8 and 9). The Tm in PCR reaction was 50°C. M – Marker 50bp DNA, N – water control.

4.1.3.3 Dilution of genomic DNA for PCR

- **Detection of *L. fungicola* in soil samples using Yeates *et al.* (1998) DNA extraction method**

The DNA was extracted from different conidia concentrations of *L. fungicola* (0-8.95 × 10^7 conidia/g soil) mixed with 1 gram of soil using the method of Yeates *et al.* (1998). The soil, (1 g soil) was mixed with conidia suspensions and DNA was extracted. All samples showed high molecular weight quality genomic DNA (**Figure** 4-31).

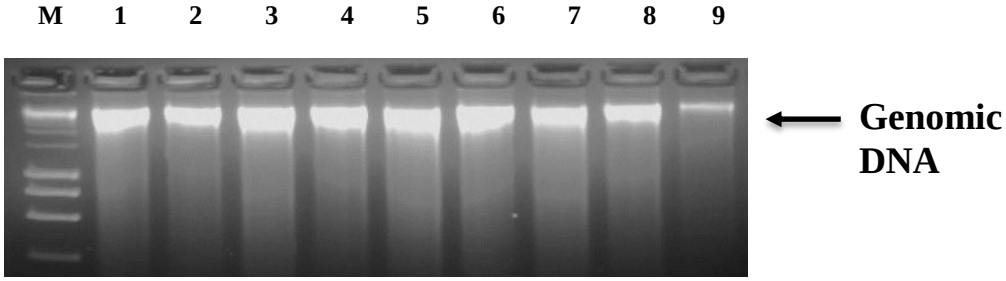


Figure 4-31: Electrophoresis profile of genomic DNA extracted using the method of Yeates *et. al.* (1998). Genomic DNA of *L. fungicola*. Lines: M – DNA marker 100 bp, 1 – 8.95 × 10^7 conidia/g, 2 – 10^7 conidia/g, 3 – 10^6 conidia/g, 4 – 10^5 conidia/g, 5 – 10^4 conidia/g, 6 –10^3 conidia/g, 7 – 10^2 conidia/g, 8 – 10^1 conidia/g, 9 – (soil without *L. fungicola* conidia) 0 conidia/g.

The extracted DNA was analysed by PCR assay using 1 µl of template. The PCR product visualised on electrophoresis gel. Samples containing soil mixed with different conidia concentrations of *L. fungicola* did not show an amplification product. Only samples containing clean *L. fungicola* DNA (positive control) showed an amplification product at 102 bp size (**Figure** 4-32).

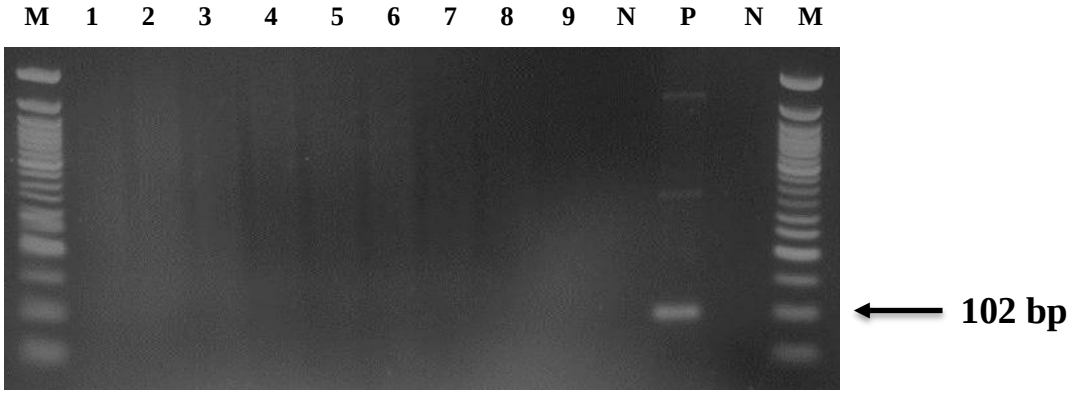


Figure 4-32: Electrophoretic profile of PCR products. DNA was extracted using the method of Yeates *et al.* (1998). For PCR reaction 1 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola*. Lines: M – DNA marker 50 bp, 1 – 8.95 × 10^7 conidia/g, 2 – 10^7 conidia/g, 3 – 10^6 conidia/g, 4 – 10^5 conidia/g, 5 – 10^4 conidia/g, 6 – 10^3 conidia/g, 7 – 10^2 conidia/g, 8 – 10^1 conidia/g, 9 – soil without *L. fungicola* conidia. The Tm in PCR reaction was 50°C. N – Water control, P – positive control of *L. fungicola* DNA.

In this PCR assay DNA template was diluted 1/50 and for PCR reaction 1 µl extracted DNA was used. Samples containing 8.95×10^7 conidia/g and 10^7 conidia/g and showed an amplification product of 102 bp. In samples with lower concentrations of *L. fungicola* conidia the amplification product was not present (**Figure** 4-33).

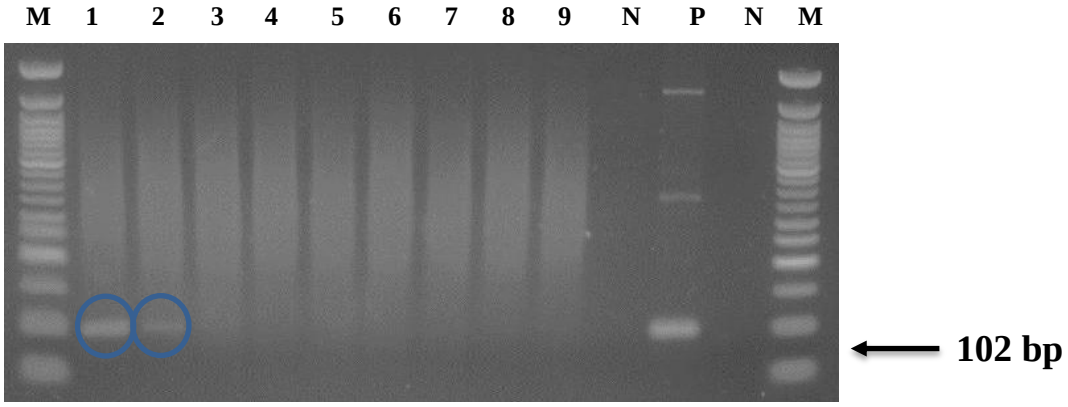


Figure 4-33: Electrophoretic profile of PCR products. DNA was extracted using the method of Yeates *et al.* (1998). For PCR reaction 2 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola*. Lines: M – DNA marker 50 bp, 1 – 8.95×10^7 conidia/g, 2 – 10^7 conidia/g, 3 – 10^6 conidia/g, 4 – 10^5 conidia/g, 5 – 10^4 conidia/g, 6 – 10^3 conidia/g, 7 – 10^2 conidia/g, 8 – 10^1 conidia/g, 9 – soil without *L. fungicola* conidia. N – Water control, P – positive control of *L. fungicola* DNA. The Tm in PCR reaction was 50 °C.

In this PCR assay DNA template was diluted 1/20 and 1/10 and for PCR reaction 1 µl of template was used for PCR assay. Both diluted samples contained 10^6 conidia/g and showed an amplification product of 102 bp. In samples with 10^5 conidia/g of *L. fungicola* the amplification product was not present (**Figure** 4-34).

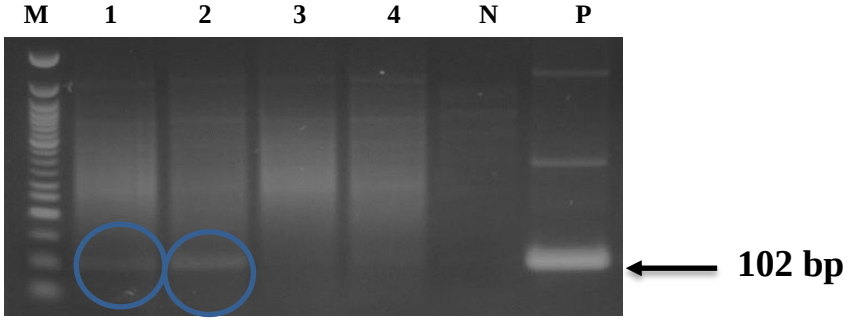


Figure 4-34: Electrophoretic profile of PCR products. DNA was extracted using the method of Yeates *et al.* (1998). For PCR reaction 1 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola*. Lines: M – DNA marker 50 bp, 1 – 10^6 conidia/g (template diluted by 1/20), 2 – 10^6 conidia/g (template diluted by 1/10), 3 – 10^5 conidia/g (template diluted by 1/20), 4 – 10^5 conidia/g (template diluted by 1/10). N – Water control, P – positive control of *L. fungicola* DNA. The Tm in PCR reaction was 50 °C.

- **Use of different conidia concentration with Yeates *et al.* (1998) method**

DNA was extracted from different conidia concentrations of *L. fungicola* (10^6-10^7 conidia/g or ml) using the method of Yeates *et al.* (1998). The 1g of casing or soil, 1 ml of casing extracts, dust extract were mixed with *L. fungicola* conidia and DNA was extracted. The high molecular weight quality genomic DNA was present in casing, casing extract and soil samples. Samples with dust extract did not show good quality genomic DNA (**Figure** 4-35).

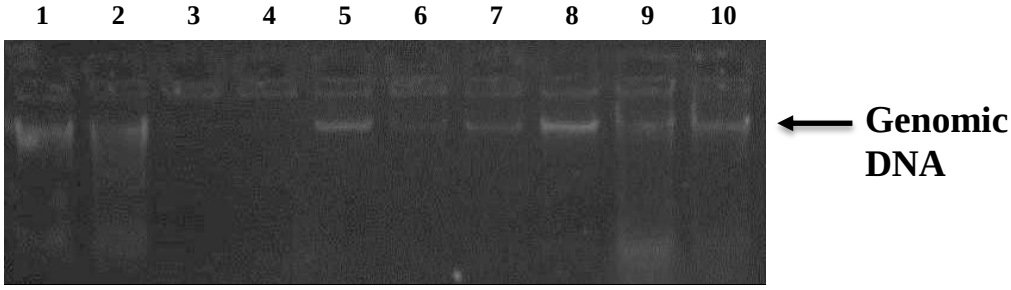


Figure 4-35: Electrophoresis profile of genomic DNA extracted using the method of Yeates *et. al,* (1998). Genomic DNA of different conidia concentrations of *L. fungicola* mixed with casing, soil, casing extract and dust extract. Lines: 1 – casing mixed with *L. fungicola* conidia 10^7 conidia/g casing, 2 – casing mixed with *L. fungicola* conidia 10^6 conidia/g casing, 3 – water dust mixed with *L. fungicola* conidia 10^7 conidia/ml, 4 – water dust mixed with *L. fungicola* conidia 10^6 conidia/ml, 5 – casing extract mixed with *L. fungicola* conidia 10^7 conidia/ml, 6 – casing extract mixed with *L. fungicola* conidia 10^6 conidia/g, 7 – casing mixed with *L. fungicola* conidia 10^7 conidia/g casing, 8 – casing mixed with *L. fungicola* conidia 10^6 conidia/g casing, 9 – soil mixed with *L. fungicola* conidia 10^7 conidia/g. 10 – soil mixed with *L. fungicola* conidia 10^6 conidia/g.

The extracted DNA was analysed by PCR assay with 3µl of DNA template. The PCR product was visualised on electrophoresis gel. Samples with casing extract and dust extract containing 10^6 and 10^7 conidia/ml of *L. fungicola* did show an amplification product on 102 bp. In samples that contained casing or soil did not show any amplification product (**Figure** 4-36).

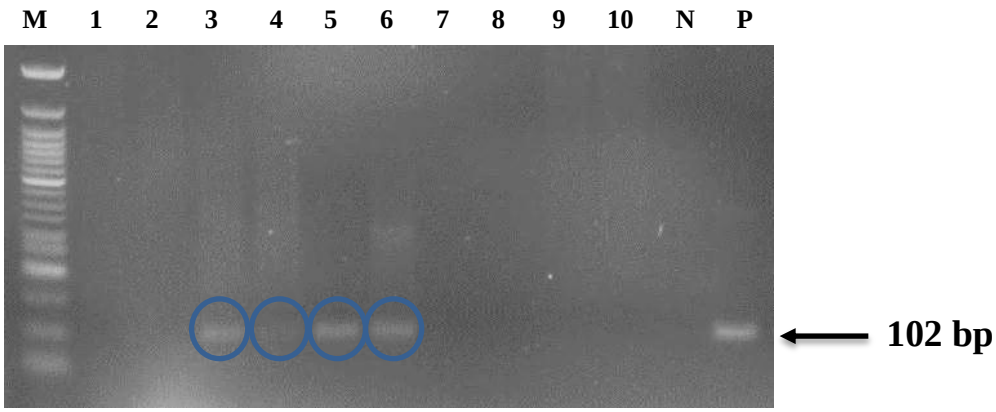


Figure 4-36: Electrophoretic profile of PCR products. DNA was extracted using the method of Yeates *et al.* (1998). For PCR reaction 3 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola*. Lines: M – Marker 50 bp, Lines: look Figure 3.36. N – Water control, P – positive control of *L. fungicola* DNA. The Tm in PCR reaction was 50°C.

- **Use of different conidia concentrations mixed with soil and isolated by ZR Fungal/Bacterial DNA extraction kit**

Casing soil (100mg) was mixed with 100 µl of different conidia concentrations of *L. fungicola* (10^4-10^7 conidia/g casing). The DNA was extracted using ZR Fungal/Bacterial DNA kit. In all samples high molecular weight quality genomic DNA was present (**Figure** 4-37).

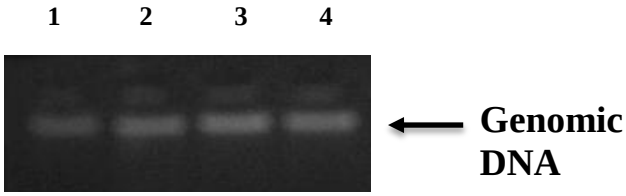


Figure 4-37: Electrophoresis profile of genomic DNA extracted using ZR Fungal/Bacterial DNA kit from *L. fungicola* and casing. Lines: 1 – 10^7 conidia/g, 2 – 10^6 conidia/g, 3 – 10^5 conidia/g, 4 – 10^4 conidia/g.

The extracted DNA was analysed by PCR assay. The PCR product was present on electrophoresis gel. Sample containing 10^6 conidia per 100 mg casing showed a weak amplification product on 102 bp, but lower conidia concentrations did not show an amplification product (**Figure** 4-38).

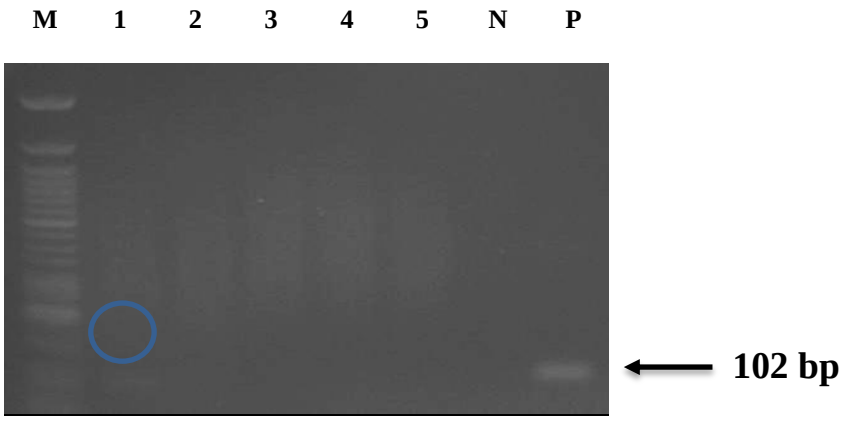


Figure 4-38: Electrophoretic profile of PCR products. DNA was extracted using ZR Fungal/Bacterial DNA kit from *L. fungicola* and casing. For PCR reaction 1 µl of genomic DNA was used. The amplification product of isolates of *L. fungicola*. Lines: M – DNA marker 50 bp, 1 – 10^7 conidia/g, 2 – 10^6 conidia/g, 3 – 10^5 conidia/g, 4 – 10^4 conidia/g, 5 – 10^4 conidia/g (2µl of genomic DNA was used for PCR reaction). The Tm in PCR reaction was 50 °C. N – Water control, P – positive control of *L. fungicola* DNA.

- **Different conidia concentrations mixed with casing and isolated by DNeasy Plant Mini Kit**

DNeasy Plant Mini Kit was used for DNA extraction from casing soil (100mg) mixed with 100 µl of different conidia concentrations of *L. fungicola* (10^4-10^7 conidia/g casing). The DNA was extracted but first step was changed and glass bead were used for cell disruption. In the all samples high molecular weight quality genomic DNA was present (**Figure** 4-39).

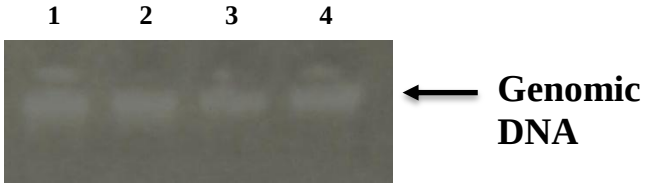


Figure 4-39: Electrophoresis profile of genomic DNA extracted using DNeasy Plant Mini Kit from *L. fungicola* and casing. Lines: 1 – 10^7 conidia/g, 2 – 10^6 conidia/g, 3 – 10^5 conidia/g, 4 – 10^4 conidia/g.

DNeasy Plant Mini Kit DNA - in this PCR assay DNA template was diluted 1/10 and for PCR reaction 1 µl DNA template was used. The amplification product was not present in any samples of *L. fungicola* conidia mixed with casing (**Figure** 4-40 **A**). PCR was repeated with these same results (**Figure** 4-40 **B**).

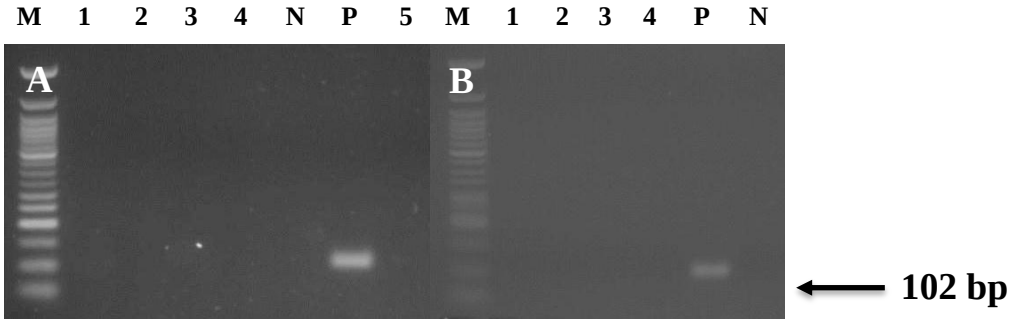


Figure 4-40: A and B – Electrophoretic profile of PCR products. DNA was extracted using DNeasy Plant Mini Kit from *L. fungicola* and casing. For PCR reaction 1 µl of genomic DNA was used. Lines: M – DNA marker 50 bp, 1 – 10^7 conidia/g, 2 – 10^6 conidia/g, 3 – 10^5 conidia/g, 4 – 10^4 conidia/g, 5 – 10^7 conidia/g (2µl of genomic DNA was used for PCR reaction). The Tm in PCR reaction was 50 °C. N – Water control, P – positive control of *L. fungicola* DNA.

4.1.3.4 Analysis of mushroom farm samples using Yeates *et al.* (1998) DNA extraction method

Casing and dust samples were collected from a mushroom farm (Section 2.6.15.5 and 2.6.17). DNA from these samples was extracted using Yeates *et al.* (1998) protocol. Some samples showed high molecular weight quality genomic DNA (**Figure** 4-41).

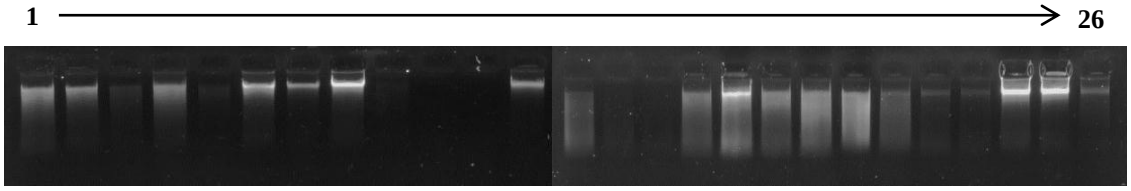


Figure 4-41: Electrophoresis profile of genomic DNA extracted using the method of Yeates *et. al*. (1998). Genomic DNA of *L. fungicola*. Lines: A (1 – 3) casing samples , B(4 – 6) casing samples, C (7 – 9) casing samples, D (10 – 12) casing samples, E (13 – 15) dust from floor, F (16 – 18) dust from floor, G (19 – 21) dust from floor, H (22 – 23) dust from floor, I (24 – 26) dust from floor.

The extracted DNA was analysed by PCR assay using 1 µl or 3 µl of template. No amplification product was present in any samples. (**Figure** 4-42 and **Figure** 4-43).

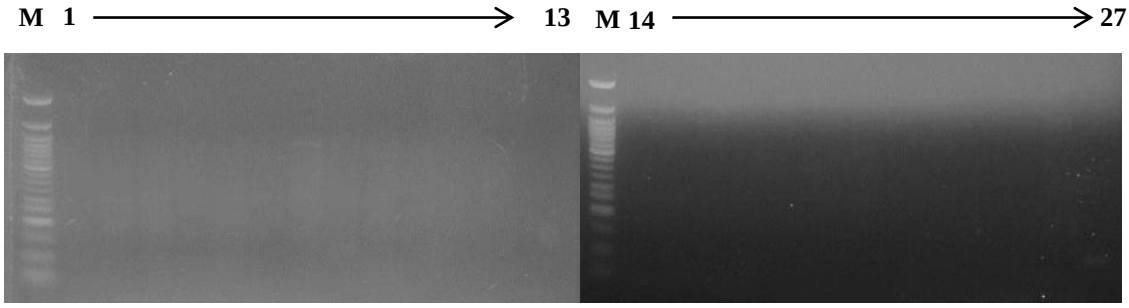


Figure 4-42: Electrophoretic profile of PCR products. DNA was extracted using the method of Yeates *et al.* (1998). For PCR reaction 1 µl of genomic DNA was used. The amplification product of *L. fungicola*. Lines: M – DNA marker 50 bp, Lines:: A (1 – 3) casing samples , B (4 – 6) casing samples, C (7 – 9) casing samples, D (10 – 12) casing samples, E (13 – 15) dust from floor, F (16 – 18) dust from floor, G (19 – 21) dust from floor, H (22 – 23) dust from floor, I (24 – 26) dust from floor. 13 – Water control, 27 – positive control of *L. fungicola* DNA. The Tm in PCR reaction was 50 °C.

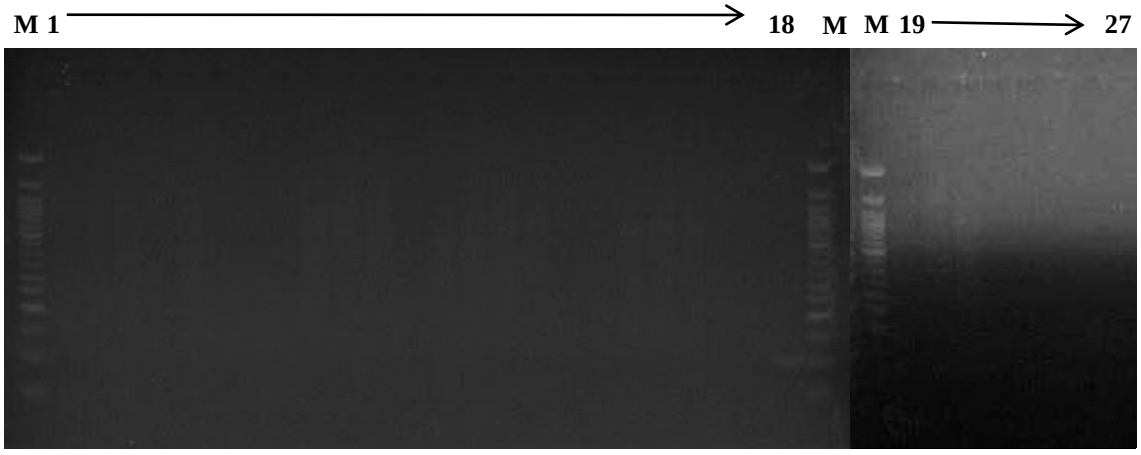


Figure 4-43: Electrophoretic profile of PCR products. DNA was extracted using method of Yeates *et al.* (1998). For PCR reaction 3 µl of genomic DNA was used. The amplification product of *L. fungicola*. Lines: M – DNA marker 50 bp, Lines: 1- 3 casing samples , 4- 6 casing samples, 7-9 casing samples, 10-12 casing samples, 13- 15 dust from floor, 16- 18 dust from floor, 19- 21 dust from floor, 22-23 dust from floor, 24-26 dust from floor. 27 – Water control, 18 – positive control of *L. fungicola* DNA. The Tm in PCR reaction was 50 °C.

4.1.3.5 DNA extraction of *L. fungicola* from autoclaved and non-autoclaved soil

Different concentrations of *L. fungicola* (9.7×10^6, 1.94×10^7 and 2.91×10^7 conidia/g soil) conidia were mixed with non-autoclaved and autoclaved soil collected from the NUIM grounds. The DNA was extracted using a ZR Fungal/Bacterial DNA kit. In all samples (6) high molecular weight quality genomic DNA was present (**Figure** 4-44).

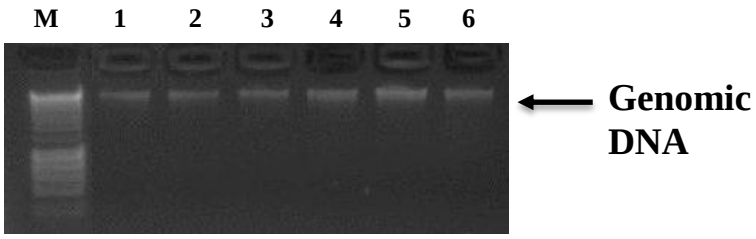


Figure 4-44: Electrophoresis profile of genomic DNA of *Lecanicillium fungicola* mixed with soil isolated by ZR Fungal/Bacterial DNA kit (Line 1-6). Autoclaved soil and live *L. fungicola* conidia (Line 1-3) and non-autoclaved soil and live *L. fungicola* conidia (Line 4-6) where 2 µl of genomic DNA was loaded for each sample. Genomic DNA of *L. fungicola* was isolated from conidia concentration (Line 1 – 9.7×10^6 conidia/g soil, 2 – 1.94 x10^7 conidia/g soil, 3 – 2.91×10^7 conidia/g soil.

The extracted DNA was analysed by PCR assay. The PCR product was present in all tested samples. The electrophoresis gel showed one amplification product which is due to *L. fungicola* rRNA region and has a 102 bp amplicon. The primers did not amplify any non-specific targets in soil samples (**Figure** 4-45)

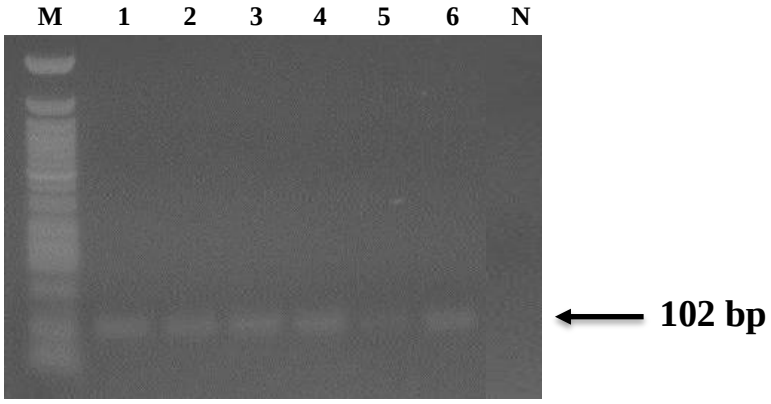


Figure 4-45: Electrophoretic profile of PCR products. DNA was extracted using ZR Fungal/Bacterial DNA kit, where 3.5 µl of genomic DNA was used for PCR reaction. The amplification product of isolates of *L. fungicola* autoclaved soil and live *L. fungicola* conidia (Line 1-3) and not autoclaved soil and live *L. fungicola* conidia (Line 4-5). *L. fungicola* was isolated from conidia concentration (Line 1 – 9.7×10^6 conidia/g soil, 2 – 1.94×10^7 conidia/g soil, 3 – 2.91×10^7 conidia/g soil where 2 µl of genomic DNA was loaded for each sample. GoTaq polymerase – Promega and Zijlstra primers. The Tm in PCR reaction was 50 °C. M – Marker 50 bp DNA, N – water control.

4.1.3.6 Summary

The experiments described in this part of the thesis were performed with the help of GoTaq polymerase supplied by Promega and selective primers used for this part were designed by Zijlstra *et al.* (2007, 2008 and 2009). This set of primers amplified an rRNA region of *L. fungicola* giving a 102 bp amplication product.

The comparison of three DNA extraction methods from soil samples in this study has highlighted differences in the quality and quantity of genomic DNA depending on the method. The **ZR Fungal/Bacterial** DNA kit was considered the best

because it always gave high molecular weight quality genomic DNA from soil samples. The second tested DNA extraction method from soil samples was manual method described of **Yeates *et al.* (1998).** This method gave high molecular weight quality DNA which is good for use in PCR assay. The third DNA extraction method from soil samples was manual method described by **Aljanabi and Martinez (1997)**. This method did not show good results for *L. fungicola* DNA from soil samples. Genomic DNA was not good quality and PCR product was not present.

DNA from soil isolated by **ZR Fungal/Bacterial DNA** gave an amplification product when conidia concentrations were 10^6 and 10^7 g/casing. When DNA extraction was performed from other fungi mixed with soil, genomic DNA was present but PCR product was absent. When PCR reaction was performed using 3.5 µl of template the amplification product of *L. fungicola* DNA was not present, but when PCR reaction was performed with 1 µl of template the amplicon of 102 bp was present. ZR Fungal/Bacterial DNA kit was also used for extraction of DNA from *L. fungicola* conidia (10^4 and 10^7 conidia/ml) mixed with 100 mg casing only 10^6 conidia per 100 mg casing showed an amplicon of 102bp when PCR reaction was performed using 1 µl of template.

The second DNA extraction method from soil samples which gave good quality DNA was manual method described by **Yeates *et al.* (1998).** In this method extraction of genomic DNA from samples contained in soil mixed with fungi was successful and extraction of DNA showed high molecular weight quality genomic DNA. When PCR reaction was performed with 3.5 µl of DNA template the amplification product was not present, but when genomic DNA was diluted 1/50 times and for PCR reaction 1 µl was used the amplicon was present when conidia concentrations were 8.95×10^7 conidia/g and 10^7 conidia/g soil. DNA template was diluted 1/20 and 1/10 and, for PCR reaction 1 µl was used the amplicon was present when conidia concentration was 10^6 conidia/g soil. When *L. fungicola* conidia (10^6 and 10^7 conidia/ml) were mixed with casing, soil, casing extract and dust extract and PCR reaction was performed with 3 µl of template the PCR product was present in both conidia concentrations of *L. fungicola* mixed with casing extract and dust extract, casing and soil did not show an amplification product.

Yeates *et al.* (1998) method was used for DNA extraction from samples collected from a mushroom farm. Almost all samples showed high molecular weight quality genomic DNA, but PCR assay performed with two options of template for PCR reaction (1 µl or 3 µl) did not show any amplification product.

The last tested extraction of *L. fungicola* conidia mixed with casing was **DNeasy Plant Mini Kit.** In this kit the genomic DNA was present in all samples, but PCR product was not present when PCR template was diluted 1/10.

4.1.4 Test of different primers sets – DNA extraction, PCR reaction and visualisation by agarose gel electrophoresis.

The genomic DNA was extracted from *L. fungicola* var. *fungicola*, *L. fungicola* var. *aleophilum*, *A. bisporus*, *M. perniciosa*, *C. mycophilum*, *A. fumigatus* and *T. aggressivum* and from clean casing and casing extract. The DNA was stored in -20 °C and used when the PCR reaction was performed (**Table** 4-1).

Table 4-1: Summary of species, isolation code, date of DNA extraction and DNA extraction method.

No	Species/ Sample	Isolate code	Date DNA extraction	DNA kit supplier
1	*Lecanicillium fungicola var. fungicola*	L.15	18.09.2008	Zymo Research
2	*Lecanicillium fungicola var. fungicola*	L.16	18.09.2009	Zymo Research
3	*Lecanicillium fungicola var. fungicola*	CBS 992.68	10.03.2010	Zymo Research
4	*Lecanicillium fungicola var. fungicola*	CBS 648.79	10.03.2010	Zymo Research
5	*Lecanicillium fungicola var. aleophilum*	CBS 357.79	10.03.2010	Zymo Research
6	Clean casing	n/a	03.12.2008	Promega
7	Casing extract	n/a	13.01.2009	Promega
8	Casing extract	n/a	11.08.2009	Fujifilm
9	*Agaricus bisporus* (contaminated)	Ab.1	02.10.2007	Qiagen
10	*Agaricus bisporus*	Ab.3	02.10.2008	Qiagen
11	*Agaricus bisporus*	21.08.09	21.08.2008	Zymo Research
12	*Mycogone perniciosa*	M.11	14.12.2007	Zymo Research
13	*Mycogone perniciosa*	M.31	21.08.2008	Zymo Research
14	*Cladobotryum mycophilum*	D.1	13.12.2007	Qiagen
15	*Aspergillus fumigatus*	As.	04.12.2007	Zymo Research
16	*Trichoderma agressivum*	CBS 433.95	10.03.2010	Qiagen

n/a – not avaliable

The PCR reaction was performed. The PCR assay reaction contained 25 µl PCR reaction system could be summarized as follows: 1x PCR buffer, 2 mmol/l $MgCl_2$, 2 mmol/l dNTP's, 8.5 µl of 50 % glycerol, 0.52 µmol/l each primers, 2 Units Taq and 3 µl DNA template. The annealing temperature was 58 °C and this was chosen as a good temperature for most tested primers.

The PCR reaction was: 5 min 95 °C for template denaturation and enzyme activation, amplification was obtained with 35 cycles of denaturation at 95 °C for 15 sec., annealing at 58 °C for 30 sec. and extension at 72 °C for 30 sec. followed to 72 °C for 1 min. The PCR product was put to 2 % agarose gel electrophoresis with ethidium bromide prepared in 1x TAE buffer.

4.1.5 Primer sets from the MAT1-2-1 region compared to Zijlstra *et al.* set of primers

4.1.5.1 Primer set I – Ay 124053 Forward 116 – Reverse 205 (F116-R205)

Primer set I (Ay 124053 (F116-R205)) gave a 90 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from casing samples, *A. fumigatus*, *C. mycophilum* and *T. aggressivum*. Using this set of primers it was possible to identify *L. fungicola* from diseased tissue of *A. bisporus* in *in vitro* experiment. This set of primers amplified primer dimers which are present in water control this makes this primer not very good for use (**Figure** 4-46).

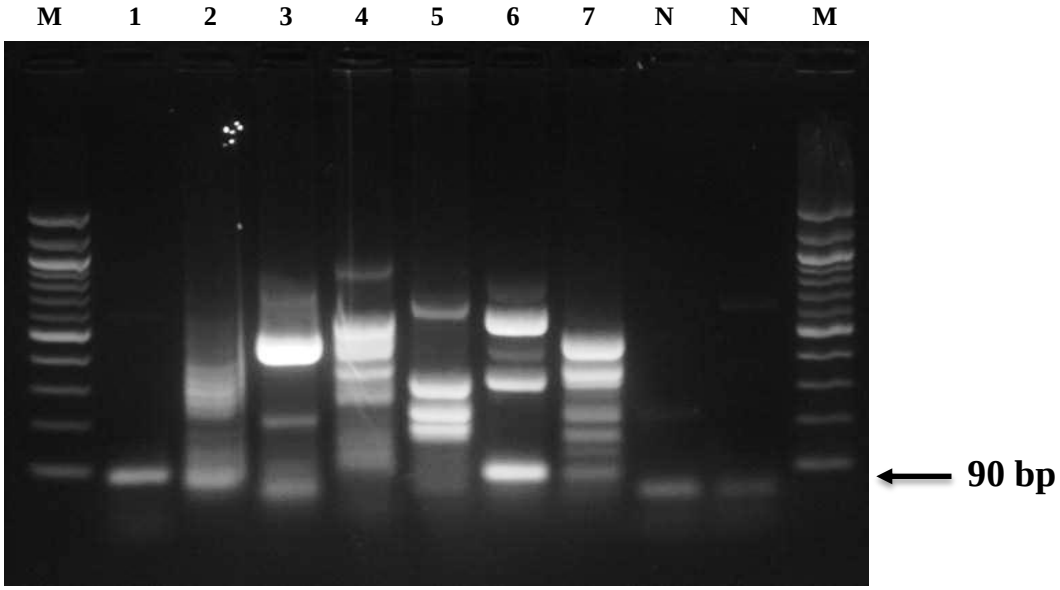


Figure 4-46: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), Clean casing (Line 2), *A. bisporus* (21.08.09)(Line 3), *M. perniciosa* (M.31)(Line 4), *C. mycophilum* (D.1)(Line 5), *A. fumigatus* (As.) (Line 6), and *T. aggressivum* (CBS 433.95) (Line 7). Set of primers amplified a product of 90 bp (Ay 124053 F and R). M – Marker 100 bp DNA, N – water control. PCR reaction was performed on 13.11.2009.

The PCR assay was repeated with other isolates of *L. fungicola* var. *fungicola* and var. *aleophilum*. This set of primers amplified DNA from both varieties of *L. fungicola* giving a 90 bp amplicon. Using this set of primers it is possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro*. This set of primers also amplified *A. fumigatus*, *C. mycophilum* and *T. aggressivum*. Casing extract used in this experiment was stored sometimes at -20 °C and the DNA may have degraded during this time and may explain why no amplification band was seen (**Figure** 4-47).

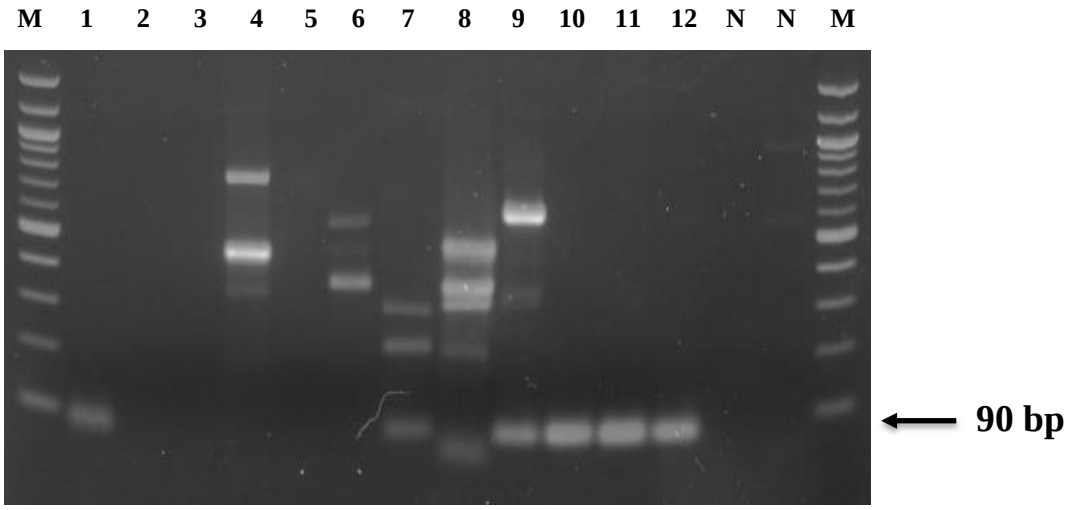


Figure 4-47: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), casing extract – Fujifilm(Line 2), casing extract – Promega (Line 3), *A. bisporus* (21.08.09) (Line 4-5), *M. perniciosa* (M.31)(Line 6), *C. mycophilum* (D.1)(Line7), *T. aggressivum* (CBS 433.95) (Line 8) and *A. fumigatus* (As.) (Line 9), *L. fungicola* var. *fungicola* – CBS 992.69 (Line 10), *L. fungicola* var. *fungicola* – CBS 648.80 (Line 11) and *L. fungicola* var. *aleophilum* – CBS 357.80 (Line 12). Set of primers amplified a product of 90 bp (Ay 124053). M – Marker 100 bp DNA, N – water control. PCR reaction was performed on 16.06.2010.

4.1.5.2 Primer set II – Ay 124053 – Forward 151–Reverse 205 (F151-R205)

Primer set II (Ay 124053 (F151-R205) gave a 50 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from all tested fungi (*L. fungicola*, *A. bisporus*, *A. fumigatus*, *M. perniciosa* and *C. mycophilum*) and casing samples (**Figure** 4-48).

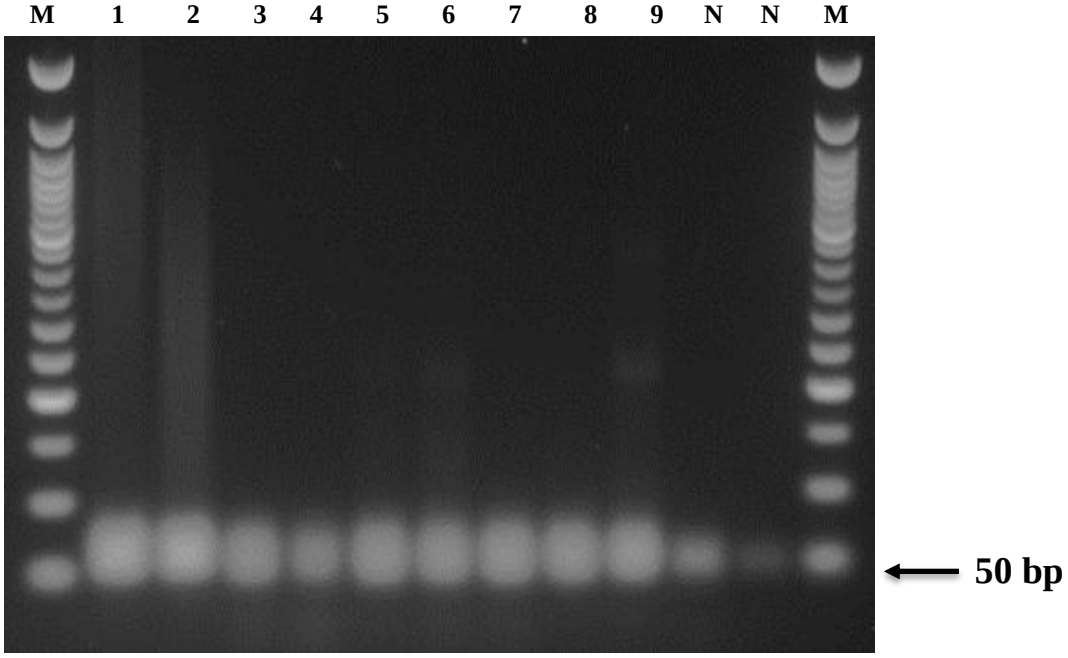


Figure 4-48: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15-L.16) (Line 1-2), Clean casing 2(Line 3), Casing extract – Promega (Line – 4), *A. bisporus* (Ab.1 and Ab.3)(Line 5-6), *A. fumigatus* (As.) (Line 7), *M. perniciosa* (M.11)(Line 8) and *C. mycophilum* (D.1)(Line 9). M – Marker 50 bp DNA, N – water control. PCR reaction was performed on 17.7.2009.

4.1.5.3 Primer set III – Forward 9-Reverse 167 (F9-R167)

Primer set III (F9-R167) gave a 159 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from *A. fumigatus* and *C. mycophilum*. The sample of *A. bisporus* (Ab.1) DNA was contaminated by *L. fungicola* DNA during storage what was discovered using different primers designed by Zijlstra primers. Fresh *A. bisporus* DNA was isolated and this set of primers (F9-R167) did not amplify a 159 bp amplicon. The amplification product from *A. bisporus* is very close in size to *L. fungicola* amplicon and agarose gel has low resolution for this separation and it might have been good to use a polyacrylamide gel (**Figure** 4-49).

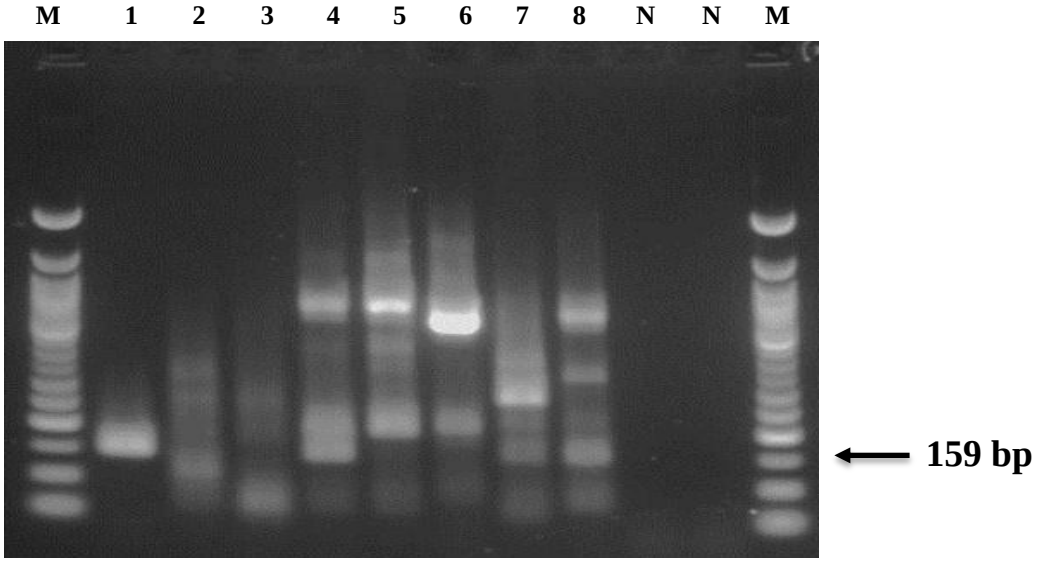


Figure 4-49: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* – contaminated by *L. fungicola* DNA (Ab.1)(Line 4), *A. bisporus* (21.08.09)(Line 5), *M. perniciosa* (M.1)(Line 6), *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 50 bp DNA, N – water control. PCR reaction was performed on 25.08.2009.

4.1.5.4 Primer set IV – Forward 9-Reverse 201 (F9-R201)

Primer set IV (F9-R20) gave a 193 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from *A. fumigatus* and *A. bisporus* (contaminated – Ab.1) and clean *A. bisporus* DNA (21.08.09) so it is not a useful primer set for detection of *L. fungicola*. This set of primers amplified primer dimers which are present in water control which makes these primers not very good for use. This set of primers did not give a 193 bp amplicon from *M. perniciosa* or *C. mycophilum* DNA (**Figure** 4-50).

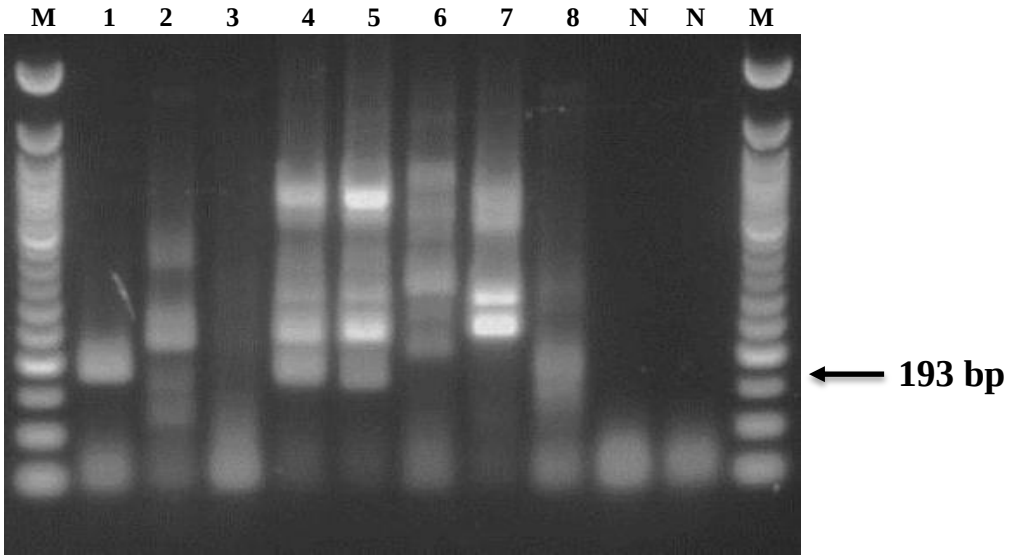


Figure 4-50: Electrophoretic profile of PCR products. The amplification product *for L. fungicola* (L.15)(Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* – contaminated by *L. fungicola* DNA (Ab.1)(Line 4), *A. bisporus* (21.08.09)(Line 5), *M. perniciosa* (M.1)(Line 6), *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 50 bp DNA, N – water control. PCR reaction was performed on 25.08.2009.

4.1.5.5 Primer set V – Forward 79-Reverse 167 (F79-R167)

Primer set V (F79-R167) gave an 89 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from *C. mycophilum* but efficiency level was very low. Using this set of primers it is possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro* test. This set of primers did not amplify an 89 bp amplicon from *A. bisporus* DNA. The amplification product from *A. bisporus* using these primers was around 250 bp. This set of primers amplified primer dimers which are present in water control which makes these primers not very good for use (**Figure** 4-51).

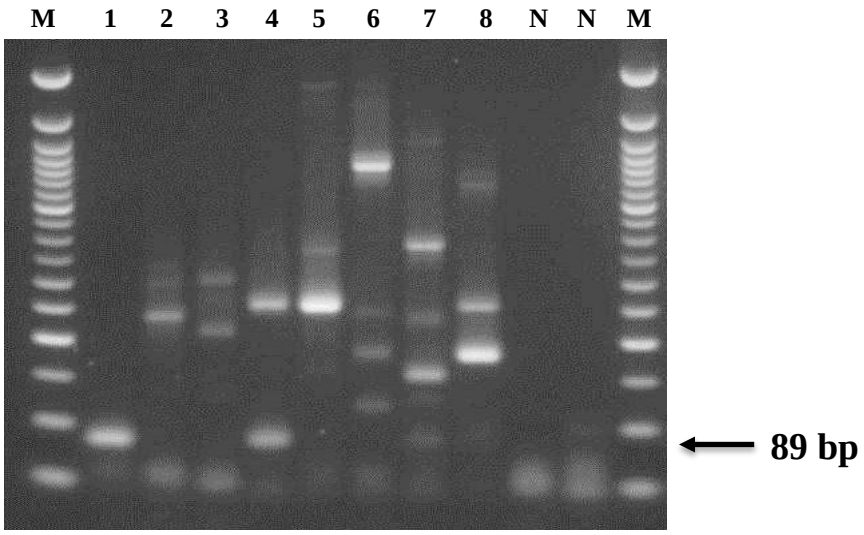


Figure 4-51: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* – contaminated by *L. fungicola* DNA (Ab.1)(Line 4), *A. bisporus* (21.08.09)(Line 5), *M. perniciosa* (M.1)(Line 6), *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). . The Tm in PCR reaction was 58°C. M – Marker 50 bp DNA, N – water control. PCR reaction was performed on 25.08.2009.

The PCR assay was repeated. This set of primers gave an 89 bp amplification product for *L. fungicola*, but the amplicon of these primers was present from *T. aggressivum*. Using this set of primers it is possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro*. This set of primers also did not amplify other *A. bisporus* mycoparasites such as *M. perniciosa* and *C. mycophilum*. This set of primers look promising for detection of *L. fungicola* and future study (**Figure** 4-52).

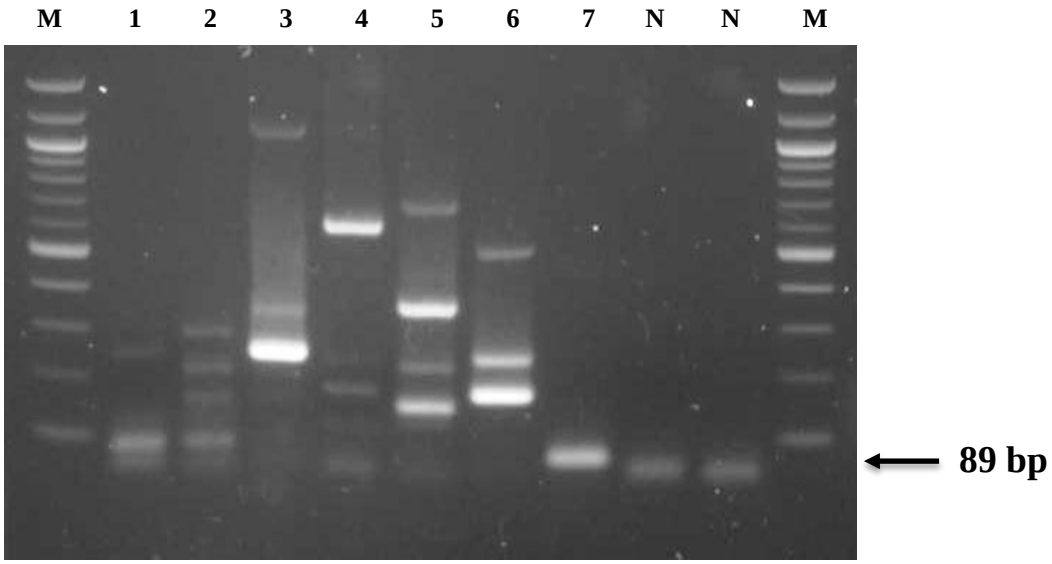


Figure 4-52: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), Clean casing (Line 2), *A. bisporus* (21.08.09)(Line 3), *M. perniciosa* (M.31)(Line 4), *C. mycophilum* (D.1)(Line 5), *A. fumigatus* (As.) (Line 6), and *T. aggressivum* (CBS 433.95) (Line 7). Taq DNA polymerase in stored buffer A – Promega set of primers amplified a product of 90 bp (Ay 124053 F and R). M – Marker 100 bp DNA, N – water control. PCR reaction was performed on 13.11.2009.

The PCR assay was repeated again to test other isolates of *L. fungicola* var. *fungicola* and var. *aleophilum*. This set of primers amplified both varieties of *L. fungicola* giving an 89 bp amplicon. Using this set of primers it is possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro* test. This set of primers did not amplify any tested fungi which will give an 89 bp amplicon and on this occasion *T. agrressivum* did not produce an amplicon (**Figure** 4-52). Casing extract used in this experiment was stored some times in -20 °C and the DNA may have been degraded during this time and this is why no amplification band was shown. This set of primers looks very promising for detection of *L. fungicola* from dirty material (**Figure** 4-53).

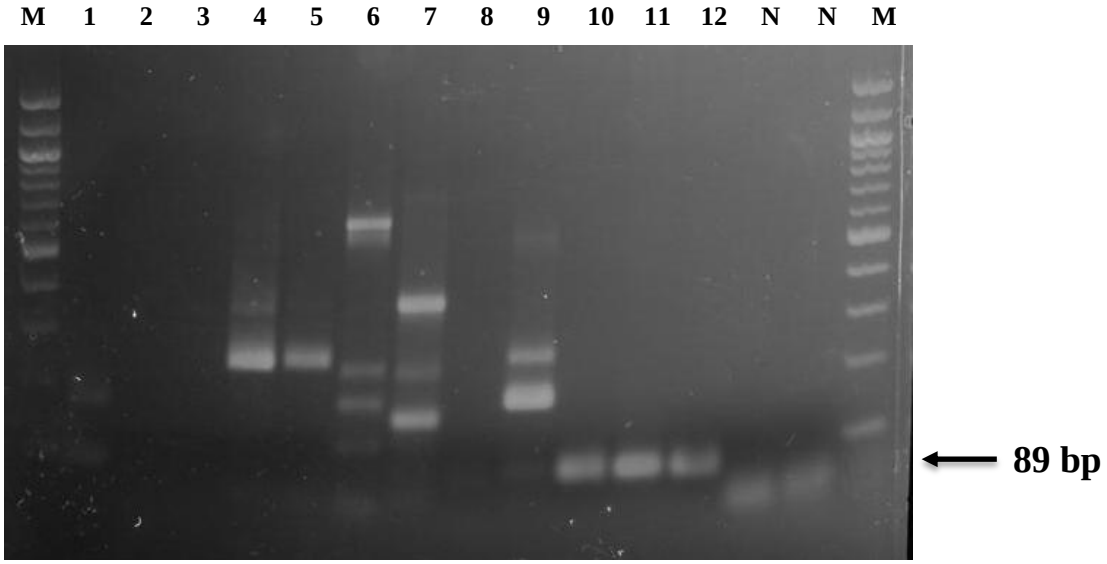


Figure 4-53: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), casing extract – Fujifilm (Line 2), casing extract – Promega (Line 3), *A. bisporus* (21.08.09) (Line 4-5), *M. perniciosa* (M.31)(Line 6), *C. mycophilum* (D.1)(Line 7), *T. aggressivum* (CBS 433.95) (Line 8) and *A. fumigatus* (As.) (Line 9), *L. fungicola* var. *fungicola* – CBS 992.69 (Line 10), *L. fungicola* var. *fungicola* – CBS 648.80 (Line 11) and *L. fungicola* var. *aleophilum* – CBS 357.80 (Line 12). Taq DNA polymerase in stored buffer A – Promega set of primers amplified a product of 89 bp (Ay 124053). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 16.06.2010.

4.1.5.6 Primer set VI – Forward 79-Reverse 201 (F79-R201)

Primer set VI (F79-R201) gave a 123 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from *A. fumigatus*. Using this set of primers it is possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro*. This set of primers did not amplify a 123 bp amplicon from *A. bisporus* DNA. The amplification products from *A. bisporus* using these primers have 4-5 amplification products with different sizes. This set of primers amplified primer dimers which are present in water control which makes these primers not very good for use (**Figure** 4-54).

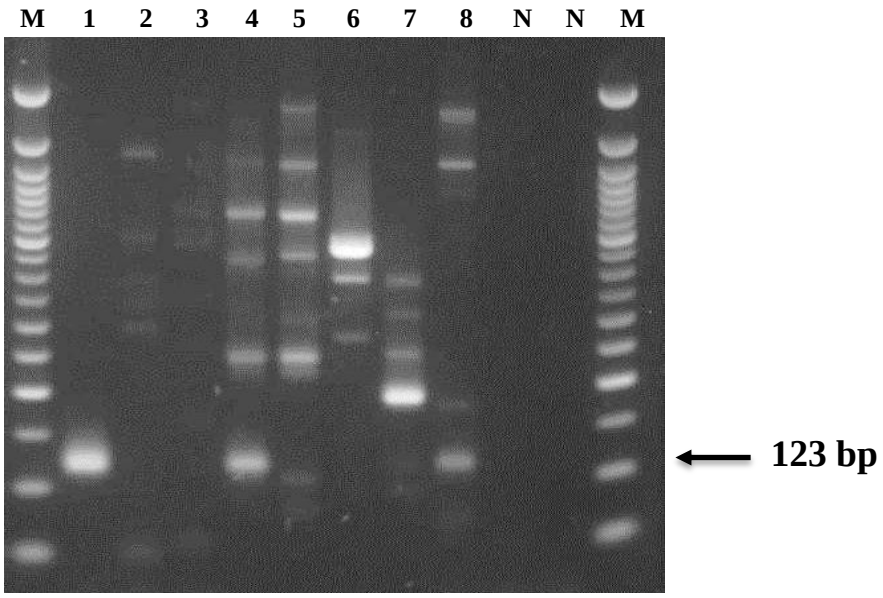


Figure 4-54: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* – contaminated by *L. fungicola DNA* (Ab.1)(Line 4), *A. bisporus* (21.08.09)(Line 5), *M. perniciosa* (M.1)(Line 6), *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 50 bp DNA, N – water control. PCR reaction was performed on 25.08.2009.

The PCR assay was repeated with other isolates of *L. fungicola* var. *fungicola* and var. *aleophilum*. This set of primers amplified both varieties of *L. fungicola* giving a 123 bp amplicon. This set of primers also detected *A. fumigatus* giving this same size amplicon (123 bp) but it was weak. Casing extract used in this experiment was stored for 2 months in -20 °C and the DNA may have been degraded during this time which is why no amplification band was visible. This set of primers looks promising for detection of *L. fungicola* from diseased tissue of *A. bisporus* (**Figure** 4-55).

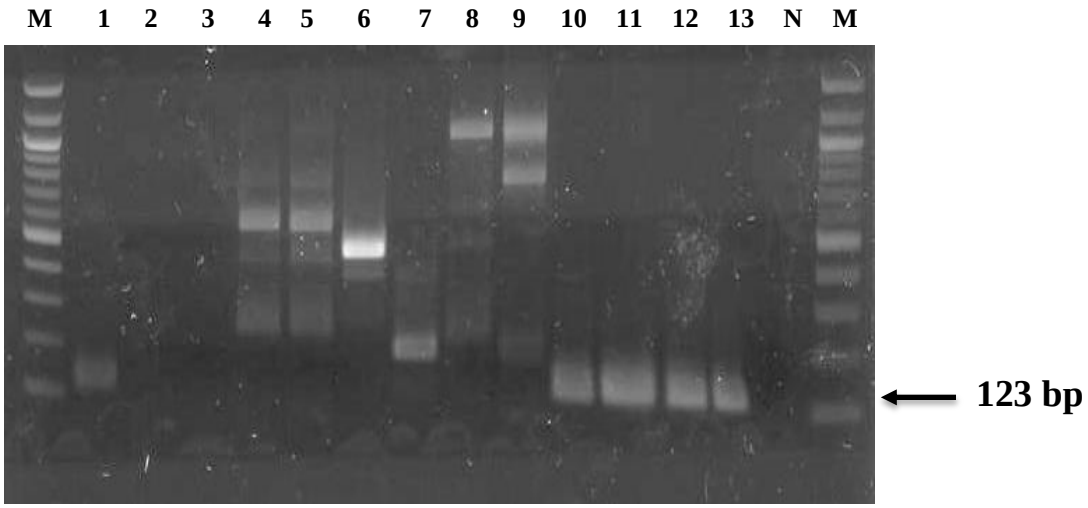


Figure 4-55: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.09) (Line 4-5), *M. perniciosa* (M.31)(Line 6), *C. mycophilum* (D.1)(Line 7), *T. aggressivum* (CBS 433.95) (Line 8) and *A. fumigatus* (As.) (Line 9), *L. fungicola* var. *fungicola* – CBS 992.69 (Line 10), *L. fungicola* var. *fungicola* – CBS 648.80 (Line 11) and *L. fungicola* var. *aleophilum* – CBS 357.80 (Line 12-13). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 16.06.2010.

4.1.5.7 Primer set VII – Forward (P) 87-Reverse 167 (F87-R167)

Primer set VII (F87-R167) gave a 80 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from *A. fumigatus*, *M. perniciosa* and *C. mycophilum*. The efficiency for amplification from other fungi was very low but this set is good for identification of *L. fungicola* from *A. bisporus* tissue. *Agaricus bisporus* DNA gave only one amplification product in size around 175 bp. This set of primers amplified primer dimers which are present in water control which makes these primers not very good for use (**Figure** 4-56).

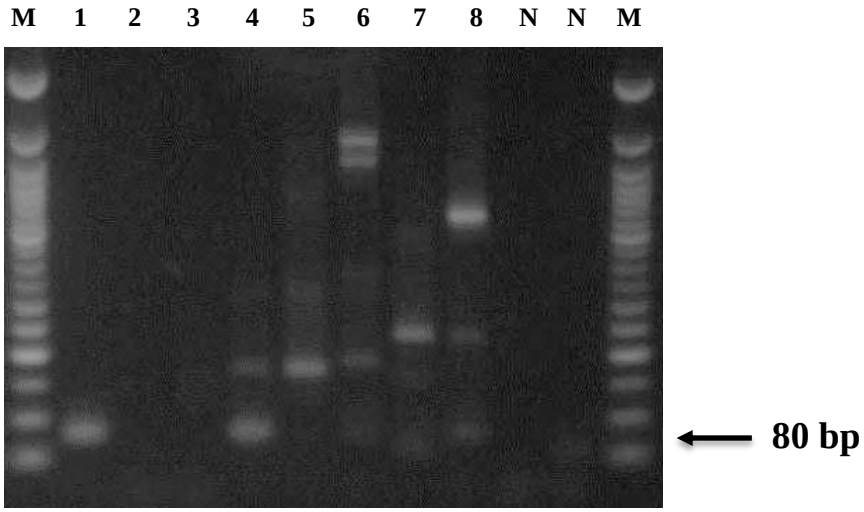


Figure 4-56: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* – contaminated by *L. fungicola* DNA (Ab.1)(Line 4), *A. bisporus* (21.08.09)(Line 5), *M. perniciosa* (M.1)(Line 6), *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 50 bp DNA, N – Water control. PCR reaction was performed on 25.08.2009.

4.1.5.8 Primer set VIII – Forward (P) 87-Reverse 201 (F87-R201)

Primer set VIII (F87-R201) gave a 115 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from *A. fumigatus, M. perniciosa* and *C. mycophilum*. This set of primers can detect and identify *L. fungicola* from diseased tissue of *A. bisporus in vitro*. *Agaricus bisporus* DNA gave two amplification products in size around 150 bp and 200 bp. This set of primers amplified primer dimers which are present in water control which makes these primers not very good for use (**Figure** 4-57).

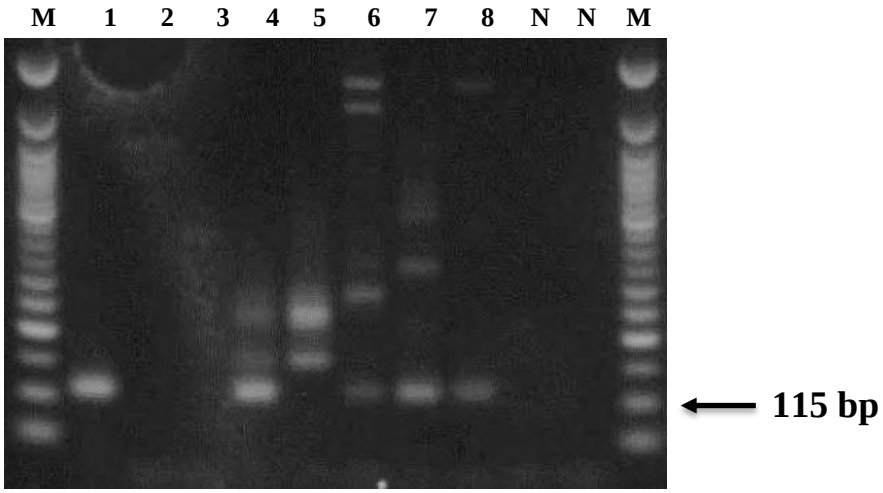


Figure 4-57: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* – contaminated by *L. fungicola DNA* (Ab.1)(Line 4), *A. bisporus* (21.08.09)(Line 5), *M. perniciosa* (M.1)(Line 6), *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 50 bp DNA, N – Water control. PCR reaction was performed on 25.08.2009.

The PCR assay was repeated. This set of primers gave a 115 bp amplification product for *L. fungicola*, but these primers also amplified *A. fumigatus* and *C. mycophilum* but *T. aggressivum* did not show an amplification product of this size. Using this set of primers it is possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro*. *Agaricus bisporus* DNA gave two amplification products in size around 150 bp and 200 bp (**Figure** 4-58).

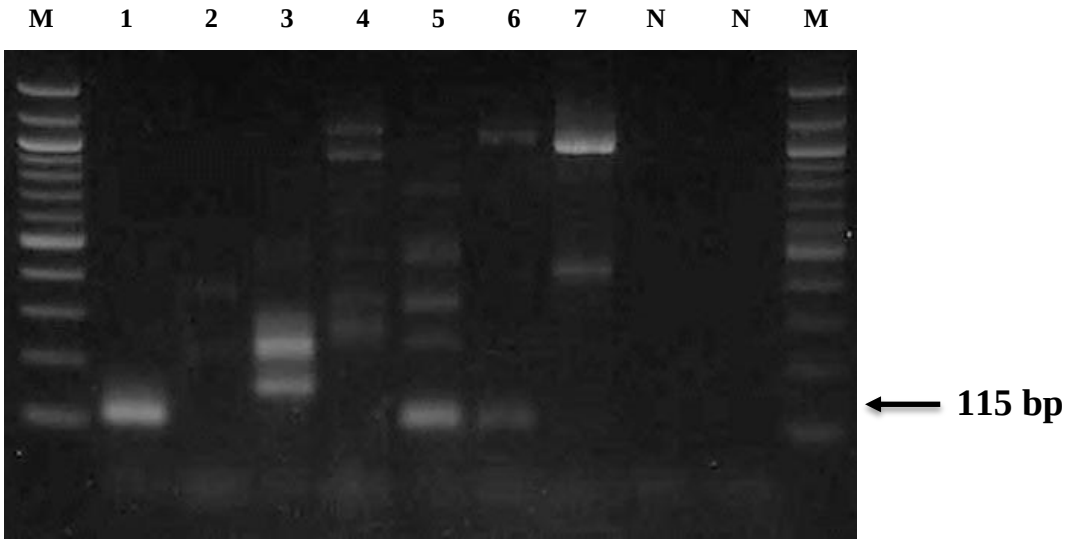


Figure 4-58: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), Clean casing (Line 2), *A. bisporus* (21.08.09)(Line 3), *M. perniciosa* (M.31)(Line 4), *C. mycophilum* (D.1)(Line 5), *A. fumigatus* (As.) (Line 6), and *T. aggressivum* (CBS 433.95) (Line 7), M – Marker 100 bp DNA, N – water control. PCR reaction was performed on 13.11.2009.

4.1.5.9 Primerw set IX – Forward (P) 142 – Reverse 201 (F142-R201)

Primer set IX (F142-R201) gave a 60 bp amplification product for *L. fungicola*, but the same size of amplification product was also present in DNA isolated from *A. fumigatus, M. perniciosa* and *C. mycophilum*. Using this set of primers it was possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro*. *Agaricus bisporus* DNA gave three amplification products bigger than 60 bp. This set of primers amplified primer dimers which are present in water control. This makes these primers not very good for use (**Figure** 4-59).

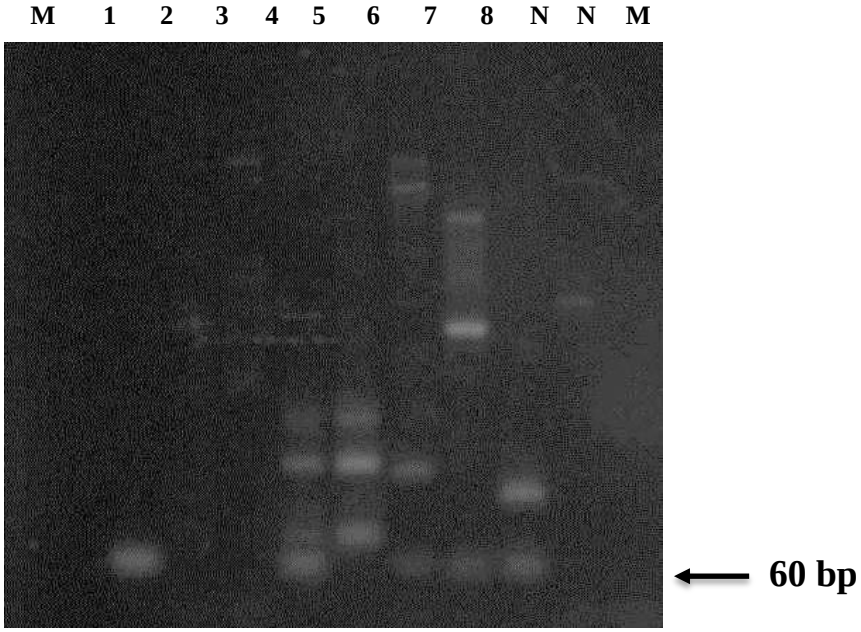


Figure 4-59: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* – contaminated by *L. fungicola* DNA (Ab.1)(Line 4), *A. bisporus* (21.08.09)(Line 5), *M. perniciosa* (M.1)(Line 6), *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 50 bp DNA, N – Water control. PCR reaction was performed on 25.08.2009.

4.1.5.10 Primers designed by Zijlstra *et al.* amplified rRNA region

The primers designed by Zijlstra *et al.* (2007, 2008 and 2009) (Zijlstra primers) were tested on different fungi and casing extract to check specificity for detection of *L. fungicola* from dirty samples. This set of primers gave a 102 bp amplification product for *L. fungicola* DNA. This set of primers did not amplify this size of amplicon (102 bp) in any tested fungi, but they gave many non-specific bands in *A. bisporus* DNA and other fungi. The *A. bisporus* isolate (Ab.1) gave a positive amplification product indicating that sample was contaminated with *L. fungicola* during storage. All information and experiments performed with this set of primers are presented in Chapter 3 and Chapter 6 (**Figure** 4-60).

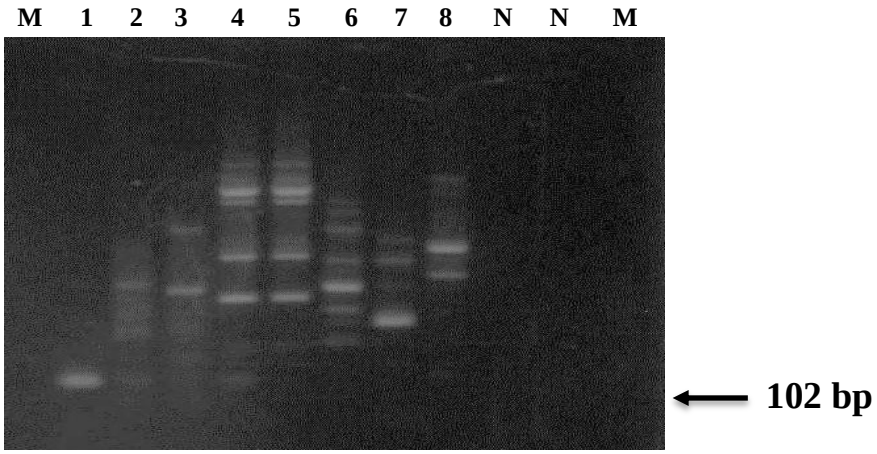


Figure 4-60: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* – contaminated by *L. fungicola* DNA (Ab1)(Line 4), *A. bisporus* (21.08.09)(Line 5), *M. perniciosa* (M.1)(Line 6), *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 50 bp DNA, N – Water control. PCR reaction was performed on 25.08.2009.

The PCR assay was repeated with other isolates of *L. fungicola* var. *fungicola* and var. *aleophilum*. This set of primers amplified both varieties of *L. fungicola* giving a 102 bp amplicon. This set of primers did not amplify other tested fungi, but they gave many non-specific amplification products for other organisms. The A. *bisporus* DNA amplified using this set of primers gave 6 or more non-specific bands. The *M. perniciosa* DNA gave 4 non-specific amplicons, *C. mycophilum* DNA has only 1 or 2 amplification products, *T. agrressivum* gave 2 amplicons, *A. fumigatus* DNA gave 3 non-specific amplicons, but none of these amplification products were 102 bp size. Casing extract used in this experiment was stored sometimes at -20 °C and the DNA may have been degraded during this time compared with previous figure where fresh casing gave several amplification bands. These set of primers designed by Zijlstra *et al.* (2007, 2008 and 2009) were designed for Real Time PCR – TaqMan probe and must amplify only this region of DNA where/when TaqMan probe is present (**Figure** 4-61).

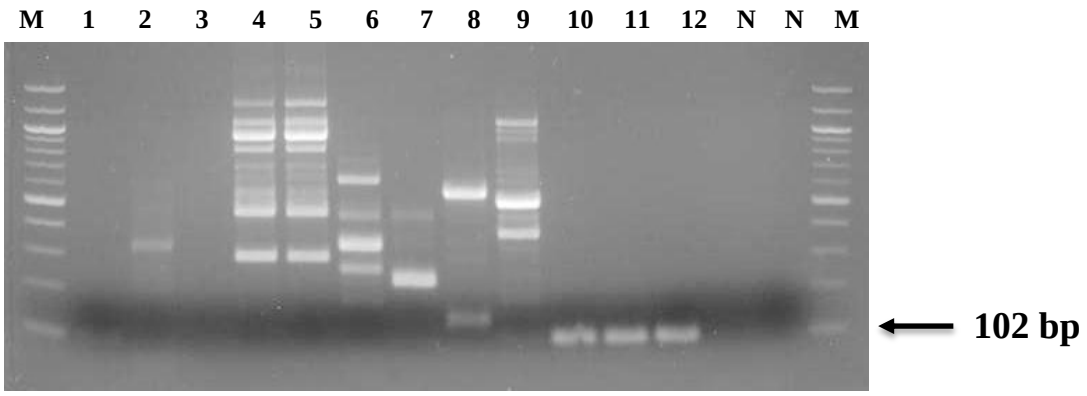


Figure 4-61: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), casing extract – Promega (Line 2), casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.09) (Line 4), *M. perniciosa* (M.31)(Line 5), *C. mycophilum* (D.1)(Line 6), *T. aggressivum* (CBS 433.95) (Line 7) and *A. fumigatus* (As.) (Line 8), *L. fungicola* var. *fungicola* – CBS 992.69 (Line 9), *L. fungicola* var. *fungicola* – CBS 648.80 (Line 10) and *L. fungicola* var. *aleophilum* – CBS 357.80 (Line 11). Set of primers amplified a product of 90 bp (Ay 124053). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 16.06.2010.

4.1.5.11 Summary

This experiment tested different sets of primers designed in MAT1-2-1 gene region described by Yokoyama and Hara (2000). The MAT1-2-1 is conserved gene in all Ascomycetes fungi.

All designed and tested primers gave amplification products in other filamentous fungi. One set of designed and tested primer set F9-R201 gave the same size of amplicon in *L. fungicola* and *A. bisporus* and was therefore not useful. Other set of

primers III (F9-R167) gave a very similar size amplicon in *L. fungicola* and *A. bisporus*. One set of designed primers VI (F79-R201) amplified a 123 bp product of *L. fungicola* and *A. fumigatus* DNA could be used for identification of *L. fungicola* from samples containing other mycoparasites of *A. bisporus* (**Table** 4-2).

No designed or tested set of primers for MAT1-2-1 region gave only *L. fungicola* amplification product that could be used for detection of *L. fungicola* in samples containing other fungi. Some of these sets of primers are useful for identification of *L. fungicola* from *A. bisporus* tissue in *in vitro* experiment.

A set of primers (rRNA) designed by Zijlstra *et al.* (2007, 2008 and 2009) amplified a *L. fungicola* DNA only giving 102 bp amplicon. The other tested fungi did not show an amplification product of this size, but tested fungi showed many non-specific amplicons (**Table** 4-2).

Table 4-2: Different sets of primers designed on MAT1-2-1 gene region tested on different fungi.

Fungi, sample/ Primer set	**I Ay124053 (F116-R205**	**II Ay124053 (F151-R205**	**III F9-R167**	**IV F9-R201**	**V F79-R167**	**VI F79-R201**	**VII F87-R167**	**VIII F87-R201**	**IX F142-R201**	**Zijlstra *et al.***
Agaricus bisporus	No	Yes	No	Yes	No	No	No	No	No	No
Aspergillus fumigatus	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	No
Cladobotryum mycophilum	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No
Casing extract	No	nt	No	No	No	No	No	No	No	No
Clean casing	No	Yes	nt	nt	nt	nt	nt	nt	nt	No
Lecanicillium fungicola var. *aleophilum*	Yes	Yes	nt	nt	Yes	Yes	nt	nt	nt	Yes
Lecanicillium fungicola var. *fungicola*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Mycogone perniciosa	No	Yes	No	No	No	No	Yes	Yes	Yes	No
Trichoderma aggressivum	Yes	Yes	nt	nt	Yes	No	nt	nt	nt	No

nt – not tested, red – amplified the same size amplicon as *L. fungicola* DNA, green – the amplification product had different size compared to *L. fungicola* product.

4.1.6 PCR of internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS1-5.8S-ITS2)

4.1.6.1 Primer set A – Af 324874 (Forward 57-Reverse 191)

Primer set A (Af 324874 (Forward 57 – Reverse 191) gave a 135 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from *A. fumigatus* and *C. mycophilum*. Using this set of primers it is possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro*. *Agaricus bisporus* DNA gave three amplification products bigger than a 135 bp. This set of primers amplified primer dimers which are present in water control which makes these primers not very good for use (**Figure** 4-62).

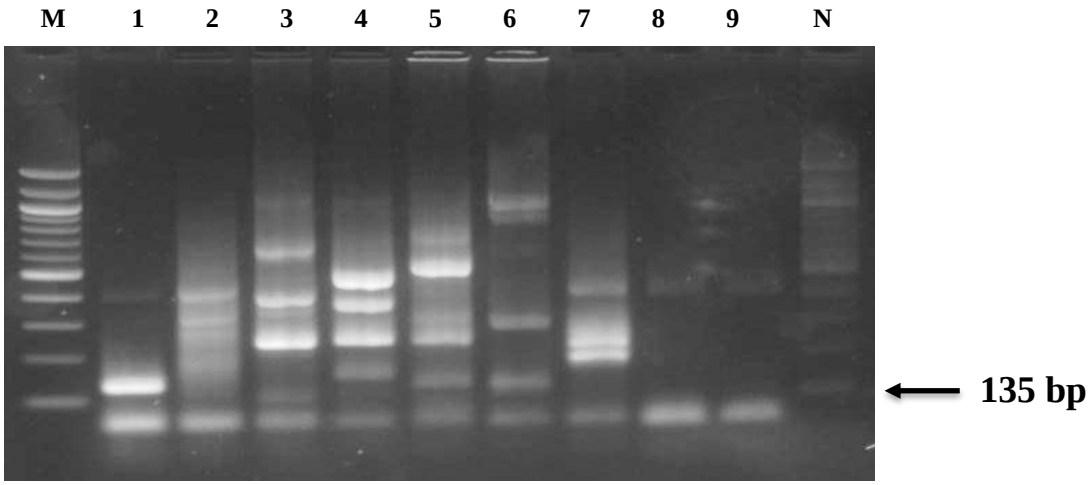


Figure 4-62: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15)(Line 1), Clean casing (Line 2), *A. bisporus* (21.08.09)(Line 3), *M. perniciosa* (M.31)(Line 4), *C. mycophilum* (D.1)(Line 5), *A. fumigatus* (As.) (Line 6), and *T. aggressivum* (CBS 433.95) (Line 7). Taq DNA polymerase in stored buffer A – Promega set of primers amplified a product of 135 bp (Af 324874). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 13.11.2009.

The PCR assay was repeated with other isolates of *L. fungicola* var. *fungicola* and var. *aleophilum*. This set of primers amplified both varieties of *L. fungicola* giving a 135 bp amplicon. This set of primers also detected *A. fumigatus* and *C. mycophilum* giving the same size amplicon (135 bp) but the amplification products gave a very weak signal. Casing extract used in this experiment was stored sometimes at -20 °C and the DNA may have been degraded. The *A. bisporus* DNA gave 3 to 6 non-specific amplicons. This set of primers look promising for detection of *L. fungicola* from diseased tissue of *A. bisporus*. This set of primers amplified primer dimers which are present in water control this makes these primers not very good for use (**Figure** 4-63).

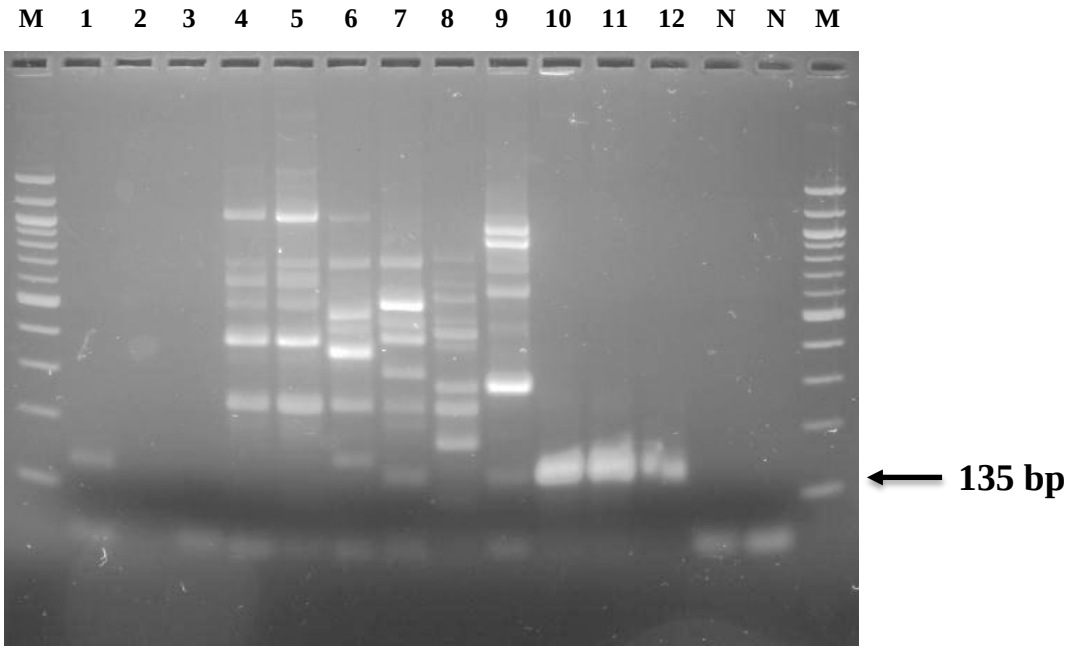


Figure 4-63: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), casing extract- Fujifilm (Line 2), casing extract – Promega (Line 3), *A. bisporus* (21.08.09) (Line 4-5), *M. perniciosa* (M.31)(Line 6), *C. mycophilum* (D.1)(Line 7), *T. aggressivum* (CBS 433.95) (Line 8) and *A. fumigatus* (As.) (Line 9), *L. fungicola* var. *fungicola* – CBS 992.69 (Line 10), *L. fungicola* var. *fungicola* – CBS 648.80 (Line 11) and *L. fungicola* var. *aleophilum* – CBS 357.80 (Line 12). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 16.06.2010.

4.1.6.2 Primer set B – Af 324874 (Forward 138-Reverse 191)

Primer set B (Af 324874 (F138-R191) gave a 50 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from all tested fungi (*L. fungicola*, *A. bisporus*, *A. fumigatus*, *M. perniciosa* and *C. mycophilum*) and casing samples (**Figure** 4-64).

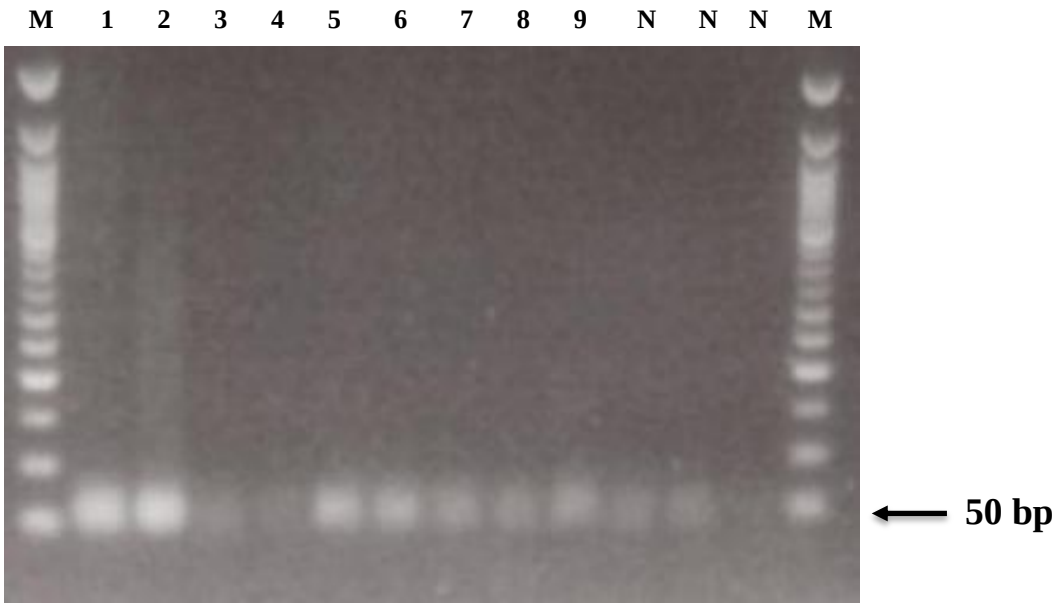


Figure 4-64: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15-L.16) (Line 1-2), Clean casing 2(Line 3), Casing extract – Promega (Line – 4), *A. bisporus* (Ab.1 and Ab.3) (Line 5-6), *A. fumigatus* (As.) (Line 7), *M. perniciosa* (M.11) (Line 8) and *C. mycophilum* (D.1) (Line 9). M – Marker 50 bp DNA, N – Water control. PCR reaction was performed on 17.07.2009.

4.1.6.3 Summary

These experiments tested different sets of primers designed in ITS1-5.8S-ITS2 rDNA region described by Collopy *et al.* (2000). This sequence ITS1-5.8S-ITS2 is a highly conserved region.

The Primer set A – Af 324874 (F57-R191) were not specific enough for identification and detection of *L. fungicola* in samples containing other tested fungi such as *A. fumigatus* and *C. mycophilum*. This set of primers can detect and identify *L. fungicola* from diseases tissue of *A. bisporus in vitro*. This set gave many non-specific amplification products so they are not good to use with dirty samples. These primers may be suitable for use with clean culture of *L. fungicola* (**Table** 4-3).

A second set of primers B – Af 324874 (F138-R191) failed to identify *L. fungicola* since all tested fungi showed the same size of amplification product (**Table** 4-3).

Table 4-3: Different sets of primers designed in ITS1-5.8S-ITS2 rDNA region tested on different fungi.

Fungi, sample/Primers	**A – Af 324874 (F57-R191)**	**B – Af 324874 (F57-R191)**
Agaricus bisporus	No	Yes
Aspergillus fumigatus	Yes	Yes
Cladobotryum mycophilum	Yes	Yes
Casing extract	No	nt
Clean casing	No	Yes
Lecanicillium fungicola var. *aleophilum*	Yes	Yes
Lecanicillium fungicola var. *fungicola*	Yes	Yes
Mycogone perniciosa	No	Yes
Trichoderma aggressivum	No	Yes

nt – not tested, red – amplified the same size amplicon as *L. fungicola* DNA, green – the amplification product had different size compare to *L. fungicola* product

4.1.7 PCR of 18 S-ITS1-5.8 S-ITS2- 28S regions

4.1.7.1 Primer set 1 – Forward 1540-Reverse 1723 (18S ribosomal RNA)

Primer set 1 (F1540-R1723) gave a 184 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from all tested fungi (*L. fungicola*, *A. bisporus*, *A. fumigatus*, *M. perniciosa* and *C. mycophilum*) and casing samples (**Figure** 4-65). PCR was repeated and results were the same (gel not shown).

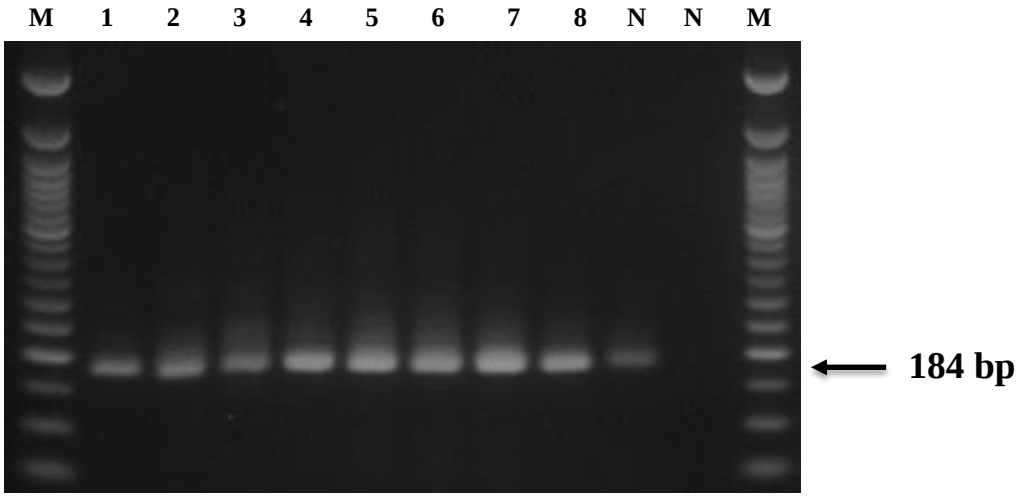


Figure 4-65: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – water control. PCR reaction was performed on 28.08.2009.

4.1.7.2 Primer set 2 – Forward 1540-Reverse 2042 (18S-ITS1-5.8S)

Primer set 2 (F1540-R2042) gave a 485 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from casing samples, *A. fumigatus*, *C. mycophilum* and *T. aggressivum*. This set of primers was not good for identification of *L. fungicola* from diseased tissue of *A. bisporus*. The amplification product from *A. bisporus* is very close to the size of *L. fungicola* amplicon and agarose gel has low resolution for this type of separation (**Figure** 4-66).

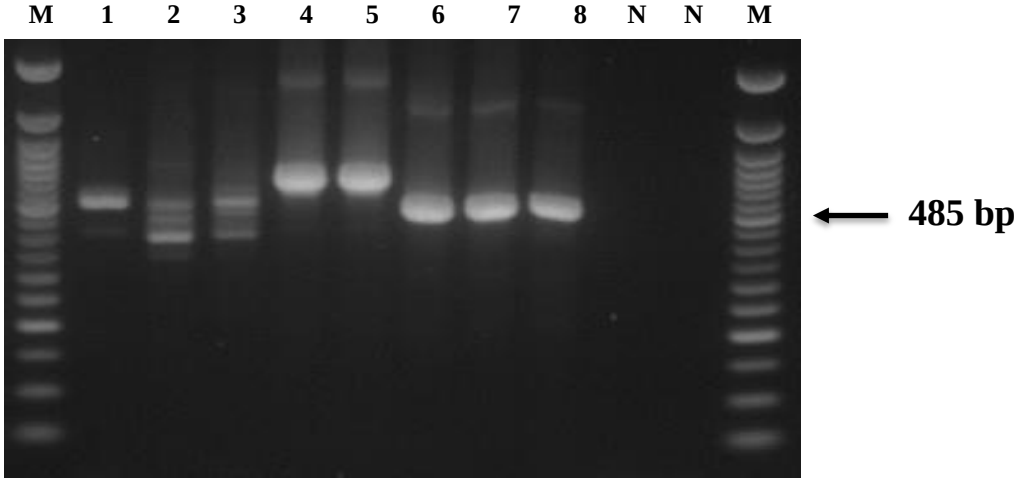


Figure 4-66: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 28.08.2009.

4.1.7.3 Primer set 3 – Forward 1934-Reverse 2042 (5.8S)

Primer set 3 (F1934-R2042) gave a 109 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from all tested fungi (*L. fungicola*, *A. bisporus*, *A. fumigatus*, *M. perniciosa* and *C. mycophilum*) and casing samples. The water control showed an amplification product also with this same size amplicon as *L. fungicola* (**Figure** 4-67). PCR was repeated and results were the same (gel not shown).

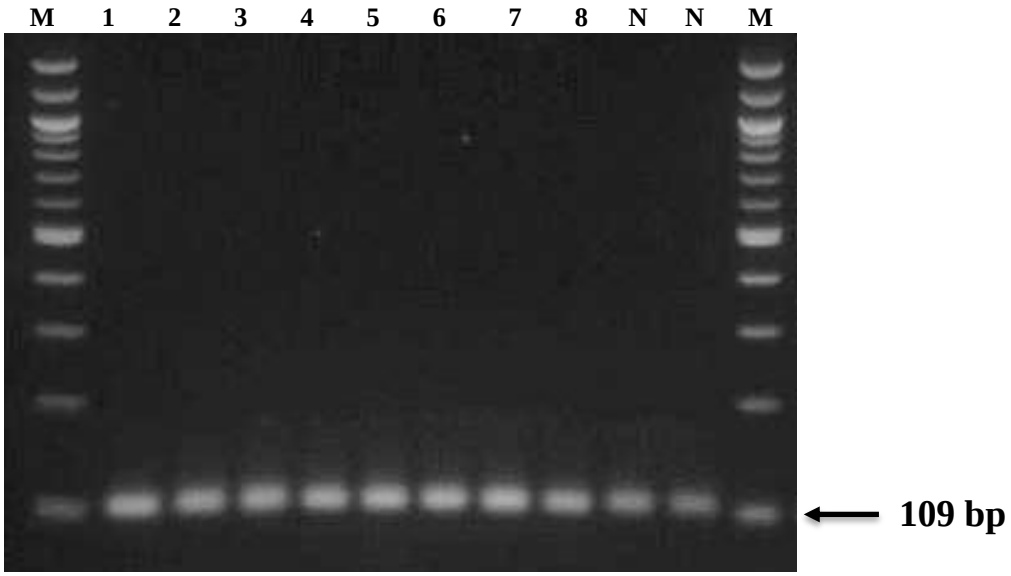


Figure 4-67: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 28.08.2009.

4.1.7.4 Primer set 4 – Forward 1958-Reverse 2042 (18S-ITS1-5.8S)

Primer set 4 (F1958-R2042) gave an 85 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from all tested fungi (*L. fungicola*, *A. bisporus*, *A. fumigatus*, *M. perniciosa* and *C. mycophilum*) and casing samples. The water control showed an amplification product also with the same size amplicon as *L. fungicola* but here it could be due to primer dimers (**Figure** 4-68). PCR was repeated and results were the same (gel not shown).

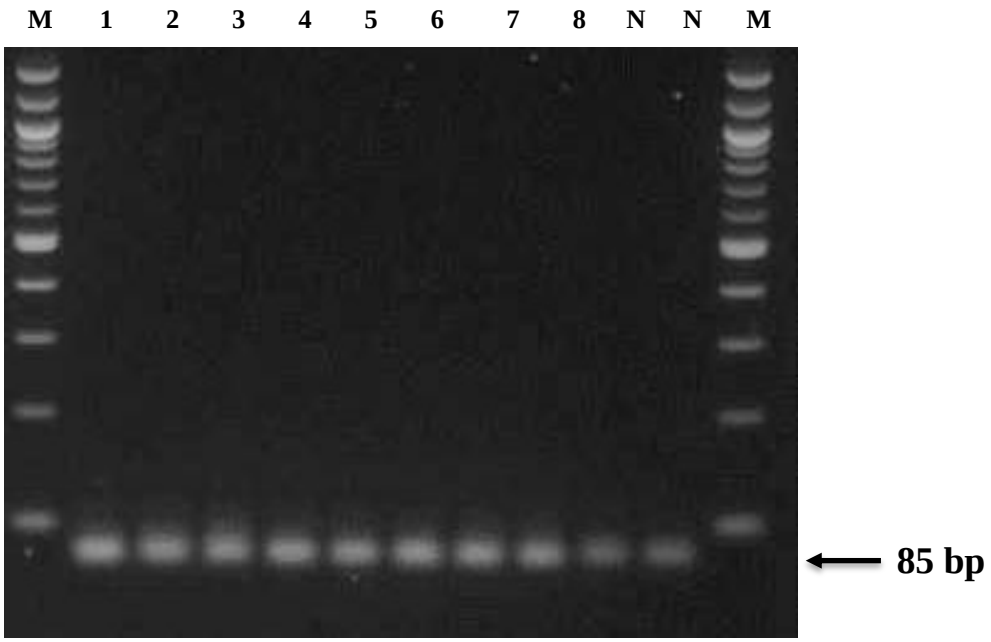


Figure 4-68: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 28.08.2009.

4.1.7.5 Primer set 5 – Forward 1540-Reverse 2100 (18S-ITS1-5.8S-ITS2)

Primer set 5 (F1540-R2100) gave a 384 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from other fungi such as *A. fumigatus*, *M. perniciosa* and *C. mycophilum*. Using this set of primers it is possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro* test. *Agaricus bisporus* DNA gave two amplification products bigger and smaller than a 561 bp. (**Figure** 4-69). PCR was repeated and results were the same.

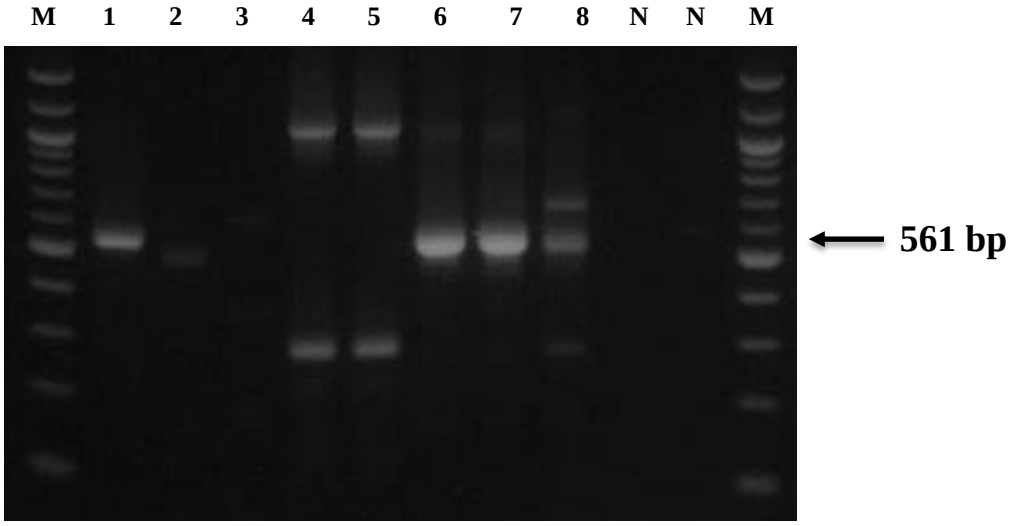


Figure 4-69: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 28.08.2009.

4.1.7.6 Primer set 6 – Forward 1934-Reverse 2100 (5.8S-ITS2)

Primer set 6 (F1934-R2100) gave a 167 bp amplification product for *L. fungicola*, but this same size of amplification product was present in all tested fungi (*L. fungicola*, *A. bisporus*, *A. fumigatus*, *M. perniciosa* and *C. mycophilum*) and casing samples (**Figure** 4-70).

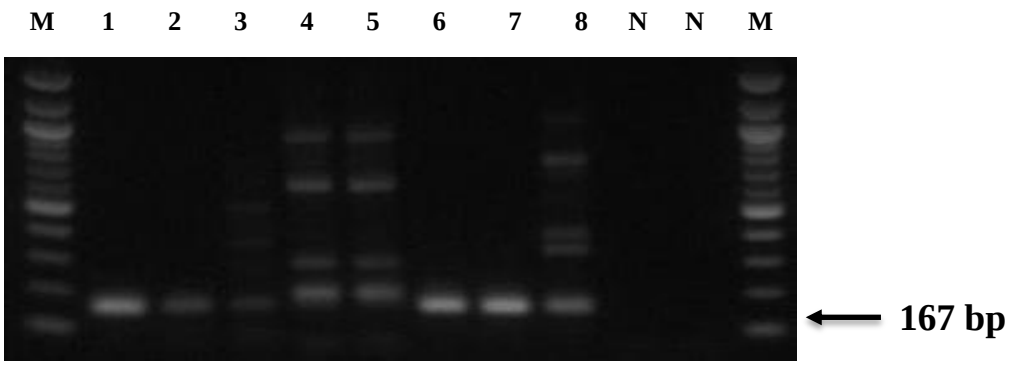


Figure 4-70: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 28.08.2009.

4.1.7.7 Primer set 7 – Forward 1958-Reverse 2100 (5.8S-ITS2)

Primer set 7 (F1958-R2100) gave a 143 bp amplification product for *L. fungicola*, but this same size of amplification product was all tested fungi (*L. fungicola*, *A. bisporus*, *A. fumigatus*, *M. perniciosa* and *C. mycophilum*) and casing samples (**Figure** 4-71).

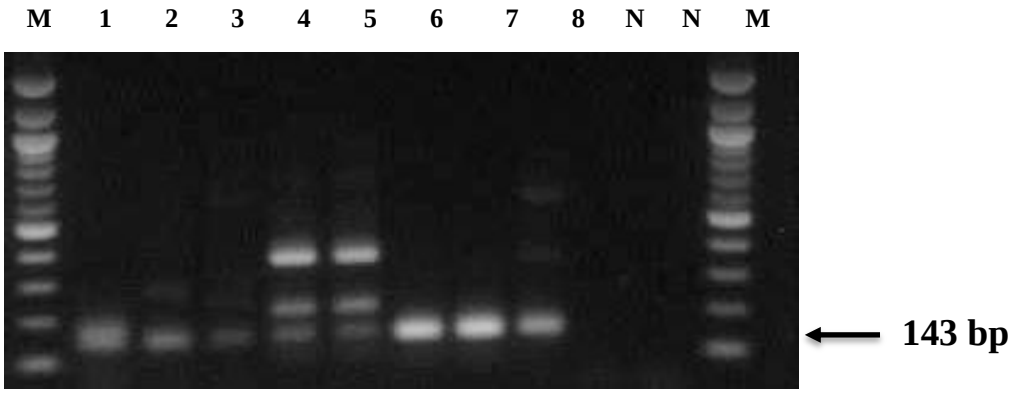


Figure 4-71: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 28.08.2009.

4.1.7.8 Primer set 8 – Forward 2017-Reverse2100 (5.8S-ITS2)

Primer set 8 (F2017-R2100) gave an 84 bp amplification product for *L. fungicola*, but this same size of amplification product was present in all tested fungi (*L. fungicola*, *A. bisporus*, *A. fumigatus*, *M. perniciosa* and *C. mycophilum*) and casing samples

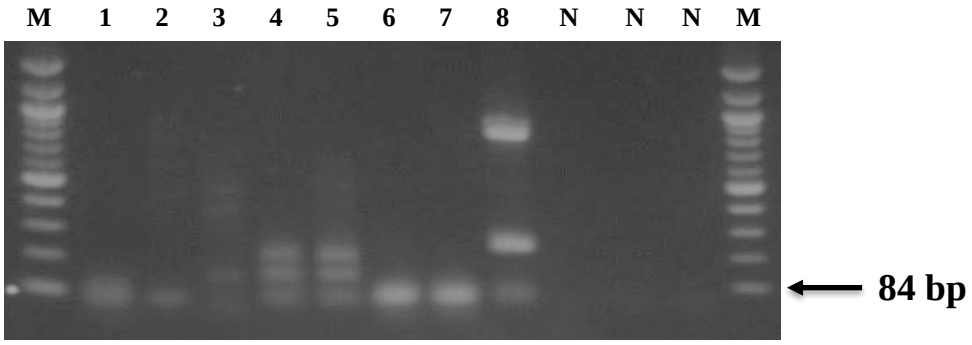


Figure 4-72: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 28.08.2009.

4.1.7.9 Primer set 9 – Forward 1659-Reverse 1723 (18S)

Primer set 9 (F1659-R1723) gave an 65 bp amplification product for *L. fungicola*, but this same size of amplification product was also present in DNA isolated from all tested fungi (*L. fungicola*, *A. bisporus*, *A. fumigatus*, *M. perniciosa* and *C. mycophilum*) and casing samples (**Figure** 4-73). PCR was repeated and results were the same (gel not shown).

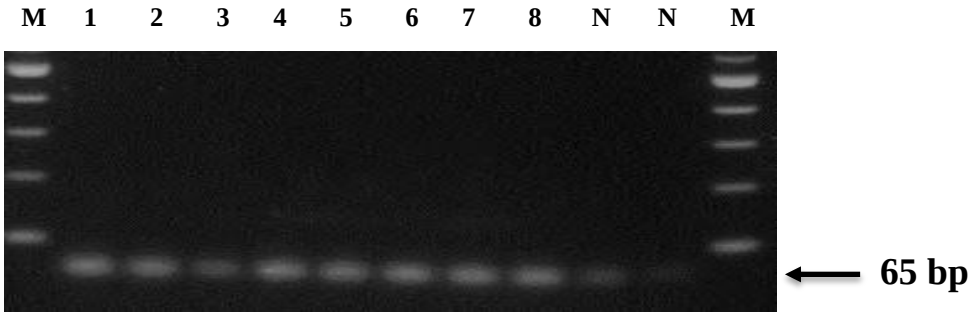


Figure 4-73: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 28.08.2009.

4.1.7.10 Primer set 10 – Forward 1659-Reverse 2042 (18S-ITS1-5.8S)

Primer set 10 (F1659-R2042) gave a 384 bp amplification product for *L. fungicola*, but the same size of amplification product was also present in DNA isolated from other fungi such as: *A. fumigatus*, *M. perniciosa* and *C. mycophilum*. Using this set of primers it is possible to identify *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro* test. *Agaricus bisporus* DNA gave one amplification products bigger than 384 bp. This set of primers amplified primer dimers which means these primers set not good for use (**Figure** 4-74).

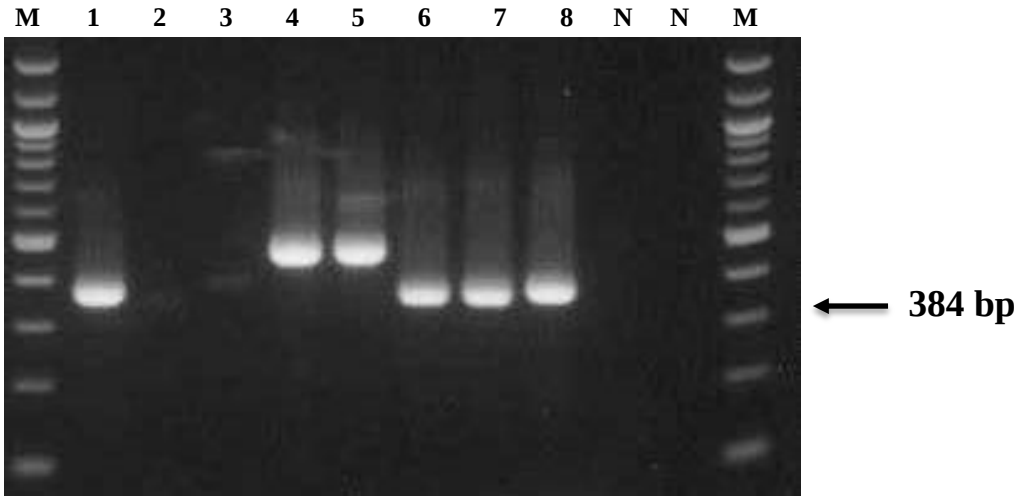


Figure 4-74: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – water control. PCR reaction was performed on 28.08.2009.

4.1.7.11 Primer set 11 – Forward 1659-Reverse 2100 (18S-ITS1-5.8S-ITS2)

Primer set 11 (F1659-R2100) gave a 442 bp amplification product for *L. fungicola* DNA but amplification product was very weak. This same size of amplification product was also present in DNA isolated from *M. perniciosa* and *C. mycophilum*. This set of primers look promising for identifying *L. fungicola* from diseased tissue of *A. bisporus* when clean cultures are used *in vitro*. *Agaricus bisporus* DNA gave one amplification products bigger than 442 bp. PCR optimisation is required to produce a better signal from *L. fungicola* DNA (**Figure** 4-75).

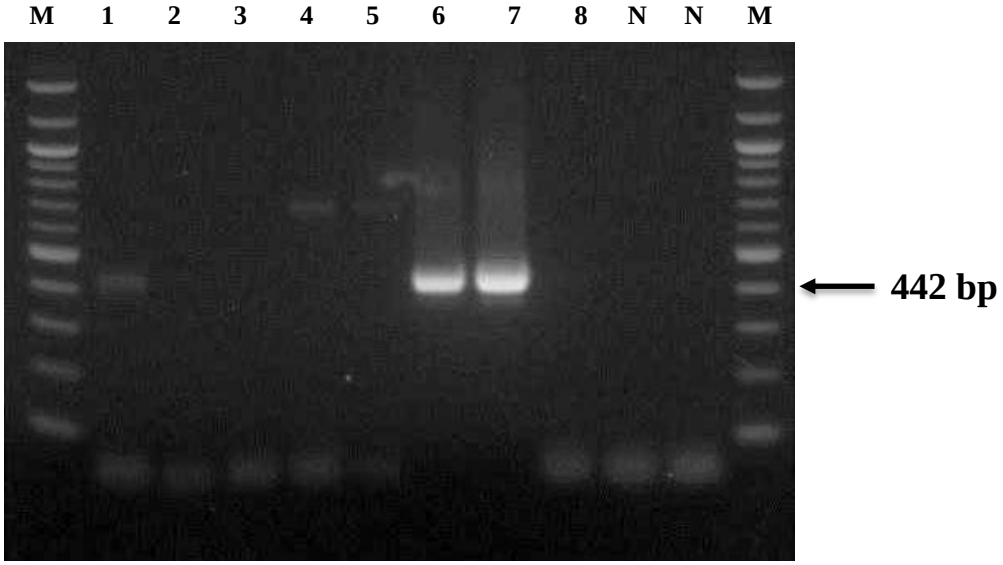


Figure 4-75: Electrophoretic profile of PCR products. The amplification product for *L. fungicola* (L.15) (Line 1), Casing extract – Promega (Line 2) Casing extract – Fujifilm (Line 3), *A. bisporus* (21.08.2009)(Line 4-5), *M. perniciosa* (M.1)(Line 6) *C. mycophilum* (D.1)(Line 7) and *A. fumigatus* (As.) (Line 8). M – Marker 100 bp DNA, N – Water control. PCR reaction was performed on 28.08.2009.

4.1.7.12 Summary

The work described examined different sets of primers designed in the ITS1-5.8S-ITS2 and 28S rRNA region previously described by Yokoyama and Hara (2003). This sequence of rDNA is conserved and it was very difficult to find selective primers. Primer sets 1, 3, 4, 9 (F1540-R1723; F1934-R2042; F1958-R2042; F1659-R1723) were not specific and all organisms and casing or casing extract produced the same amplicon size as *L. fungicola*. The primer set 6, 7, 8 (F1934-R2100; 1958-R2100; F2017-R2100) were not specific for *L. fungicola* DNA but some non-specific amplicons were present from other fungi and casing extract. The same size amplicon was present in other samples especially other mushroom pathogens so they would be unsuitable for identification and detection of *L. fungicola* (**Table** 4-4).

The primer set 2 (F1540-R2042) was not specific enough for identification and detection of *L. fungicola* from samples in casing and other fungi such as: *A. fumigatus, M. perniciosa* and *C. mycophilum*. This set is not good for identification of *L. fungicola* from *A. bisporus* tissue using agarose gel electrophoresis.

The primer set 5 (F1540-R2100) was not specific enough for identification and detection of *L. fungicola* from samples contained in casing and other fungi such as: *A. fumigatus, M. perniciosa* and *C. mycophilum*.

The primer set 10 (F1659-R2042) failed to detect and identify of *L. fungicola* from other fungi such as *A. fumigatus, M. perniciosa* and *C. mycophilum.*

The last primer set 11 (F1659-R2100) gave a 442 bp amplicon from *L. fungicola* DNA but also the same amplification product was present in other *A. bisporus* mycoparasites *M. perniciosa* and *C. mycophilum*. The efficiency of amplification of *L. fungicola* was very low (**Table** 4-4).

The main conclusions for this section are: the rDNA region is conserved in filamentous fungi (Ascomycetes) and almost all tested fungi showed the same size amplicon product as *L. fungicola* (White *et al.*, 1990; Bruns *et al.*, 1991, Richard *et al.*, 2008). *Agaricus bisporus* is a Basidiomycete and some of the designed primers did not show amplification product of the same size as with *L. fungicola*. These differences in amplicon size between *A. bisporus* and *L. fungicola* could be used for identification and detection of *L. fungicola* from diseased tissue of *A. bisporus* in *in vitro* experiments.

Table 4-4: Different sets of primers designed in ITS1-5.8S-ITS2 and 28S rRNA region of rDNA tested on different fungi.

Fungi, sample/ Primer set	**1 (F1540-R1723)**	**2 (F1540-R2042)**	**3 (F1934-R2042)**	**4 (F1958-R2042)**	**5 (F1540-R2100)**	**6 (F1934-R2100)**	**7 (F1958-R2100)**	**8 (F2017-R2100)**	**9 (F1659-R1723)**	**11 (F1659-R2042)**	**10 (F1659-R2100)**
Agaricus bisporus	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No	No
Asperigillus fumigatus	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Cladobotryum mycophilum	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Casing extract	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
Clean casing	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	No	No
Lecanicillium fungicola var. *aleophilum*	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
Lecanicillium fungicola var. *fungicola*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Mycogone perniciosa	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Trichoderma aggressivum	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt

nt – not tested, red – amplified the same size amplicon as *L. fungicola* DNA, green – the amplification product had a different size as compare to *L. fungicola* product.

4.2 Discussion

In this study four DNA extraction methods for use with clean cultures of *L. fungicola* DNA extraction were compared: one manual method (Aljanabi and Martinez, 1997) and three commercial DNA isolation kits: Nucleon Phytopure Genomic DNA Extraction, ZR Fungal/Bacterial DNA kit and DNeasy Plant Mini Kit. All tested extraction methods gave high quality genomic DNA which is potentially suitable for PCR assay.

The best method for *L. fungicola* DNA extraction is DNA extraction kit **ZR Fungal/Bacterial**. In this kit the first step of cell breakage is performed by glass beads in special lysing buffer. The genomic DNA was isolated very well and quality of DNA was good for PCR assay. This method allowed the preparation of 30 samples per session without loss of quality or quantity of DNA.

The **Aljanabi and Martinez** (1997) DNA extraction method worked well but required the addition of RNase A for removing RNA. This extraction method is a low cost DNA extraction method, but this method is time consuming and 10 to 15 samples per working day.

The two methods **Nucleon Phytopure Genomic DNA Extraction**, and **DNeasy Plant Mini Kit** required a first step (cell breakage) in liquid nitrogen which is time consuming and expensive. In these kits it is possible to do no more than 10 isolations per working day larger numbers of samples per day would be difficult because of the grinding step with liquid nitrogen.

For PCR assay of *L. fungicola* primers described by Largeteau *et al.* (2007) designed for Real Time PCR with SYBR green, and primers designed by Zijlstra *et al.* (2007, 2008 and 2009) for Real Time PCR with TaqMan Probe were used. The Largeteau *et al.* (2007), set of primers amplified a 130 bp amplicon of *L. fungicola,* but these primers also amplified a similar size amplicon of *A. bisporus* DNA but with lower efficiency. This set of primers is not *L. fungicola* specific and cannot be used for *L. fungicola* detection from mushroom farm samples (Largeteau *et al.* (2007). The Zijlstra primers set of primers amplified a 102 bp amplicon of *L. fungicola* DNA only. Zijlstra primers also give non-specific amplified products from other fungi such as *C. mycopilum, M. perniciosa, A. fumigatus* and *A. bisporus*.

The important thing in PCR assay is optimisation and for this sensitive polymerases were employed. Conditions of PCR reaction proposed for Zijlstra primers

were optimised for Real Time PCR with MGB TaqMan probe and the Tm depends on the MGB TaqMan probe. For normal PCR assay reaction optimization was pivotal for *L. fungicola* DNA amplification from clean culture and also from soil and casing what was another objective of the project.

The next part of this study compared four DNA extraction methods for use with soil and casing. Two manual: **Aljanabi and Martinez** (1997) and **Yeates *et al.*** (1998) and two commercial DNA extraction kits: **ZR Fungal/Bacterial** and **DNeasy Plant Mini Kit** were tested for their efficiency, sensitivity and rapidness in extracting *L. fungicola* DNA from soil, casing, casing extract and dust extract. Problems with PCR can arise when samples contain soil and casing because the soil and casing contains many PCR inhibitors such as humic acids that can reduce the Taq polymerase activity. Finding a good DNA extraction method/kit and a sensitive polymerase were pivotal for the detection of *L. fungicola* DNA in casing and soil samples (Tebbe and Vahjen, 1993; Tsai and Olson, 1992; Wilson *et al.*, 1990; Wilson, 1997). DNA extraction from soil or casing samples must be inexpensive, practical and rapid for processing large number of samples required for epidemiological studies of *L. fungicola* on mushroom farms. The quantification of DNA in samples containing humic acids is problematic because humic acids have similar chemical characteristics to DNA. They exhibit considerable absorbance at the wavelength used to quantify DNA (260 nm) (Vazquez-Marrufo *et al.*, 2002). But the quality of extracted DNA can be checked by agarose electrophoresis gel.

The **Aljanabi and Martinez** (1997) DNA extraction method did not gave good results for genomic DNA from soil and in PCR assay the amplification product was not present. The **DNeasy Plant Mini Kit extraction DNA** kit gave good results from casing samples when DNA extraction protocols was modified and glass beads were used for cell breakage, but PCR assay did not show any amplification product.

The **Yeates *et al.*** (1998) and **ZR Fungal/Bacterial DNA** extraction kit gave high quality genomic DNA when conidia of *L. fungicola* were mixed with soil and casing samples. The ZR Fungal/Bacterial DNA extraction kit gave a good quality DNA and PCR amplification product was present when the amount of template for PCR reaction was 1 µl and 0.5 µl and amount of conidia suspension of *L. fungicola* used for extraction was 1.94×10^7 conidia per 200 µl mixed with 0.26-0.28 gram soil. The higher amount of DNA template (3.5 µl and 10 µl) used for PCR reaction did not give an amplicon. The reaction was repeated using casing *L. fungicola* conidia (10^4-10^7 conidia/g casing) and positive amplification was seen when amount of conidia for isolation was 10^6 conidia/g casing and for PCR reaction 1 µl of template was used.

The **Yeates *et al.*** (1998), DNA extraction method did not give a PCR amplification product when the amount of template for PCR reaction was 3.5 µl and conidia concentration of *L. fungicola* used for extraction was 1.94×10^7 conidia per 200 µl mixed with 0.26-0.28 gram soil. Only amplification product was present when *L. fungicola* was isolated from clean culture. When DNA extraction of different conidia concentrations of *L. fungicola* was repeated and 1 gram of soil was used and for PCR reaction 2 µl template was used the amplification product was present when conidia concentration was 8.95×10^7 conidia/g and 10^7 conidia/g soil. The amplification product was present when DNA template was diluted 1/20 and 1/10 and conidia concentrations were 10^6 conidia/g soil. When this DNA extraction method was repeated for extraction of *L. fungicola* conidia (10^6-10^7 conidia/ml) from casing, soil, casing extract and dust was used for extract the genomic DNA was not present in dust extract, but casing extract showed good high molecular weight quality genomic DNA. The amplification product was present in casing extract and dust extract when PCR reaction used 3 µl, the casing and soil samples did not show any amplification product.

The **Yeates *et al.*** (1998) DNA extraction method was tested on samples collected from mushroom farms for detection of *L. fungicola*. All samples contained some amount of casing. The genomic DNA was extracted and in some samples high molecular weight quality DNA was extracted. The PCR assay was performed with 1 µl and 3 µl of template, but no amplification product was present. The problem was possibly due to inhibition of polymerase activity for amplification of DNA target. The most common inhibitions present in casing and soil are: humic acids, phenolic compounds and heavy metals (Wilson, 1997).

The next tested DNA extraction method was **DNeasy Plant Mini Kit**. The protocol in this method was modified and glass bead were used for cell breakage in place of liquid nitrogen. The genomic DNA was good quality in all casing samples mixed with *L. fungicola* conidia (10^4-10^7 conidia/g casing). The PCR assay was performed using 1/10 diluted DNA template, but no amplification product was present.

This study demonstrated specificity of PCR by reaction performing small experiment with dead (autoclaved) and live (not autoclaved) conidia of *L. fungicola*. The extraction from *L. fungicola* conidia was made by **ZR Fungal/Bacterial DNA**. Genomic DNA was not detected in autoclaved material but in live material high molecular weight quality genomic DNA was present. The PCR assay was performed and both autoclaved conidia (dead) and non-autoclaved (live) showed an amplification product on 102 bp. The PCR reaction is therefore able to detect live and dead material.

In this study a small amplicon 102 bp of rRNA region was amplified which is many times present in genomic DNA (Griffin, 1994). The proposition for detection live material was introduced by Beaulieu *et al.*, (2011). They demonstrated how to quantify active *T.harzianum* in peat and compost. Those same techniques could be used by detection *L. fungicola* from samples from mushroom farms. Beaulieu *et al.*, (2011) isolate DNA obtained from non-active fungal material (conidia, dead mycelia, etc.) and RNA obtained from active material (live mycelium) to demonstrate the ability to quantify active T. harzianum. They used ITS region of *T. harzianum*.

The present study presents four DNA extractions methods. All methods used for extraction DNA from pure culture of *L. fungicola* gave high quality genomic DNA which was suitable for PCR but the best method was the ZR Fungal/Bacterial DNA extraction kit. This study also presents differences between four DNA extraction methods from soil and casing samples. This comparative study shows that the only 3 out of 4 gave high quality genomic DNA which is suitable for PCR, but only 2 out of 4 gave a PCR amplification product when conidia concentrations was 10^6-10^7 conidia/g soil, casing and amount of template used for PCR reaction was 1 or 2 µl.

Polymerase chain reaction (PCR) is a very powerful tool to detect pathogens and sources of pathogens in agriculture. The PCR method is a very useful and fast method for the detection of fungi. This diagnostic method requires unique target-sequences of DNA. Today the full sequences of several fungal genomes are available (http://www.broad.mit.edu/annotation/fungi/fgi/; http://www.genome.gov/). The second important aspect of detection is the isolation of DNA from different ‘’dirty and /or contaminated’’ samples. The PCR method is very sensitive and can detect 1 to 10 molecules, but practically the sensitivity depends on the level of contamination, the DNA extraction method and the sensitivity of the enzymes used (Geisen, 2007). The most difficult samples for detection of microorganisms using the PCR system are samples that contain inhibitors of polymerases, such as soil samples. Picard *et al.* (1992) reported that detection of *Agrobacterium tumefaciens* was routinely obtained when soil was inoculated with 10^3 to 10^7 cells/g soil, but the reliable detection was 10^4 conidia per gram soils. Tsai and Olson (1992) were able to detect 2×10^5 cells of *E. coli* per gram soil rich in humic acids.

The first important step for good PCR detection is to design selective primers which amplify a unique sequence present in the pathogen. The unique target sequences are important for the development of PCR. This part is very difficult if all the genome is not available, but many researchers use ribosomal or ITS sequences (Spiess *et al.*,

2007). For *L. fungicola* available sequenced regions are rDNA (ITS1-5.8S-ITS2 and 28S rRNA and 5S) and RNA (MAT1-2-1), but the rDNA region is a conserved region in fungi, as is the MAT locus. Polymorphism of short sequences of the ITS region is often to low to distinquish between several Ascomycetes or Basidiomycetes. Fungi in which we want to use these sequences must be characterized by diversity between related taxa. If this difference is not present then the correct identification is not possible for example *Fusarium culmorum, F. graminearum* and *F. crookwellense* could not be separated based on the ITS region of the rDNA (Bateman *et al.*, 1996). Pedersen *et al.* (1997) reported that the rDNA sequences within the *Penicillium* subgenus *Penicillium* are too conserved for identification between individual species.

In the experiments reported here many sets of primers were designed and tested, based on the known sequences of *L. fungicola* – RNA sequence of MAT1-2-1 region and rDNA sequence. The PCR test was cross-checked against *A. bisporus* and other *A. bisporus* mycopathogens such as *M. perniciosa, C. mycophilum* and *T. aggressivum,* as well as *A. fumigatus* and an extract from casing and clean casing.

All tested primers designed for MAT1-2-1 region and rDNA sequence failed to amplify only *L. fungicola* DNA. Identification of *L. fungicola* var. *fungicola* was not possible using MAT1-2-1 region. Primers designed in this region also amplified *L. fungicola* var. *aleophilum*. Some sets of primers were able to amplify *L. fungicola* and did not give the same size of amplification product for the host *A. bisporus* DNA.

The rDNA sequence looks to be more conserved in Ascomycete fungi than in basidiomyctes. The MAT1-2-1 region looks very promising for future research and the designed selective primers especially primer set F79-R201 which amplified a 123 bp of *L. fungicola* and *A. fumigatus* DNA, could be used for identification of *L. fungicola* from samples containing other *Agaricus* mycoparasites and *A. bisporus*. This of set of primers requires more work to make them a more specific.

The set of primers presented and designed by Zijlstra *et al.* (2007, 2008 and 2009) amplified *L. fungicola* DNA only giving 102 bp amplicon. The other tested fungi such as *M. perniciosa, C.mycophilum* and *Trichoderma* spp., *A. fumigatus* did not show an amplification product of this same size, but tested fungi showed many non-specific amplicons. This makes the Zijlstra *et al.* (2007, 2008 and 2009) primer set the most selective at present for identification and detection of *L. fungicola* from dirty samples, which indicated the use of these primers and probes for detection of *L. fungicola* from mushroom farm samples.

Chapter 5 Molecular tests (Real Time PCR – TaqMan) for *Lecanicillium fungicola* detection on mushroom farms

Real Time PCR was introduced in 1991 by Russell Higuchi and colleagues (Higuchi *et al.*, 1992 and 1993) who used an ethidium bromide (EtBr) dye to show an increasing amount of DNA during a reaction. When EtBr is bound to double-stranded DNA and excited by UV light it fluoresces therefore an increase in fluorescence in such a PCR indicates positive amplification. After that they presented the idea of Real Time PCR product quantitation or kinetic PCR, by monitoring the increase in fluorescence caused by the intercalation of EtBr during the reaction. Quantitative Real Time PCR methods started to be commercially available in 1996-1998.

Today Real Time PCR is a versatile technique for rapid analysis of multiple samples. The use of fluorescent dyes that intercalate with any and all double-stranded DNA product (e.g. SYBR® Green I, SYBR® Green II, SYBR Gold, SYTO 9) and fluorogenic oligoprobes that detect only specific sequences Real Time PCR avoids detection of non-specific amplification products because of its stringent design to bind to the target gene sequence, (e.g. TaqMan®, BHQplusTM, Molecular beacons, ScorpionTM primers, PlexorTM primers) (Mackay *et al.*, 2007 a).

Real Time PCR is a very sensitive method and has the ability to detect, identify and quantify microbial pathogens. This method eliminates post-PCR processing of PCR products saving time (Cockerill and Smith 2002) and it is more saver, faster and more sensitive method.

Molecular work with fungi started after White *et al.* (1990). They designed universal primers for detection of fungal ribosomal RNA genes (rRNA) for phylogenetic analysis. This region of rDNA started to be used by many researchers for mycological studies, particularly in systematics and detection, and identification of fungi and fungal pathogens in the environment (Borneman and Hartin 2000, Frederick *et al.*, 2000, Ferrer *et al.*, 2001, Bridge 2002). But sometimes the rRNA or ITS sequences show homogeneity between related taxa and it was not possible to differentiate between *Fusarium culmorum*, *F. graminearum* and *F. crookwellense* (Bateman *et al.*, 1996). *Penicillium* genus has the same conserved rDNA sequences

which make differentiation between individual species more difficult or impossible (Pedersen *et al.*, 1997).

The first information about molecular methods for *L. fungicola* detection comes from Romaine *et al.* (2002). In this work they used a PCR technique to detect *L. fungicola* from cultivated mushroom, *Agaricus bisporus*. They used primers which detect only *L. fungicola* var. *aleophilum* and do not detected *L. fungicola* var. *fungicola* or *Agaricus bisporus*.

Largeteau *et al.* (2007) first used Real Time PCR for detection of the residual pathogen of *A. bisporus* infected mushrooms – *L. fungicola* using rDNA sequence (ITS1, 5.8S, ITS2). They used a SYBR green dye for quantification, but the primers detected both the fungal pathogen and its host. The *A. bisporus* DNA was amplified with a far lower efficiency than *L. fungicola* DNA, but without quantification aspect of Real Time PCR was lost.

Zijlstra *et al.* (2007, 2008 and 2009), used Real Time PCR with a TaqMan probe. The "TaqMan" Real Time PCR measured PCR-product accumulation during the exponential phase of the PCR reaction. TaqMan assay used a dual-labeled fluorogenic probe (referred to as "TaqMan probe"). The TaqMan assay is based on the 5'-3' exonuclease activity of Taq DNA polymerase to cleave a dual-labeled probe, which is designed to hybridize to a target sequence during amplification. Disintegration of the probe during PCR releases reporter fluorescence and the intensity of the fluorescence signal measured during the exponential phase of the PCR reaction is proportional to the amount of input target DNA according to Gangisetty and Reffy (2009). Zijlstra *et al.* (2007, 2008 and 2009) designed primers and specific probes using rRNA region (18S ribosomal RNA gene). A TaqMan test detected both varieties of *L. fungicola*: *L. fungicola* var. *fungicola* and *L. fungicola* var. *aleophilum*. This test is able to detect *L. fungicola* conidia when conidia concentration was 10 conidia per 1 gram casing; quantification was possible when conidia concentration was 10^4 conidia per 1 gram. The TaqMan test enabled the reliable quantification of 10^4 conidia/g casing or higher. DNA isolation from soil is very difficult because soil contains a lot of inhibitors and other contaminants, such as humic acids and heavy metal ions. The casing soil contains a lot of humic materials which have similar size and charge characteristics as DNA. Humic acids limit the sensitivity and inhibit PCR reactions (Tsai and Olson, 1992 a, b; Yeasts *et al.*, 1998, Watson and Blackwell, 2000).

The aim of this study was to test a TaqMan Real Time PCR assay for the quantification and detection of *L. fungicola* from mushroom farm samples. One

objective of this study was to learn a novel molecular method – Real Time PCR for detecting fungus from dirty samples and to find a good commercial kit for DNA extraction from dirty samples and also to the check sensitivity of different reagents and Real Time machines LightCycler 480 – Roche. The primers and probe (TaqMan) used were designed by Zijlstra *et al.* (2007, 2008 and 2009), and they were tested with clean *L. fungicola* DNA and inoculated by *L. fungicola* conidia by casing soil extract for specificity.

5.1 Results

5.1.1 Reaction condition for Real Time PCR using a LightCycle 480 Roche machine

Following a Real Time PCR training course in the Netherlands, the optimised method was identified as that of Zijlstra *et al.* (2007, 2008 and 2009). The Dutch Real Time PCR reaction was tested to see if it worked well with a LightCycle 480 Roche machine in NUI, Maynooth. The Real Time PCR reaction was carried out to search for the amplification conditions that gave the lowest crossing-point (CP) value and the highest amplification curve plateau for a given amount of DNA template. The Dutch Real Time PCR reaction protocols worked well with LightCycle 480 Roche machine and with Polymerase Lightcycler 480 Probes Master (Roche).

The standard curve was prepared using wild isolate *L. fungicola* (L.15), identified as a *L. fungicola* var. *fungicola*. DNA was isolated using the ZymoResearch Fungal/Bacterial DNA kit. The quality was checked in 1 % agarose gel with the ethidium bromide prepared in TAE buffer. The quantity was checked by nanodrop spectrophotometer (OD_{260}/OD_{280} ratio for an indication of nucleic acid purity). *L. fungicola* genomic DNA showed high molecular weight quality genomic DNA and quantity was 94.5 ng/µl.

The Real Time PCR amplification curves and the corresponding fluorescent quantitative Real Time PCR standard curve were generated by employing successively diluted amounts of *L. fungicola* DNA for Real Time PCR reaction under the optimized conditions. Water control did not give a signal, therefore the results were reliable. Samples with *L. fungicola* gave a positive signal; the primers and probe were specific for *L. fungicola*. A standard curve was generated using a serial dilution of known

amount of DNA of *L. fungicola*. The dilutions were 10-fold dilutions. The crossing point (CP) value between serial dilutions was around 3 cycles (**Table** 5-1 and **Figure** 5-1). The total voulum of Real Time PCR reaction was 25 µl.

Table 5-1: CP value of different concentrations of *L. fungicola* DNA – standard curve.

No.	Amount *of L. fungicola* DNA (pg/µl)	*L. fungicola* DNA per reaction (pg)	Mean CP (cycles)	Slope
1	1	2	30.03	n/a
2	0.1	0.2	33.84	-3.36
3	0.001	0.02	37.37	-3.24
4	0.0001	0.002	n/s	n/a
5	0 (Negative control)	0	n/s	n/a

n/a – not available, n/s – no signal

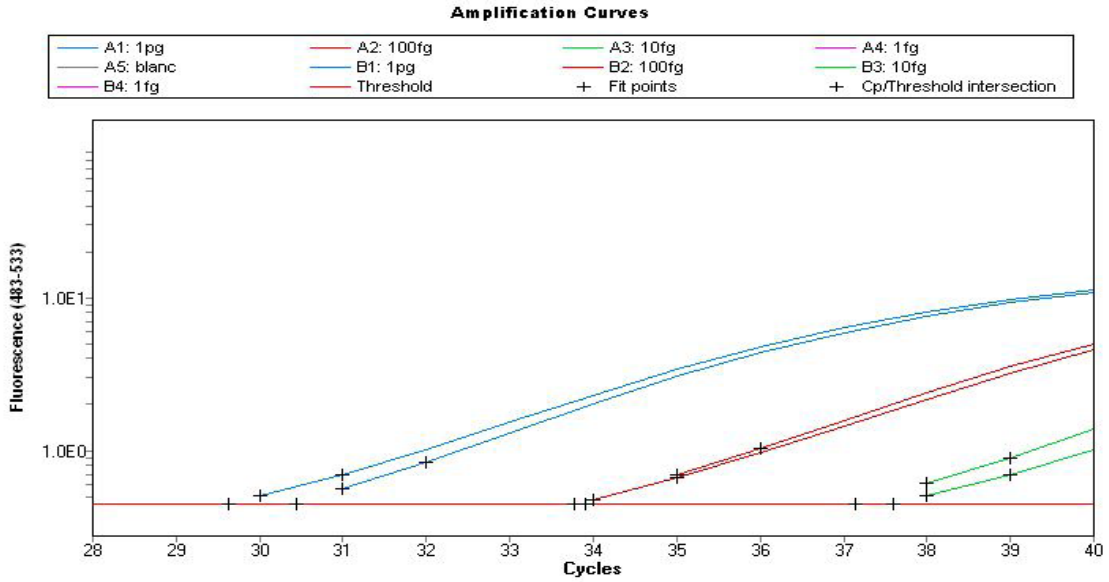
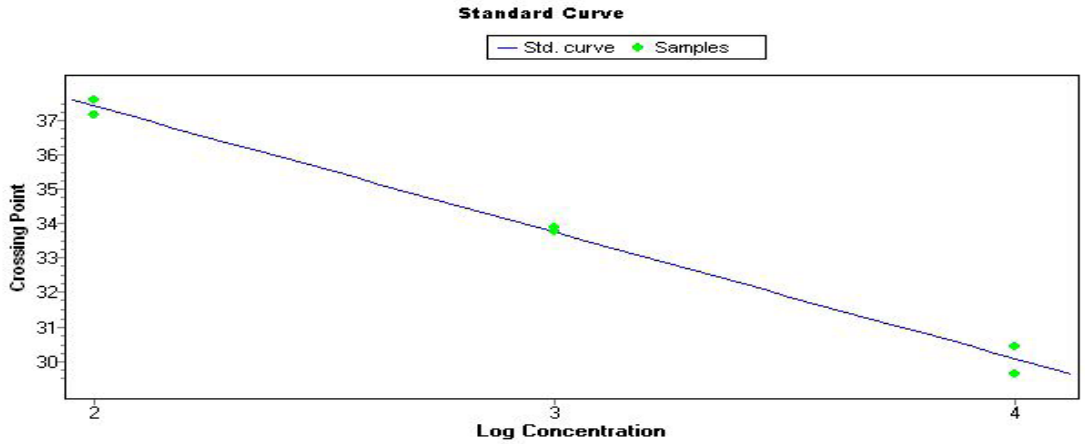


Figure 5-1: Amplification curves of *L. fungicola* DNA. The DNA template of *L. fungicola* per reaction was 2 µl.

The minimum number of samples to make a standard curve is three. All samples were repeated twice and all repeats gave very similar CP value. The slope was -3.67. The slope should be between -3.58 and -3.10 for accurate and reproducible results. The slope of standard curve described the kinetics of the PCR amplification and indicates how quickly the amount of target nucleic acid can be expected to increase with the amplification cycles. The slope of the standard curve is also referred to as the efficiency of the amplification reaction. The PCR efficiency (E) can easily be calculated using the formula were ($E = 10^{-1/slope}$), $E = 10^{(-1/-3.669)}$; E =1.87 copies per cycle. The reaction had efficiency of 1.87 copies per cycle. The perfect amplification reaction would produce a

standard curve with an efficiency of "2", because the amount of target nucleic acid would double with each amplification cycle. The Y-intercept was 44.75, values around 40 indicate good sensitivity of the assay. The Y-intercept value corresponds to the CP value for a single copy of the target molecule. The error value (mean squared error of the single data points fit to the regression line) was 0.038. Compared to maximum possible error equal 0.2 – above which results are regarded as unreliable according to the LightCycler® 480 instrument Operator's Manual – Roche as well as Real Time PCR: from Theory to Practice – Invitrogen. The correlation coefficient (R^2) was 1. The standard curve and the established Real Time PCR are excellent at performance (**Figure** 5-2).



Error: 0.038 Efficiency: 1.87 Slope: -3.67 Y-intercept: 44.70 R^2: 1

Figure 5-2: Establishment of the fluorescent quantitative Real Time PCR standard curve**.** Standard curve of the *L. fungicola* fluorescent quantitative Real Time PCR. Four dilutions of standard DNA ranging from 1 pg to 10 fg DNA/µl were used, as indicated on the x-axis, whereas the corresponding cycle threshold (CP) values are presented on the y-axis. Each dot represents the result of duplicate amplification of each dilution. The correlation coefficient, slope, Y-intercept and error of the regression curve were calculated of the reaction.

It was decided to repeat the standard curve to include a bigger number of samples. The standard curve results were similar to the previous one. A standard curve was performed using a serial dilution of known amounts of DNA of *L. fungicola*. The dilutions were 10-fold dilutions. The crossing point (CP) value between serial dilutions was around 3 cycles. Water control was without signal indicating the results are reliable. Samples with *L. fungicola* DNA gave a positive signal. The clean DNA of *L. fungicola* was prepared by serial diluting the slope value between serial dilutions and was around -2.74 to -3.81 (**Table** 5-2 and **Figure** 5-3).

Table 5-2: CP value of different concentrations of *L. fungicola* (L.15) DNA – standard curve.

No.	Amount *of L. fungicola* DNA (pg/µl)	*L. fungicola* DNA per reaction (pg)	Mean CP (cycles)	Slope
1	10,000	20,000	19.35	n/a
2	1,000	2,000	23.16	-3.81
3	100	200	26.70	-3.54
4	10	20	30.20	-3.50
5	1	2	33.73	-3.53
6	0.1	0.2	37.36	-3.63
7	0.01	0.02	41.09	-3.73
8	0.001	0.002	43.83	-2.74
9	0 (Negative control)	0	n/s	n/a

n/a – not available, n/s – no signal

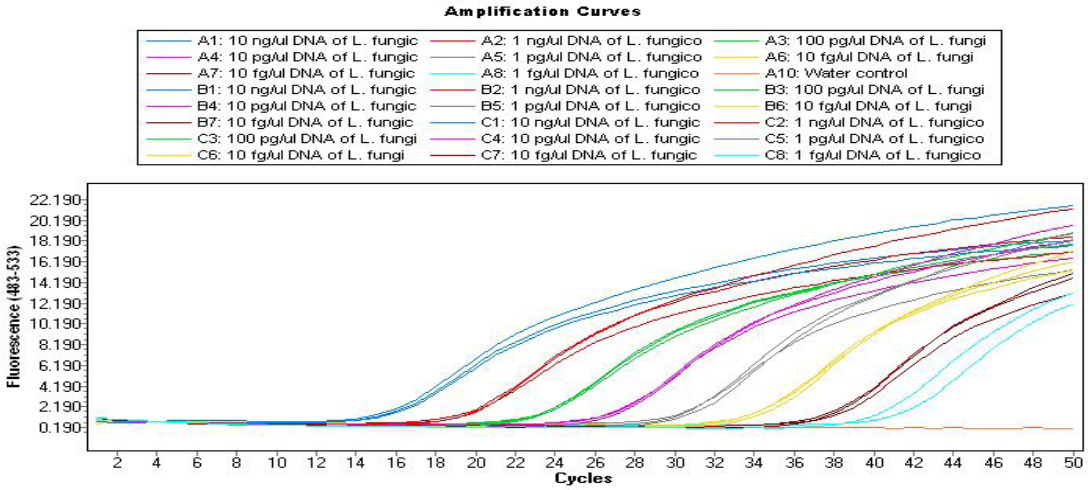
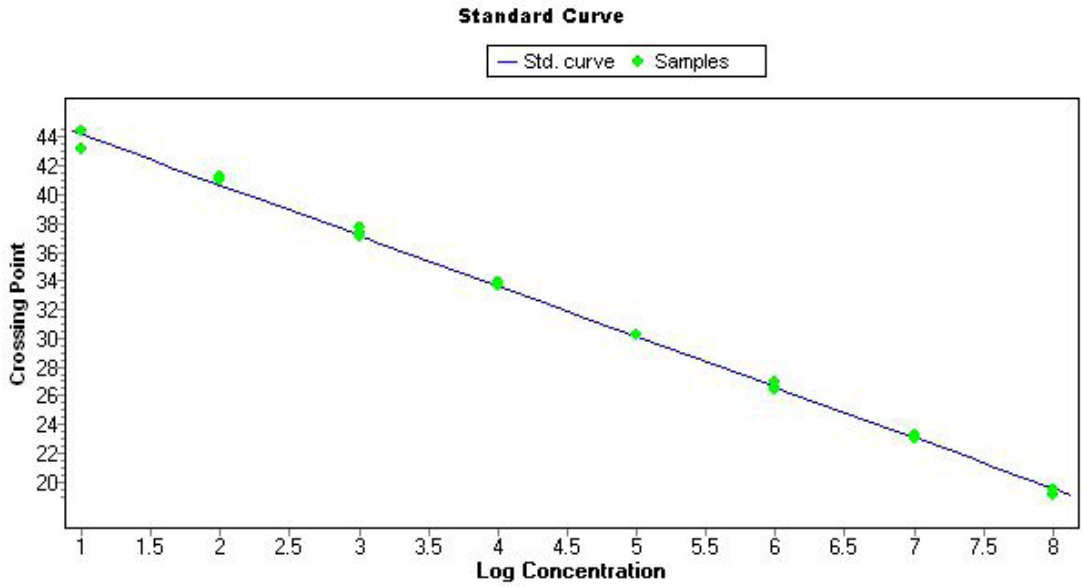


Figure 5-3: Amplification curves of *L. fungicola* DNA. The DNA template of *L. fungicola* per reaction was 2 µl.

The standard curve was calculated by Real Time PCR LightCycler 480 software. The slope was -3.519. The reaction had efficiency of 1.924 copies per cycle. The Y-intercept was high at 47.72 as a value of around 40 indicates good sensitivity of the assay. The correlation coefficient (R^2) was 0.999, ideally $R^2 = 1$, although 0.999 is generally the maximum value. (LightCycler® 480 instrument Operator's Manual – Roche as well as Real Time PCR: from Theory to Practice – Invitrogen). The standard curve and the established Real Time PCR are good at performance. The reaction conditions are appropriate and good for this LightCycle 480 Roche machine.



Slope -3.519, Y-intercept 47.72, Efficiency: 1.924, Correlation coefficient (R^2) 0.999

Figure 5-4: Establishment of the fluorescent quantitative Real Time PCR standard curve**.** Standard curve of the *L. fungicola* fluorescent quantitative Real Time PCR. Three dilutions of standard DNA ranging from 10 ng to 1 fg DNA/µl were used, as indicated on the x-axis, whereas the corresponding cycle threshold (CP) values are presented on the y-axis. Each dot represents the result of single amplification of each dilution. The correlation coefficient, slope, Y-intercept and error of the regression curve were generated of the reaction.

5.1.2 DNA extraction from casing soil for use with Real Time PCR

DNA was extracted from casing soil extract and soil extract samples collected from NUIM grounds using Wizard Magnetic DNA Purification System for Food recommended by Zijlstra *et al.* (2007, 2008 and 2009). *Lecanicillium fungicola* DNA samples were also included to check primers and probe work well and water samples (negative samples) did not give a signal what indicated the reagents were free from *L. fungicola* DNA. The results showed that only *L. fungicola DNA* gave a signal and that no signal was recorded for the casing extract and soil extract samples what was expected (**Figure** 5-5).

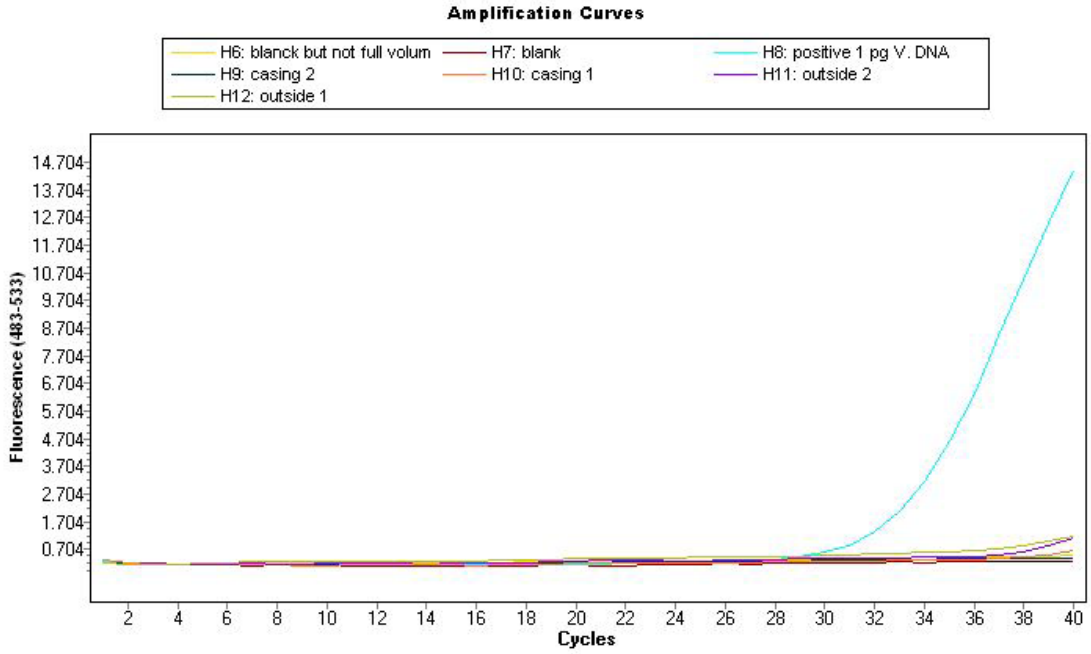


Figure 5-5: Results of amplification plots showing the testing of casing extract and soil extract samples using the TaqMan PCR. The DNA template of *L. fungicola* (L.15) per reaction was 2 µl.

5.1.2.1 Real Time PCR on casing extract with *L. fungicola* conidia

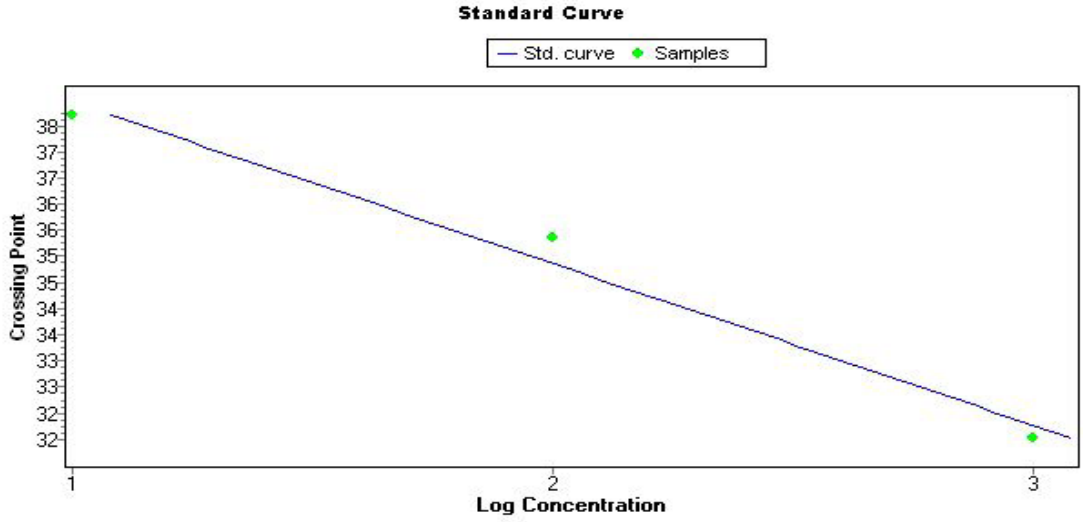
DNA was extracted from casing soil extract (Section 2.5.19) and casing soil extract mixed with different conidia concentrations of *L. fungicola* (10-10^6 conidia/ ml casing extract) using a Wizard Magnetic DNA Purification System for Food. The DNA extraction was performed from 1 ml material which is a limitation of the DNA extraction kit.

The standard curve gave a slope -3.09. The reaction had an efficiency of 2.1 copies per cycle. The Y-intercept was 41.04 values. The error value was 0.204. Compared to maximum possible error equal to 0.2 (above which results are regarded as unreliable) the obtained results are satisfactory (**Table** 5-3 and **Figure** 5-6).

Table 5-3: CP value of different concentrations of *L. fungicola* (L.15) DNA – standard curve.

No.	Amount *of L. fungicola* DNA (pg/µl)	*L. fungicola* DNA per reaction (pg)	Crossing point (CP) (cycles)	Slope
1	1	2	31.52	n/a
2	0.1	0.2	35.36	-3.84
3	0.001	0.02	37.70	-2.34
4	0 (Negative control)	0	n/s	n/a

n/a – not available; n/s – no signal



Error: 0.204 Efficiency: 2.107 Slope: -3.090 Y-Intercept: 41.04

Figure 5-6: Establishment of the fluorescent quantitative Real Time PCR standard curve**.** Standard curve of the *L. fungicola* fluorescent quantitative Real Time PCR. One dilutions of standard DNA ranging from 1 pg to 10 fg DNA/µl were used, as indicated on the x-axis, whereas the corresponding cycle threshold (CP) values are presented on the y-axis. Each dot represents the result of single amplification of each dilution. The correlation coefficient, slope, Y-intercept and error of the regression curve were generated of the reaction.

Specificity of the TaqMan PCR (FAM signal) showed positive CP values on samples containing 10^1 to 10^6 conidia/ml extract casing. The crossing point (CP value) had different values between samples. When conidia concentration decreased the CP value started to be reliable and quantitative aspect of Real Time PCR was established. The differences between different amounts of conidia gave different CP value. The correlation coefficient (R^2) was 0.989 (**Table** 5-4 and **Figure** 5-7).

Table 5-4: Detection of *L. fungicola* in casing extract. The DNA template was 2 µl.

No.	Conidia concentration per 1 ml casing extract	Crossing point (CP) (cycles)
1	0 (casing extract)	n/s
2	1	n/s
3	10	39.74
4	10^2	36.62
5	10^3	34.28
6	10^4	32.70
7	10^5	28.84
8	10^6	26.02

n/s – no signal

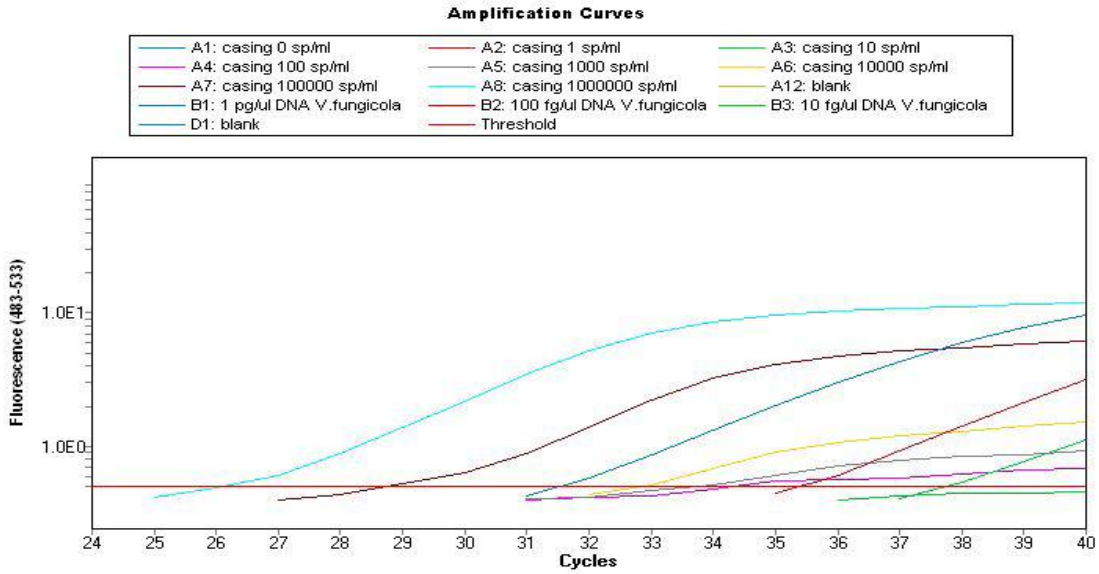


Figure 5-7: Results of amplification plots showing testing of genomic DNA of *Lecanicillium fungicola* using TaqMan PCR. Conidia concentration was 10 to 10^6 conidia per ml casing extract.

5.1.2.2 Comparison of Real Time PCR on *L. fungicola* detection in samples of casing extract and water

DNA was extracted from casing soil extract (Section 2.5.19) and casing soil extracts mixed, or clean water with different conidia concentrations of *L. fungicola* (10-10^6 conidia/ ml casing extract or water) using a Wizard Magnetic DNA Purification System for Food. The DNA extraction was performed from 1 ml material.

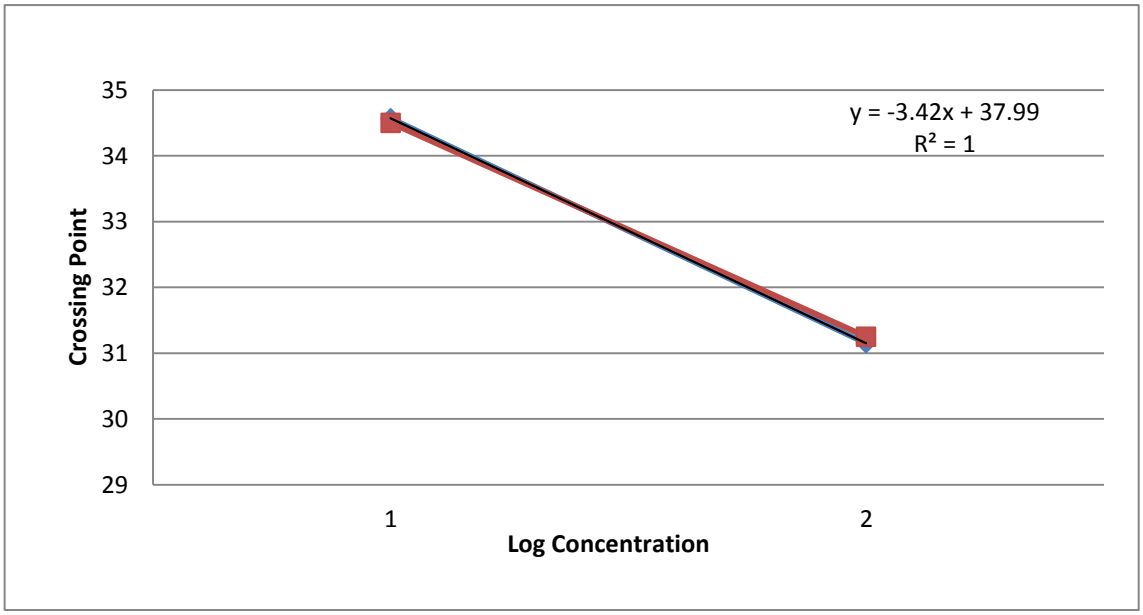
The *L. fungicola* was included to calculate a standard curve and to check the reaction was performing well. The standard curve gave a slope - 3.42. The reaction had efficiency of 1.96 copies per cycle. The Y-intercept was 37.99. The correlation coefficient (R^2) was 1 and this is generally the maximum value. The lower DNA (10 fg/µl) concentration may have been due to freeze/thawing during defrosting leading to

disintegration which had an impact and the LightCycle 480 Roche software was not able to calculate a standard curve. The standard curve was calculated manually (**Table** 5-5 and **Figure** 5-8).

Table 5-5: Detection of *L. fungicola* in casing extract. The DNA template was 2 µl.

No.	Amount *of L. fungicola* DNA (pg/µl)	*L. fungicola* DNA per reaction (pg)	Crossing point (CP) (cycles)	Slope
1	1	2	31.20	n/a
2	0.1	0.2	34.53	-3.33
3	0.001	0.02	n/a	n/a
4	0 (Negative control)	0	n/s	n/a

n/a – not available; n/s – no signal



Efficiency: 1.96, Slope -3.42, Y-intercept 37.99, Correlation coefficient (R^2) =1

Figure 5-8: Establishment of the fluorescent quantitative Real Time PCR control point**.** Control point of the *L. fungicola* fluorescent quantitative Real Time PCR. One dilutions of standard DNA ranging from 1 pg to 100 fg DNA/µl were used, as indicated on the x-axis, whereas the corresponding cycle threshold (CP) values are presented on the y-axis. Each dot represents the result of single amplification of each dilution. The correlation coefficient, slope, Y-intercept and error of the regression curve were generated of the reaction.

DNA was extracted from freshly prepared samples of *L. fungicola* (0-10^6 conidia/ml casing extract or water) and casing extract and water using a Wizard Magnetic DNA Purification System for Food. One sample with casing extract gave a positive result but the other one gave a negative result. All samples containing conidia of *L. fungicola* gave a positive signal and there was a correlation between CP value and conidia concentration. Conidia detected in casing extract had a similar CP value as conidia detected in water. The detection level was 10 conidia per ml of casing extract or water but there was no correlation between CP value and conidia concentrations. The quantitative aspect of Real Time PCR was lost. The Results are not as expected with no clear quantitative effect. Real Time PCR is a highly technical and skilled operation and there is a possibility that human error occurred here. The results are inconclusive. The positive control with clean *L. fungicola* DNA gave a signal (**Table** 5-6 and **Figure** 5-9).

Table 5-6: Detection of *L. fungicola* in casing extract or water. Results of CP values of different conidia concentrations of *L. fungicola* mixed with casing extract or water. The DNA template was 2 µl.

No.	Spore concentration per ml	Spore concentration per reaction	CP Casing extract (cycles)	CP Water (cycles)
1	0	0	36.82	37.13
2	0	0	n/s	n/s
3	10^1	2×10^{-2}	37.09	37.25
4	10^2	2×10^{-1}	35.42	37.67
5	10^3	2	32.95	36.3
6	10^4	2×10^1	29.14	29.45
7	10^5	2×10^2	25.54	25.9
8	10^6	2×10^3	22.35	22.78

n/s – not signal

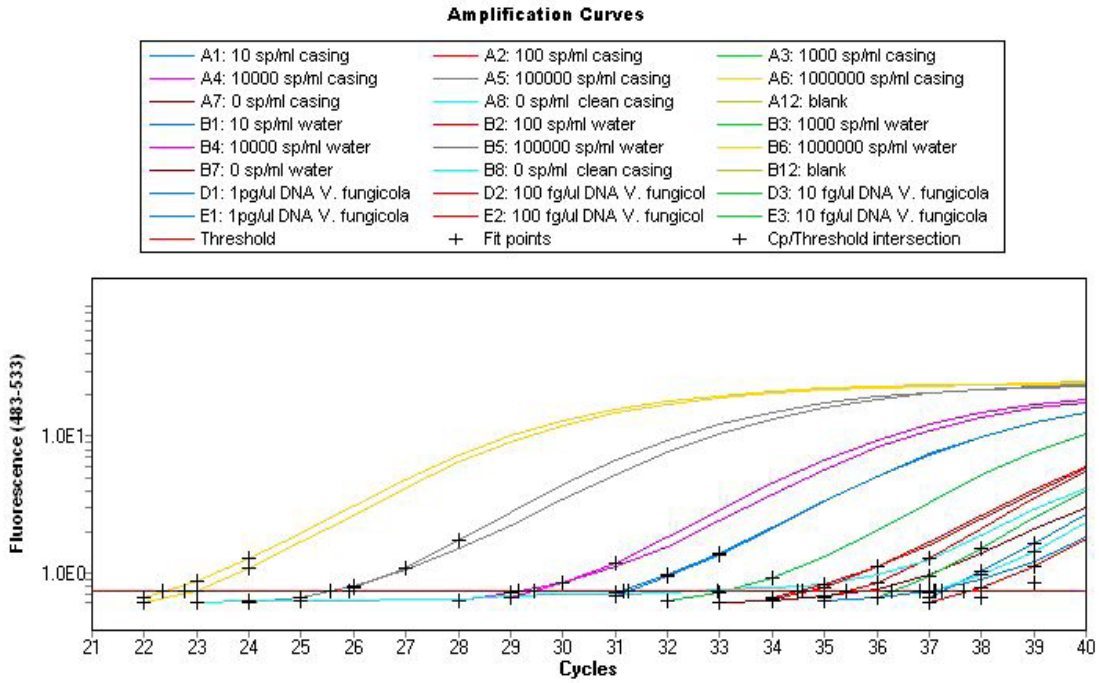


Figure 5-9: Results of amplification plots showing testing of genomic DNA of *Lecanicillium fungicola* using TaqMan PCR. Conidia concentration was 10 to 10^6 conidia per ml casing extract and water.

Amplification products were checked on 2 % agarose gel. The casing soil extract and water without *L. fungicola* conidia did not show a signal. Samples containing *L. fungicola* conidia or DNA gave a band of size 102 bp. All samples from casing extract and *L. fungicola* conidia gave many non-specific amplification products. The water samples with *L. fungicola* gave only one PCR product of 102 bp. The amount of 102 bp product does not correlate with the concentration of conidia or the amount of product after 40 cycles. The difference between amplified samples was too small to show on a gel. The primer set was not specific and also amplified non-specific products. The TaqMan probe was designed as a specific probe for *L. fungicola* as a target and gave a positive signal (**Figure** 5-10).

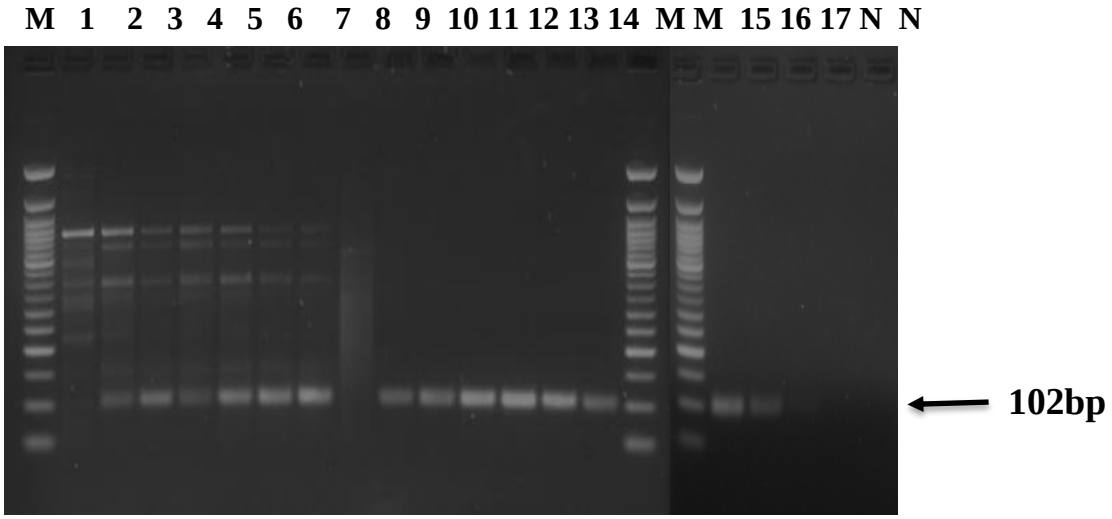


Figure 5-10: Real Time PCR identification of *L. fungicola*. Lanes: M – Marker 50 bp, 1 casing extract 1, 2 – 10^1 conidia/ml; 3 – casing extract 10^2 conidia/ml, 4 – casing extract 10^3 conidia/ml, 5 – 10^4 conidia/ml, 6 – casing extract 10^5 conidia/ml, 7 – casing extract 10^6 conidia/ml, 8 – casing extract 2, 9 – water 10^1 conidia/ml, 10 – water 10^2 conidia/ml, 11 – water 10^3 conidia/ml, 12 – water 10^4 conidia/ml, 13 – water 10^5 conidia/ml, 14 – water 10^6, 15 – 1 pg/µl *L. fungicola* DNA, 16 – 100 fg/µl *L. fungicola* DNA, 17 – 10 fg/µl *L. fungicola* DNA, N – water control. Amplified product (10 µl = 2µl loading buffer and 8µl Real Time PCR product) were subjected to electrophoresis – 2 % agarose gel with the ethidium bromide prepared in 1x TAE buffer.

5.1.2.3 Summary

The experiments in this part of the thesis were performed with a LightCycler 480 Roche as a Real Time Machine (SFI Equipment Grant (SFI/07/RFP/GEN/F571/ECO7) using the high quality hot start polymerase Lightcycler 480 Probes Master polymerase. DNA was isolated using a Wizard Magnetic DNA Purification System for Food (Promega). The casing soil comes from Irish mushroom farms and a *L. fungicola* (L.22) wild isolate was used.

These experiments described the reaction conditions for Real Time PCR reaction on Lightcycler 480 Roche which worked well. In this test, the primers and probe amplified a 102 bp amplicon. The reaction optimisation for LightCycler 480 Roche gave an optimal standard curve. The concentration and composition of primers and probe used in the reaction gave optimal results which was observed by correlation coefficient (0.99), slope (-3.67), Y-intercept 44.70 and error 0.038.

Designed primers used for *L. fungicola* detection gave no specific amplification products in casing samples. The primers and probe were specific only for *L. fungicola* DNA. Casing extract and soil samples did not show a FAM signal. However casing samples were contaminated by *L. fungicola* during reaction preparation. This highlights the need to be extremely careful when working with Real Time PCR. The conidia isolation from casing extract gave better results than conidia isolation from water

contrary to expected. On agarose gel samples from casing extract showed a few non-specific amplicons. The designed primers were non-specific. They amplify an rRNA region which is much conserved in many other fungi present in casing extract. The detection level of *L. fungicola* conidia mixed with casing extract or water was 10 conidia/ml. However two out of three experiments demonstrated good correlation between conidia concentration and CP values which means that Real Time PCR may be a suitable method to use with mushroom farm samples. The reliable detection level and successfully quantified by Real Time PCR was 10^4 conidia/ml casing extract.

5.1.3 Comparison of two DNA extraction kits for use with casing extract samples

5.1.3.1 *Lecanicillium fungicola* DNA isolated from casing extract using a QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA isolation kits (Fujifilm)

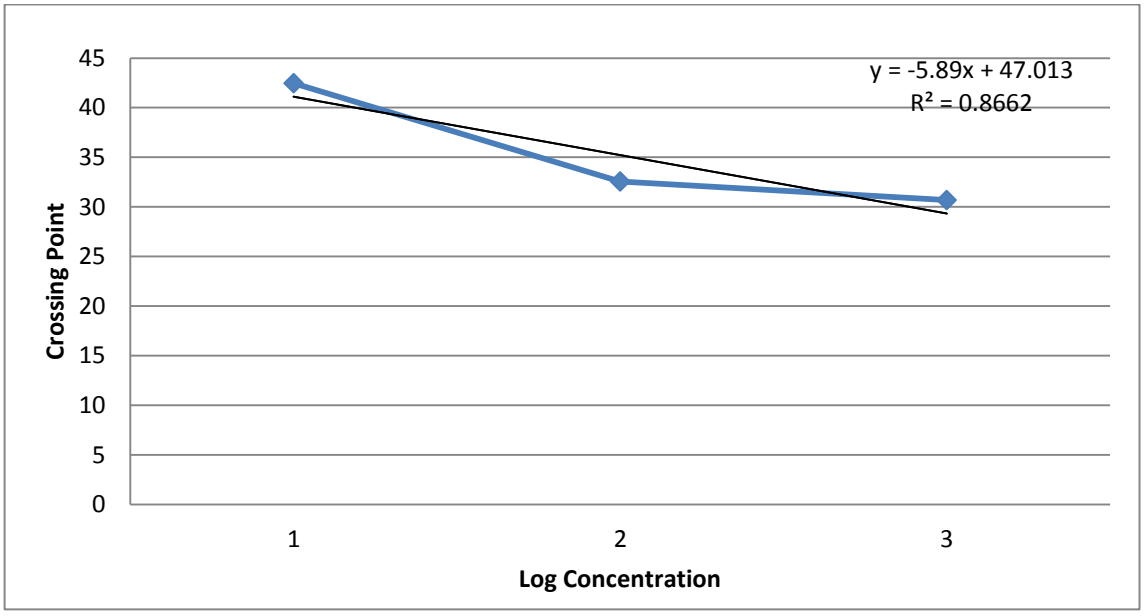
DNA was extracted from casing soil extract (Section 2.5.19) and casing soil extracts or clean water mixed with different conidia concentrations of *L. fungicola* (5-10^5 conidia/ ml casing extract or water) using a QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA isolation kits (Fujifilm). The DNA extraction was performed from 1 ml material which is a limitation of the DNA extraction kit.

The standard curve gave a slope -5.89. The reaction had efficiency of 1.48 copies per cycle. The Y-intercept was 47.013. The correlation coefficient (R^2) was 0.8662. This poor result of Real Time PCR standard curve was preperd from old dilution of pure *L. fungicola* DNA which is disintegrated during storage but Real Time PCR reagents work well (**Table** 5-7 and **Figure** 5-11).

Table 5-7: Detection of *L. fungicola* in casing extract. The DNA template of *L. fungicola* (L.15) was 2 µl.

No.	Amount *of L. fungicola* DNA (pg/µl)	*L. fungicola* DNA per reaction (pg)	Crossing point (CP) (cycles)	Slope
1	10	20	30.68	n/a
2	1	2	32.56	-1.88
3	0.1	0.2	42.46	-9.90
4	0 (Negative control)	0	n/s	n/a
5	0 (Negative control)	0	n/s	n/a

n/a – not available; n/s – no signal



Slope -5.89, Y-intercept 47.013, Efficiency: 1.48, Correlation coefficient (R^2) 0.8662

Figure 5-11: Establishment of the fluorescent quantitative Real Time PCR control point**.** Control point of the *L. fungicola* fluorescent quantitative Real Time PCR. One dilutions of standard DNA ranging from 10 pg to 100 fg DNA/µl were used, as indicated on the x-axis, whereas the corresponding cycle threshold (CP) values are presented on the y-axis. Each dot represents the result of single amplification of each dilution. The correlation coefficient, slope, Y-intercept and error of the regression curve were generated of the reaction.

Specificity of the TaqMan PCR (FAM signal) showed results on all samples containing *L. fungicola* conidia. Samples without *L. fungicola* conidia did not show a signal. The different conidia concentrations of *L. fungicola* DNA gave a positive CP value (signal) corresponding to the DNA amount. The crossing point – CP value had a different value between different conidia concentrations. The quantitative aspect of Real Time PCR was present, because different amounts of conidia gave different CP values. The detection limit was found to be 5 conidia/ml casing extract of *L. fungicola*. However, with the lower conidia concentrations quantitation was not always reproducible compared to other conidia concentrations. Therefore the dynamic range of the method was between 5 and 10^5 conidia/ ml of *L. fungicola*, which is relatively broad. The reliable detection level successfully quantified by Real Time PCR was 10^2 conidia/ml casing extract (**Table** 5-8 and **Figure** 5-12).

Table 5-8: CP value of different concentrations of *L. fungicola* DNA isolated by QuickGene (Fujifilm).

No.	Conidia concentration per 1 ml casing extract	Conidia concentration per reaction (2 µl)	QuickGene Mini 80 (Fujifilm) CP (cycles)
1	casing extract	n/s	n/s
2	casing extract	n/s	n/s
3	5	10^{-2}	40.66
4	10^{1}	2×10^{-2}	39.96
5	$5x10^{1}$	10^{-1}	38.94
6	10^{2}	2×10^{-2}	37.67
7	$5x10^{2}$	1	34.65
8	10^{3}	2	34.31
9	$5x10^{3}$	10^{1}	33.9
10	10^{4}	2 x10	29.8
11	$5x10^{4}$	10^{2}	31.16
12	10^{5}	2×10^{2}	26.36

n/s – no signal

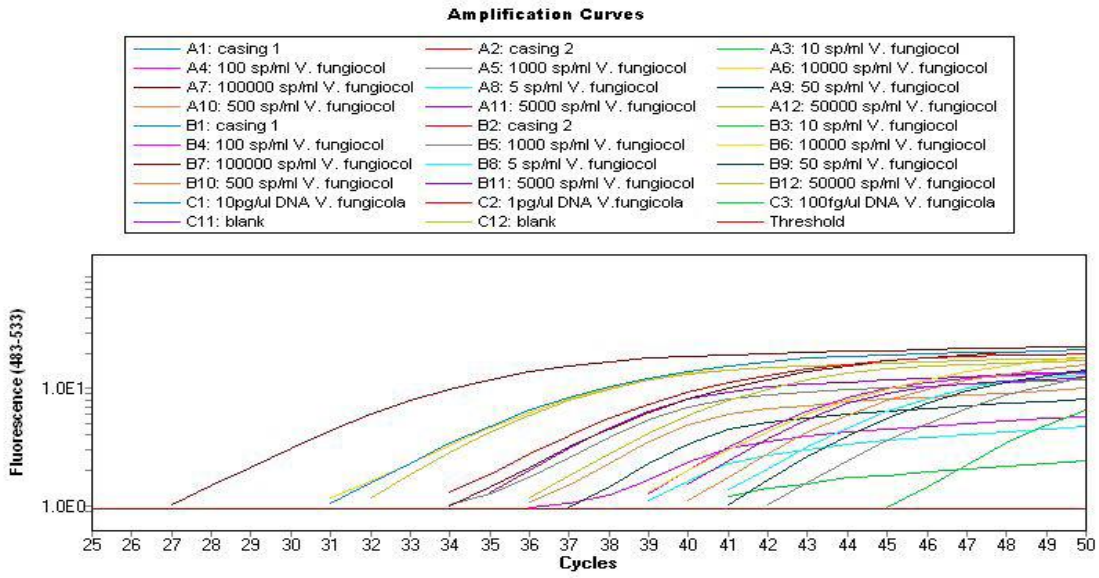


Figure 5-12: Results of amplification plots showing testing of genomic DNA of *Lecanicillium fungicola* using TaqMan PCR. Conidia concentration was tested from 10 to 10^{5} conidia per ml casing extract using a QuickGene (Fujifilm).

5.1.3.2 *Lecanicillium fungicola* DNA extraction from casing extract samples using two commercial DNA extraction kits.

Two DNA extraction kits were compared for use with *L. fungicola* DNA extracted from casing extract. For this experiment QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA isolation kits (Fujifilm) were used and gave good results in pervious experiment. Wizard Magnetic DNA Purification System for Food (Promega) which was recommended by Dutch researchers (Zijlstra *et al.* 2007, 2008 and 2009) was also used.

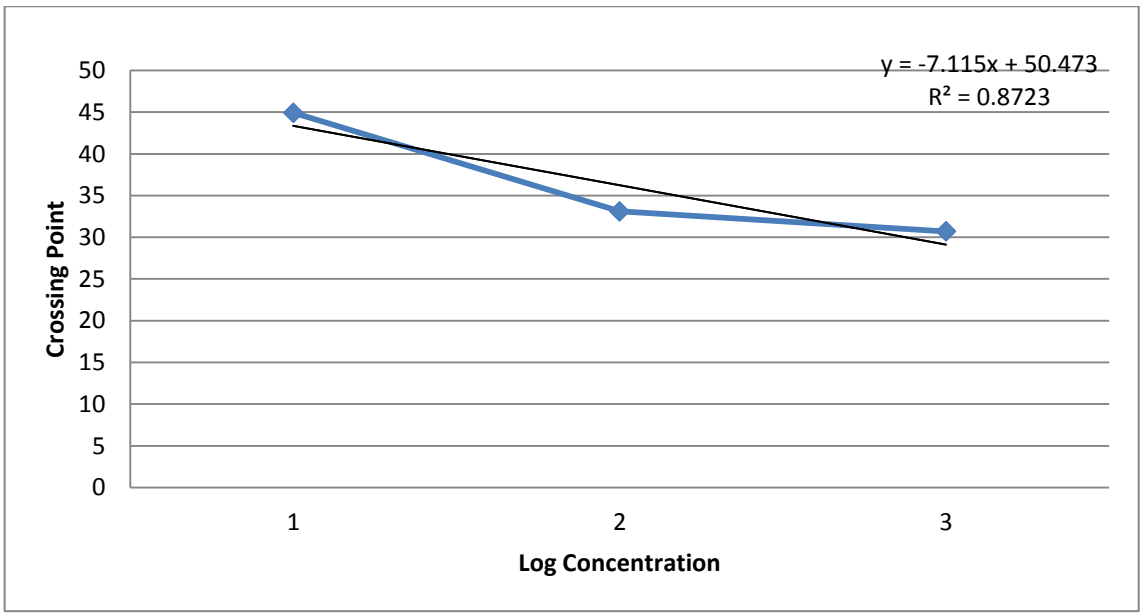
DNA was extracted from casing soil extract (Section 2.5.19) and casing soil extracts mixed with different conidia concentrations of *L. fungicola* (5-10^5 conidia/ ml casing extract or water) and extracted. The DNA extraction was performed from 1 ml material.

The standard curve gave a slope of -7.12. The reaction had efficiency 2.6. The Y-intercept was 50.473. The correlation coefficient (R^2) was 0.8723. That poor result of Real Time PCR has due to old dilution of pure *L. fungicola* DNA which may have disintegrated during storage but Real Time PCR reagents worked well (**Table** 5-9 and **Figure** 5-13).

Table 5-9: Detection of *L. fungicola* in casing extract. The DNA template of *L. fungicola* (L.15) was 2 µl.

No.	Amount *of L. fungicola* DNA (pg/µl)	*L. fungicola* DNA per reaction (pg)	Crossing point (CP) (cycles)	Slope
1	10	20	30.70	n/a
2	1	2	33.10	-2.4
3	0.1	0.2	44.93	-11.83
4	0 (Negative control)	0	n/s	n/a
5	0 (Negative control)	0	n/s	n/a

n/a – not available; n/s – no signal



Efficiency: 2.6, Slope -7.115, Y-intercept 50.473, Correlation coefficient (R^2) 0.8723

Figure 5-13: Establishment of the fluorescent quantitative Real Time PCR control point**.** Control point of the *L. fungicola* fluorescent quantitative Real Time PCR. One dilutions of standard DNA ranging from 10 pg to 100 fg DNA/µl were used, as indicated on the x-axis, whereas the corresponding cycle threshold (CP) values are presented on the y-axis. Each dot represents the result of single amplification of each dilution. The correlation coefficient, slope, Y-intercept and error of the regression curve were generated of the reaction.

Specificity of the TaqMan PCR (FAM signal) showed results for all samples containing *L. fungicola* conidia. Samples without *L. fungicola* conidia, casing extract and negative control did not show a CP value. The different conidia concentrations of *L. fungicola* DNA gave a positive CP value corresponding to the conidia amount although it is not as pronounced as expected. CP value of *L. fungicola* conidia isolated by QuickGene gave better sensitivity than Wizard Magnetic DNA Purification System for Food. The QuickGene DNA isolation kit gave a CP value for all conidia concentrations of lower than 40 cycles. When conidia concentration was 5, 10, 50 and 1,000 per ml – Wizard Magnetic DNA Purification System for Food gave a CP value higher than 40 cycles, and CP values for other conidia concentrations were similar to excepted at the highest concentrations. As a result, the quantitative aspect of Real Time PCR was lost when DNA was isolated by QuickGene DNA isolation kit, because different amounts of conidia gave different CP values. The differences between conidia amounts (10^1, 10^2, 10^3, 10^4, 10^5 conidia/ml casing extract) were 2.15 to 3.83 cycles, but detection limit was found to be 5 conidia/ml of *L. fungicola*. The conidia concentration 5, 5×10^2, 5×10^3, 5×10^4 per ml casing extract isolated by QuickGene DNA isolation kit showed a

differences between cycles of 1.28 to 2.77 and quantitative aspect of Real Time PCR was lost.

However, at the lower conidia concentrations quantitation was not always reproducible compared to the higher conidia concentrations. Therefore the dynamic range of the method was between 5 and 10^5 conidia/ ml of *L. fungicola*, which is relatively broad. The reliable detection level and successful quantification by Real Time PCR was 10^1 conidia/ml casing extract when DNA was isolated by QuickGene DNA isolation kit and 10^4 conidia/ml casing extract when DNA was isolated by Wizard Magnetic DNA Purification System for Food (**Table** 5-10 and **Figure** 5-14).

Table 5-10: CP value of different concentrations of *L. fungicola* DNA isolated by QuickGene (Fujifilm) and Wizard Magnetic DNA Purification System for Food (Promega).

No.	Conidia concentration per 1 ml casing extract	Conidia concentration per reaction (2µl)	QuickGene CP Value (cycles)	Wizard Magnetic DNA Purification System for Food (Promega) CP value (cycles)
1	0 (Casing extract)	0	n/s	n/s
2	0 (Casing extract)	0	n/s	n/s
3	5	10^{-2}	38.52	40.09
4	10^1	2×10^{-2}	39.58	n/s
5	$5x10^1$	10^{-1}	36.93	40.8
6	10^2	2×10^{-2}	35.75	38.31
7	$5x10^2$	1	35.54	39.61
8	10^3	2	33.6	41.79
9	$5x10^3$	10^1	34.26	39.01
10	10^4	2×10	30.27	38.21
11	$5x10^4$	10^2	31.49	35.49
12	10^5	2×10^2	26.76	33.85

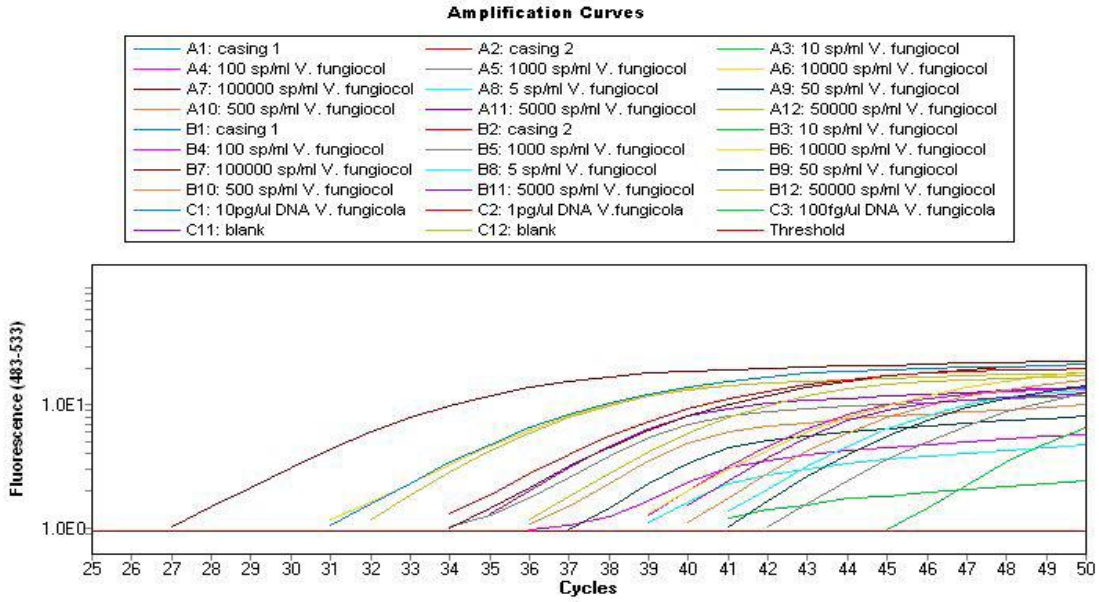


Figure 5-14: Results of amplification plots showing testing of genomic DNA of *Lecanicillium fungicola* using TaqMan PCR. Conidia concentration was tested from 10 to 10^5 conidia per ml casing extract using a (A1-A12) QuickGene (Fujifilm) and (B1-B12) Wizard Magnetic DNA Purification System for Food (Promega).

5.1.4 Comparison of polymerases on samples of casing extract

The three hot start polymerases were tested for sensitivity and specificity of samples extracted from casing extract with different concentrations of *L. fungicola* conidia.

The following polymerases were used for this comparison experiment:

1. LightCycler 480 – Roche
2. Maxima^{TM} Probe/ROX – Fermentas
3. Maxima^{TM} Probe/qPCR – Fermentas

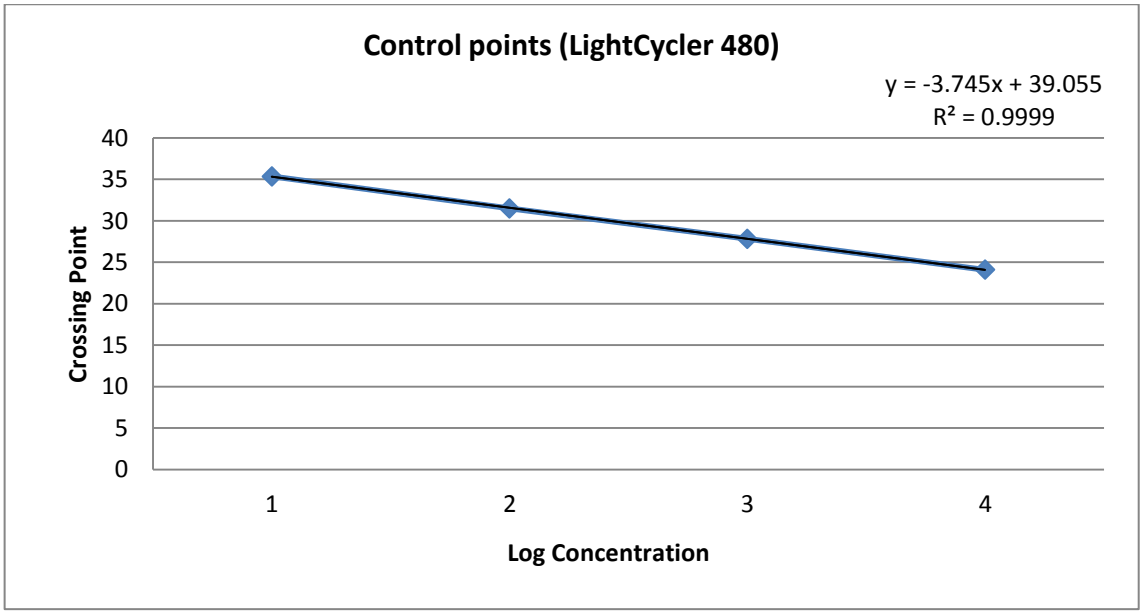
The Real Time PCR amplification curves and the corresponding fluorescent quantitative Real Time PCR standard curve were generated by employing the successively diluted known concentration of *L. fungicola* DNA for Real Time PCR reaction under the optimized conditions.

The results for **LightCycler 480** polymerase with pure *L. fungicola* DNA gave a correlation coefficient (0.999), slope -3.745, PCR efficiency of 1.85 and Y-intercept was 39.055 of the standard curve by the established Real Time PCR. The standard curve and the established Real Time PCR showed good performance (**Table** 5-11 and **Figure** 5-15).

Table 5-11: Detection of *L. fungicola* in casing extract. The DNA template of *L. fungicola* (L.15) was 2 µl.

No.	Amount *of L. fungicola* DNA (pg/µl)	*L. fungicola* DNA per reaction (pg)	LightCycler 480 (Roche) CP value (cycles)	Maxima™ Probe/ROX qPCR CP value (cycles)	Maxima™ Probe qPCR CP value (cycles)
1	100	200	24.10	22.18	22.52
2	10	20	27.82	26.29	28.14
3	1	2	31.49	29.78	30.97
4	0.1	0.2	35.36	34.68	34.88
5	0 (Negative control)	0	n/s	n/s	n/s
6	0 (Negative control)	0	n/s	n/s	n/s

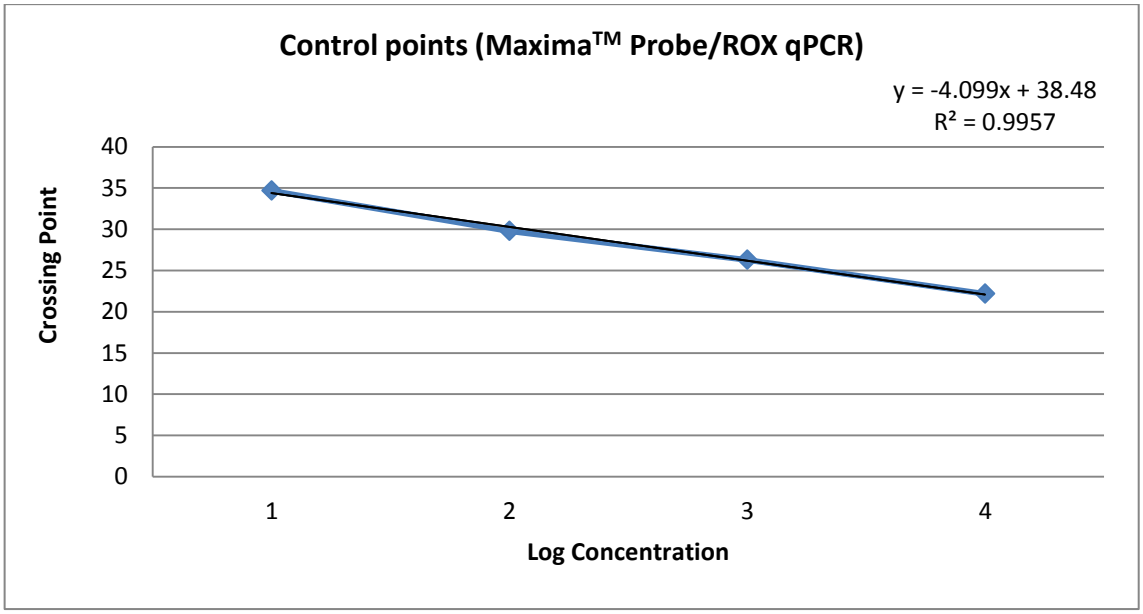
n/a – not available; n/s – no signal



Efficiency: 1.85, Slope -3.745, Y-intercept 39.055, Correlation coefficient (R^2) 0.999

Figure 5-15: Establishment of the fluorescent quantitative Real Time PCR control point**.** Control point of the *L. fungicola* fluorescent quantitative Real Time PCR. One dilutions of standard DNA ranging from 100 pg to 100 fg DNA/µl were used, as indicated on the x-axis, whereas the corresponding cycle threshold (CP) values are presented on the y-axis. Each dot represents the result of single amplification of each dilution. The correlation coefficient, slope, Y-intercept and error of the regression curve were generated of the reaction.

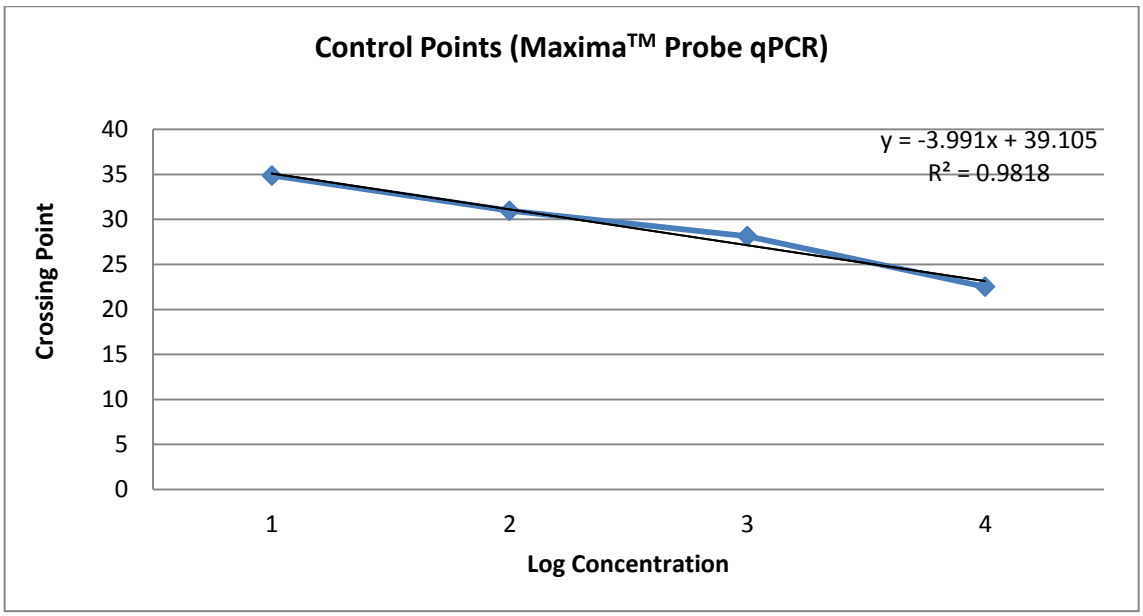
The results for **Maxima^{TM} Probe/ROX qPCR** polymerases with pure *L. fungicola* DNA gave a correlation coefficient (R^2) 0.9957, slope -4.099, efficiency 1.75 and Y-intercept 38.48 of the standard curve by the established real time PCR. The standard curve and the established Real Time PCR showed good performance (**Table** 5-11 and **Figure** 5-16).



Efficiency: 1.75, Slope -4.099, Y-intercept38.48, Correlation coefficient (R^2) 0.9957

Figure 5-16: Establishment of the fluorescent quantitative Real Time PCR control point**.** Control point of the *L. fungicola* fluorescent quantitative Real Time PCR. One dilutions of standard DNA ranging from 100 pg to 100 fg DNA/µl were used, as indicated on the x-axis, whereas the corresponding cycle threshold (CP) values are presented on the y-axis. Each dot represents the result of single amplification of each dilution. The correlation coefficient, slope, Y-intercept and error of the regression curve were generated of the reaction.

The results for **Maxima^{TM} Probe/qPCR** polymerases with pure *L. fungicola* DNA gave a correlation coefficient (R^2) 0.9818, slope -3.991, efficiency 1.78 and Y-intercept 39.105 of the standard curve by the established Real Time PCR, it can be seen that the standard curve and the established Real Time PCR give good performance (**Table** 5-11 and **Figure** 5-17).



Efficiency: 1.78, Slope -3.991, Y-intercept 39.105, Correlation coefficient (R^2) 0.9818

Figure 5-17: Establishment of the fluorescent quantitative Real Time PCR control point**.** Control point of the *L. fungicola* fluorescent quantitative Real Time PCR. One dilutions of standard DNA ranging from 100 pg to 100 fg DNA/µl were used, as indicated on the x-axis, whereas the corresponding cycle threshold (CP) values are presented on the y-axis. Each dot represents the result of single amplification of each dilution. The correlation coefficient, slope, Y-intercept and error of the regression curve were generated of the reaction.

All three tested polymerases gave acceptable control points which indicated that the reaction conditions were suitable and gave a good performance.The three hot start polymerases were tested for sensitivity and specificity of samples extracted from casing extract with different concentrations of *L. fungicola* conidia.

DNA was extracted by QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA isolation kits (Fujifilm) and Wizard Magnetic DNA (Promega). DNA was extracted from casing soil extract (Section 2.5.19) and casing soil extracts mixed with different conidia concentrations of *L. fungicola* (5-10^6 conidia/ ml casing extract or water) and extracted. The DNA extraction was performed from 1 ml material.

When DNA was extracted by QuickGene DNA extraction kit the LightCycler 480 polymerase gave a lower sensitivity and the CP value was a little higher compared to Maxima™ Probe/ROX qPCR and Maxima™ Probe qPCR. When the conidia concentrations were 50 conidia/ml the CP values was negative (0). The Maxima™ Probe/ROX qPCR and Maxima™ Probe qPCR polymerases gave a very similar sensitivity when conidia concentrations was 500 conidia/ml and higher, but when conidia concentration was 10 conidia/ml the positive signal was only obtained with Maxima™ Probe/ROX qPCR. Samples without *L. fungicola* conidia gave a negative signal with all tested polymerases.

However, with the lower conidia concentrations quantitation was not always reproducible compared to the higher conidia concentrations. Therefore the dynamic range of the method was between 5 and 10^5 conidia/ml of *L. fungicola*, which is relatively broad. The reliable detection level and successful quantification by Real Time PCR was 10^2 conidia/ml casing extract. The detection limit was 5 conidia per ml casing extract with all tested polymerases but the CP value was very high and varied from 40.92-43 cycles (**Table** 5-12).

Table 5-12: CP value of different concentrations of *L. fungicola* DNA extraction by QuickGene (Fujifilm) and tested by LightCycler 480 (Roche), Maxima™ Probe/ROX qPCR (Fermentas) and Maxima™ Probe qPCR (Fermentas).

No.	Conidia concentration per 1 ml casing extract	Conidia concentration per reaction (2µl)	LightCycler 480 (Roche) CP value (cycles)	Maxima™ Probe/ROX qPCR CP value (cycles)	Maxima™ Probe qPCR CP value (cycles)
1	0 (casing extract)	n/s	n/s	n/s	n/s
2	0 (casing extract)	n/s	n/s	n/s	n/s
3	5	10^{-2}	43.52	43.90	40.92
4	10^1	2×10^{-2}	40.02	40.79	n/s
5	$5x10^1$	10^{-1}	0	37.77	38.54
6	10^2	2×10^{-1}	38.41	39.49	37.86
7	$5x10^2$	1	36.96	35.93	36.28
8	10^3	2	35.52	34.78	35.16
9	$5x10^3$	10^1	34.38	33.44	33.98
10	10^4	2×10^1	31.52	30.28	30.83
11	$5x10^4$	10^2	31.98	30.22	30.87
12	10^5	2×10^2	27.59	26.50	26.93

n/s – no signal

When DNA was extracted by Wizard Magnetic DNA Purification System for Food isolation kit all three polymerases worked equally well when the *L. fungicola* conidia concentrations was 10^3 conidia/ml casing extract, but no signal was obtained for conidia concentration < 10^3 conidia/ml casing extract. The lower level of detection with all tested polymerases was 10^3 conidia/ml casing extract and successful quantification by Real Time PCR was 10^3 conidia/ml casing extract (**Table** 5-13).

Table 5-13: CP value of different concentrations of *L. fungicola* DNA extraction Wizard Magnetic DNA Purification System for Food (Promega) by and tested by LightCycler 480 (Roche), Maxima™ Probe/ROX qPCR (Fermentas) and Maxima™ Probe qPCR (Fermentas).

No.	Conidia concentration per 1 ml casing extract	Conidia concentration per reaction (2µl)	LightCycler 480 (Roche) CP value (cycles)	Maxima™ Probe/ROX qPCR CP value (cycles)	Maxima™ Probe qPCR CP value (cycles)
1	Casing extract	0	n/s	n/s	n/s
2	10^1	2×10^{-2}	n/s	n/s	n/s
3	10^2	2×10^{-1}	n/s	n/s	n/s
4	10^3	2	39.87	39.94	40.95
5	10^4	2×10^1	37.34	36.89	37.28
6	10^5	2×10^2	34.35	32.87	32.96
7	10^6	2×10^3	30.5	29.72	28.58

n/s – no signal

There were little differences between all three polymerases, but the QuickGene DNA extraction kit gave better results (more efficient) than a Wizard Magnetic DNA Purification System for Food (Promega).

5.1.4.1 Summary

The experiments described in this part of the thesis were performed with the help of LightCycler 480 Roche as a Real Time Machine, casing soil came from Irish mushroom farms, and a wild isolate of *L. fungicola* (L. 46) was used. To delimit the standard curve DNA of *L. fungicola* (L. 15) was used and this isolated DNA was extracted by ZymoResarch DNA isolation kit. The casing soil comes from Irish mushroom farms and *L. fungicola* (L.46) DNA was used to calculate a standard curve.

This assay illustrated the isolation of DNA using a QuickGene DNA isolation kit. With this kit the detection level of *L. fungicola* conidia was 5 conidia but the reliable detection level was 10^2 conidia per ml casing extract. Next experiment compared QuickGene and Wizard Magnetic DNA Purification System for Food. The DNA isolated by QuickGene gave better sensitivity and lower CP value at all conidia concentrations (5 - 10^5 per ml casing extract) compared to Wizard Magnetic DNA Purification System for Food. The detection level of both kits was 5 conidia per ml casing extract. With the Wizard Magnetic DNA Purification System for Food reliable detection was when conidia concentrations was 10^4 per ml casing extract and higher compared to QuickGene when a reliable detection level was 10^2 conidia/ml casing extract.

These assays illustrate specificity and sensitivity of three different polymerases compatible with LightCycler® 480 Real Time PCR machine supplied by Roche. High quality hot start Taq DNA polymerase could minimize non-specific amplification and increase the PCR cycling efficiency which is important with soil samples containing humic acid. When DNA of *L. fungicola* was extracted using QuickGene positive CP values were present with all three tested polymerases (LightCycler 480, Maxima™ Probe/ROX qPCR and Maxima™ Probe qPCR) when conidia concentrations were 10^2 ml casing extract and higher. The LightCycler 480 polymerase gave lower sensitivity than Maxima™ Probe/ROX qPCR and Maxima™ Probe qPCR. The Maxima™ Probe/ROX qPCR gave a positive signal in all lower conidia concentrations i.e. 5, 10^1 and $5x10^1$ ml casing extract.

When DNA of *L. fungicola* conidia was isolated by Wizard Magnetic DNA Purification System for Food the detection level for all tested polymerases was 10^3 conidia/ml casing extract. The three polymerases did not show significant differences between them.

5.2 Discussion

Real Time PCR is a method which permits the direct online determination of the created PCR product during the reaction by an increase in the fluorescence of the reaction mixture. The Real Time PCR reaction has many applications as a quantitative method, but also as a very sensitive qualitative method. The preparation of the Real Time PCR reaction is not so easy because this method is very sensitive to any contamination. TaqMan tests are designed to increase the specificity of Real Time PCR tests and contamination may come from the organism. In microbiology, especially for microorganism detection from different sources (food, soil, plant, human, animals), the quantitative side is not present. Usually Real Time PCR a test in microbiology is used for detection of microorganisms and for qualitative side and can give only answers yes (present) or no (absent) (Geisen, 2007, Mackay *et al.*, 2007 b).The detection level of Real Time PCR using TaqMan probe is 10 conidia per ml but reliable quantification is 10^3 and/or 10^4 conidia/ml (Selma *et al.*, 2008 and Zijlstra *et al.*, 2007, 2008 and 2009).

In this test, the primers and probes (TaqMan) designed by Zijlstra *et al.* (2007, 2008 and 2009) have been designed using of rRNA sequence of *L. fungicola* genome. Zijlstra *et al.* (2007, 2008 and 2009) used a DNA hydrolysis probe TaqMan conjugated with Minor Groove Binding (MGB™). The MGB increases the Tm of the hybridized probe and facilitates highly specific binding to the targeted sequence, especially when a mismatch is present (Kutyavin *et al.*, 2000). This probe contains a quencher dye which does not emit fluorescence within the detectable wavelength range and results in greater accuracy in measurement. This upgrading reduces spectral similarities with fluorescence emitted by the reporter dye, and results in greater precision in the measurement of reporter-specific signals (Guo *et al.*, 2009). The development of a TaqMan MGB-based Real Time PCR probe gave sensitivity of signal for detection and quantitation of *L. fungicola* from samples collected from mushroom farm containing casing, soil and other debris.

The first part of the experiment was to compare different commercially available DNA extraction kits. The most important thing for microorganism detection from different materials is sample preparation and isolation of a high molecular weight quality genomic DNA without PCR inhibitors contained in tested material (e.g. soil, food and casing). DNA isolation is an important and critical part of good quantitative and qualitative aspects of Real Time PCR. Casing soil contains large amounts of humic acid which can inhibit and stop a PCR reaction (Tebbe and Vahjen, 1993; Tsai and

Olson, 1992 a, b; Wilson *et al.*, 1990; Wilson, 1997). The Real Time PCR reaction is a very sensitive method and requires clean samples.

The aim of this Chapter was to test Real Time PCR detection system using a LightCycle 480 supplied by Roche and test detection and reliable quantification level of *L. fungicola* diluted in casing extract.

The optimization of the *L. fungicola* assay was focused on the concentration of primers and probe. The standard curve had a correlation coefficient $R^2 > 0.995$ and efficiency 1.87. The amount of primers and probe gave good results.

TaqMan MGB probe was specific only for *L. fungicola* DNA, casing soil and soil from outside the NUIM grounds gave a negative result. The detection level of *L. fungicola* conidia was 10^1 per ml casing extract, but quantification level was 10^4 conidia per ml casing extract and higher when DNA of *L. fungicola* was extracted by Wizard Magnetic DNA Purification System for Food. When the conidia of *L. fungicola* (10-10^6) were extracted from casing extract or water, better sensitivity was recorded when conidia were mixed with casing extract rather than in water. The detection level in both samples was 10^1 conidia per ml casing extract or water, but reliable results were obtained when conidia concentrations were higher than 10^4 conidia/ml casing extract or water.

The aim of the next part of this Chapter was to evaluate a less expensive DNA extraction kit and new polymerases. The QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA isolation kits (QuickGene) gave very good results when conidia concentrations were 10-10^5 per ml casing extract. The detection level of *L. fungicola* conidia was 5 conidia per ml casing extract but a more reliable result was obtained when conidia concentrations were 10^2 and higher. When two DNA isolation kits were compared the QuickGene and Wizard Magnetic DNA Purification System for Food the QuickGene gave better sensitivity than Wizard Magnetic DNA Purification System for Food. The CP value was lower and more reliable with all conidia concentrations. The detection level for both DNA kits was 5 conidia per ml casing extract but reliable results for QuickGene were obtained with 10^2 conidia per ml casing extract compared to Wizard Magnetic DNA Purification System for Food when reliable results were obtained with conidia concentrations of 10^4 and higher. The comparison of DNA extraction from difficult samples is described by many researchers (Pinto *et al.*, 2005; Demeke *et al.*, 2009; Mori *et al.*, 2007).

The next part of the assay was to compare three polymerases able to work with LightCycle 480 Roche machine. The high quality hot start Taq DNA polymerase is a

very important part of Real Time PCR especially when tested materials contain contaminations such as humic acids. The use of appropriate polymerase could minimize unspecific amplifications and increase the PCR cycling efficiency. All tested polymerases gave good results when conidia concentrations was 10^2 per ml casing extract or higher and DNA was isolated by QuickGene and Wizard Magnetic DNA Purification System for Food. The differences between polymerases occurred when conidia concentrations were lower than 10^2 conidia per ml casing extract. The Maxima^{TM} Probe/Rox qPCR gave a positive CP in all tested conidia concentrations (5-10^5 per ml casing extract), but the sensitivity was similar in other tested polymerases by LightCycler 480 and Maxima^{TM} Probe qPCR.

The Real Time PCR assay was highly reproducible and linear over a range of 10^4 to 10^6 conidia per ml casing extract or 1 gram casing when DNA was extracted by Wizard Magnetic DNA Purification System for Food, but QuickGene DNA extraction kit gave better results and reproducible data were obtained when conidia concentrations were 10^2 to 10^5 per ml casing extract.

The Real Time PCR method is a very sensitive test. Good equipment, reagents and experience in preparation of Real Time PCR reaction are essential.

The final result is a molecular diagnostic method that is not only rapid and reliable, but one that is also easy to perform and applicable to use for testing large numbers of samples. Real Time PCR presents the benefits of increased speed due to reduced cycle time and removal of post-amplification processes, offering considerable labor savings and allowing higher throughput analysis.

In conclusion, the TaqMan MGB Real Time PCR method tested in this study is highly specific and sensitive with good ability to detect *L. fungicola* from moderately dirty samples contain not so much casing soil. The method described in this study will be helpful for detecting *L. fungicola* from different samples collected from mushroom farms and characterising distribution and sources of mushroom mycoparasites.

The present information could be used for routine diagnostic use for samples collected from mushroom farms.

Chapter 6 Measuring sources of *Lecanicillium fungicola* on mushroom farms in Ireland

Agaricus bisporus, the common button mushroom, has a long cultivation tradition. The first information on the cultivation of *Agaricus* comes from France and after that cultivation spread to other European countries such as England, the Netherlands, Germany, Italy, Russia and Poland. Mushroom cultivation also commenced in USA in the early 20th century (Van Griensven 1988; Szymański 1997; Van Griensven and Roestel, 2004).

Today *Agaricus bisporus* is cultivated in more than 70 countries in the world (Cappelli 1984). This monoculture is affected by many pathogens and pests. The most important pathogen of *A. bisporus* is *L. fungicola* which causes the disease called "dry bubble". The symptoms of dry bubble disease are: dry bubble – not differentiated mass of tissue, split stipe and spotting. When *A. bisporus* shows symptoms of dry bubble the mushrooms cannot be sold or used for consumption which affects mushroom growers economically. *Lecanicillium fungicola* produces large numbers of conidia which are held in sticky mucilage and these conidia can be very easily spread around the mushroom farm. Conidia are spread and dispersed in many ways via water, flies, humans and machinery (Beyer *et al.*, 2005; Fletcher and Gaze, 2008). The conidia can also survive for a long time (7-12 month) in dry or moist casing soil mixture (Cross and Jacobs, 1969; Brady and Gibson, 1969). All these factors make *L. fungicola* a very serious pathogen of the mushroom industry that is difficult to eliminate.

The first information about the spread of *L. fungicola* on a mushroom farm by human factor was demonstrated by Fekete (1967) and confirmed by Cross and Jacobs (1969) in an *in vitro* experiment. Cross and Jacobs (1969) took a finger imprint from a 7 day old *L. fungicola* clean culture and impressed it one hundred times in succession on the surface of malt agar in Petri dishes. They incubated the Petri-dishes and colonies of *L. fungicola* were present on all the imprinted areas indicating conidia are easily spread by touch. They also demonstrated that the conidia of *L. fungicola* were not transported by a blast of air with a speed of 10.75m/s^{-1} (the typical range of air speed across the crop is 0.003-0.03 m/s^{-1}), (Grant and Staunton, 1999), indicating that *L. fungicola* conidia are not easily dislodge by air movement.

Gandy (1972), stated that *L. fungicola* conidia are spread in mushroom houses by watering operations, but water splash dispersal of conidia occurred only over short distances, compared to dispersal of *L. fungicola* conidia in contaminated debris. Contaminated debris increased the concentration of *L. fungicola* conidia in the atmosphere which was inferred from many primary outbreaks of dry bubble. Dry bubble disease often first appears near the "exit holes" and near the doors where air can enter. Experiments with phorid flies indicated that *L. fungicola* is present on the bodies of flies, which can spread the disease to new mushrooms. Gandy (1972) also reported that pickers did not affect the spread of dry bubble disease in growing houses, but mushroom pickers gloves and tools were a source of *L. fungicola* conidia.

Wong and Preece (1987) performed a comprehensive study to search for sources of *L. fungicola* on mushroom farms. They used a microbiological method consisting of two different microbiological media for the detection of *L. fungicola* from samples on one large mushroom farm in the UK which was seriously affected by dry bubble disease. The samples were collected from 1979 to 1981. At that time the preparation of compost and casing soil was different to those of today. They collected many different types of sample. They did not isolate *L. fungicola* from the spawn, compost or water used in watering the crop, but they isolated *L. fungicola* from symptomless white mushrooms, diseased blotched mushrooms, casing mixture (peat and limestone), pickers' hands, shoes and ladders, the hands of growers and watering equipment, the floors and doors of buildings. *Lecanicillium fungicola* can be also isolated from the bodies of the principal pests of the crop on this farm, – sciarid flies and mites. Wong and Preece (1987) also tested air samples for *L. fungicola* conidia and only 4 out of 40 samples were positive which indicated that *L. fungicola* is not primary air born fungus, but *L. fungicola* conidia were caught in the air of the production areas of the farm at almost all times. Wong and Preece (1987) also demonstrated that the main and primary source of *L. fungicola* on the mushroom farm was fresh peat and limestone (arriving or stored on the mushroom farm) used for making the casing soil. *Lecanicillium fungicola* was also spread by water splash, and people's hands. The fungus was present on many surfaces such as ladders and floors.

Nair and Macauley (1987) reported that the most common source of *L. fungicola* var. *fungicola* was soil from around mushroom farms. The peat moss and water which was tested were not a source of the pathogen. For this experiment they detected *L. fungicola* by preparing a serial dilution and plating it out on potato dextrose agar with antibiotics.

The next information about sources of *L. fungicola* on mushroom farms was by Rinker *et al.* (1993), who designed a selective medium for *L. fungicola* to test samples from mushroom farms. They tested debris from floors and structural surfaces, casing material, flies and mushrooms. *Lecanicillium fungicola* was present on mushrooms, casing material (mixture of sphagnum and peat moss and $CaCO_3$), debris from floor and structural surfaces.

Grogan (2001) confirmed Gandy's (1972) information about the spread of *L. fungicola* by the debris-dust fraction which is present on a mushroom farm. Samples collected from inside and outside mushroom houses, which were infected by *L. fungicola* caused dry bubble disease when they were added to casing. Grogan (2001) concluded that dust containing *L. fungicola* material can be a potential source for spreading this pathogen on mushroom farms.

The objectives of this work were to detect *L. fungicola* on commercial mushroom farms in Ireland. From 2008 to 2010 samples were collected during 18 visits to 9 mushroom farms with different levels of dry bubble disease. In total 438 samples were collected from different locations and stages of the crop cycle from spawn running to 3rd flush. For detection of *L. fungicola* two methods are used which were described in Chapter three and Chapter six. Two selective media were used with 438 samples. The first selective media was Novel (PDA) selective medium (NPDASM) based on PDA. The second selective medium was modified version of Rinker's selective medium for *L. fungicola* (Rinker *et al.* 1993), called modified Rinker's selective medium (MRSM). The RT PCR method was also used and 375 samples were tested using Zijlstra primers and probe (Zijlstra *et al.*, 2007, 2008 and 2009). Identification of the possible sources of *L. fungicola* on mushroom farm could provide useful information for managing dry bubble disease.

6.1 Results

6.1.1 Hygiene levels on mushroom farms

Eighteen mushroom farm visits were performed from 2008 to 2010. The samples were collected from 9 commercial mushroom farms with different levels of dry bubble disease. The level of hygiene was assessed for each mushroom farm based on mushroom grower information (MGI). A second assessment was made based on a

personal observation of hygiene (POH). The personal observation of hygiene was based on my own observation and on mushroom growers' interview about presence or absence of dry bubble disease and flies.

The MGI recorded the presence or absence of dry bubble and flies, use or not of fungicides, and cook-out or chemical disinfection at crop termination. One point was given for use of fungicide, one point for cook-out or chemical disinfection, one point for absence of flies and one point for absence of dry bubble at each crop stage. The maximum score was 5 (when crop was finished after 2nd flush) or 6 (when crop was finished after 3rd flush). When fungicide and cook-out or chemical disinfection was not used and flies and dry bubble were present for each crop the score was zero. The MGI score was counted where the maximum score (5 or 6) = 100 % (**Table** 6-1). The POH assessment ranged from 1 to 3 with 1 = low level of hygiene, 2 = medium and 3 = high level of hygiene.

The personal observation assessment of hygiene POH and mushroom growers information (MGI) for hygiene level were different, when the POH was low the MGI scale was between 33-60 %, when POH was medium hygiene level the MGI scale was between 33 %-83 %, and when POH was high the MGI scale was between 67 %-100 %, where 100 % means a very high hygiene level and dry bubble disease is not present. These results indicated that there is a weak correlation so it would be necessary to look at gathering more information to quantify the level of hygiene on a mushroom farms (**Figure** 6-1).

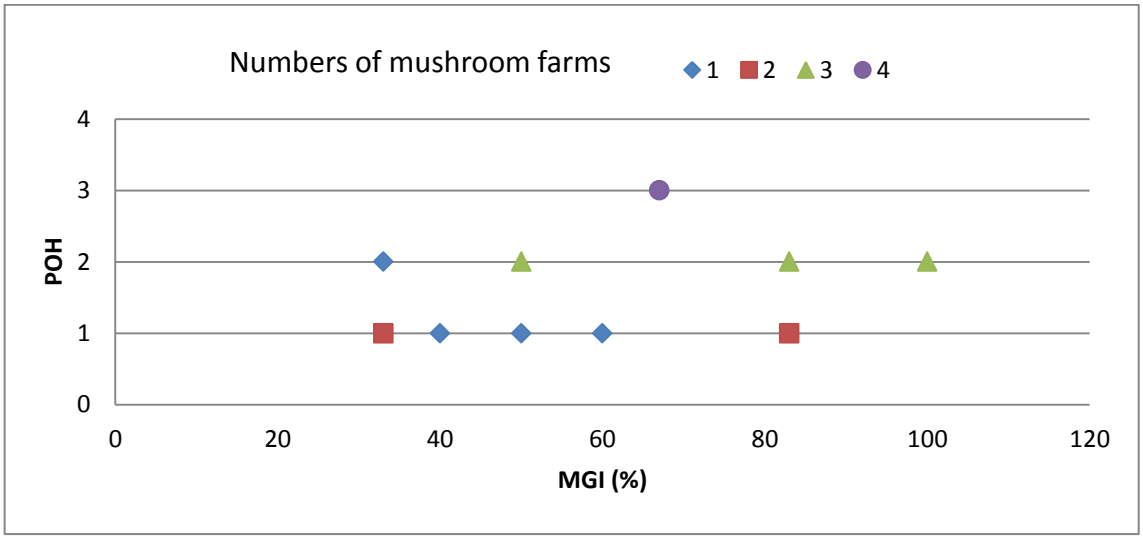


Figure 6-1: Comparison of mushroom grower information (MGI) and personal observation of hygiene level (POH) for mushroom farms.

Table 6-1: Mushroom farms visits from 2008 to 2010 with a personal observation of hygiene (POH) and mushroom growers information (MGI).

Visit number	Mushroom farm code	County	Repeat visits	Season	Date of visits			Dutch system of growing mushrooms	Mushroom grower information				Dry bubble - present			Mushroom growers information	Personal observation of hygiene
					2008	2009	2010		Sporgon	Flies	Cook-out	Chemical disinfecti on	1st flush	2nd flush	3rd flush	Hygiene scale	Assessment level
1	EQ	Monaghan	1	autumn	yes	no	no	yes	yes	yes	yes	no	yes	yes	yes	33%	low
2	EQ	Monaghan	2	autumn	yes	no	no	yes	yes	no	yes	no	yes	yes	yes	50%	low
3	EQ	Monaghan	3	winter	yes	no	no	yes	yes	no	yes	no	yes	yes	finish	60%	low
4	EQ	Monaghan	4	winter	yes	no	no	yes	yes	yes	yes	no	yes	yes	finish	40%	low
5	MMcG	Leitrim	1	spring	no	yes	no	no	yes	yes	no	yes	yes	yes	yes	33%	low
6	MC	Cavan	1	spring	no	yes	no	yes	no	no	no	no	no	no	no	67%	high
7	EK	Cavan	1	spring	no	yes	no	yes	no	yes	yes	no	no	no	yes	50%	medium
8	DG	Cavan	1	spring	no	yes	no	yes	yes	no	yes	no	no	no	no	100%	high
9	JK	Westmeath	1	summer	no	yes	no	yes	yes	yes	yes	no	no	yes	yes	50%	medium
10	GR	Westmeath	1	summer	no	yes	no	yes	no	yes	yes	no	no	yes	yes	33%	medium
11	JH	Tipperary	1	summer	no	yes	no	no	yes	yes	no	yes	no	no	no	83%	high
12	JQ	Tipperary	1	summer	no	yes	no	yes	yes	yes	yes	no	no	yes	yes	50%	medium
13	EQ	Monaghan	5	winter	no	no	yes	yes	yes	no	yes	no	no	no	yes	83%	high
14	EK	Cavan	2	winter	no	no	yes	yes	no	no	no	no	no	no	no	67%	high
15	JH	Tipperary	2	winter	no	no	yes	no	yes	no	no	yes	no	no	yes	83%	medium
16	JQ	Tipperary	2	winter	no	no	yes	yes	no	no	no	no	no	no	no	67%	high
17	JK	Westmeath	2	winter	no	no	yes	yes	yes	no	yes	no	no	no	no	100%	high
18	GR	Westmeath	2	winter	no	no	yes	yes	no	no	no	no	no	no	no	67%	high

6.1.1.1 Detection of *L. fungicola* using selective media and RT PCR

During 18 visits to 9 different mushroom farms a total of 438 samples were collected. All samples were tested on two selective media, a novel PDA-based selective medium (NPDASM) and modified Rinkers's selective medium (MRSM) (**Table** 6-2). Real Time PCR was used for 375 samples.

Lecanicillium fungicola was detected 77 times using selective media (17 %) and 238 times using Real Time PCR (65 %) after 45 cycles. On selective media *L. fungicola* were detected on 14 farm visits, but with Real Time PCR *L. fungicola* was detected on all 18 farm visits (**Table** 6-2).

Table 6-2: Number of samples collected for each mushroom farm and number of samples tested from each mushroom farm on selective media and RT PCR and number of positive isolations of *L. fungicola* on both selective media and RT PCR after 40 and 45 cycles.

Visit number	Mushroom farm code	Selective media					Real Time PCR				
		No. samples tested on selective media	*L. fungicola* detected on NPDASM	*L. fungicola* detected on MRSM	*L. fungicola* detected on NPDASM and MRSM	% samples positive on both selective media	No. samples tested on Real Time PCR	*L. fungicola* recorded after 40 cycles	% samples positive on RT PCR after 40 cycles	*L. fungicola* recorded after 45 cycles	% samples positive on RT PCR after 45 cycles
1	EQ	24	7	8	9	38%	24	16	67%	17	71%
2	EQ	18	0	0	0	0%	15	10	67%	12	80%
3	EQ	36	5	5	5	14%	31	12	39%	24	77%
4	EQ	29	6	3	6	21%	18	7	39%	11	61%
5	MMcG	24	5	5	6	25%	24	10	42%	11	46%
6	MC	11	1	1	1	9%	11	1	9%	1	9%
7	EK	38	9	12	12	32%	36	6	17%	16	44%
8	DG	7	0	0	0	0%	7	3	43%	6	86%
9	JK	20	1	3	3	15%	18	12	67%	15	83%
10	GR	23	2	1	3	13%	21	13	62%	16	76%
11	JH	31	0	0	0	0%	11	6	55%	10	91%
12	JQ	33	2	1	2	6%	19	13	68%	13	68%
13	EQ	26	0	0	0	0%	24	1	4%	12	50%
14	EK	26	6	5	6	23%	24	2	8%	9	38%
15	JH	17	6	6	6	35%	17	14	82%	15	88%
16	JQ	33	3	3	3	9%	33	12	36%	22	67%
17	JK	24	8	8	8	33%	24	10	42%	17	71%
18	GR	18	7	6	7	39%	18	8	44%	11	61%
	Summary	**438**	**68**	**67**	**77**	**17%**	**375**	**156**	**44%**	**238**	**65%**

6.1.1.2 Comparison of selective media by McNemar's Test

All 438 samples were tested using two media – NPDASM and on MRSM. *Lecanicillium fungicola* was detected 68 times on NPDASM and 67 times on MRSM. The detection on both media was analysed by McNemar's test. There was a non-significant difference between two media of detection *L. fungicola*. The P value by McNemar's Test was given by P > 0.8185 which means both media work to same (**Table** 6-3).

Table 6-3: *Lecanicillium fungicola* detection using selective media NPDASM media and MRSM media.

Table of Result: Result NPDASM by Result MRSM				
		Positive Results Selective medium MRSM		
		0 (No)	1 (Yes)	Total
Positive Results Selective medium NPDASM	0 (No)	361	9	370
	1 (Yes)	10	58	68
	Total	371	67	438
P>0.8185				

The percentage of samples positive on both selective media showed no association of mushroom growers' information (MGI) for hygiene level (**Figure** 6-2).

The results (**Figure** 6-2) showed there is no correlation between detection of *L. fungicola* on selective medium and MGI scale so it was necessary to look at gather more information to quantify the level of hygiene on mushroom farms.

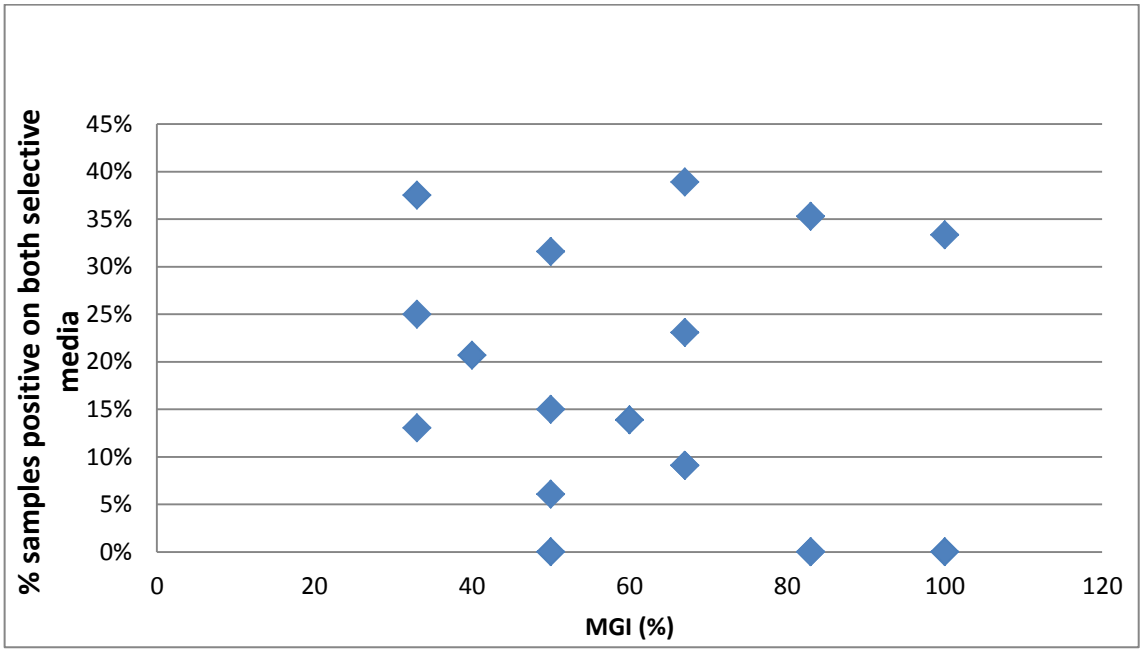


Figure 6-2: Comparison of % samples positive on both selective media and mushroom growers' information (MGI).

6.1.1.3 Comparison of RT PCR after 40 and 45 cycles

The 375 samples were tested by Real Time PCR. The data were analysed after 40 and 45 cycles of PCR reaction. Positive results after 40 cycles were recorded in 156 samples and after 45 cycles positive signal was present in 238. There was a significant difference between the detection of *L. fungicola* after 40 and after 45 cycles, P value by McNemar's Test was given by P < 0.0001 (**Table** 6-4). There were significantly more positive results after 45 cycles.

Table 6-4: *Lecanicillium fungicola* detection after 40 and 45 cycles.

Table of Result: Positive 45 by Positive 40				
		Positive Result 40		
		0 (No)	1 (Yes)	Total
Positive Results 45	0 (No)	137	0	137
	1 (Yes)	82	156	238
	Total	219	156	375
P<.0001				

The percentage number of samples positive after 40 and 45 cycles on RT PCR and mushroom growers information (MGI) for hygiene did not have any connection (**Figure** 6-3 and **Figure** 6-4).

There in a non-correlation between detection of *L. fungicola* on RT PCR after 40 and after 45 cycles and MGI scale so it would be necessary to look at gathering more information to quantify the level of hygiene on a mushroom farms.

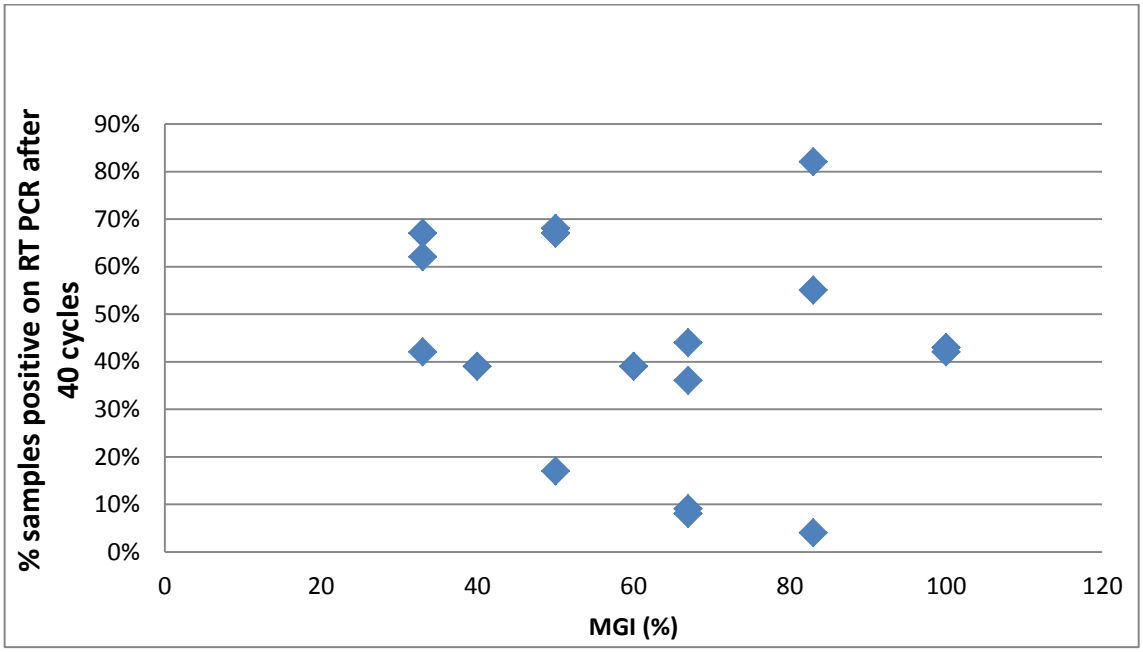


Figure 6-3: Comparison of % samples positive on RT PCR after 40 cycles and mushroom grower information (MGI).

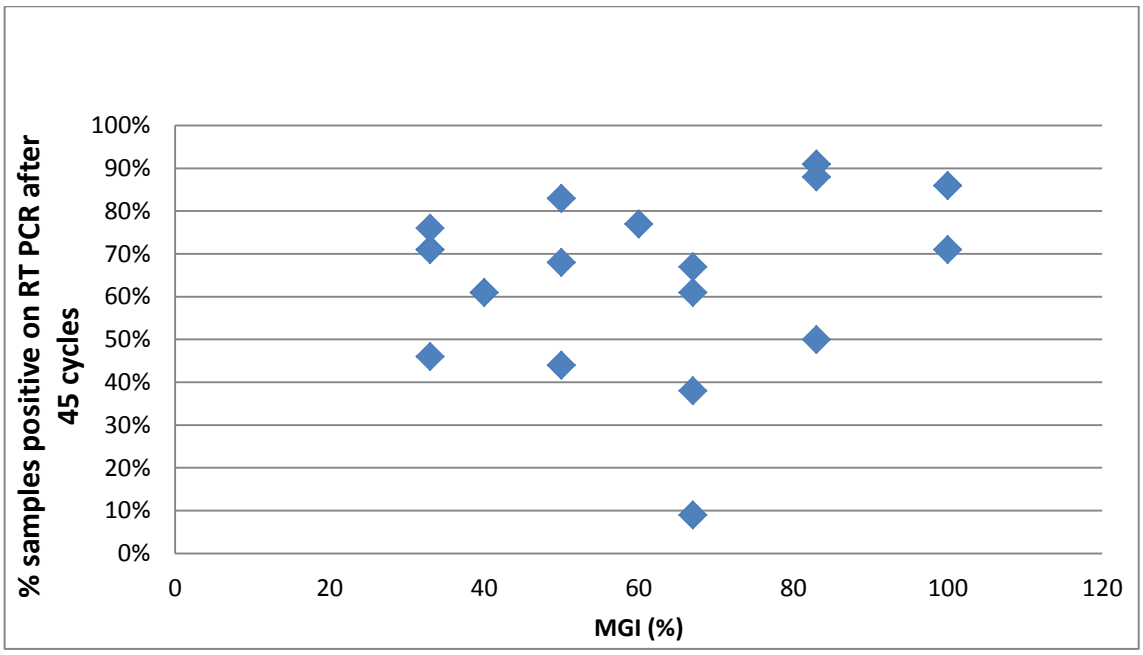


Figure 6-4: Comparison of % samples positive on RT PCR after 45 cycles and mushroom grower information (MGI).

6.1.1.4 Comparison of selective media with RT PCR after 40 and 45 cycles

When the selective media (NPDASM and MRSM) and Real Time PCR after 40 cycles were compared there was significant difference between two methods. The P value by McNemar's Test was given by $P < 0.0001$ (**Table** 6-5). There were significantly more positive results by Real Time PCR than on selective media.

Table 6-5: *Lecanicillium fungicola* detection compared between selective media and RT PCR after 40 cycles.

Table of Result: Positive Selective media by Positive 40 cycles				
		Positive Result 40		
		0 (No)	1 (Yes)	Total
Positive Results Selective media	0 (No)	187	114	301
	1 (Yes)	32	42	74
	Total	219	156	375
P<.0001				

When the results for selective media (NPDASM and MRSM) and Real Time PCR after 45 cycles, were compared there was significant difference between numbers of cycles. The P value by McNemar's Test was given as $P < 0.0001$

There were significantly more positive results on Real Time PCR than on selective media, but almost 20 samples were positive on selective media and negative by RT PCR it is almost 26 % of all positive detections of *L. fungicola* on selective

medium. Those samples came from places where a large amount of casing was present such as floor inside growing room or picker's platform (**Table** 6-6).

Table 6-6: *Lecanicillium fungicola* detection compared between selective media (NPDASM and MRSM) and RT PCR after 45 cycles.

Table of Result: Positive Selective media by Positive 45 cycles				
		Positive Result 45		
		0 (No)	1 (Yes)	Total
Positive Results Selective media	0 (No)	117	184	301
	1 (Yes)	20	54	74
	Total	137	238	375
P<.0001				

6.1.1.5 Summary

The results presented in this section, demonstrate two ways for assessing hygiene level and presence of *L. fungicola* on commercial mushroom farms. The subjective assessment and mushroom growers information after calculation showed a weak correlation. *Lecanicillium fungicola* was 3 times more frequently detected by Real Time PCR (65 %) than on selective medium (17 %). There was no significant difference between the two media tested. On both selective media *L. fungicola* was detected 77 out of 438 samples. On Real Time PCR *L. fungicola* gave a positive FAM signal in 238 out of 375 tested samples. The selective medium is able to detect only live material in contrast to Real Time PCR when positive FAM signal is obtained from live and dead material.

6.1.2 Detection *L. fungicola* using two selective media and Real Time PCR on samples collected on mushroom farms.

6.1.2.1 Stage of cropping

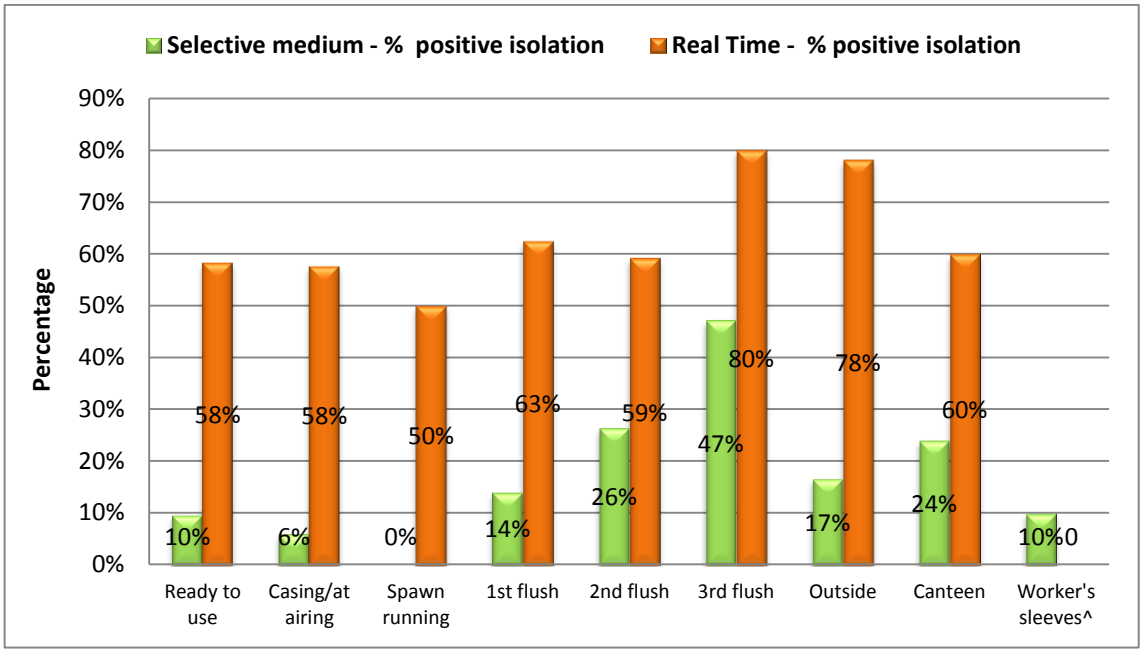
On selective media (NPDASM and MRSM) *L. fungicola* was not detected during the spawn running stage of cropping but in other crop stages and samples *L. fungicola* colonies were recorded. The *L. fungicola* was very often detected during 2nd and 3rd flush compared to 1st flush. On Real Time PCR positive FAM signal was recorded in all crop stages and samples after 45 cycles. Real Time PCR detected significantly more *L. fungicola* than selective media (**Table** 6-7).

Lecanicillium fungicola was very often detected in outside samples such as debris from bin trash (picker's gloves, hair net), samples from common area in mushroom farm (scales, crates), crates outside, water tank and soil close to mushroom farm. The Real Time PCR identified the pathogen in all crop stages. Real Time PCR gives more positive detection of *L. fungicola* than selective media (**Figure** 6-5).

Table 6-7: Detection of *L. fungicola* using Selective media and Real Time PCR after 45 cycles.

No.	Crop stage	Samples tested – selective media	Selective media – positive isolation	samples tested RT PCR	Real Time PCR – positive isolation	McNemar's Test comparison of selective media and RT PCR
1	Ready to use	31	3	24	14	*
2	Spawn running	8	0	8	4	^^
3	Casing/at airing	116	7	104	60	**
4	1st flush	100	14	88	55	**
5	2nd flush	53	14	49	29	**
6	3rd flush	53	25	45	36	**
7	Outside	42	7	32	25	**
8	Canteen	25	6	25	15	**
9	Worker's sleeves^	10	1	n/t	n/t	n/a
	Summary	**438**	**77**	**375**	**238**	*****

* – Significant at P < 0.05; ** – significant at P < 0.01; ns – not significant; ^^ – There are no discordant pairs (not enough samples for a valid comparison) n/a – not available; n/t – not tested; ^ – only selective medium tested;



^ – only selective media tested.

Figure 6-5: The percentage detection of *L. fungicola* using selective media and Real Time PCR after 45 cycle.

6.1.2.2 Origin of samples collection

On selective media (NPDASM and MRSM) *L. fungicola* was detected at a low level or not at all on canteen samples, casing equipment, machines, and water equipment but in other tested places and equipment *L. fungicola* was detected (**Table** 6-8). The *L. fungicola* was very often detected on picker's equipment and items such as trolleys, platforms, hair net and gloves, scales and number rolls. The *L. fungicola* colonies were also very often present in samples collected from floor inside the growing room close to the door and crates which are used to transport mushrooms (**Figure** 6-6). A very interesting place where *L. fungicola* was detected was door handle where the number of colonies was usually > 20 per plate. These samples were only collected in Winter 2009/2010.

Figure 6-6: Samples collection from mushroom farm: A – shelves; B – Picker's equipment; C – Growing room structure inside; D – Inside of growing room – floor; E – Water hose; F – Outside floor close to the door, crates, door handle.

Table 6-8: Detection of *L. fungicola* using selective media and Real Time PCR after 45 cycles.

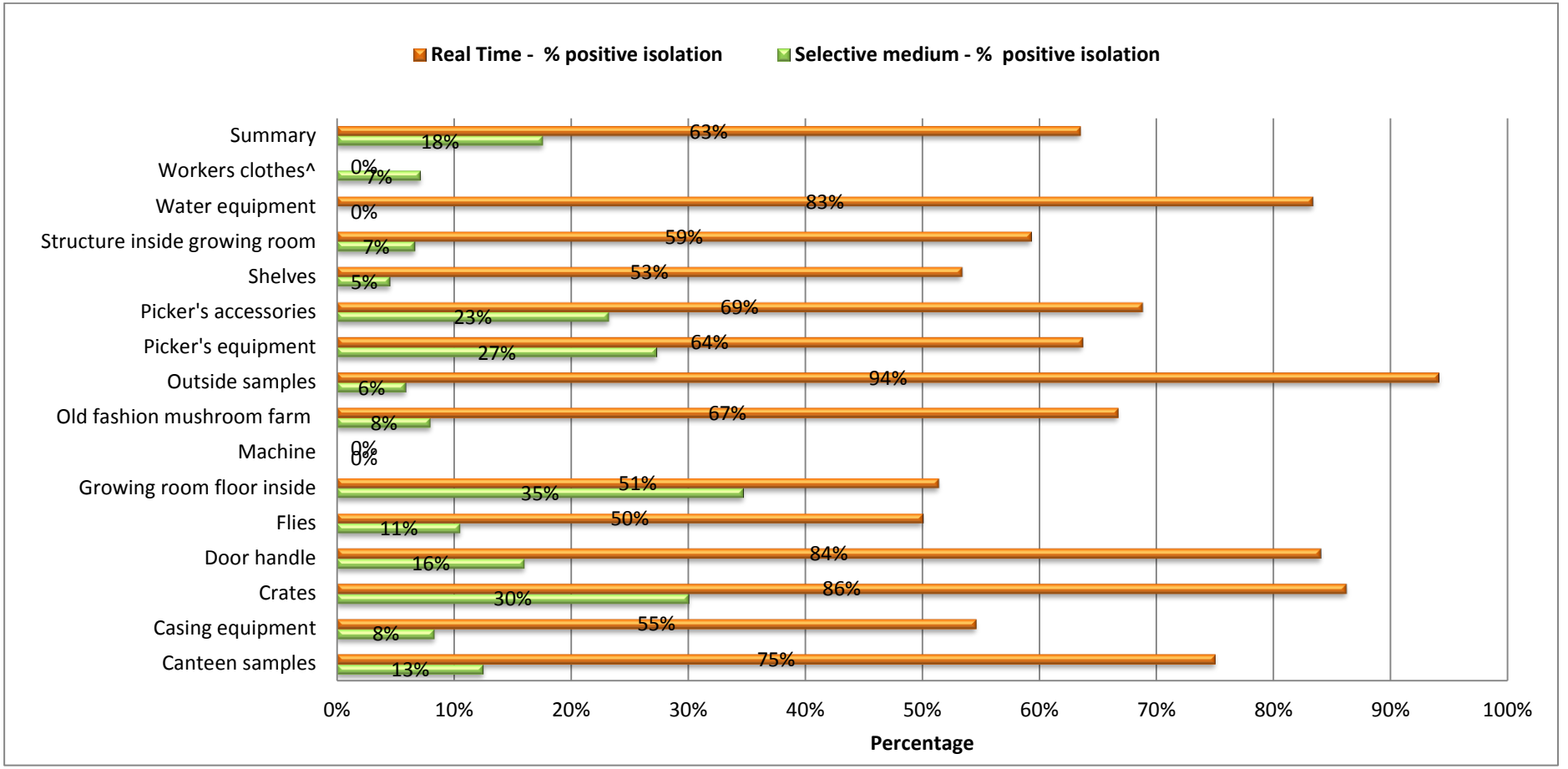
No.	Samples	Samples tested – selective media	Selective media – positive isolation	samples tested RT	Real Time PCR – positive isolation	McNemar's Test comparison of selective media and RT PCR after 45 cycles
1	Canteen samples	8	1	8	6	*
2	Casing equipment	12	1	11	6	*
3	Crates	30	9	29	25	**
4	Door handle	25	4	25	21	**
5	Flies	19	2	18	9	**
6	Growing room floor inside	75	26	74	38	*
7	Machine	7	0	4	0	^^
8	Old fashion mushroom farm	25	2	15	10	**
9	Outside samples	17	1	17	16	**
10	Picker's equipment	33	9	33	21	**
11	Picker's accessories	69	16	48	33	**
12	Shelves	66	3	60	32	**
13	Structure inside growing room	30	2	27	16	**
14	Water equipment	8	0	6	5	^^
15	Workers clothes*	14	1	n/t	n/t	n/t
	Summary	438	77	375	238	**

* – Significant at $P < 0.05$; ** – significant at $P < 0.01$; ns – not significant; ^^ – There are no discordant pairs (not enough samples for a valid comparison); n/a – not available; n/t – not tested; ^ – only selective medium tested;

On Real Time PCR Positive FAM signal was recorded in all sample categories except for machines. *Lecanicillium fungicola* was very often detected in crates, door handle (Winter 2009/2010), from floor inside of growing room close to the door, outside samples such as floor close to the growing room, picker's equipment and items such as trolleys, platforms, hair net and gloves, scales and number rolls and shelves (**Table** 6-8).

When the effectiveness of detection of *L. fungicola* on NPDASM and on MRSM selective medium was compared there was no significant difference between the media, but when effectiveness of selective media and Real Time PCR after 45 cycles of detection of *L. fungicola* were tested, a significant difference was present (**Table** 6-8).

The very dirty samples contained lots of casing, such as those from the floor inside of growing room close to the door and pickers platform, and on a few occasions did not show a positive signal on Real Time PCR but colonies of *L. fungicola* were recorded on selective media. These kinds of samples are not good for Real Time PCR and the best way to test is with selective media. *Lecanicillium fungicola* was detected outside and in common areas where it can be moved around the farm (**Figure** 6-7).



* – only selective medium tested, n/a – not available

Figure 6-7: Percentage detection of *L. fungicola* using selective media and Real Time PCR after 45 cycles.

6.1.2.3 Summary

On selective media *L. fungicola* was not detected during spawn running, but the number of sample was small (8). Real Time PCR detected *L. fungicola* 4 times in spawn run samples. The samples collected for other stages of crops showed the presence of *L. fungicola*. The *L. fungicola* was frequently discovered on selective media on 2nd and 3rd flush, but the pathogen was also detected many times on 1st flush. By Real Time PCR *L. fungicola* was frequently discovered in 2nd and 3rd flush. *Lecanicillium fungicola* was also recorded on outside samples and 1st flush.

When data were analysed and organised by origin of samples *L. fungicola* was not detected on machines by either method, but in other places pathogen was detected by both methods (selective media and Real Time PCR). Samples containing some amount of casing (inside, floor close to the door, picker's platform, casing etc.) often gave positive results on selective media but negative results on Real Time PCR. The samples (flies, door handle, water equipment, crates, etc.) which did not contain polymerase inhibitors gave much better positive results on Real Time PCR than on selective media and this kind of sample is better to use with Real Time PCR.

The high level of positive detection by RT PCR could mean that it picks up dead and live material or maybe the primer and probe is not selective enough. If that is the case then they are not good for diagnostic of detection of *L. fungicola* on mushroom farms.

6.2 Discussion

The results presented here demonstrate graduation of different levels of hygiene of dry bubble disease on mushroom farms. The personal observation of hygiene (POH) did not have a correlation with mushroom growers' information (MGI). But most time where POH was high the MGI was 67-100%. In low and medium POH the MGI had a range 33-83 %. The POH and MGI did not show a correlation between detection of *L. fungicola* on selective media and Real Time PCR.

The selective media had some limitations such as ability to detect only live material which has enough energy for germination to give a colony on artificial media. The selective media were good for samples containing organic matter such as casing. That kind of sample was collected from inside growing room from floor, picker's platform, and casing. The differences between detection on NPDASM and MRSM were not significant and both media worked well but *L. fungicola* colonies were easier to find on NPDASM than on MRSM. *Lecanicillium fungicola* colonies on NPDASM had a white colour and were easy to locate and count compared to MRSM where colonies were transparent and difficult to find.

When comparing the two detection methods (selective media and Real Time PCR) the difference between methods was significant and Real Time PCR detected four times more *L. fungicola* than selective media. But Real Time PCR method also has some limitations and the main limitation is in sample preparation (DNA extraction) especially when the sample contain lots of different reaction inhibitors such as humic acids and heavy metals. The other restriction on the use of Real Time PCR is the need for expensive equipment and technical expertise. The difference between detection after 40 and after 45 cycles was also significant. Many samples showed a positive signal after 45 cycles compared with 40 cycles. This may be due to polymerase inhibitors in the samples. The amount of cycles for Real Time PCR does not have a standard but PCR starts with 2 DNA target molecules, during exponential amplification (2n) millions of molecules can be detected after 20-50 cycles (Landgraf, 2006). Some researchers using RT PCR techniques for detection of different microorganisms such as bacteria, fungi and protozoan parasite in a difficult samples such as blood and urine have used 45 cycles (Cuenca-Estrella *et al.*, 2009; Pascual *et al.*, 2010), or 50 cycles (Hardick *et al.*, 2003; Pounder *et al.*, 2007) but Fink *et al.* (1998) did RT PCR tests up to 60 cycles but polymerase failed at this point.

Lecanicillium fungicola was detected on selective media and Real Time PCR in all three flushes. The detection of *L. fungicola* in the 1st and 2nd flush is not a good signal for mushroom growers, because the mushroom grower must expect an outbreak of dry bubble disease in the 2nd and 3rd flush, which can have an impact on yield and economic losses. The *L. fungicola* was also recorded on other stages of mushroom cropping such as casing/at airing and growing room after disinfection which can suggest the *L. fungicola* is present in all crop stages in mushroom farm. For the crop stage – ''spawn running'' a small number of samples were collected but *L. fungicola* was detected by Real Time PCR. When *L. fungicola* is added to compost it does not cause dry bubble disease when the amount of conidia is low. When samples were collected in canteen area (canteen samples), the *L. fungicola* was detected almost at the same frequency as on 2nd flush. This suggests that the common area which is the canteen and equipment used by pickers contain *L. fungicola* material. An interesting result was the presence of the fungus on outside samples where *L. fungicola* was 80 % time's positive by Real Time PCR but on selective media the positive detection rate was only 17 %. Those relationships suggest that the outside environmental condition may terminate the *L. fungicola* live material which was not detected on selective media but by Real Time PCR. *Lecanicillium fungicola* was positively detected on 1 out of 10 samples on worker's sleeves and it can be proposed the human factor is a source of spreading of *L. fungicola* on mushroom farm.

Lecanicillium fungicola was detected almost in all places except machine samples but the number of collected machine samples was small (7). Other places showed positive detection of *L. fungicola* by both methods – selective media and Real Time PCR. The samples from floor inside a growing room, picker's platform and picker's equipment were a source of *L. fungicola* and these results confirmed Wong and Preece (1987). Rinker *et al.* (1993) also reported the presence of *L. fungicola* on debris collected from floor and structural surfaces. Another source of *L. fungicola* was also mushroom crates and door handles of growing room. The human factor is an important aspect of source and spreading of *L. fungicola* on mushroom farms and this has been discussed by many researchers previously (Fekete 1967; Cross and Jacobs 1969; Wong and Preece 1987).

Lecanicillium fungicola was more often detected by Real Time PCR than by selective medium. Level of hygiene did not have any correlation between presence of *L. fungicola* on Real Time PCR and selective medium. In conclusion these experiments illustrate the different effectiveness of detection of *L. fungicola* in mushroom farm

sample using two methods; selective media and Real Time PCR. On Real Time PCR *L. fungicola* was detected more often than on selective media but, Real Time PCR can detect live and dead material. Selective media can only detect live material.

The high level of *L. fungicola* detection by RT PCR at all crops stages is a cause for concern. It is unlikely that all RTPCR results reflect live *L. fungicola* therefore its use as a diagnostic tool on mushroom farms might be limited. The best locations for detection *L. fungicola* on selective media were: picker's equipment and accessories, growing room inside – floor close to the door and crates. The best places for detection of *L. fungicola* by RT PCR are clean samples or samples which do not contain a lot of casing soil, also crates, door handle, picker's accessories such as gloves and flies. The samples which contain casing could be also used for RT PCR but sometimes the results on this kind of sample were negative contrary to selective medium where *L. fungicola* was detected.

Both of the presented methods could be used for routine diagnostis of *L. fungicola* on mushroom farms. The selective medium is better for use on samples which contain lots of casing debris and other material. Selective medium is not an expensive method for detection of *L. fungicola* sources on mushroom farm, but this method required few days to show results, but this method detects only live material such as spores and mycelium which could start a disease outbreak. The Real Time PCR is a fast but expensive method for detection of *L. fungicola* from samples from mushroom farms. RT PCR is good for clean samples which do not contain lot of casing debris. This method required a high tech machines and a person who knows how to prepare and read the results. This technique is very sensitive and could also be used as a routine diagnostic test for detection of *L. fungicola* on mushroom farm but this method is able to detect live and dead material of *L. fungicola.* The dead material is not able to start dry bubble disease.

Chapter 7 General discussion

The filamentous fungus *Lecanicillium fungicola* is an important pathogen of the cultivated mushroom *Agaricus bisporus* and causes dry bubble disease (Van Zaayen and Gams, 1982). *Lecanicillium fungicola* is a significant mycoparasite which causes loss of yield in many mushroom farms where the white mushroom is cultivated. In this research, microbiological and molecular methods for the detection of *L. fungicola* on mushroom farms were developed. Methods to find possible sources of *L. fungicola* in mushroom farms were devised so that a more systematic method of control might be developed.

The aim of this study was to modify and design a selective medium for the detection of *L. fungicola* from complex samples which can be collected from different places on mushroom farms. The first information about a microbiological method for the detection of *L. fungicola* on mushroom farms was presented by Wong and Preece (1987) and Rinker *et al.* (1993), but only Rinker *et al.* (1993) designed a selective medium for *L. fungicola*. Rinker's medium was modified in this study and a novel selective medium was designed. Both of these media contain nutrients, dyes, fungicides and antibiotics. The dye which is used in both media is malachite green. This organic compound can be used as a dye or an anti-fungal. The anti-fungal specificity of malachite green was used in Rinker's selective medium (Rinker *et al.*, 1993), but high concentrations of malachite green inhibited the growth of *L. fungicola*. In the modified and novel selective medium the concentration of malachite green was decreased to allow for growth of *L. fungicola* and stop growth of competitive fungi such as *Mucor* sp. The second important element used in this selective medium are two fungicides: Sporgon (a.i. prochloraz-manganese) and KapChem (a.i. carbendazim). The sensitivity of *L. fungicola* to prochloraz-manganese and carbendazim was tested for Irish, Polish and Serbian isolates, isolated from diseased fruiting body of white mushrooms. All tested isolates were resistant to carbendazim but all tested isolates showed sensitivity to prochloraz-manganese where EC_{50} had a range 1.16 to 6.28 mg/l. For selective media the concentration of carbendazim did not have an impact on growth of *L. fungicola* and the 100 mg/l was chosen for subsequent work. The prochloraz-manganese had an effect on *L. fungicola* and the concentration for Irish, Polish and Serbian isolates was 1 mg/l. The other tested isolates (such as Canadian and American) had to be tested because they

were collected many years ago. The Irish, Polish, Serbian, Spanish, Canadian and USA isolates were also identified by the temperature test. The European isolates were identified as *Lecanicillium fungicola* var. *fungicola*, but North American isolates were identified as *Lecanicillium fungicola* var. *aleophilum*.

These selective media were used for the detection of *L. fungicola* on mushroom farms to find possible sources of *L. fungicola* and to help measure and manage dry bubble disease in mushroom farms.

The second task of this study was to investigate the DNA extraction method and evaluation of PCR reaction for the detection *L. fungicola* in samples originating from mushroom farms. DNA extraction from clean culture of *L. fungicola* has been demonstrated by many *L. fungicola* researchers (Bonnen and Hopkins, 1997; Bidochka *et al.*, 1999 b; Collopy *et al.*, 2001 and 2010; Amey *et al.*, 2002, Juarez del Carmen *et al.*, 2002; Romain *et al.*, 2002; Largeteau *et al.*, 2004 and 2007; Zare and Gams, 2008). The DNA extraction from pure culture of *L. fungicola* was not found difficult in this study. The aim was to evaluate four different DNA extraction methods and all methods gave good high molecular quality genomic DNA for PCR reaction. Some of the methods required small modification to get DNA free from RNA. The optimum method used was ZR Fungal/Bacterial kit which is a less time-consuming and less technically demanding DNA extraction kit, next is a manual method described by Aljanabi and Martinez (1997) which required a RNase A during extraction but this method is relatively inexpensive but it is time consuming and uses toxic reagents. Third and fourth DNA extraction methods were a Nucleon Phytopure Genomic DNA Extraction Kit and DNeasy Plant Mini Kit, these methods required grinding of samples in liquid nitrogen for extraction. The Nucleon Phytopure Genomic DNA Extraction Kit is a chloroform DNA extraction method whereas DNeasy Plant Mini Kit is a typical column DNA extraction method. Both these kits were time consuming and equipment demanding.

The problem with DNA extraction of *L. fungicola* starts when *L. fungicola* material (conidia) was mixed with soil and casing soil and DNA was extracted. The DNA extraction from soil must eliminate interfering substances contained in soil and casing soil (Tebbe and Vahjen, 1993; Tsai and Olson, 1992; Wilson *et al.*, 1990; Wilson, 1997). In this study four DNA extractions method were compared, only two gave good high molecular weight quality DNA suitable for PCR reaction. These methods were manual method of Yeates *et al.* (1998) and commercial DNA extraction kit ZR Fungal/Bacterial; other two kits did not give good results for DNA extraction from soil and casing samples. The Yeates *et al.* (1998) and ZR Fungal/Bacterial DNA

extraction kits were able to detect *L. fungicola* DNA in PCR when suspension of *L. fungicola* used for extraction was 1.94×10^7 conidia per 200 µl mixed with 0.26-0.28 gram soil. Positive amplification product was seen when the amount of conidia used for DNA isolation was 10^6 conidia/g casing. The positive amplification product was seen when amount of DNA template per PCR reaction was 1 µl or was diluted 1/20 or 1/10 times and the volume used for PCR reaction was 1 µl.

After DNA extraction the important task was to find selective primers for *L. fungicola* detection from dirty samples. The first researchers to design selective primers for detection of *L. fungicola* from affected *A. bisporus* tissue were Romaine *et al.* (2000), but this set of primers is able only to detect *L. fungicola* var. *aleophilum* giving a 162 bp amplicon. The Romaine *et al.* (2000) set of primers does not amplify *L. fungicola* var. *fungicola* which is a pathogen of white mushroom in Ireland, UK and continental Europe. The second researchers who designed selective primers for *L. fungicola* from affected *A. bisporus* were Largeteau *et al.* (2007). Their primers for detection of *L. fungicola* var. *fungicola* also amplified *A. bisporus* giving this same size amplicon as *L. fungicola* DNA. The first set of primers and probe for detection of *L. fungicola* from casing samples was designed by Zijlstra *et al.* (2007, 2008 and 2009). They designed a probe which is able to detect only *L. fungicola*, but this set of primers amplified many non-specific products from casing soil and from other fungi.

The next task of this study was to design selective primers for PCR assay for detection of *L. fungicola* from samples collected from mushroom farms. Primers were designed for a mating type (MAT1-2-1) locus and rDNA sequences from *L. fungicola*. The MAT locus has been identified in a number of filamentous fungi and fits in to fungal groups that are widely separated in evolutionary terms (Varga, 2003). This locus was identified in *L. fungicola* by Yokoyama *et al.* (2004 and 2006) and the sizes of sequences have around ≈ 210 bp. The designed primers were from MAT locus – MAT1-2-1 region of *L. fungicola* RNA. The identification of only *L. fungicola* var. *fungicola* using MAT1-2-1 was not possible as the designed primers also amplified *L. fungicola* var. *aleophilum*, but some sets of primers were capable of detecting *L. fungicola* from infected *A. bisporus* tissue giving a different size of amplicon of *L. fungicola* than *A. bisporus*. The rDNA region is an attractive target for PCR-based *L. fungicola* detection methods. The multicopy nature of rDNA in fungal genomes is an ideal target for PCR reactions which should increase the sensitivity at which these fungi can be detected (Garber *et al.*, 1988; Howlett *et al.*, 1997). The number of rDNA copies in the *L. fungicola* genome is unknown but reported analyses of several other fungal

species have given values range from ≈ 50 to > 200 (Clare *et al.*, 1986; Garber *et al.*, 1988; Howlett *et al.*, 1997). In this study the selected target for PCR primers was rDNA region of *L. fungicola* which was sequenced and has around ≈ 2300 bp. The primer designed for the rDNA region was more conserved and all designed primers for *L. fungicola* detection also amplified this same size amplicon for other ascomycetes fungi. For future work it would be better to use the MAT1-2-1 region to design specific primers for detection of *L. fungicola* only. This region is very promising for future work for detection of *L. fungicola* from contaminated samples.

The design of selective primers of *L. fungicola* for PCR is incomplete. Primers and probe designed by Zijlstra *et al.* (2007, 2008 and 2009) work well and this set of primers and probe was used for detection of *L. fungicola* from mushroom farm samples. The first task was to develop a method for Real Time PCR. It is a new technique which requires high-tech equipment and experience. The Real Time PCR was performed on LightCycle 480 – Roche machine following method of Zijlstra *et al.* (2007, 2008 and 2009), Real Time PCR protocol. The amount of reagents and reaction conditions were suitable and amplification curve gave a good value of slope, efficiency, Y-intercept, error and correlation coefficient according to the LightCycler® 480 instrument Roche – Operator's Manual and Invitrogen – Real Time PCR: from Theory to Practice, when pure *L. fungicola* DNA was diluted 10-fold. The primers and probe for Real Time PCR was also tested for specificity against Irish casing extract and soil from ground and the signal was negative this mean that the probe was specific only for *L. fungicola* DNA. After those different concentrations of *L. fungicola* conidia were mixed with casing extract and isolated following Dutch information about DNA extraction. Positive FAM signal was recorded when the amount of conidia mixed with casing extract was 10 conidia/ml casing extract, but reliable detection was when conidia concentrations were 10^3-10^4 conidia per ml casing extract and higher (Selma *et al.*, 2008 and Zijlstra *et al.*, 2007, 2008 and 2009). Very similar detection was recorded when different conidia concentrations of *L. fungicola* were mixed with casing extract and water and DNA was extracted. The results of Real Time PCR were not affected differently by water samples than casing extract sample. The detection level in both samples was 10 conidia per ml casing extract or water, but reliable results were obtained when conidia concentrations were higher than 10^4 conidia/ml casing extract or water. The next part of this section was to compare two DNA extraction kits. The samples contained different conidia concentrations mixed with casing extract. Both tested kits worked well and detection level was 5 conidia per ml casing extract, but reliable results were obtained when

conidia concentrations were 10^2 and higher per ml casing extract when sample was isolated by QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA isolation kits (QuickGene). The Wizard Magnetic DNA Purification System for Food gave also good results but reliable results were only obtained with conidia concentrations of 10^4 and higher. The DNA extraction and purification methods had a significant influence on quality and quantity of DNA. Good DNA extraction method improved the accuracy of Real Time PCR results. The comparison of DNA extraction methods and kits for use with difficult samples is described by many researchers (Pinto *et al.*, 2005; Demeke *et al.*, 2009; Mori *et al.*, 2007). The second important thing for good accuracy of Real Time PCR is an effective polymerase. Polymerase plays a crucial role in PCR reaction especially when DNA template contains potential inhibitors. In this study three polymerases from two different producers was compared. All three polymerases gave similar sensitivity on samples containing different conidia concentrations of *L. fungicola* mixed with casing and DNA extracted by QuickGene Mini 80 device and QuickGene DNA tissue DT-S DNA isolation kits (QuickGene) and Wizard Magnetic DNA Purification System for Food. The Real Time PCR technique is a very sensitive test which requires good equipment, reagents and experience in preparation.

Finally, the results presented in Chapter 6 demonstrate results of experiments for testing unknown samples collected from mushroom farms. At this time only a few researchers using microbiological methods have tested the sources and spreading of *L. fungicola* on mushroom farms (Cross and Jacobs, 1969; Wong and Preece 1987; Nair and Macauley, 1987). Rinker *et al.* (1993) designed a selective medium for detection of *L. fungicola* from mushroom farm samples. In this study a survey of mushroom farms was conducted to collect samples and analyse samples using microbiological diagnostic tests – selective media, and molecular method Real Time PCR to search for the presence of *L. fungicola* in the collected samples. The samples were collected from 2008 to 2010 during 18 visits to mushroom farms with different levels of hygiene and dry bubble disease. The samples were tested on selective media (NPDASM and MRSM) and by Real Time PCR (after 45) using primers and probes designed by Zijlstra *et al.* (2007, 2008 and 2009). Neither selective medium showed significant differences for detection of *L. fungicola* and both media worked well. Significant differences were recorded when detection of *L. fungicola* on selective media and by Real Time PCR were compared. Usually Real Time PCR detected *L. fungicola* 4 with times more sensitivity than selective media, but in some samples containing casing soil the selective medium

had better results than Real Time PCR; this may have been caused by polymerase inhibitors. The Real Time PCR gave good results for clean samples without polymerase inhibitors. Using 45 cycles 30 % more positive samples were obtained compared to 40 cycles. This may have been due to contaminations or polymerase inhibitors in samples. That number of cycles is popular (Cuenca-Estrella *et al.*, 2009 and Pascual *et al.*, 2010). In this study the level of hygiene and dry bubble present on mushroom farms was graduated by POH and MGI, but detection on selective media and Real Time PCR correspond for the POH hygiene scale contrary to MGI scale. The obtained results indicated that there was a weak correlation between POH and MGI it would be necessary to look at gathering more information to quantify the level of hygiene on mushroom farms.

The selective media had some limitations such as ability to detect only live material which has enough energy for germination to give a colony on artificial media. The selective media were good for samples containing organic matter such as casing. That kind of sample was collected from inside growing room from floor, picker's platform, and casing. The differences between detection on NPDASM and MRSM were not significant and both media worked well but *L. fungicola* colonies were easier to find on NPDASM than on MRSM. *Lecanicillium fungicola* colonies on NPDASM had a white colour and were easy to locate and count compared to MRSM where colonies were transparent and difficult to find.

When comparing the two detection methods (selective media and Real Time PCR) the difference between methods was significant and Real Time PCR detected four times more *L. fungicola* than selective media. Real Time PCR method also has some limitations and the main limitation is in sample preparation (DNA extraction) especially when the sample contain lots of different reaction inhibitors such as humic acids and heavy metals. The other restriction on the use of Real Time PCR is the need for expensive equipment and technical expertise. The difference between detection after 40 and after 45 cycles was also significant. Many samples showed a positive signal after 45 cycles compared with 40 cycles. This may be due to polymerase inhibitors in the samples. This may be because the Real Time PCR recorded a signal from dead material of *L. fungicola* giving a positive signal.

Analysis of different crop cycles and other locations found that *L. fungicola* was most often detected by both methods in 2nd and 3rd flush. The interesting sources of *L. fungicola* were canteen samples and outside samples. The presence of *L. fungicola* in 1st flush is not a good signal for mushroom growers because it can lead to an outbreak of dry bubble disease on flush 2nd and 3rd flush of course depending to for an environmental conditions. Samples collected from mushroom farms machines did not show *L. fungicola*. On selective media and Real Time PCR *L. fungicola* was detected on samples from floor inside growing room, picker's platform and picker's equipment and these results confirm the results of Wong and Preece (1987). The debris from floor and from structural surface were also a source of *L. fungicola* which was also found by Rinker *et al.* (1993). The interesting information on spread and source of *L. fungicola* is the positive detection of this mycoparasite on crates and door handle of growing room. The staff who work on mushroom farms were also a source of *L. fungicola* but it was difficult to collect samples from staff and few researchers identify staff as an important factor for spreading and as a source of *L. fungicola* on mushroom farms (Fekete 1967; Cross and Jacobs 1969; Wong and Preece 1987).

The future work for detection and measuring of dry bubble disease caused by *L. fungicola* on mushroom farms could generate more selective primers for PCR. The second task could to be profile secondary metabolites of *L. fungicola*. This profile of secondary metabolites could help to detect and identify *L. fungicola* from samples on mushroom farms using HPLC or/and GS connected to Mass Spectrometry. The first information about profiles of secondary metabolites of *L. fungicola* is introduced by Farrag *et al.* (2009).

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