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Importance of microbial pest control agents and their metabolites In relation to the natural microbiota on strawberry

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Content

Preface

The research presented in this report on the importance of applied microbial pest control agents (MPCAs) and their metabolites as compared to the natural microbiota on strawberry was carried out from February 2006 to February 2010.

Institutions involved in the project were: University of Copenhagen, Department of Plant Biology and Biotechnology, Aarhus University, Department of Agroecology and Technical University of Denmark, Department of Systems Biology.

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Finally, we thank the members of the steering committee and the chairman Jørn Kirkegaard for their constructive and positive involvement through out the project period. Especially we want to thank the referees: Carsten Suhr Jacobsen (GEUS), Anne Mette Madsen (National Reseach Center for the Working Environment), Michael Nielsen (Knowledge Center for Agriculture) and Louise Grave Larsen (Danish Ministry of the Environment) for their valuable comments during the writing process of this report.

Summary

The main objectives of the this project were to examine the abundance of applied microbial pest control agents (MPCAs) and their metabolites compared to that of the natural microbiota and to examine the compatibility between MPCAs and conventional fungicides and their combination effects in disease control.

A series of laboratory, growth chamber, semi-field and field experiments using strawberry as a model plant focusing on commercial microbial pest control products (MPCPs) or laboratory MPCAs expected to be on the market within 10 years served as our experimental platform.

Initially the background level of indigenous microbial communities and their mycotoxins/metabolites on strawberries was examined in a field survey with 4 conventional and 4 organic growers with different production practise and geographic distribution. Culturable bacteria, yeasts and filamentous fungi were isolated and identified using both chemotaxonomy (fatty acids and metabolite profiling) and morphological characteristics.

Microbial communities on strawberries were complex including potential plant pathogens, opportunistic human pathogens, plant disease biocontrol agents and mycotoxin producers. Bacteria were the most abundant and diverse group of strawberry microbiota followed by yeasts and filamentous fungi. Grower practice did not seem to correlate with the strawberry microbiota. Limited difference between microbial communities on strawberries from conventional and organic production systems was observed.

Mycotoxins were not detected in strawberries from any of the 8 different growers covering both fresh and worst-case (semi-rotted) berries. However, filamentous fungi from the genera ***Penicillium*** and ***Aspergillus*** isolated from the field survey produced high amounts of carcinogenic mycotoxins when applied to strawberries ***in vitro***.

A broad range of microbial biocontrol agents (MPCAs) including the commercial ***Trichoderma*** based products TRI003, Binab-T and Supresivit and the laboratory MPCAs ***Clonostachys rosea*** and ***Ulocladium atrum*** were examined concerning their target and non-target effects, production of mycotoxins, fungicide sensitivity and performance (establishment, growth and survival).

Among the MPCAs tested only the laboratory MPCAs ***C. rosea*** and ***U. atrum*** demonstrated biocontrol effects against the strawberry pathogen ***Botrytis cinerea*** and the background microbial community was unaffected by both ***C. rosea*** and ***U. atrum***.

None of the fungal MPCAs produced any mycotoxins when applied to flowers in semi-field and field experiments, but strawberries artificially inoculated with ***Trichoderma***-based MPCPs ***in vitro*** contained biologically active fungal metabolites of the peptaibol family.

In general, fungicides employed in conventional strawberry production had no fungicidal effects on any of the MPCAs. Establishment of ***Trichoderma*** (TRI003) on berries following flower application was poor and applying fungicides prior to the MPCA application did not result in improved MPCA performance.

In conclusion, the relative importance of deliberately released fungal MPCAs and their metabolites in relation to that of the natural strawberry microbiota seem to be limited when considering the potential risk from the natural microbiota including mycotoxin producers and opportunistic human pathogens. In addition, our results suggest that it is possible to combine MPCAs and fungicides in an integrated strategy, potentially reducing the levels of fungicide applications.

Sammenfatning

Hovedformålet med projektet var at undersøge betydningen af mikrobiologiske bekæmpelsesorganismer (MBO) og deres metabolitter i forhold til den naturlige mikrobiota og at undersøge foreneligheden af MBO og fungicider samt deres kombinationseffekter overfor plantesygdomme.

Projektets eksperimentelle platform bestod af en lang række forsøg i laboratorium, vækstkammer, semifield og mark med jordbær som modelafgrøde og fokus på kommercielle mikrobiologiske bekæmpelsesmidler (MBM) eller laboratorium MBO, der forventes markedsført indenfor de næste 10 år.

Indledningsvis blev baggrundsmikrofloraen og deres mykotoksiner/metabolitter på jordbær undersøgt under markforhold hos 4 konventionelle og 4 økologiske jordbæravlere med forskellig dyrkningspraksis og geografisk beliggenhed. Dyrkbare bakterier, gærsvampe og filamenetøse svampe blev isoleret og identificeret med både kemotaksonomiske (fedtsyrer- og metabolitprofiler) og morfologiske karakteristika.

Samfund af mikroorganismer på jordbær var kompleks omfattende potentielle plantepatogener, opportunistiske humanpatogener, MBO og mykotoksin dannere. Bakterier var den mest mangfoldige og diverse gruppe i jordbærmikrobiotaen efterfulgt af gær og filamentøse svampe. Der syntes ikke at være sammenhæng mellem dyrkningspraksis og jordbærmikrobiotaen, og forskelle i jordbærmikrobiotaen mellem konventionelle og økologiske avlere var begrænset.

Mykotoksiner blev ikke detekteret hos nogen af de 8 forskellige avlere omfattende både friske, sunde og halvrådne (værste situation) bær. Dog producerede filamentøse svampe fra slægten ***Penicillium*** og ***Aspergillus***, isoleret fra markforsøget, store mængder af kræftfremkaldende mykotoksiner, når de blev inokuleret på jordbær under ***in vitro*** forhold.

En bred vifte af MBO omfattende kommercielle ***Trichoderma***-baserede MBM (TRI003, Binab-T og Supresivit) og laboratorium MBO (***Clonostachys rosea*** and ***Ulocladium atrum***), blev testet for deres mål og ikke-mål effekter, dannelse af mykotoksin, fungicid følsomhed og udvikling (etablering, vækst og overlevelse).

Blandt de testede MBO var kun ***C. rosea*** og ***U. atrum*** effektive til at bekæmpe jordbærpatogenet ***Botrytis cinerea***, og baggrundsmikrofloraen var ikke påvirket af hverken ***C. rosea*** eller ***U. atrum.*** Ingen af de testede MBO producerede mykotoksiner efter applikation til blomster/grønne bær i semifield eller markforsøg, men jordbær kunstigt inokuleret med ***Trichoderma***-baserede MBM ***in vitro*** indeholdt biologisk aktive svampemetabolitter fra peptaibol familien.

Generelt havde fungicider, der anvendes i konventionel jordbærdyrkning ingen virkning på de testede MBO. Etablering og vækst af ***Trichoderma*** (TRI003) på jordbærblomster med efterfølgende overlevelse på bær var

begrænset, og applikation af fungicider før applikation af MBM gav ikke som forventet en øget etablering af MBM.

Projektets konklusion er, at betydningen af MBO og deres metabolitter er begrænset i forhold til den naturlige baggrundsmikroflora på jordbær, når potentielle mykotoksin dannere og opportunistiske humanpatogener tages i betragtning. Derudover viser vores resultater, at det er muligt at kombinere MBO og fungicider i en integreret strategi til plantebeskyttelse, der potentielt vil kunne reducere anvendelsen af fungicider.

1 Introduction

1.1 Objectives

The overall motivation of this project was to generate information useful in risk assessment of microbial pest control agents (MPCAs) and in developing integrated disease control measures (MPCAs + fungicides). Main objectives were (1) to examine the relative abundance of applied MPCAs and their metabolites compared to the natural microbiota and (2) to examine the compatibility between MPCAs and conventional fungicides and their combination effects in disease control. A series of lab, growth chamber, semi-field and field experiments using strawberry as a model plant focusing on MPCAs formulated into microbial pest control products (MPCPs) or laboratory MPCAs expected to be on the market within 10 years served as our experimental platform examining:

- Background level of microbial communities and mycotoxins/metabolites on strawberries from a field survey
- Fate, biocontrol efficacy and non-target effects of MPCAs and their interactions with naturally occurring fungi
- ***In vivo*** production of metabolites by naturally occurring fungi and MPCAs under suboptimal storage condition
- Fungicide effects on MPCAs and background microbial communities

1.2 Plant disease control by MPCAs

Biological control of plant diseases based on pathogen antagonists is an important alternative to conventional disease control with pesticides (Cook and Baker, 1983; Andrews, 1992; Kvistgård, 1994; Green, 2001; Jensen ***et al.***, 2008; Sharma ***et al***., 2009). In most cases biological plant disease control relies on prophylactic applications of the antagonists preventing the pathogen to develop and infect the plant, but disease control may also be achieved through conservation of populations of indigenous microbial antagonists. Microbial antagonists can be applied as a seed-coat, foliar spray, mixed into the growth substrate or applied by vectors such as pollination insects.

Several commercial microbial antagonists are available, but only few have been registered for use in plant disease protection (Table 1). Five microbial biocontrol products have been included in Annex I of Directive 91/414/EEC and thereby obtained registration in EU. In Denmark, presently eight MPCPs are marketed of which five are based on ***Trichoderma*** ssp. (Table 1).

MPCAs as plant disease control measures are used mainly in the horticultural sector especially against root diseases except the MPCPs Cedomon and Contans WG, which are also marketed for the agricultural sector focusing on cereals and oil seed rape, respectively. In other European countries AQ10 is marketed for control of mildew, which is an important foliar disease in several crops. Concerning foliar disease control in Denmark different ***Trichoderma*** products are used to some extent against grey mould in ***e.g***. strawberries.

Still the practical use of MPCAs in plant disease control is limited, but MPCAs are expected to play a stronger role in future plant disease control strategies as an important component in IPM protocols aiming at reducing the use of fungicides in plant production systems.

Table 1. Microbial pest control agents (MPCAs) on EU Annex 1, MPCAs marketed in DK and selected laboratory MPCA isolates. Information on their mode of action is given.

Organism	Product	EU	DK	Mode of action
Bacteria				
Streptomyces griseoviridis[1)]	Mycostop		+	Antibiosis, competition, induction of plant defense
Pseudomonas chlororaphis	Cedomon	+	+	
Bacillus subtilis	Serenade	+		
Fungi				
Ampelomyces quisqualis	AQ10	+		Competition, parasitism
Coniothyrium minitans	Contans	+	+	Parasitism
Trichoderma harzianum[1]	TRI002		+	Competition, antibiosis, parasitism, cell wall degrading enzymes (CWDE), induction of plant defense
	TRI003		+	
	Supresivit		+	
	Aperto		+	
T.harzianum+T. polysporum	Binab T		+	
Clonostachys rosea f. catenulata	Prestop	+		
C. rosea	Laboratory[2]			See above
Ulocladium atrum	Laboratory[3]			Competition

[1)] Notification for inclusion in Annex I inclusion submitted in November 2005
[2)] Laboratory isolate developed by Birgit Jensen *et al.*, KU-Life
[3)] Laboratory isolate developed by David Yohalem *et al.*, ÁU-DJF

MPCPs based on yeasts and bacteria have been developed to manage foliar and post harvest diseases such as Aspire based on ***Candida oleophila.*** The hyperparasitic yeast ***Pseudozyma flocculosa*** marketed as Sporodex has been registered in the US on the EPA list for control of mildew in roses and cucumber:
www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/brad_119196.pdf

Different modes of action of MPCAs have been suggested including competition for nutrient and space, antibiosis, parasitism and induction of host plant defence system. For ***Trichoderma***-based MPCPs most likely a combination of different mode of actions are involved in their biocontrol activity (Table 1).

Besides the commercial MPCPs many laboratory MPCAs have been identified of which the saprotrophic fungi ***Clonostachys rosea*** (Jensen, 1999) and ***Ulocladium atrum*** (Yohalem, 2004) have shown strong plant disease biocontrol efficacy in a broad range of crops.

The biocontrol mode of action of ***C. rosea*** seems mainly to be achieved through nutrient competition, mycoparasitsm involving cell wall degrading enzymes (CWDEs) and antibiosis (Mamarabadi ***et al.***, 2008; Sutton ***et al.,*** 1997). ***U. atrum*** is more specifically active against ***Botrytis cinerea*** the causal agent of grey mold, which is a serious disease in many horticultural crops. Competition for nutrients has been found to be the biocontrol mode of action of ***U. atrum***. Pre-emptive niche exclusion through competition for nutrients in terms of plant debris seems to play an important role in the biocontrol effects of ***U. atrum*** against ***B. cinerea*** (Table 1).

1.3 Phyllopshere microbiology

Plants are hosting a broad range of epiphytic and endophytic microorganisms both fungi and bacteria, which play an important role in their growth, health and fitness. Most plant inhabiting microorganisms are non-pathogenic with beneficial traits, but plants also host pathogenic and/or parasitic microorganisms (Kinkel, 1997; Andrews and Harris, 2000; Lindow and Brandl, 2003). The phyllosphere is an extreme environment with limited nutrients available, UV radiation and highly variable temperature and humidity conditions. The natural phyllosphere microbiota is dominated by pigmented bacteria and yeasts (Lindow and Brandl, 2003).

1.4 Methods to study phyllopshere microbiology

Studies of microbial ecology involve a broad range of methods both classical, biochemical and molecular. Classical methods focus on microbial population density using selective and/or semi-selective media and subsequently isolates can be identified employing morphological, biochemical and/or molecular tools.

Fatty acid based methods are useful for identification of culturable bacteria and yeasts and also in examination of microbial communities both in qualitative and quantitative terms.

Employing molecular culture independent methods in phyllosphere microbiology has revealed a higher microbial diversity, than found when using classical culture dependent methods (Yang ***et al.,*** 2001).

Secondary metabolites from MPCAs and/or indigenous microorganisms can be identified and quantified using different chromatography methods both liquid (LC) and gas (GC) based coupled with mass spectrometer (MS) and chemical ID libraries and/or comparison with known standards.

Monitoring of MPCAs released to the plant-soil environment can be achieved by employing specific molecular PCR based tools (Lübeck ***et al.,*** 2002; Jensen ***et al.,*** 2004) and/or using genetically transformed isolates of the MPCAs with fluorescent reporters such as gfp and DsRed, which combined with laser confocal microscopy can be used in detailed studies of microbial growth and interaction (Lübeck ***et al.,*** 2002; Mikkelsen ***et al.,*** 2003; Jensen and Schulz, 2004).

1.5 Strawberry production and disease management

Strawberry is an important horticultural crop in Denmark with conventional and organic production in both open field and tunnels. In 2004 the total Danish strawberry production was 4500 t (Danmarks Statistik). Pesticide use in conventional strawberry is high with a pesticide treatment frequency value on 5.9 (Ørum and Christensen, 2001), of which fungicides (Euparen, Teldor, Signum etc.) contribute substantially with 3-6 applications during flowering mainly against grey mould caused by the pathogen ***B. cinerea***. This high level of pesticide application may result in undesired pesticide residues in the strawberries. Pesticide residues were found in more than half of the nurseries included in a survey (>12) in the period 2003-2007, but all below the allowed

maximum residue value (Fødevarestyrelsen, 2007, gl.foedevarestyrelsen.dk/FDir/Publications/2008004/Rapport.pdf).

Strawberry was chosen as a model plant in the present project due to the above-mentioned importance and relevance, but also because strawberries are consumed fresh and hence represent a worst case scenario if application of MPCAs result in increased levels of microbial toxins.

Use of MPCPs in the Danish horticultural sector is overall limited, but in strawberry production, where post harvest diseases are also common, MPCPs are used to some extent, especially ***Trichoderma***-based products. Lindhardt ***et al.*** (2003) suggest that the fungi ***C. rosea*** (synonym ***Gliocladium roseum***) (Peng ***et al.***, 1992; Sutton ***et al.***, 1997) and ***U. atrum*** (Boff ***et al.***, 2002) represent potential efficient MPCAs against grey mold in strawberry. Both ***U. atrum*** (Yohalem, 2004; Yohalem and Kristensen, 2004; Yohalem ***et al.***, 2007; Møller ***et al***., 2009) and ***C. rosea*** (Knudsen ***et al.***, 1995; Knudsen ***et al.,*** 2004; Jensen ***et al.,*** 2002; Jensen ***et al.,*** 2004; Møller, 1999; Møller ***et al.,*** 2003) have been found to possess strong biocontrol affects in several crops both in field and greenhouse production.

1.6 IPM

An integrated strategy combining MPCAs and fungicides can facilitate a reduction in fungicide use. Both additive and synergistic effects on disease control have been reported when combining MPCAs and compatible fungicides (Harman ***et al.***, 1996; Buck, 2004; Van den Boogert and Luttikholt, 2004) suggesting that integrated disease control may offer an alternative to full dose fungicide application. The underlying mechanism for such combination effects may relate to an improved establishment of the MPCA when their natural competitors have been adversely affected by the fungicide application. However, it is important to also consider non-target effects when combining MPCAs and fungicides in relation to the indigenous microbial community and their toxin production.

1.7 The natural strawberry microbiota and their secondary metabolites

In general, fruit and vegetables are exposed to microrganims (Jensen, 1998). However, microorganisms developed for biocontrol of foliar diseases such as ***Trichoderma*** are not common phyllosphere inhabitants (McLean and Sutton, 1992; Elad and Kirshner, 1993; Latorre ***et al.***, 1997) and for ***Trichoderma*** strains applied to leaves and flowers, populations of ***Trichoderma*** spp. decrease by a factor 10-100 within a 2 weeks period (Jensen, 1998; Freeman ***et al.***, 2004). However, the knowledge on post harvest level of applied MPCAs is missing.

The natural community of filamentous fungi on mature strawberries is dominated by ***B. cinerea***, ***Penicillium*** spp., ***Alternaria*** spp., ***Cladosporium*** spp. ***Rhizopus*** spp., ***Aureobasidium pullulans*** and yeasts dominated by ***Cryptoococcus*** spp. (Dennis, 1976; Tronsmo, 1986; Weidenboerner ***et al.***, 1995; Aziz and Moussa, 2002; Tournas and Katsoudas, 2005). Bacterial populations on strawberry plants are dominated by ***Psuedomonas***, ***Stenotrophomas***, ***Bacillus*** and ***Arthrobacter*** (Krimm ***et al.***, 2005). In total the population density of filamentous fungi and yeasts have been shown to be 10^5-10^6 cfu/g and 10^6-10^7

cfu/g berry, respectively in the above-mentioned studies. Danish studies of the microbial community on strawberries are not avaible.

Among the fungi isolated from strawberries ***Penicillium, Rhizopus*** and ***Alternaria*** are known to be potential mycotoxin producers (Samson ***et al.,*** 2002; Frisvad and Thrane, 2002; Jennessen ***et al.***, 2005; Andersen ***et al.***, 2004; Christensen ***et al***., 2005; Andersen ***et al.***, 2002). Few studies have examined the mycotoxin production in strawberry, but Aziz and Moussa (2002) have detected penicillic acid, patulin and cyclopiazionic acid in several marketed strawberries. MPCAs such as ***Trichoderma*** are known to produce several biologically active compounds including peptaibols, pyrons, iso-nitrils and other secondary metabolites. Among these compounds gliotoxin, trichodermin and harzianum A are known as mycotoxins (Frisvad and Thrane, 2002; Nielsen ***et al.***, 2005). Metabolite profiles of ***Ulocladium*** species have been described by Andersen and Hollensted (2008).

It is important to examine the influence of applied MPCAs on the residue levels of mycotoxins in foodstuff for consumption such as strawberries both in terms of their own contribution and their effects on the mycotoxin production of the natural microbiota. Mycotoxins produced during postharvest disease development may be controlled by MPCAs such as ***Trichoderma*** and ***Clonostachys*** (Cooney ***et al.***, 2001; Takahashi-Ando ***et al.***, 2002).

1.8 Risk assessment of MPCAs

As for the pesticides it is also crucial to make proper risk assessment analyses of MPCAs before their practical applications (Folker-Hansen ***et al***., 1993; Lübeck, 1994; Jensen, 1998). However, data on background and natural exposure levels of MPCAs or potential MPCAs are often not available. Such data will help to estimate the risk caused by an artificial application of MPCAs to the environment improving the possibilities for safety implementation of MPCPs.

Possible non-target effects of MPCAs include adverse effects on human health in relation to food poisoning from toxic microbial secondary metabolites, allergy and lung diseases imposed on the gardener applying the MPCPs and effects on the indigenous microbiota and their grazers.

Non-target studies of MPCAs on the indigenous microbiota are few (reviewed by Brimner and Boland, 2003), but recently the topic has received more attention both in the rhizosphere (Johansen ***et al***., 2004; Scherwinski ***et al***., 2007; Cordier and Alabouvette, 2009; Corrrea ***et al***., 2009) and in the phyllosphere (Zhang ***et al***., 2008). In general, most of the above-mentioned studies showed limited non-target effects.

2 Materials and methods

2.1 Naturally occurring microbiota on strawberry

2.1.1 Agar media for microbiota isolation and identification

Fungi: For CFU counts and isolation of fungi the following substrates were used: Dichloran 18% Glycerol agar (DG18), V8-juice agar with antibiotics (V8), Spezieller Nährstoffarmer Agar with antibiotics (SNA), Oat Meal agar (OM) and Dichloran Rose bengal Yeast Extract Sucrose agar (DRYES) were prepared according to (Samson ***et al.***, 2002). Chloramphenicol (0.05%), chlortetracycline (0.05%) and Novobiocin (0.05%) were used as antibiotics. Glucose media, Rose Bengal agar (GM-RB) and Glucose media Triton-X (GM-T) were prepared according to Inacio ***et al.*** (2002). For identification isolates were cultured on V8, SNA or Potato Carrot agar (PCA) (Samson ***et al.***, 2002). PDA (Scharlau Microbiology) was amended with 0.22% Triton-X (PDA-T) and antibiotics. SNA, PDA-C, PDA-T, GM-T and V8 plates were incubated for 7 days at 21-23ºC under Black Light Blue (Phillips TLD 36W/08) and Cool Daylight (Phillips TLD 36W/95) tubes, 12 hrs light-12 hrs dark. OM and PCA plates were incubated for 7 days at 21-23ºC under Cool Daylight (Phillips TLD 36W/95) tubes (8 hrs light-16 hrs dark) and DG18, DRYES and GM-RB plates were incubated 7 days at 25ºC in the dark.

Yeast: For CFU counts and isolation of yeast, Saboraud Dextrose Broth Agar (SDBA) was used.Plates for bacteria and yeast isolation were incubated for three and two days, respectively, at 20-23ºC in darkness.

Bacteria: For CFU counts and isolation of bacteria the following substrates were used: Tryptic Soy Broth Agar (TSBA) and Nutrient Agar (NA).

For sample wash, homogenization and 10-fold dilutions autoclaved Milli-Q (Millipore, Bedford, MA) purified water with 0.1% Triton-X (Sigma-Aldrich, St. Louis, MO) was used.

2.1.2 Initial screening experiment

The twelve semi-selective solid media listed in Table 2 were used for quantification of filamentous fungi, yeast and bacteria on strawberry in order to select the best media and dilutions for the survey. Berries of the cultivar Florence were collected from an organic grower (Grower 5, see Table 3) on the 26th June 2006 a few hours after heavy rain fall. Four samples (A,B,C,D) each consisting of about 200 g berries was mixed with 400 ml sterile water (0.1% Triton-X) in a plastic bag. Calyx was removed from the berries before washing. The bag was placed in an ultrasonic bath (Branson 3210, Danbury, CT) for 20 minutes. The isolation procedure is summarized in figure 1.

Table 2. Media and incubation conditions tested in a pre-experiment in order to select the best media for quantification of the strawberry microbiota.

Tested media	Abbr.	Target organisms	Incubation
PDA + Triton-X+ Ca[1)]	PDA-C	Fungi, yeast	21-23˚C, 12 h light
PDA + Triton-X+ Novobiocin	PDA-N	Fungi, yeast	21-23˚C, 12 h light
Glucose media + rose bengal + Ca	GM-RB	Fungi, yeast	25˚C, darkness
Glucose media + Triton-X + Ca	GM-T	Fungi, yeast	21-23˚C, 12 h light
V8 agar + Ca+Ct[2)]	V8	Fungi, yeast	21-23˚C, 12 h light
Special Nutrient-poor agar + Ca+Ct	SNA	Fungi, yeast	21-23˚C, 12 h light
Dichloran rose bengal yeast extract sucrose agar + Ca+Ct	DRYES	Fungi, yeast	25˚C, darkness
Dichloran 18% glycerol agar + Ca+Ct	DG18	Fungi, yeast	25˚C, darkness
Oat meal agar + Ca+Ct	OM	Fungi	21-23˚C, 8 h light
Tryptic Soy Broth Agar	TSBA	Bacteria	20-23˚C, darkness
Saboraud Dextrose Broth Agar	SDBA	Yeast	20-23˚C, darkness
Nutrient agar	NA	Bacteria	20-23˚C, darkness

[1)]Chloramphenicol, [2)]Chlortetracycline

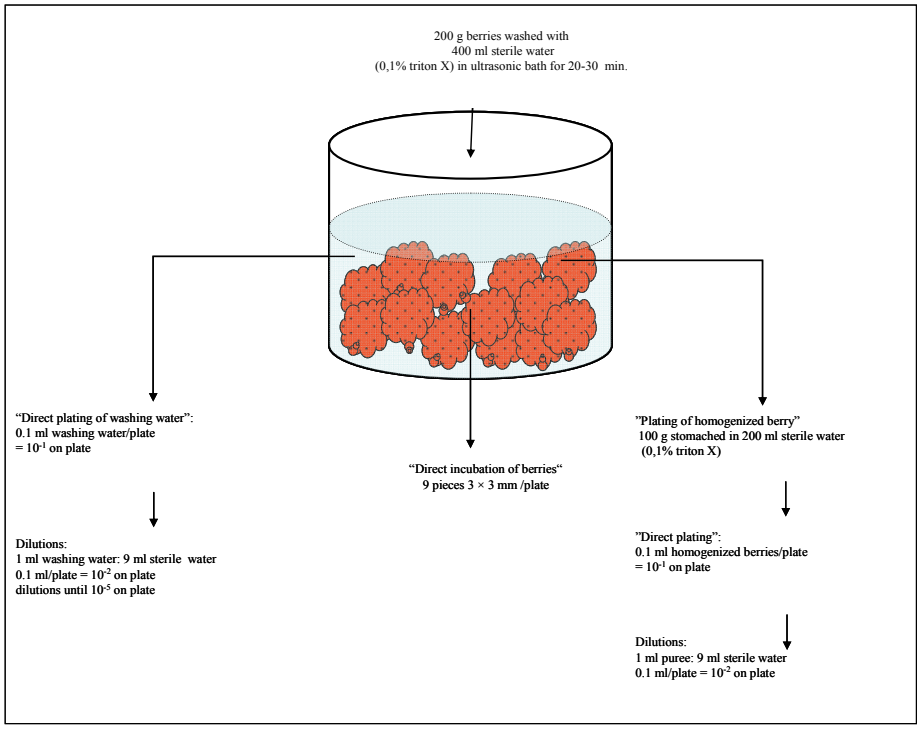

Figure 1. Procedure for isolation of microorganisms from strawberry.

From each bag of washed strawberries three series of isolations were made: 1) The washing water was plated in 10-fold dilutions (10^{-1} to 10^{-5}) onto the isolation substrates, 2) Nine pieces of strawberries (approx. 3 × 3 mm) were also plated directly onto all isolation substrates except TSBA, SDBA and NA, 3) One hundred gram of washed strawberries was homogenized with 200 ml of sterile water (0.1% Triton-X) for two minutes in a Stomacher 400 (Seward Medical, London, UK). The homogenate (pure) was plated in 10-fold dilutions (10^{-1} to 10^{-2}) onto all isolation substrates. Plates were incubated according to the conditions listed in Table 2. After 7 days incubation fungal cultures were identified and counted directly on the isolation plates when possible. Problematic fungi were identified by microscopy. For identification of the fungal cultures the identification routines in Samson ***et al.*** (2002) were used. Total number of bacterial and yeast colonies were counted after 3 and 2 days of incubation, respectively.

2.1.3 2006 survey of the strawberry microbiota

2.1.3.1 Sampling of berries

Four organic and four conventional growers all producing the cultivar Florence were identified. See Table 3 for geographic distribution of growers. Berries from all 8 growers were collected on the same day (17-07-2006). High quality berries were collected randomly into boxes and kept separately in order to avoid cross contamination between berries. In addition 25 berries with disease symptoms or otherwise low quality berries were selected from each grower and kept strictly separated from the high quality berries. Sampled berries were stored at 4°C until isolation of microorganisms the following day.

Table 3. Conventional and organic growers participating in a survey of strawberry microbiota 2006.

Cropping system	Grower No.	Geographic placement of field
Conventional	1	Gørløse
	2	Klippinge
	3	Skælskør
	4	Skælskør
Organic	5	Klippinge
	6	Odder
	7	Lille Skensved
	8	Års

2.1.3.2 Isolation of the microbiota from strawberry

Isolations from berries from all 8 growers were performed 18-07-06. From each grower three samples (A, B and C) each containing 200 g of berries were prepared. The two hundred gram of strawberries was mixed with 400 ml sterile water (0.1% Triton-X) in a plastic bag. The bag was placed in an ultrasonic bath (Branson 3210, Danbury, CT) for 30 minutes. The water in the ultrasonic bath was cooled by ice cubes to minimize heating of the samples.

Table 4. Media and dilutions selected for qualitative and quantitative investigation of the naturally occurring microbiota on strawberries from 8 growers.

Sample type	Media	Dilutions	Target organisms
Washing water	PDA-C	10^{-1}, 10^{-2}	Fungi
	DG-18	10^{-2}, 10^{-3}, 10^{-4}	Fungi
	TSBA	10^{-2}, 10^{-3}, 10^{-4}	Bacteria
	SDBA	10^{-2}, 10^{-3}, 10^{-4}	Yeasts
Homogenized berries	PDA-C	10^{-1}, 10^{-2}	Fungi
	DG-18	10^{-2}, 10^{-3}	Fungi
	V8	10^{-2}, 10^{-3},	Fungi
	SNA	10^{-2}, 10^{-3},	Fungi
	TSBA	10^{-2}, 10^{-3}, 10^{-4}	Bacteria
	SDBA	10^{-2}, 10^{-3}, 10^{-4}	Yeasts
Strawberry pieces	PDA-C	9 pieces/plate	Fungi
	DG-18	9 pieces/plate	Fungi
	V8	9 pieces/plate	Fungi
	SNA	9 pieces/plate	Fungi

From each bag of washed strawberries three series of isolations were made: 1) The washing water was plated in 10-fold dilutions (10^{-1} and 10^{-2}) onto PDA-C and (10^{-2} to 10^{-4}) onto the isolation substrates DG18 for isolation of fungi while 10-fold dilutions (10^{-2} to 10^{-4}) were plated onto TSBA and SDBA for isolation of bacteria and yeast, respectively,2) Twenty-seven pieces of strawberries (approx. 3 × 3 mm) were plated directly onto the isolation substrates, DG18, V8, PDA-C and SNA (three plates of each per sample), 3) One hundred gram of washed strawberries was homogenized with 200 ml of sterile water (0.1% Triton-X) for two minutes in a Stomacher 400 (Seward

Medical, London, UK). The homogenate was plated in 10-fold dilutions (10^{1} to 10^{-2}) onto the isolation substrates DG18, V8, PDA-C and SNA, while 10-fold dilutions (10^{-2} to 10^{-4}) were plated onto TSBA and SDBA. Media, dilutions and target organisms are summarized in Table 4.

2.1.3.3 Identification of fungi

After seven days of incubation; according to the conditions listed in Table 2, fungal cultures were identified to genera directly on the isolation plates, when needed microscopy was used. For identification to species level, isolates representing mycotoxin producing genera (***Penicillium, Fusarium***, ***Aspergillus, Alternaria***), genera of MPCA candidate fungi such as ***Trichoderma, Clonostachys, Ulocladium*** and other unidentified genera frequently occurring were selected and purified by subsequent culturing on SNA, V8 and PCA, in total 117 isolates. For identification of the fungal cultures the identification routines in Samson ***et al.*** (2002) were used. Fungi identified to species level, in particular those belonging to mycotoxin producing genera, were also characterized by metabolite profiles (see details on methods in paragraph 2.7).

Selected fungi, in particular ***Trichoderma*** isolates, were identified by ITS1-5.8S-ITS2 sequencing information. In order to identify ***Trichoderma*** isolates to the species level, the ITS1 and ITS2 region was amplified from DNA extracted from the strains and sent to sequencing using MWG sequence service. Sequences were blasted in NCBI and TrichoKey for species identification.

2.1.3.4 Identification of bacteria and yeast

From each of the eight growers ten isolates of bacteria and yeasts were isolated randomly from each of the three replicates giving a total of 30 bacteria and 30 yeasts from each grower. Pure cultures were kept on agar plates at 4°C containing the same medium as they were originally isolated on.

Both yeasts and bacteria were identified by using the Microbial Identification System (MIDI). Fatty acid profiles of each of the isolates obtained from 24 hrs cultures according to the MIDI system were obtained using a four-step fatty acid extraction procedure (Sasser, 1990; Mansfeld-Giese ***et al.***, 2002): 1) saponification, 2) methylation, 3) extraction and 4) base wash. Fatty acid methyl ester analyses were performed with an Agilent 6890 Plus Chromatograph and the Sherlock System Software 4.0 with the recommended libraries for aerobic heterotrophic bacteria and yeasts (Parsley, 1996).

2.1.3.5 Metabolite analyses of isolates and berries from the 2006 survey.

From each replicate 100 g of berries were stored at -20ºC, in total 24 samples. Samples of low quality berries were also stored at -20ºC until metabolite analysis (see details on methods in paragraph 2.7).

2.1.3.6 Grower questionnaire concerning production systems

Even though the microbiota investigation from the 8 growers was based on the same strawberry cultivar, several other factors, apart from climate, varied between growers. Therefore a questionnaire with supplementary information on the individual production methods was worked out in collaborating with the strawberry consultant at Frugt & Grønt Rådgivningen. The growers were asked for information on pest and disease management, fertilization irrigation etc. as outlined in Table 5.

Table 5. Questionnaire to strawberry growers participating in the microbiota survey. The questions asked concern pest management, fertilization, irrigation etc.

Main questions	Details
Pest and disease management fungicides herbicides insectidices	Product, dosages and dates of spraying
Fertilization organic inorganic foliar	Product, type, amount
Irrigation drip spray canon	Frequency, amount
Other practices Row size and distance Cover material	Type
General culture development	

2.2 Establishment of *Trichoderma* on strawberries in the field

2.2.1 Experimental set-up and sampling

Two field experiments were performed to examine the establishment of ***Trichoderma*** on strawberries in the field after application of the MPCP TRI003 (***T. harzianum,*** isolate 1295-22) to flowers and green berries. Two organic strawberry growers were selected among the growers involved in the initial survey on microbiota communities on strawberries: grower 5 and grower 7 (see Table 3). The two experiments had the same experimental design with and without application of TRI003 each with 3 replicates. The six experimental units consisted each of a 5-m double row located in approximately 100-m^2 isolated field areas (Figure 2). First and second year plants of the strawberry cultivar Florence was used in the study with grower 5 and grower 7, respectively.

Figure 2. Left-side picture shows field experiment area at the grower number 5 nursery. Right-side picture shows green berries (blue sticks) and flowers (white sticks) labelled after TRI003 application.

The treatments with ***T. harzianum*** were applied June 7th 2007 (grower 5) and June 11th 2007 (grower 7). Two gram of TRI003 was dissolved in 50 ml sterile water, which was sprayed uniformly with a hand pressure pump over the plants in a 5-m row. Treatments without ***T. harzianum*** received a similar amount of pure sterile water. Twenty flowers and green berries were sampled from each row and stored in a cooling bag until further processing the same day. Other twenty flowers and berries from each row were labelled with white

and blue plastic strips respectively (Figure 2). When green berries and flowers had developed into red berries these were sampled 20 and 32 days after spraying, respectively. At grower 5 only flowers were considered, which were sampled as red berries 28 days after TRI003 application.

2.2.2 Isolation and identification of *Trichoderma* spp and dominating fungi

The sampled flowers and berries were homogenized with sterile water in a stomacher as described in 2.1.2. Flower samples were homogenized in 100 ml water. For each replicate of red berries only one half of the berries were used for microbiota determination the remaining halves were stored at -20ºC until metabolite analysis (in total 18 samples). Both green and red berries were weighed and the double amount of water was added for homogenization. Washing water from the homogenized strawberry tissue was plated on DG18 and PDA-C and incubated as described in Table 2. In addition, all replicates were plated on a ***Trichoderma*** semi-selective medium and incubated at 25ºC in darkness for 8 days.

Purified isolates for DNA extraction were cultivated for six days in PDB. The fungal biomass was filtered, washed with sterile water and subsequently freeze dried. DNA was extracted from approximately 100 mg freeze-dried biomass using the Fungal DNA Mini Kit (Omega, Bio-tek, USA). UP-PCR followed the procedure described in Lübeck ***et al.*** (2000) and Lübeck and Poulsen (2001) using the primer L15AS19 (5'-GAGGGTGGCGGCTAG-3'). The UP-PCR products were separated on 1.7 % agarose gels, which were stained with ethidium bromide, and scanned using the image Master VSD (Pharmacia Biotech, Uppsala, Sweden). Banding profiles of the test isolates were compared to the banding profiles of the following reference strains: ***T. harzianum*** 1295-22, ***C. rosea*** IK726 and ***U. atrum.*** Selected ***Trichoderma*** isolates were also identified based on ITS1-ITS2 sequences as described in 2.1.3.3.

2.3 Establishment of MPCAs on strawberries in Semi-field

Establishment of microbial MPCAs on strawberries was examined in a pot experiment in a semi-field controlled area with plastic roof, fence and concrete floor (figure 3). The main objective of the experiment was to study the establishment of fungal MPCAs on strawberries when applied alone or in combination with a grey mould fungicide. The main hypothesis was that a combined application of MPCAs with a fungicide with no effect on the MPCAs would improve the establishment of the MPCAs. Compatible combinations of fungicide and MPCAs were selected from the ***in vitro*** agar and flower assays. The fungicide Teldor was selected for the present study, as it had no effect on any of the tested MPCAs. The MPCAs selected were ***C. rosea***, ***U. atrum*** and the commercial ***T. harzianum*** based MBCA TRI003. The experiment had a randomised three-factorial design with two main factors fungicide with two levels (without and Teldor) and MPCAs with four levels (water, ***C. rosea***, ***U. atrum*** and TRI003) divided in two blocks each with 4 replicates so that each of the 8 main treatments had six replicates giving a total of 48 experimental units. Each experimental unit consisted of sixteen 2-l pots each with one strawberry plant. The sixteen pots were placed in a square and the central four plants of the plot were treated according to the experimental design and the surrounding 12 plants served as buffer zones between different plots and were left untreated. The experiment was arranged with six rows each with one replicate of each treatment. Elite strawberry frigoplants of the

variety ***Florence*** were planted May 16th 2007 in 2-l pots with fully fertilized coarse peat (Unimuld 2, pH 6). Pots were placed on the semi-field concrete floor covered by a layer of thick plastic and latex-covered cloth to sustain water so that watering could be done from below to prevent MPCAs and fungicide to be washed off flowers when applied. Plants were watered according to their needs. Five weeks later June 20th at maximum flowering four central plants in each plot were treated according to the experimental design. First the fungicide Teldor was applied in a standardized pesticide chamber according to ½ x recommended dosage. Secondly, the MPCAs were applied indoor as aqueous solutions with 10^6 spores per ml to run off using a hand pressure pump.

Figure 3. Semi-field experiment at Research Centre Flakkebjerg one week before application of MPCAs and of fungicide.

Plants were harvested in two blocks each with three rows July 17th and July 19th four weeks after the treatments had been applied. Following analyses were made based on sum of four central plants in each of the 48 experimental units: numbers and weight of mature (red) and immature (green) strawberries, numbers of rotted strawberries, numbers of runner plants, shoot dry weight and microbiota analyses were performed as in grower survey 2006 (CFU and community analyses of bacteria and yeast, total microbial community and community of filamentous fungi with focus mainly on the applied MPCAs). Isolation and identification of MPCAs and the naturally occurring microbiota followed the procedures previously describes

The total microbial community on strawberries was examined using fatty acid profiling of 48 hrs enrichment cultures. Half a strawberry from each treatment was incubated in sterile water in a 50 ml test tube for 48 hrs on a rotary bench (180 rpm) at room temperature. Test tubes were stored in the freezer until further processing. Before fatty acid analyses strawberry tissues from defrost samples were removed and the enrichment cultures were centrifuged at 10.000 rpm. Pellets were subjected to fatty acid extraction according to the four step procedure described by Sasser (1996). Fatty acid methyl ester analyses were performed with an Agilent 6890 Plus Chromatograph and the Sherlock System Software 4.0 with the recommended libraries for aerobic heterotrophic bacteria and yeasts (Parsley, 1996).

2.4 Activity of MPCAs and naturally occurring fungi on strawberry

2.4.1 Wild-type strains and marked strains

In order to study activity of potential MPCAs and fungal species naturally occurring on strawberry tissue, strains marked with a gene coding for green fluorescent protein (GFP) or with a red fluorescent protein (Dsred) were used. By fluorescence microscopy conidia germination, hyphal growth and sporulation can be directly observed on the surface of leaves, flowers and berries. Isolates used are listed in Table 6.

Stocks of all strains are maintained in 10% glycerol at -80ºC. Conidia for assays were produced on PDA and harvested in sterile water. Conidia concentrations were adjusted to 10^6 or 10^7 conidia/ml.

Table 6. Fungal strains transformed with the *gfp* or the *Dsred* gene.

Fungal specie	Wildtype isolate	Transformed isolate	Type of organism	Rererence
Clonostachys rosea	IK726	IK726***d11***	MBCA	Lübeck et al., 2002
Clonostachys rosea	IK726	IK726***Dsred5***	MBCA	Birgit Jensen, unpubl.[1)]
Trichoderma harzianum	1295-22	1295-22***gfp1***	MBCA	Birgit Jensen, unpubl.
Penicillium expansum	IBT 21525	IBT 21525***gfp4***	pathogen	Birgit Jensen, unpubl.
Botrytis cinerea	IK2018	IK2018***gfp5***	pathogen	Birgit Jensen, unpubl.

[1)] Has not yet been published in refereed scientific journal

2.4.2 *Clonostachys rosea* activity on whole plants

Six strawberry plants of the cultivar ***Florence*** were grown in greenhouse for four weeks. Four leaves and four mature berries on each plant were marked with cotton strings. Marked leaves and berries were sprayed with a conidia suspension of ***C. rosea*** IK726***d11*** (1 x 10^7 conidia/ml) using an atomizer. Two control plants were sprayed with distilled water. Inoculated plants were grown at 18ºC under cycles of 12 h light cool daylight/12 h of darkness. Immediately after spraying the density of conidia on tissue was estimated. One berry and one leaf, respectively, from each plant was weighed and then shaken for 1 min. in 10 ml sterile water +0.1% Triton X. Subsequently the washing water was plated onto PDA+C. Survival/sporulation of ***C. rosea*** IK726***d11*** was also determined after 1 and 5 days of incubation. Conidia germination was assessed on one berry and one leaf per plant after 1 and 5 days of incubation. On each of three tissue pieces per leaf or berry the germination of 100 conidia were evaluated by fluorescence microscopy. Berries were also studied by Confocal laser scanning microscopy (TCS4d; Leica Laser Tecknik) 6 days after inoculation. The set-up of plant assay is shown in Figure 4.

Figure 4. Plant assay to evaluate growth of fungi on strawberry plants in growth chamber assays. Leaves and berries marked with cotton strings are inoculated with fungal conidia.

2.4.3 Conidia germination on detached berries, leaves and flowers

2.4.3.1 Experiment 1: germination of C. rosea on flowers and berries

Detached berries and flowers of cultivar ***Senga sengana*** were sprayed with a conidia suspension (1 x 10^7 conidia/ml) of the ***gfp*** marked ***C. rosea*** isolate IK726***d11*** using an atomizer. Berries were washed tree times in sterile water and air dried before inoculation. Control berries and flowers were sprayed with sterile water. Flowers were placed with the stalk inserted into 1.5% water agar in polystyrene boxes and berries were placed on alufoil rings on moist filter paper in polystyrene boxes (see Figure 5). Berries and flowers were incubated at 20ºC. Conidia germination was assessed on 3 flowers and 3 berries after 24 h and 48 h, respectively. On each of three tissue pieces per flower or berry the germination of 100 conidia were evaluated by fluorescence microscopy as described in 2.4.2.

Figure 5. Assay to estimate fungal germination and growth on detached strawberries (a) and detached flowers (b).

2.4.3.2 Experiment 2: germination and growth of C. rosea, P. expansum and B. cinerea on berries stored at 4ºC and 20ºC

Detached berries and flowers of cultivar ***Symphony*** from grower 7 were sprayed with conidia suspensions of the ***gfp*** marked strains using an atomizer. ***C. rosea*** isolate IK726***d11*** (1 x 10^7 conidia/ml), ***P. expansum*** IBT 21525***gfp4***, (1 x 10^7 conidia/ml) and ***B. cinerea*** IK2018***gfp5*** (1 x 10^6 conidia/ml) were used. Berries were washed three times in sterile water and air dried before inoculation. Control berries were sprayed with sterile water. All berries were placed on alufoil rings on moist filter paper in polystyrene boxes. Berries were incubated at 4ºC (12 berries/treatment) and 20ºC (18 berries/ treatment). Conidia germination was assessed after 1 day for berries incubated at 20ºC and after 2 and 4 days for berries incubated at 4ºC. Fungal growth on berries was quantified by dilution plating at inoculation (day 0) and after 4 days at both incubation temperatures. Three berries per treatment were selected for each analysis. Methods for conidia germination assessment and quantification by plate dilution are described in 2.4.2.

2.4.3.3 Interaction between fungi on flowers and on berries

Interaction between two fungi can be studied directly on plant surfaces if the fungi constitutively express DsRed and GFP, respectively. Interactions between a MPCA and a pathogen and between two MPCAs were studied in dual inoculation assays. The GFP marked ***B. cinerea*** (IK2018***gfp***5) was co-inoculated with the ***Dsred*** marked ***C. rosea*** (IK726***Dsred***) and the ***gfp*** marked ***T. harzianum*** (1295-22***gfp1***) was co-inoculated with the Dsred marked C. ***rosea.*** The fungi were also applied singly to flowers. Conidia of the fungi were spayed onto strawberry flower placed in polystyrene boxes and incubated at 20°C as described in 2.2.3.1. Each treatment had 3 replicates each consisting of six flowers. After 1 day, germination of 200 conidia per replicate was evaluated by fluorescence microscopy. Disease development (pct browning of

petals) was scored 2 and 6 days after inoculation on the remaining flowers. The germination assay was repeated except that only 100 conidia per replicate were evaluated.

The result of the interaction between ***C. rosea*** and ***B. cinerea*** was also studied on berries using real-time PCR for quantification of the fungi. Berries were surface sterilized and inoculated as described in 2.6.2 except that sporesuspensions were $1x10^7$ and 3×10^6 conidia/ml for ***C. rosea*** and ***B. cinerea***, respectively. The experiment was set up with three inoculation treatments (***C. rosea, B. cinerea*** and dual inoculation with the two fungi). Water treatment served as control. Inoculated berries were incubated for four days in darkness at 4˚C and 20˚C as described in 2.4.3.2. Each treatment had 3 replicates each consisting of 2 berries. At sampling, two half berries originating from different berries were used for CFU quantification as described above while the two other halves were frozen in liquid nitrogen and lyophilized.

Various methods for DNA extraction were evaluated. Genomic DNA was extracted from lyophilized berry material using the DNAeasy Plant Mini Kit (Qiagen, Germany)± 1% polyvinylpyrrolidone-10 (PVP) in the extraction buffer, E.Z.N.A. fungal DNA kit (Omega Bio-tek) and E.Z.N.A plant DNA kit (Omega Bio-tek) following the manufactures instructions. Furthermore the method described by Doyle and Doyle (1987) including 1% (PVP) in the extraction buffer for extraction of DNA from strawberry (Mehli ***et al.***, 2005) was tested. Generally the highest DNA yield (>100 ng/µl) was obtained with the latter extraction method and problems with inhibitors in the PCR reactions were also reduced with the Doyle procedure.

Specific real-time primers for ***B. cinerea*** tested by others and primers specifically designed for ***C. rosea*** IK726 detection at our department (Mette Lübeck, pers.com) but not evaluated in real-time PCR assays were applied (Table 7).

Table 7. Primer pairs evaluated for real-time PCR detection of *B. cinerea* and *C. rosea* in strawberry.

Primer name	Primer sequence 5´- 3´	Reference
B. cinerea		
CG11 CG12	AGC CTT ATGTCC CTT CCC TTG GAA GAG AAA TGG AAA ATG GTG AG	Gachon and Saindrenan, 2004
Bc tuba Bc tuba	TTT GGA GCC AGG TAC CAT GG GTC GGG ACG GAA GAG TTG AC	Mehli *et al.*, 2005
C. rosea		
Cr.for 726.rev.LNA	TGA CGC CTC TGA ... AACA CTGTGT CAG TTA CCT CCC GT	Mette Lübeck, pers. comm.
726.for.LNA Cr.rev	AAG TTA GTT CCA TGC ATG ATT CTA AT CTG CCT GGT AAC TAT TTG TTT CT	Mette Lübeck, pers. comm.
Glio36a Cr.rev	GTT AGT TCCATTGCATGATTC CTA TGT GTC AGT TAC CTC CCG T	Mette Lübeck, pers. comm.

PCR was performed using the real-time PCR system Mx3000P (Stratagene, USA). Each reaction of 25 µl contained 5 ng genomic DNA (amounts from 0.1 to 100 ng was tested), 10 pmol of each primer, 12.5 µl 2 x SYBR Green master mix (Stratagene, USA) and 0.4 µl of a 1000 x diluted reference dye (Stratagene, USA). The PCR reaction was set up in duplicate for each DNA sample. For primer pairs with satisfactory efficacy a standard curve was made with pure serial diluted DNA and included on each plate. Prior to amplification an initial denaturation step was performed (95° for 10 min) ensuring complete denaturation of the DNA and activation of the Taq

polymerase. This was followed by 40 cycles of 15 sec. at 95°C, 30 sec. at 58°C and 30 sec. at 72°C. Fluorescence was detected after each cycle. After amplification a melting curve analysis was performed in order to ensure that only one PCR product was amplified.The amount of ***C.rosea*** DNA was expressed in pg DNA.

2.5 Compatibility between MPCAs and fungicides

2.5.1 *In vitro* compatibility on agar

The sensitivity of four MPCAs towards fungicides used in strawberry production (Table 8) was tested in vitro using 24-well microtiter plates. The fungicides were tested in six dosages (0, x1/4, x1/2, x3/4, x1 and x10 of recommended dosage), which were mixed in to autoclaved corn meal agar before solidification when the solution had a temperature around 50 C.

The commercial MPCAs were all ***Tricoderma*** based. Supresivit and TRI 003 are based on ***T. harzianum*** and BINAB-T is based on a combination of ***T. harzianum*** and ***T. polysporum***. In addition to the commercial MPCAs, the laboratory isolate ***C. rosea*** was included. The target fungus ***B. cinerea*** (isolate MG4), the causal agent of grey mould, was included as a control for the activity of the tested fungicides and finally a treatment without fungal application was included as a control for external contamination.

Table 8. Data on fungicides used in strawberry production in Denmark.

Trade name	Active compound	Target disease	Dosage ha^{-1}
Amistar	Azoxystrobin	Mildew	1 l
Candit	Kresoxim-methyl	Mildew	0.2 kg
Euparen Multi	Tolylfluanid	Grey mould	3 kg
Scala	Pyrimethanil	Grey mould	2 l
Signum WG	Pyraclostrobin + boscalid	Mildew+ grey mould	1.8 kg
Teldor WG 50	Fenhexamid	Grey mould	1.5 kg

Conidial suspensions of each of the six fungi were applied to the surface of the agar wells. The commercial MPCAs were applied according to the recommended dosage. Conidia of ***B. cinerea*** and ***C. rosea*** were prepared from washing of one-week old cultures grown on corn meal agar. After 5 days of incubation at 20 °C in darkness the plates were scored for plus/minus growth.

2.5.2 *In vitro* compatibility on flowers

In order to study the possibility of combining MPCAs and fungicides as a strategy to manage grey mould in strawberries an ***in vitro*** flower assay was developed. Six-well microtiter plates with water agar and one flower of the variety ***Florence*** in each well were used as experimental units. Disease development was scored as percent discolouration of white petals during a one-week incubation period (Figure 6). Plates were incubated at room temperature in sealed transparent plastic bags to maintain high humidity. After three days of incubation flower discolouration was measured. Each treatment had three replicates.

Experiment 1: Grey mould fungicides were tested against ***B. cinerea*** at different dosages with and without artificial inoculation with ***B. cinerea***. In treatments with ***B. cinerea*** floweres were sprayed to run-off with a suspension of 10^5 spores of ***B. cinerea***. The experiment had a three-factorial design with three replicates per treatment. Main treatments were ***B. cinerea*** with two levels

(without and with), fungicide with four levels (without, Teldor, Scala, Euparen) and fungicide dosage with six levels (0, 10, 25, 50, 75 and 100% of recommended dosage).

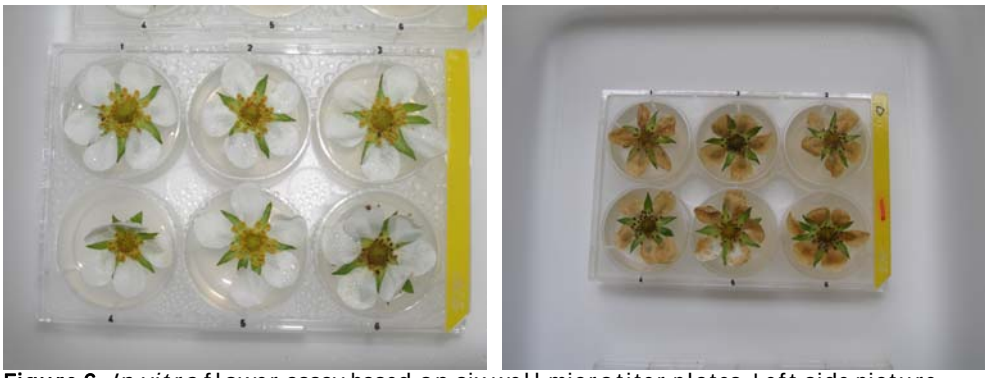

Figure 6. *In vitro* flower assay based on six well microtiter plates. Left-side picture shows control treatment without *Botrytis cinerea* and the right-side picture shows flowers infected with *B. cinerea*.

Experiment 2: The effect of different MPCAs alone and in combination with the grey mould fungicide Teldor was examined. The experiment had a two-factorial design with MPCA and fungicide as main factors. The factor MPCA had seven levels (without, TRI003, Binab, Supressivit, ***U. atrum*** and ***C. rosea***) and the factor fungicide had three levels (0, 50, 100% of recommnended dosage). MPCAs were applied to run-off to flowers by spraying solutions of MPCAs with 10^6 spores per ml. Fungicide applications were performed using a standardized spray chamber. Prior to MPCA and fungicide application all experimental units were inoculated with a 10^5 spore per ml suspension of ***B. cinerea***.

Experiment 3: Based on experience from experiment 1 and 2 a third experiment was performed to study the combination-effects of selected MPCAs and the fungicide Teldor. The experiment had a three factorial design with ***B. cinerea*** (two levels, without and with), MPCA (three levels, without, ***C. rosea*** and ***U. atrum***) and fungicide (two levels, 0 and 50% of recommended dosage) as main factors. The MPCAs and ***B. cinerea*** were applied in solutions of 10^4 and 10^3 spores per ml, respectively.

2.6 Worst case inoculation of strawberries

2.6.1 Worst case inoculation using MPCAs

Mature and sound strawberries were inoculated with the commercial products TRI003, Supresivit and Binab-T (suspended in water) and with spore suspensions of ***C. rosea*** and ***U.atrum.*** The procedures for sterilization, inoculation and incubation described in 2.4 were used. Inoculated berries were stored at 4°C and 20°C for four days. The experiment was repeated.

2.6.2 Worst case inoculation using potentially toxic field fungi

Danish organic grown strawberries were purchased in two supermarkets, which supplied ***Honeoye*** and an unknown cultivar, respectively. The berries were surface sterilized in 0.4% hypochlorite solution for 1 min, and then washed in three sets of sterile Milli Q water. They were then allowed to dry for 15 min. in a Laf bench and dipped in a 10^3 or 10^5 (duplicate from each solution concentration from each berry supplier) spore solution for ca. 2 sec

and then transferred to a small glass jar and loosely capped (see Figure 43). The 7 different fungal isolates (***Penicillium brevicompactum***, ***P. bialowiezense***, ***P. polonicum, Aspergillus niger, Alternaria infectoria*** sp-grp and ***Alternaria tenuissima*** sp-grp, are described in Table 16, After inoculation at 25°C for 4-8 d the berries were visually inspected and clearly moulded berries take for analysis and frozen at -20°C.

2.7 Metabolite analysis

Solvents were HPLC grade, and all other chemicals were analytical grade unless otherwise stated. Water was purified from a Milli-Q system (Millipore, Bedford, MA). Source of reference standards: Trichodermin was a gift from LEO Pharma A/S, (Ballerup, Denmark) and other reference standards were available from previous studies in our laboratory (Nielsen and Smedsgaard, 2003) and Sigma-Aldrich (St. Louis, MO). Harzianum A was extracted and purified as previously reported (Nielsen ***et al.***, 2005).

2.7.1 Extraction of metabolites from fungal cultures

Fungal isolates were three point inoculated on Yeast Extract Sucrose (YES) agar, Dichloran Rose Bengal Yeast Extract Sucrose (DRYES) and Czapek-Dox Yeast Autolysate (CYA) agar (Samson ***et al.***, 2002) and incubated for 7 to 14 days at 25 C in the dark. After incubation fungal culture materials was harvested by cutting out approximately 1.5 cm^2 fungal culture, transferred to a 4-mL vial and frozen until extraction (Frisvad and Thrane, 1987; Smedsgaard, 1997; Nielsen and Smedsgaard, 2003). The biomass was then extracted for 2 hr on a rotary shaker at 200 rpm with 3 mL ethyl acetate which were transferred to a new 4-mL vial and evaporated ***in vacuo***. Samples were redissolved 500 µL methanol and filtered through a 4 mm 0.45 µm PFTE syringe filter (Chromacol, Herts, UK) into a HPLC vial.

2.7.2 Extraction of metabolites from strawberries

2.7.2.1 Sample procedures

All strawberry samples from field, semi-field and laboratory experiments were stored at -20°C until analysis. High resolution LC-DAD-MS and HPLC-MS/MS analysis as described below were used for chemical analysis of strawberry samples.

2.7.2.2 Acetonitrile extraction.

Strawberries (1 g sub samples) along with 2 ml acetonitrile homogenized using five 3.2 mm Stainless steel balls in a Mini-beadbeater (Biospec Products Inc.; Bartlesville, OK, USA) for 1 min. The homogenate along with further 7 ml acetonitrile were then extracted for 1 hr on a rotary shaker at 200 rpm, centrifuged at 20 000 g, and the supernatant, up-concentrated ***in vacuo*** to ca. 2 ml. The concentrate was acidified with 3 ml water containing 2% formic acid, and the extract applied to a 60 mg Strata-X column (Phenomenex, Torrance, CA). The column was washed with 3 ml acetonitrile-water (1:9 v/v and eluted into a HPLC vial using 1 ml acetonitrile-water (9:1 v/v). The columns had previously been sequentially activated using 1 ml methanol and 1 ml acetonitrile-water (1:9 v/v).

Strawberries (5 g sub samples) along with 25 ml acetonitrile was homogenized using a Pasteur pipette for ca. 1 min, and then shaken for 1 hr on a rotary shaker at 200 rpm, centrifuged at 20 000 g. Then 12 ml was

evaporated to dryness using nitrogen flow. The sample was then redissolved in 3 ml 10% acetonitrile and the extract applied to a 30 mg Strata-X column (Phenomenex, Torrance, CA). The column was washed with 7 ml acetonitrile-water (1:19 v/v) and the sample eluted using 500 µl methanol.

2.7.2.3 Water extraction for patulin

Strawberries (1g sub samples) along with 2 ml water homogenized using five 3.2 mm Stainless steel balls in a Mini-beadbeater (Biospec Products Inc.; Bartlesville, OK, USA) for 1 min. The homogenate along with further 7 ml water were then extracted for 1 hr on a rotary shaker at 200 rpm, centrifuged at 20 000 g, and the supernatant acidified with 3 ml water containing 2% formic acid. The extract was then applied to a 30 mg Strata-X-C column (Phenomenex, Torrance, CA). The column was washed with 3 ml 2% $NaHCO_3$ solution and 3 ml 1% formic acid solution and eluted using 600 µL acetonitrile-water (3:7 v/v). The columns had previously been sequentially activated using 1 ml methanol and 1 ml acetonitrile-water (1:9 v/v).

2.7.3 Chemical analysis

2.7.3.1 HPLC-UV

Culture extracts were analyzed on an Agilent 1100 Liquid Chromatographic system equipped with a photo-diode array detector , a fluorescence detector, and a 100 × 2 mm i.d., 2.5 µm, Luna C_{18} II-HTS column (Phenomenex, Torrance, CA) fitted with a Phenomenex SecurityGuard C_{18} pre-column. Two different water-acetonitrile systems were used at a flow rate of 0.55 mL/min. The first (standard system) started at 15% CH_3CN which was increased to 100% over 14 min and then held at 100% for 3 min. The second system started at 5% acetonitrile increasing to 50% over 12 min and the to 100% in 1 min and then holding at 100% for 3 min. Sample volumes of 1-5 µL were injected onto the column. Detected metabolites were identified by comparison of UV spectra to the DTU in-house database of approx. 750 fungal metabolites.

2.7.3.2 HPLC-MS

High resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photo diode array detector and a 50×2 mm i.d., 3 µm, Luna C_{18} II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK), with a Z-spray electrospray ionization (ESI) source, a LockSpray probe (Nielsen and Smedsgaard, 2003) and controlled by the MassLynx 4.0 software.

Samples were analyzed in positive ESI (ESI^+) using a water-acetonitrile gradient system starting from 15% acetonitrile which was increased linear to 100% in 20 min holding this for 5 min or starting from 5% acetonitrile keeping this for 2 min and increasing to 100% in 18 min keeping this for 5 min. The water was buffered with 10 mM ammonium formiate and 20 mM formic acid (HCOOH) and the acetonitrile with 20 mM HCOOH as previous described (Nielsen and Smedsgaard, 2003). The only changes were that two scan functions (1 s each) were used: the first with a potential difference of 10 V between the skimmers and using a scan range of ***m/z*** 100 to 900; the second with 30 V between the skimmers and using a scan range of ***m/z*** 100 to 2000. In ESI^+ the instrument was tuned to a resolution >7000 (at half peak height).

Samples were also analyzed in negative ESI (ESI^-) mode using the same instrument and gradient systems, except that only the water was buffered (100 µL/L HCOOH). ESI^- was performed at a resolution >5000 (at half peak height), and two scan functions (1 s each) were used: the first with a potential difference of 10 V between the skimmers and second with 30 V. Desolvation temperature was 450°C, source block temperature 120ºC, and the desolvation flow (nitrogen 99.9%) was ca. 0.53 m^3/h.

The capillary was held at -1800 V and data was collected as centroid data from ***m/z*** 100 to 900. A solution of 3,4-dihydroxybenzoic acid in water-methanol (1:1 v/v) was infused (10 µL/min) into the lock spray source (second ESI^- spray) using a syringe pump. The $[M-H]^-$ ion of leucine enkephaline acid was subsequently used for on-line mass correction every 3 s.

2.7.3.3 HPLC-MS/MS analysis

Due to the purchase of a more sensitive triple-quad MS a new analytical strategy was used. In this case samples (at least 5 g sub sample) were simply homogenized with 6 volumes acetonitrile in a stomacher for 1 hr.

Samples were then centrifuged at 10 000 g, and 1 ml transferred to a autosampler vial. Samples of 0.1 to 3 µl were injected on an Agilent (Torrance, CA, USA) 1100 LC system, and separated on a Gemini C6-Phenyl 3 µm 2-mm ID ×50-mm column (Phenomenex, Torrance, CA, USA) using a constant flow of 0.3 mL/min acetonitrile-water gradient, starting at 20% acetonitrile going to 100% over 10 min, followed by a 2 min wash with 100 % acetonitrile. The water and acetonitrile were buffered with 20 mM formic acid. The LC was coupled to a Quattro Ultima triple mass spectrometer (Waters-Micromass, Manchester, UK) with Z-spray ESI source using a flow of 700 L/hr nitrogen at 350°C, hexapole 1 was held at 12 V. The system was controlled by MassLynx 4.1 (Waters-Micromass). Nitrogen was also used as collision gas and the MS was operated in the multiple reaction monitoring mode (dwell time 200 ms). Chromatography and MS/MS were optimized on pure standards of fumonisins B1 and B2; ochratoxins A, B, and alpha; alternariol, AAL toxin, mycophenolic acid, tenuazonic acid, altertoxin, infectopyrones, novo-zealandin, cyclopenol, viridicatol, viridicatin, brevianamides A and B. Crude extracts of 3 ***Trichoderma*** biocontrol strains from PDA agar were used for optimising for the 10 major peptiabiotics.

2.8 Statistics

The data obtained from the various experiments were subjected to analyses of variance. Depending on the experimental design one-way or multifactor ANOVAs were performed. Bartlett´s test for variance homogeneity was examined for all variables and when relevant data were log-transformed prior to ANOVA. Significant differences between treatment means were based on LSD values. In all analyses level of significance were fixed at alpha=0.05.

In addition, the data obtained from the strawberry survey concerning microbial communities were subjected to principal component analyses (PCA) to examine community differences between the strawberry growers.

The purpose of the analysis was to obtain a small number of linear combinations of the variables which account for most of the variability in the data. Components with an eigenvalues greater than or equal to 1,0 were extracted and 2D scatter plots of component 1 and component 2 which accounted for the highest percentages of variability in the original data were plottet.

All statistical analyses were performed using either SAS System Version 9.2 (SAS Institute Inc., Cary, NC, USA) or StatGraphics Plus 5.1 (Statpoint Inc., Virginia, USA).

3 Results

3.1 Initial screening of isolation media

The best media for identification of ***Penicillium, Fusarium, Alternaria*** and ***Aspergillus*** growing from washed strawberries were SNA, DG18 and V8 while it was difficult to identify these genera on PDA+N, PDA+C, GM-RB and GM-T, mainly due to strong inhibition of colony development on these media. According to Table 9 the highest frequency of ***Penicillium*** and ***Aspergillus*** was found on DG18, while V8 was suitable for isolation of ***Alternaria*** and ***Penicillium*** and SNA was the best medium for isolation of ***Alternaria*** and ***Fusarium***. ***Cladosporium*** and ***Botrytis*** were generally isolated to the same frequency on all three media (Table 9).

Table 9. Frequency of fungal genera growing from strawberry pieces plated on three media. Berries sampled from organic grower (No. 7).

Media / Genera	Occurrence of fungi (% pieces with growth)		
	DG18	V8	SNA
Cladosporium	81	100	100
Penicillium	60	15	24
Alternaria	-	12	24
Aspergillus	4	-	-
Fusarium	-	-	5
Botrytis	5	8	7

Fungal colonies were countable on most media at 10^{-2} and 10^{-3} dilutions of strawberry washing water while counts on 10^{-1} generally exceeded 100 and only few colonies were observed on 10^{-4} and 10^{-5} (Table 10).

Table 10. Colony counts of fungi and yeast on eight agar media from washing water and homogenized berries. Berries sampled from organic grower (No. 7).

Isolation method	Organisms counted	Colonies/plate							
		V8	SNA	DRYES	DG18	PDA-C	PDA-N	GM-RB	GM-T
Washing water, dilution 10^{-2}	Yeast	72	>200[1]	>100[1]	>100[1]	18	-	50	15
	Cladosporium	7	-	-	7	5	-	-	5
	Total fungi	-	-	20	9	14	-	21	6
	Fungi+yeast	4	-	-	-	-	>100[1]	-	-
Homogenized berries, dilution 10^{-2}	Yeast	>60[1]	>200[1]	52	43	-	-	62	44
	Cladosporium	4	-	-	6	-	-	-	6
	Total fungi	-	-	-	-	-	-	16	0
	Fungi+yeast	-	-	-	-	63	>100[1]	78	50

[1)] estimated

Surprisingly the counts from washing water and from washed homogenized berries were of the same magnitude. Since we expected that most organisms were located on the surface and that they could be detached by sonication, it was decided to increase the sonication period from 20 to 30 min. in the following survey of the berry microbiota from 8 growers (3.2). For both isolation methods (plate dilution and growth from berry pieces) ***Cladosporium*** was the most dominating fungal genus and the highest counts were observed

on DG18 and V8. ***Penicillium*** was the dominating genus amongst potentially mycotoxin producing genera with highest number counted on SNA and DG18. Using 10 fungal colonies per plate at 10^{-2} as average density, the number of fungal colony forming units on berries was estimated to 2 x 10^3 CFU/g berry. Counts for yeast were considerably higher and the densities were roughly estimated to >10^4 CFU/g berry (Table 10). Overall, the highest diversity of fungal genera was found on DG18 where ***Penicillium, Alternaria, Aspergillus, Cladosporium, Botrytis, Epicoccum*** and ***Phoma*** were identified.

For plating of washing water, the estimation of bacterial colonies on TSBA and NA was of the same magnitude and countable at 10^{-3} to 10^{-5} (Table 11). Using 10 colonies per plate at 10^{-4} as average density, the number of bacterial colony forming units on berries was estimated to >10^5 CFU/g berry from washing water. From homogenized berries counts on 10^{-2} generally were >200. On the selective yeast medium SDBA, counts of yeast and bacteria could not be separated due to technical problems with the antibiotic amendment. Nevertheless SDBA media with antibiotics is suitable for yeast counts.

Table 11. Bacterial counts on three media based on four strawberry samples per medium. Plates incubated 24 hours at 24ºC. Berries from organic grower (No. 7).

Media	Washing water					Homogenized berries	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}
TSBA	∞	∞	35-88	10	0-2	∞	∞
NA	∞	∞	65-178	1-12	0-11	∞	177- ∞
SDBA	∞	∞	13-73	4-7	0-1	∞	104-∞

Based on results of the pre-screening experiments, media shown in Table 12 were selected for quantification and isolation of the background microbiota in field experiments.

Table 12. Media for quantification and isolation of the back ground microbiota in field experiments. Addition of antibiotics to the media (see Table 1).

	DG18	PDA_C	V8	SNA	TSBA	SNA+
	Fungi				Bacteria	Yeast
Washing water	+	+			+	+
Homogenized berries	+	+	+	+	+	+
Washed berries tissue	+	+	+	+		

3.2 Survey of natural microbiota on strawberry 2006

3.2.1 Description of grower production systems

Based on a questionnaire of four conventional (1,2,3,4) and four organic growers (5,6,7,8) difference and similarities between the production systems were described and the results are summarized in Tables 13a and 13b. As expected conventional and organic grower production systems clearly differed mainly in pest management and fertilization procedures, because the organic farming protocols does not allow pesticides and inorganic fertilization, which are common among conventional growers. All of the conventional growers used pesticides but with varying intensity and one of the conventional growers also used biocontrol of spider mites. However, one of the conventional growers (grower 1) used organic fertilization and fewer pesticides than the other conventional growers.

Only one of the organic growers (grower 8) used active pest management in terms of ***Trichoderma*** application. Grower 4 was the only grower applying

foliar fertilization, which may affect the phyllopshere microbiota (Table 13a and b).

Table 13a. Result of questionnaire for conventional strawberry growers.

Pest management	Conventional growers			
	1	2	3	4
Fungicides	Euparen at 10 and 80% flowering Teldor at 50% flowering	Scala 1.5 l 26/5 Teldor 1 kg 5/6	Scala 2 L, 24/5 Candit 0.2kg, 28/5 Teldor 1.5 kg 28/5 Signum 1.25 kg 10/6 Teldor 1.5 kg 18/6	Amistar 20/4, 26/6, 20/7 Candit 3/5, 30/5 Signum 5/5 Teldor 10/5, 15/6 Euparen 28/8 Tilt 29/9
Herbicides	Flexidor 0.4 l March 06	None	None	?,1/8, 28/8
Insecticides	None	0.2 kg Karate 26/5	0.3 kg Karate 10/6	Fastac 20/4, 25/5, 20/7 Karate 3/5, 25/5
Biological control	None	None	None	Predator mites 1 mill/ha late august
Fertilization				
Organic	30 t/ha poultry manure	None	None	None
Inorganic	None	300 kg NPK (14:3:18)/ha at planting in the spring and in the autumn after harvest	Weekly application of nutrient solution in a drip irrigation system	300 kg NPK (14:3:18) /ha at planting in the spring and 250 kg NPK (14:3:18)/ha 26/7
Foliar	None	None	None	Metolasate crop-up Bor, Mg, Mn, Kali 10/5, 26/6
Irrigation				
Drip			½ hour/day	Yes
Spray canon	15-20 mm	30 mm		Yes
Other practices				
Row size	Single	Double	Single	Single
Row distance		110-60 cm	1 m	1 m
Cover material	Straw	Wheat straw	Straw	Straw
General culture development	Ordinary plant growth but good berries above average	Very good with big leaves and many flowers	Good plant growth and yield	Fair development. Slow start in May due to cold weather. Problems with spider mites

Table 13b. Result of questionnaire for organic strawberry growers.

Pest management	Organic growers			
	5	6	7	8
Biological control	None	None	None	*Trichoderma* applied via bees
Fertilization				
Organic	600 kg Binadan NPK 5-1-4 3/8 2005 and 23/7 2006	None	20-30 t/ha cow manure with straw, and 20 t/ha swine manure. Every spring 30 kg N as Binadan	20 t/ha (½cow ½swine)
Inorganic	None	None	None	None
Foliar	None	None	None	None
Irrigation		None		
Drip			3-5 mm	
Spray canon	20 mm			Twice a week in July
Other practices				
Row size	Double	Single	Double	Single
Row distance		1 m	110 – 60 cm	
Cover material	Straw	Straw	Straw	Oat straw
General culture development	OK growth. Berries big and beautiful	Plant growth not good, but berries OK. Problems with weeds	Fine	10% of normal yield. Problems with beetles *Anthonomus rubi.*

3.2.2 Fungi

3.2.2.1 Washing water

Cladosporium spp. was the most abundant fungal genus isolated from washing water of both organic and conventional strawberries. Mean densities were $>10^3$ CFU/g berry (Figure 7). Significantly higher CFU/g berry (***P***<0.04) was isolated from the organically grown berries. The ***Cladosporium*** density in washing water (Figure 8) varied between growers since grower 3 and 4 had significantly (***P***<0.0001) lower CFU levels than all other growers. ***Penicillium*** spp. was the most abundant genus with mycotoxin producing potential.There was no significant difference between the two production systems (Figure 7). The mean density of ***Penicillium*** was approximately 200 CFU/g berry. However, there were significant differences in CFU densities especially between the organic growers (***P***<0.001) as ***Penicillium*** CFU/g berry from grower 5 and 7 were at least 10-fold higher than from grower 6 and 8 (Figure 9). ***Alternaria*** spp. was isolated in low numbers and only in washing water from 6 out of 24 samples (Figure 10). On average <4 CFU /g berry was obtained from both conventional and organic growers (Figure 7). The other mycotoxin producing genera, ***Aspergillus*** and ***Fusarium***, were only detected in one sample each.

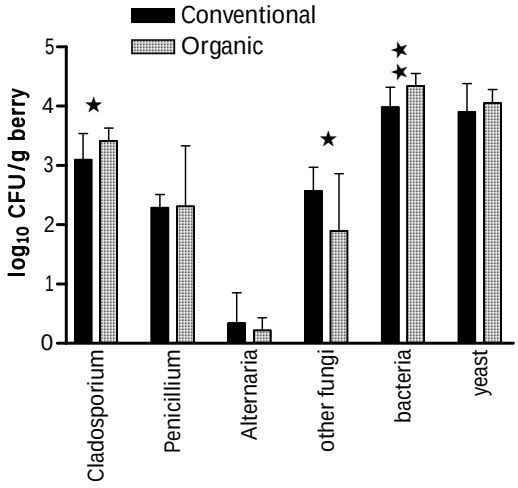

Figure 7. Quantification of the microbiota in washing water from healthy berries sampled from four organic and four conventional strawberry growers. (* = significant difference between conventional and organic growing system *P*<0.05)

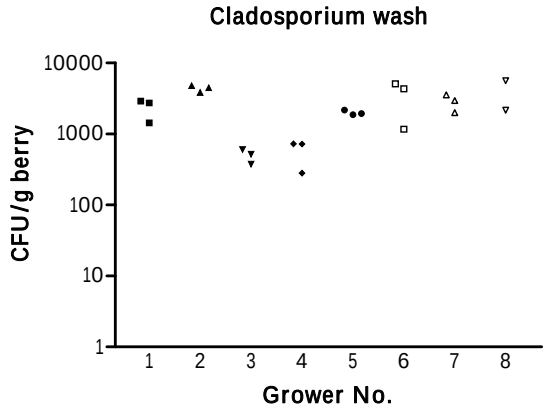

Figure 8. *Cladosporium* spp. (CFU/g berry) in washing water from berries sampled from conventional growers (No. 1 to 4) and organic growers (No. 5 to 8). Field survey 2006. Data from different growers is represented by different symbols.

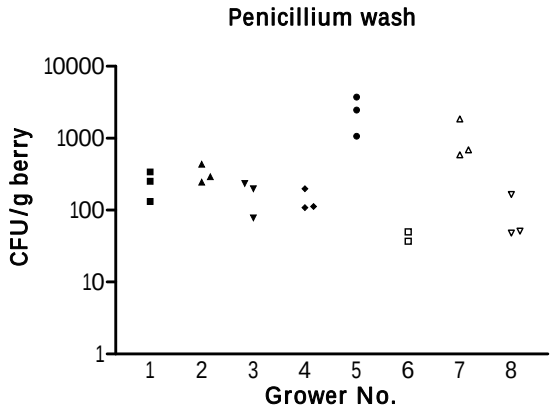

Figure 9. *Penicillium* spp. (CFU/g berry) in washing water from berries sampled from conventional growers (No. 1 to 4) and organic growers (No. 5 to 8). Field survey 2006. Data from different growers is represented by different symbols.

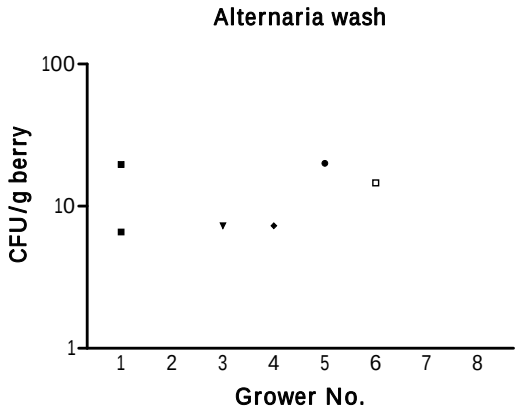

Figure 10. *Alternaria* spp. (CFU/g berry) in washing water from berries sampled from conventional growers (No. 1 to 4) and organic growers (No. 5 to 8). Field survey 2006. Data from different growers is represented by different symbols.

3.2.2.2 Homogenate of washed berries

Cladosporium spp. was also the most abundant fungal genus isolated from washed homogenized berries although CFU densities were approximately 10 fold lower than for the washing water. A significantly higher CFU density (***P***<0.05) was isolated from organic berries compared to conventional (Figure 11). Significantly lower CFU densities of ***Cladosporium*** were obtained from grower 3 and 4 compared to the other growers (Figure 12). ***Penicillium*** spp. was still present after washing of berries from all growers (Figure 13).

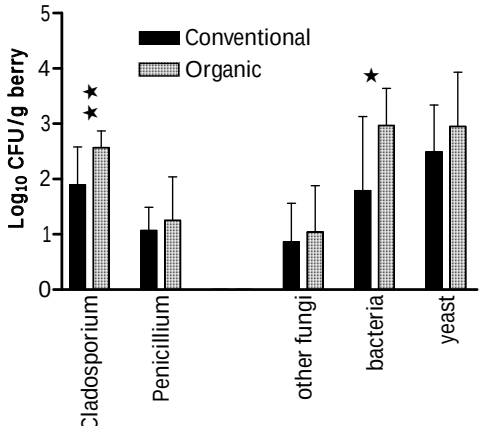

Figure 11. Quantification of the microbiota from washed healthy berries sampled from four organic and four conventional strawberry growers. (*=$P \leq 0.05$, or **= $P \leq 0.001$, significant difference between conventional and organic growing system).

However, there was no significant effect of growing system or of grower on ***Penicillium*** CFU density on washed berries and the mean density was $<10^2$ CFU/g berry (Figure 11). ***Alternaria*** spp. was also occationally isolated from homogenized berries while ***Aspergillus*** spp and ***Fusarium*** spp. were not detected.

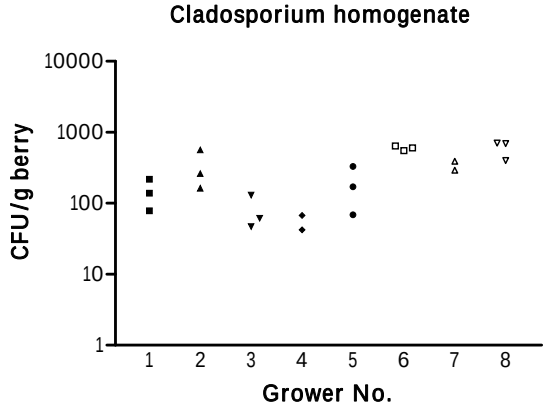

Figure 12. *Cladosporium* spp (CFU/g berry) in homogenate of washed berries sampled from conventional growers (No. 1 to 4) and organic growers (No. 5 to 8). Field survey 2006. Data from different growers is represented by different symbols.

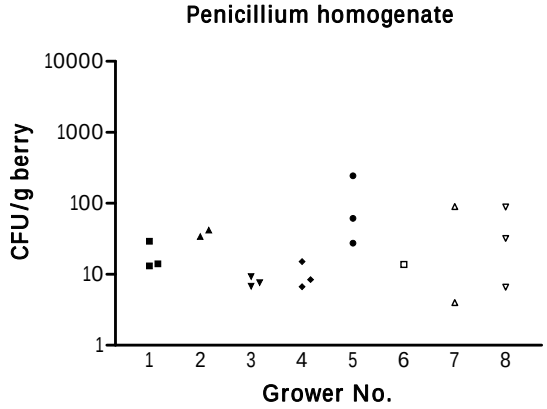

Figure 13. *Penicillium* spp. (CFU/g berry) in homogenate of washed berries sampled from conventional growers (No. 1 to 4) and organic growers (No. 5 to 8). Field survey 2006. Data from different growers is represented by different symbols.

3.2.2.3 Growth of fungi from washed berries

Surprisingly ***Penicillium*** spp. was the most frequently isolated genus growing from both conventional and organic grown berries and on average ***Penicillum*** grew from more than 70% of washed berry pieces (Figure14). Grower 3, 6 and 8 had the significantly (***P***<0.0001) lowerst ***Penicillium*** frequency (Figure 15 and 16). The percentage of berries with ***Cladosporium*** growth was also high on berries from both production systems but percentage occurrence varied significantly between growers (***P***<0.0001). Growth of ***Alternaria*** from washed berry pieces was significantly higher (***P***<0.0001) from conventional berries compared to organic berries (Figure 14). Growth of ***Fusarium*** spp., although below 4%, was only detected in conventionally grown berries and differed significantly (***P***<0.014) from organic berries (Figure 14 and 15).

Berries from two out of four growers in both production systems had growth of ***Aspergillus*** spp. (Figure 15 and 16). There was no significant effect of growing system on the frequency of ***Botrytis*** (Figure 14) while grower 1, 2, 5 and 8 had significantly lower ***Botrytis*** frequency (***P***<0.0001) than the four other growers (Figure 15 and 16).

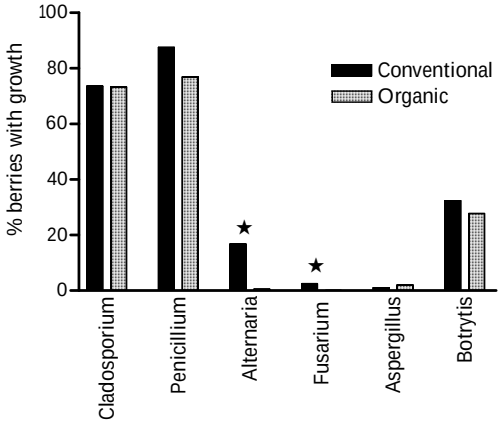

Figure 14. Frequency of fungal genera growing from washed strawberry pieces on semi-selective agar madia. Berries were sampled from four organic and four conventional strawberry growers. (* = significant difference between conventional and organic growing system at 0.05 level).

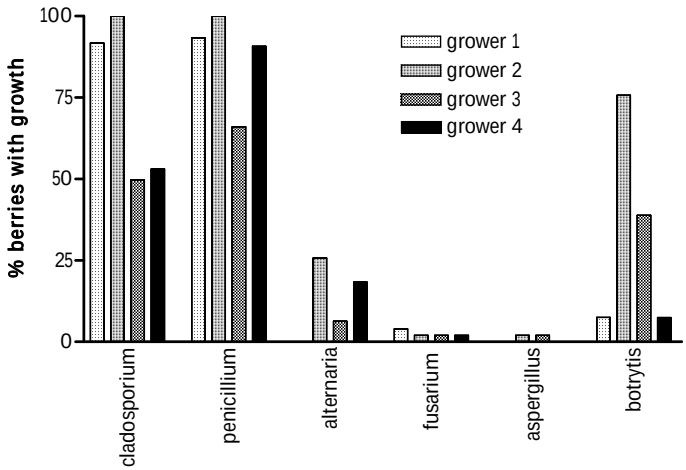

Figure 15. Frequency of fungal genera growing from washed strawberry pieces sampled from four conventional growers (grower 1-4).

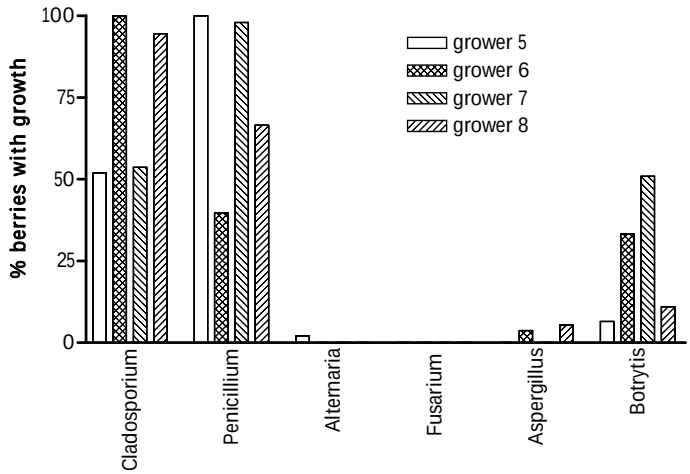

Figure 16. Frequency of fungal genera growing from washed strawberry pieces sampled from four organic growers (grower 5-8).

3.2.2.4 Identification of fungal species and their in vitro metabolite production

The identification of fungi from purified cultures (Table 14) revealed 16 species of ***Penicillium*** (***P. brevicompactum***, ***P. bialowiezense***, ***P. aurantiogriseum***, ***P. olsonii***, ***P. carneum***, ***P. purpurgenum***, ***P. freii***, ***P. polonicum***. ***P. punicae***, ***P. pulvillorum***, ***P. buchwaldii***, ***P. manginii***, ***P. thomii***, ***P. aculeatum, P. verrucosum*** and ***P. expansum***. Two species groups of ***Alternaria*** spp: ***Alt. tenuissima*** sp-grp and ***Alt. infectoria*** sp-grp, as well as ***Aspergillus niger*** and ***Aspergillus flavus*** were also identified.

Five ***Trichoderma*** strains, all isolated from washing water on PDA-C plates were identified to genera (Table 14). Sequence data of the IST1-IST2 region of the isolates revealed that the four "green" isolates were ***T. harzianum*** while the "white" isolates were ***T. viridescens. Clonostachys rosea*** was isolated from two growers. Other fungi occurring on several plate dilutions were identified to be ***B. cinerea***, ***Phoma***, ***Acremonium***-like, ***Hormomyces***-like and one isolate of ***Stemphylium herbarum*** was also identified. . Zygymycetes, especially ***Rhizopus*** sp. frequently was the dominating fugus growing from washed strawberry pieces on V8, and DG18. However, since ***Zygomycetes*** where not of major interest in the present project, ***Mucor*** and ***Rhizopus*** genera were not quantified.

Table 14. Identified fungal isolates from conventional growers (1 to 4) and organic growers (5-8) and their metabolite production in culture extracts. Identification based on morphology and metabolite profiles

Genus	Species	Grower	Location	Metabolite production
Acremonium-like	sp	1	Wash water	Nothing known
Alternaria	*tenuissima* sp-grp	1	Wash water	AOHs, Als, TeA
Alternaria	*tenuissima* sp-grp	1	Wash water	Alx, TeA
Alternaria	*tenuissima* sp-grp	1	Wash water	AOHs, Als, TeA
Mycelium sterilia		1	Wash water	Nothing known
Mycelium sterilia		1	Wash water	Nothing known
Penicillium	*bialowiezense*	1	Berry pieces	Rai, MycA, Quinolactacin
Penicillium	*brevicompactum*	1	Berry pieces	Rai, MycA, Brevianamide
Penicillium	*punicae*	1	Hom. berries	Nothing known
Penicillium	*thomii*	1	Wash water	Nothing known
Trichoderma	*harzianum*	1	Wash water	Pachybasin
Trichoderma	*viridescens*	1	Wash water	6-pentyl-alfa-pyrone
Yeast		1	Hom. berries	Nothing known
Acremonium-like	sp	2	Hom. berries	Nothing known
Acremonium-like	sp	2	Hom. berries	Nothing known
Aspergillus	sp	2	Berry pieces	Pyranonigrin A
Aspergillus	*niger*-group	2	Berry pieces	Gamma-pyrone
Penicillium	*brevicompactum*	2	Wash water	Rai, MycA, Brevianamide

Penicillium	*brevicompactum*	2	Wash water	Rai, MycA, Brevianamide
Penicillium	*brevicompactum*	2	Berry pieces	Rai, MycA, Brevianamide
Alternaria	*infectoria* sp-grp	3	Wash water	N-Z, Infec, Alx
Alternaria	*infectoria* sp-grp	3	Wash water	N-Z, Infec, Alx
Alternaria	*infectoria* sp-grp	3	Wash water	N-Z, Infec, Alx
Alternaria	*tenuissima* sp-grp	3	Wash water	AOHs, Alx, Als, TeA
Hormomyces-like	*sp*	3	Wash water	Nothing known
Mycelium sterilia		3	Wash water	Nothing known
Penicillium	*aurantiogriseum*	3	Wash water	Penicillic Acid
Penicillium	*bialowiezense*	3	Wash water	Rai, MycA, Quinolactacin
Penicillium	*carneum*	3	Wash water	MycA
Penicillium	*freii*	3	Berry pieces	Viridicatol
Penicillium	*olsonii*	3	Wash water	Asperphenamate
Penicillium	*purpurogenum*	3	Wash water	Nothing known
Trichoderma	*harzianum*	3	Wash water	Pachybasin, Chrysophanol
Alternaria	*infectoria* sp-grp	4	Wash water	N-Z, Infec, Alx
Alternaria	*infectoria* sp-grp	4	Wash water	N-Z, Infec, Alx
Alternaria	*tenuissima* sp-grp	4	Wash water	AOHs, Alx, Als, TeA
Alternaria	*tenuissima* sp-grp	4	Wash water	AOHs, Alx, Als, TeA
Penicillium	*brevicompactum*	4	Berry pieces	Rai, MycA, Brevianamide
Penicillium	*freii*	4	Wash water	Viridicatol
Penicillium	*polonicum*	4	Berry pieces	Viridicatol, Cyclopenols
Penicillium	*polonicum*	4	Berry pieces	Viridicatol, Cyclopenols
Penicillium	*polonicum*	4	Wash water	Viridicatol, Cyclopenols
Penicillium	*pulvillorum*	4	Berry pieces	Pulvillonic Acid
Penicillium	*punicae*	4	Wash water	Nothing known
Phoma	sp	4	Wash water	Nothing known
Trichoderma	*harzianum*	4	Wash water	Pachybasin
Alternaria	*infectoria* sp-grp	5	Wash water	N-Z, Infec, Alx
Alternaria	*tenuissima* sp-grp	5	Wash water	AOHs, Alx, Als, TeA
Mycelium sterilia		5	Berry pieces	Nothing known
Penicillium	*bialowiezense*	5	Wash water	Rai, MycA, Quinolactacin
Penicillium	*bialowiezense*	5	Berry pieces	Rai, MycA, Quinolactacin
Penicillium	*brevicompactum*	5	Berry pieces	Rai, MycA, Brevianamide
Penicillium	*buchwaldii*	5	Wash water	Rai, Asperentins
Penicillium	*buchwaldii*	5	Wash water	Rai, Asperentins
Stemphylium	*herbarum*	5	Hom. berries	Nothing known
Hormomyces-like	sp	6	Wash water	Nothing known
Hormomyces-like	sp	6	Hom. berries	Nothing known
Penicillium	*brevicompactum*	6	Wash water	Rai, MycA, Brevianamide
Penicillium	*manginii*	6	Wash water	Citreoviridin
Phoma	sp	6	?	Nothing known
Phoma	sp	6	Hom. berries	Nothing known
Trichoderma	*harzianum*	6	Wash water	Pachybasin
Yeast		6	Hom. berries	Nothing known
Penicillium	*aculeatum*	7	Hom. berries	Nothing known
Penicillium	*brevicompactum*	7	Wash water	Rai, MycA, Brevianamide
Penicillium	*thomii*	7	Wash water	Nothing known
Penicillium	*thomii*	7	Berry pieces	Nothing known
Penicillium	*verrucosum*	7	Berry pieces	Verrucolone
Alternaria	*infectoria* sp-grp	8	Hom. berries	N-Z, Infec, Alx
Aspergillus	*niger*-group	8	Berry pieces	Gamma-pyrone
Penicillium	*bialowiezense*	8	Berry pieces	Rai, MycA, Quinolactacin
Penicillium	*bialowiezense*	8	Berry pieces	Rai, MycA, Quinolactacin
Penicillium	*brevicompactum*	8	Berry pieces	Rai, MycA, Brevianamide
Penicillium	*expansum*	8	Berry pieces	Patulin
Penicillium	*thomii*	8	Berry pieces	Nothing known
Phoma	sp	8	Hom. berries	Nothing known
Yeast		8	Hom. berries	Nothing known

[1] Als: altersetin; Alx: altertoxins; AOHs: alternariols; Infec: infectopyrones; MycA: mycophenolic acid; N-Z: novo-zealandins; Rai: raistrick phenols; ;TeA: tenuazonic acid.

3.2.3 Bacteria

Culturable bacteria made up the largest proportion of the total microbiota on strawberries with maximum densities $>10^4$ CFU/g berry (Figure 7 and 11). The density of culturable bacteria was significantly higher on organic berries as compared to conventional berries both in washing water($\boldsymbol{P}$<0.0019) and in washed homogenized berries ($\boldsymbol{P}$<0.0128). For both quantification methods

grower 3 and 4 had significantly lower bacterial CFU/g berry (***P***<0.05) than the other six growers (Figure 17 and 18).

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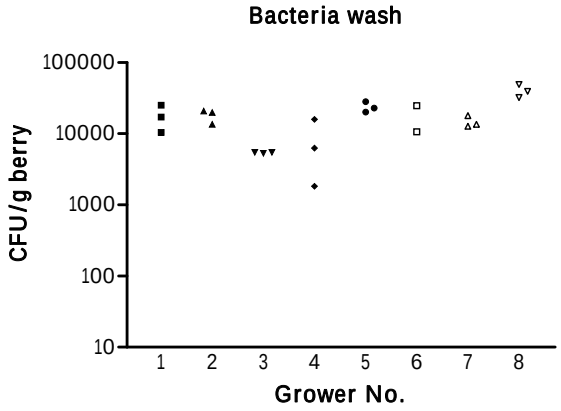

Figure 17. Quantification of bacteria in washing water from berries sampled from conventional growers (No. 1 to 4) and organic growers (No. 5 to 8). Field survey 2006. Data from different growers is represented by different symbols

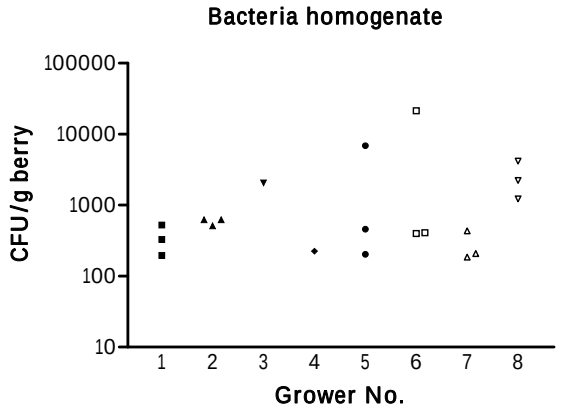

Figure 18. Quantification of bacteria in homogenate of washed berries sampled from conventional growers (No. 1 to 4) and organic growers (No. 5 to 8). Field survey 2006. Data from different growers is represented by different symbols

Bacterial communities on strawberries from conventional and organic growers are shown in Table 15. The bacteria most commonly isolated belonged to the genera ***Curtobacterium, Serratia, Pseudomonas, Enterobacter*** and ***Rahnella.*** In total 23 different genera and 34 different species were identified. The strawberry bacterial communities seem to differ between conventional and organic growers as can be seen from the separation in the PCA analysis (Figure 19). Seventeen bacterial species were only encountered from the organic growers and eight bacterial species were only isolated from conventional growers. Four plant pathogenic bacterial species were found: ***Clavibacter michiganense, Curtobacterium flaccumfaciens, Erwinia carotovora*** and ***Pseudomonas syringae,*** but these bacteria are not known as pathogens of strawberries. Six bacteria species known as potential MPCAs against different plant pathogens were found including ***Enterobacter*** agglomerans, ***Pseudomonas chlororaphis***, ***Pseudomonas putida*** and ***Serratia*** spp.

Table 15. Percent recovery of bacteria species of strawberries sampled from four conventional growers (1-4) and four organic growers (5-8) in 2006. Identification is based on signature fatty acids.

Bacteria	Grower							
	Conventional				Organic			
	(Percent recovery)							
	1	2	3	4	5	6	7	8
Alcaligenes piechaudii	-	-	-	-	-	7	-	-
Bacillus megaterium-GC subgroup B	-	-	-	-	-	13	12	-
Chromobacterium violaceum	-	3	-	-	4	-	-	-
Clavibacter michiganense nebraskense	-	-	-	7	-	-	-	8
Curtobacterium flaccumfaciens flaccumfaciens	-	-	43	50	8	-	-	-
Curtobacterium flaccumfaciens oortii	-	7	10	17	-	-	-	-
Curtobacterium flaccumfaciens poinsettiae	13	10	10	17	4	-	-	-
Enterobacter agglomerans	-	14	23	-	8	13	6	-
Enterobacter intermedius	-	-	-	-	-	7	-	-
Erwinia carotovora atroseptica	-	-	-	3	-	-	-	-
Hafnia alvei	3	-	-	-	-	-	-	-
Kluyveria ascorbata	3	-	-	-	-	-	-	-
Kocuria kristinae	-	-	3	-	-	-	-	-
Kocuria varians	3	-	-	-	-	-	-	-
Microbacterium esteraromaticum	-	-	-	-	-	-	6	-
Micrococcus lylae-GC subgroup A	-	-	-	-	-	-	6	-
Morganella morganii	3	-	-	-	-	-	-	-
Nesterenkonia halobia	-	-	-	-	-	-	-	17
Paenibacillus macerans-GC subgroup B	-	-	-	-	-	-	-	17
Pantoea agglomerans	-	-	-	-	4	27	12	-
Pseudomonas chlororaphis	7	3	-	-	8	7	-	33
Pseudomonas putida	7	14	3	-	31	7	6	-
Pseudomonas syningae phaseolicola	3	7	-	-	4	-	-	-
Pseudomonas syringae atrofaciens	-	-	-	-	4	-	-	-
Rahnella aquatilis	23	7	-	-	-	-	-	8
Rhodococcus luteus	-	-	-	3	-	-	-	-
Serratia fonticola	7	10	3	-	15	7	12	-
Serratia grimesii	13	-	-	3	-	-	-	-
Serratia liquefaciens	13	24	3	-	8	-	12	-
Staphylococcus epidermidis	-	-	-	-	-	7	-	-
Staphylococcus warneri	-	-	-	-	-	7	6	8
Staphylococcus xylosus	-	-	-	-	-	-	6	-
Stenotrophomonas maltophilia	-	-	-	-	-	-	-	8
Yersinia frederiksenii	-	-	-	-	4	-	18	-
Number of species	12	10	8	7	12	10	11	7

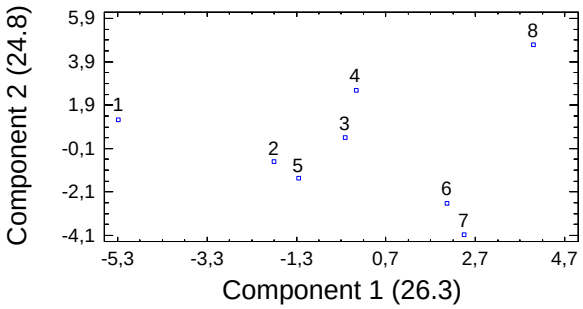

Figure 19. Principal component analysis of the bacterial communities of strawberries sampled from four conventional growers (1-4) and four organic growers (5-8) in 2006. Component 1 and 2 account for 26,3% and 24,8%, respectively, of theThe variability in the data

3.2.4 Yeast

The majority of yeasts were isolated from washing water as compared with the homogenate (Figure 20 and 21). There was no significant effect of growing system on yeast density neither from washing water (Figure 7) nor from homogenate (Figure 11). Yeast communities on strawberries from

conventional and organic growers are shown in Table 16. The yeast most commonly isolated belonged to the genera ***Candida***, ***Cryptococcus*** and ***Rhodotorula***. In total 9 different genera and 22 different species were identified. The strawberry yeast communities tended to differ between conventional and organic growers as can be seen from the separation in the PCA analysis (Figure 22), except that conventional grower 2 group with the organic growers. Six yeast species were only encountered from the organic growers and four yeast species were only isolated from conventional growers.

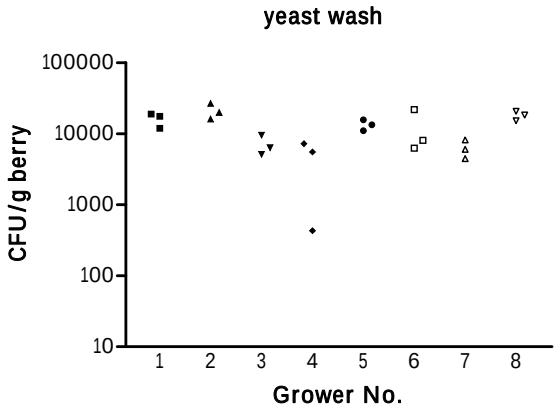

Figure 20. Quantification of yeast in washing water of strawberries sampled from conventional growers (No. 1 to 4) and organic growers (No. 5 to 8). Field survey 2006. Data from different growers is represented by different symbols

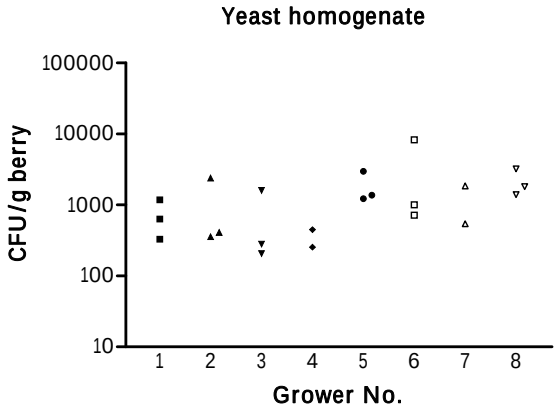

Figure 21. Quantification of yeast in from washed homogenized berries sampled from conventional growers (No. 1 to 4)and organic growers (No. 5 to 8). Field survey 2006. Data from different growers is represented by different symbols

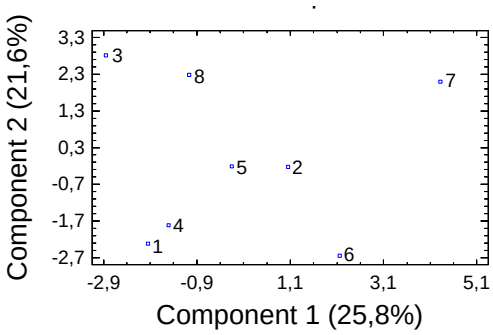

Figure 22. Principal component analysis of the yeast communities of strawberries sampled from four conventional growers (1-4) and four organic growers (5-8) in 2006. Component 1 and 2 account for 25,8% and 21,6%, respectively, of theThe variability in the data

Table 16. Percent recovery of yeast species of strawberries sampled from four conventional growers (1-4) and four organic growers (5-8) in 2006. Identification is based on signature fatty acids.

Yeast	Grower							
	Conventional				Organic			
	(Percent recovery)							
	1	2	3	4	5	6	7	8
Candida acidothermophilum	0	0	0	0	0	4	0	0
Candida cacaoi	0	4	0	0	0	0	5	0
Candida castrensis	0	0	3	0	0	0	0	0
Candida famata	15	13	14	24	11	22	24	4
Candida fragariorum	0	0	0	0	0	0	5	0
Candida glaebosa	0	0	7	0	0	0	0	0
Candida inconspicua	0	0	3	0	0	0	0	0
Candida paludigena	0	9	3	5	5	4	10	4
Candida silvae	0	0	0	0	5	0	0	0
Candida utilis	0	0	0	0	0	9	0	0
Candida zeylanoides	8	9	0	5	11	13	10	4
Cryptococcus albidus var. *Albidus*	0	9	0	0	0	4	0	0
Cryptococcus neoformans-GC subgroup B*	8	4	3	14	0	0	0	4
Cryptococcus terreus	19	4	7	19	5	9	5	11
Hanseniaspora uvarum	0	0	0	0	0	4	0	0
Hansenula anomala	0	13	0	0	0	0	5	0
Metschnikowia pulcherrima	4	0	0	0	0	0	0	0
Rhodotorula minuta-GC subgroup B	12	9	10	14	16	9	0	4
*Rhodotorula rubra**	19	9	45	5	21	9	24	54
*Sporobolomyces salmonicolor**	0	0	3	0	5	0	14	7
Trichosporon beigelii-GC subgroup B*	0	0	0	0	0	0	0	4
Zygosaccharomyces bisporus	0	0	0	0	5	4	0	0
No Match	15	17	0	14	16	9	0	7
Number of species	7	10	10	7	9	11	9	9

3.3 Establishment of *Trichoderma* in field experiments 2007

After a single TRI003 spray treatment the density of ***Trichoderma*** on exposed flowers were 7 x 10^4 CFU/flower at grower 5 (Figure 23) and 4 x 10^4 CFU/flower at grower ST (Figure 24). In the corresponding control plots, the background level of ***Trichoderma*** on flowers was below 100 and 10 CFU per berry, respectively. The recovery of ***Trichoderma*** on berries developed from sprayed flowers was very low. At grower 5, ***Trichoderma*** was recovered from all TRI003 treated plots corresponding to 100 CFU/g berry which differed significantly (***P***<0.0262) from the control treatment. At grower 7, ***Trichoderma*** was isolated from 2 out of 3 TRI003 treated plots which corresponded to 7 CFU/g berry but the density was not significantly different from the control where no ***Trichoderma*** was detected. Following a single TRI003 spray treatment to exposed green berries (Figure 25), the density of ***Trichoderma*** was 2 x 10^4 CFU/green berry, which corresponded to 8 x 10^4 CFU/g berry. However, 20 days later only a few ***Trichoderma*** isolates were recovered from the mature TRI003-exposed berries. ***Trichoderma*** spp. was not isolated from samples of red berries in the unsprayed controls except for one single isolate.

Cladosporium spp. was the dominating fungal genus on mature berries followed by ***Penicillium*** spp. but there was no significant difference between control (water treated) and TRI003 treated, except that ***Cladosporium*** density was significantly lower (***P***<0.0025) on berries developed from TRI003 sprayed green berries compared with the control berries (Figure 25). The populations of ***Penicillium*** spp., ***Fusarium*** spp. and ***Aspergillus*** spp. on mature berries did not differ between TRI003 and water treatments. The isolation of

Penicillium spp varied between 10^1 and 4 x 10^2 CFU/g berry depending on grower and sampling time. ***Fusarium*** spp. was only isolated from berries at grower 5 and the density (approximately 60 CFU/g berry) was at the same level as ***Penicillium*** and ***Trichoderma***. ***Aspergillus*** spp. was only isolated from berries at grower 7.

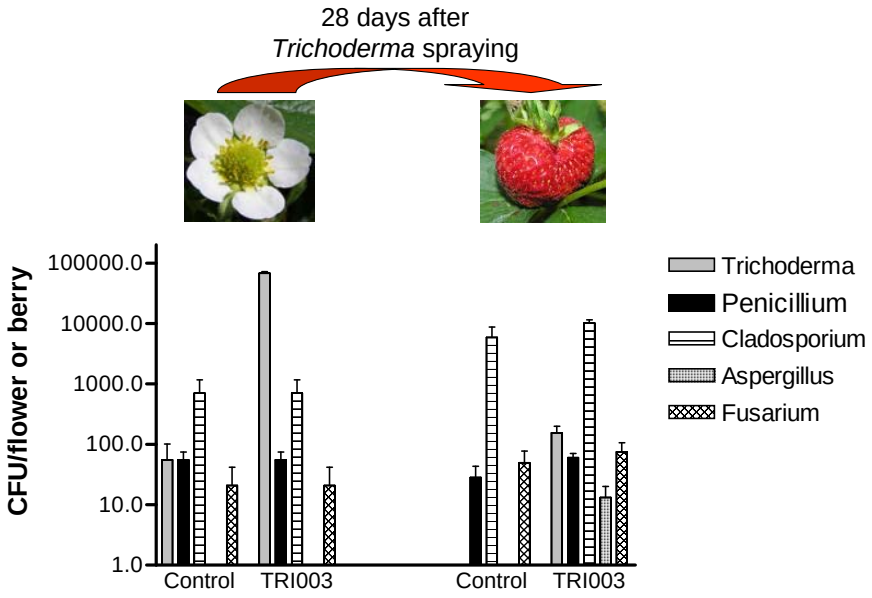

Figure 23. Quantification of fungi on flowers sprayed with *T. harzianum* (TRI003) and on berries developed from sprayed flowers 29 days after the application. Field experiment with grower 5. Plants sprayed 11th June 2007.

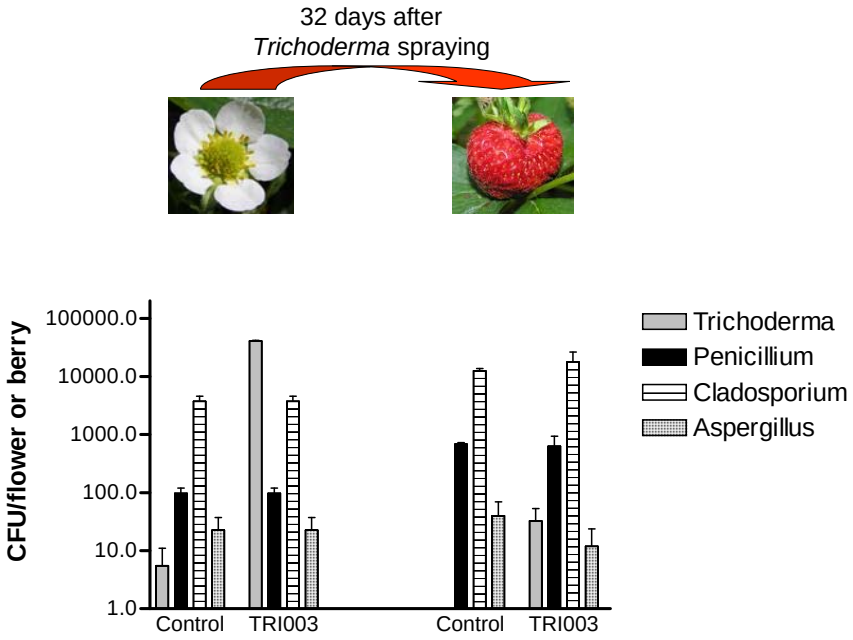

Figure 24. Quantification of fungi on flowers sprayed with *T. harzianum* (TRI003) and on mature berries developed from sprayed green berries 32 days after the application. Field experiment at grower 7. Plants sprayed 7th June 2007.

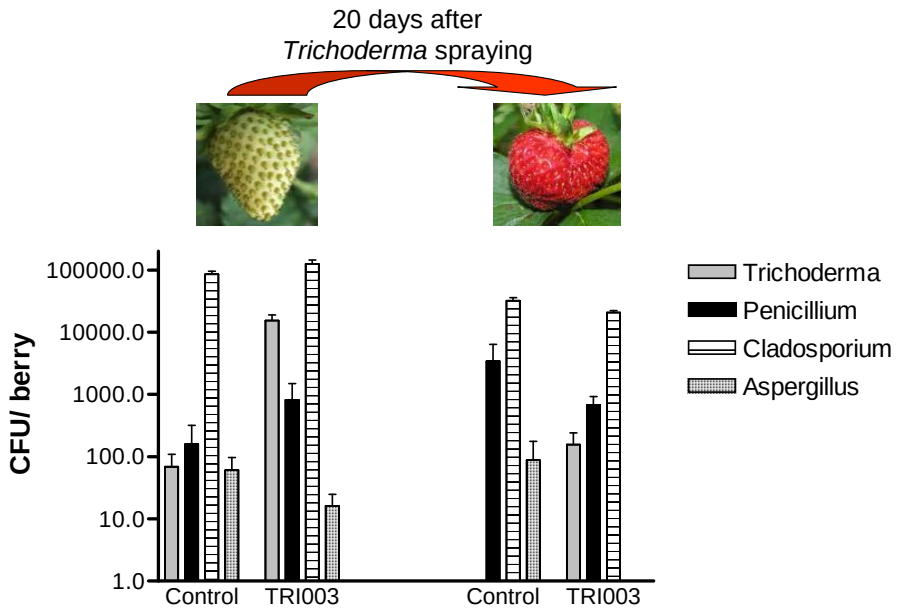

Figure 25. Quantification of fungi on green berries sprayed with *T. harzianum* (TRI003) and on mature berries developed from sprayed green berries 20 days after the application. Field experiment at grower 7. Plants sprayed 7th June 2007.

In order to determine the origin of ***Trichoderma*** strains, we reisolated and purified a total of 125 isolates identified by morphological characteristics as ***Trichoderma*** sp. (Table 17). All isolates from both control and TRI003 treatment at time of spraying had a UP-PCR profile similar to that of TRI003 (Figure 26, Table 17) and the similarity with TRI003 was confrmed by ITS1/ITS2 sequence alignement. The only isolate coming from red berries of control plots had a UP-PCR profile differing from TRI003 and sequence alignement identified it as ***T. harzianum*** with 6 bp differences to the TRI003 strain. Out of 27 isolates from red strawberries of TRI003 sprayed plots, only one isolate (TRI104) differed from the TRI003 profile and sequence alignement identified it as ***T. harzianum*** with 9 bp differences to the TRI003 strain(Figure 26, Table 17).

Table 17. Trichoderma strains isolated from flowers and berries following spray treatment with TRI003. Numbers of isolates identified mophologically, by UP-PCR profile and by sequencing are given.

		Trichoderma sp. isolated from strawberry (number)			
		Flowers/berries at application		Red berries one months after application	
		Control	TRI003	Control	TRI003
Trichoderma sp. isolated		17	35	1	72
UP-PCR profile	TRI003	15	22	0	26
	Other	0	0	1	1
Sequence similarity	TRI003	15	22	0	26
	Other	0	0	1	1

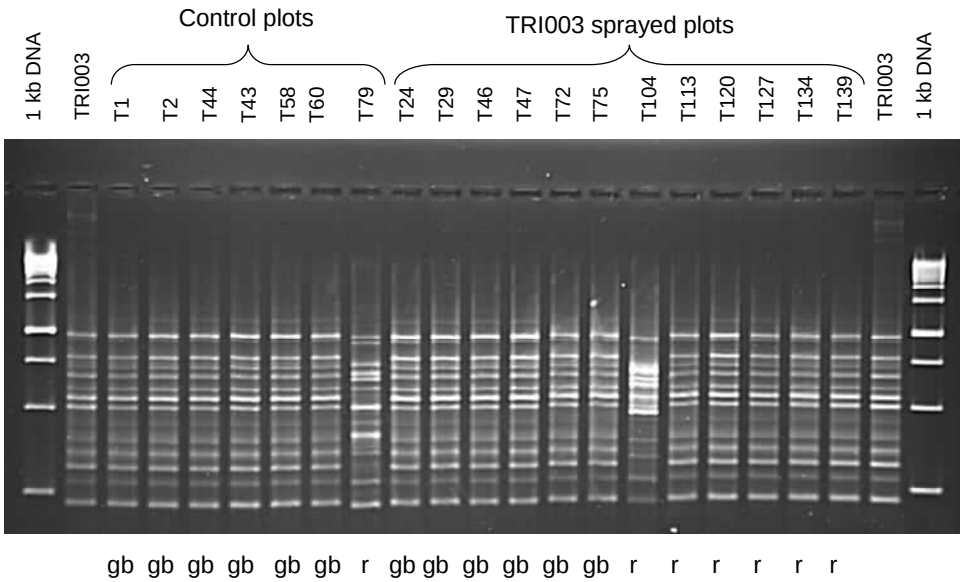

Figure 26. UP-PCR profile of *Trichoderma*-strains isolated immediately after application of TRI003 to flowers/green berries (gb) and from red berries developed from exposed flowers/green berries (r) approximately one months after application of TRI003.

3.4 Activity of *C. rosea* on strawberry plants.

The germination of conidia of ***C. rosea*** on berries and leaves of strawberry plants grown at 18ºC in growth chamber are shown in Table 18. Only a few percentages of the conidia had germinated on berries and leaves after one day. However, more than 10% of the conidia had germinated on berries after two 2 days. Number of germinated conidia varied considerably within berries as germinated conidia often were concentrated around the seed of the berry. It was not possible to assess conidia germination after 5 days since dense hyphal growth was observed especially around the seed cavities. Confocal microscopy 6 days after application of ***C. rosea*** confirmed that hyphal growth of the fungus was concentrated in the area around the seed cavities of the berry (Figure 27). Laser scanning of the uppermost cell layers also showed that the growth of ***C. rosea*** was superficial. Sporulation on berries and leaves were observed neither by fluorescence microcopy nor by confocal microscopy within 6 days. No significant change in CFU/g leaf was found from inoculation with ***C. rosea*** (day 0) until 5 days of incubation whereas on berries a significant 4-fold decrease in CFU/g berry was found (Table 18).

Table 18. Germination and sporulation activity of *C. rosea*, IK726*d11* on strawberry plants. Attached berries and leaves were inoculated with conidia of the MBCA.

Incubation period (days)	Leaves		Berries	
	Cfu/g[1)]	Conidia germination (%)	Cfu/g[1)]	Conidia germination (%)
0	3.0×10^5 a	0	1.3×10^5 a	0
1	3.5×10^5 a	1.9 a	4.4×10^4 b	3.3 a
2	-	4.4 a	-	13.0 b
5	2.8×10^5 a	-[2)]	4.2×10^4 b	-[2)]

[1)] Within columns mean values followed by the same letter are not significantly different according to Duncan´s multiple range test ($p < 0.05$)
[2)] Not possible to determine conidia germination due to heavy hyphal growth

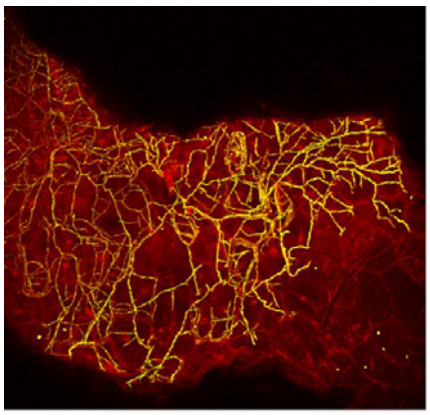

Figure 27. Growth of *C. rosea* IK726*d11* containing the green fluorescent protein gene (*gfp)* on the surfafe of a strawberry is visualized by confocal laser scanning microscopy. Hayhae are growing in the cavity around the strawberry seed on a berry stored six days at 20ºC.

3.5 Activity of fungi on detached berries and flowers

The activity of ***C. rosea*** on different strawberry tissues. Germination of conidia were compared on detached flowers and berries using the ***gfp*** transformed isolate of the fungus (IK726***D11***), respectively. Significantly more conidia germinated on flowers than on berries after 24 hours (Table 19). Furthermore ***C. rosea*** grew much faster on the flowers as hyphal growth was so dense that it was impossible to determine germination after 48 hours. In contrast, conidia germination was easily determined on berries after 48 h and no further increase in germination were noted (Table 19).

Table 19. Germination of *C. rosea* (IK726*d11*) conidia on strawberry flower petals and berries stored at 20ºC.

Incubation (hours)	Conidia germination (%)	
	Flower petals	Berry
24	21.7 a	7.6 b
48	-[2)]	8.3

[1)] Within row mean values followed by the same letter are not significantly different according to Duncan´s multiple range test ($P < 0.05$)
[2)] Not possible to determine conidia germination du to heavy hyphal growth following 48 hours incubation of flowers

Germination of ***P. expansum***, ***C. rosea*** and ***B. cinerea*** on detached strawberries was evaluated at 4º and 20ºC using ***gfp*** transformed isolates of the three fungi (Table 20, Figure 28). At 20ºC approximately 40% of the ***P. expansum*** and the ***C. rosea*** conidia had germinated while significantly more, almost 90%, of the ***B. cinerea*** conidia had germinated. At 4ºC, germination generally was slower but more than 70% of the ***B. cinerea*** conidia had germinated after 2 days. Only 1% of the ***C. rosea*** conidia were germinated which was significantly lower than seen for ***P. expansum*** after 2 days. The difference between ***P. expansum*** and ***C. rosea*** was even bigger after 4 days as 30% of the ***P.expansum*** conidia had germinated compared to only 2% of the ***C.rosea*** conidia (Figure 28). The difference in activity of the two latter fungi was confirmed from data on CFU/g berry (Table 21). For ***P. expansum*** no increase in cfu density on berries was found at 4ºC while a 2 x 10^4-fold increase in CFU/g berry was estimated at 20ºC. For ***C. rosea*** CFU/g berry decreased significantly on berries stored at 4ºC whereas no increase was seen at 20ºC. Berries inoculated with ***C. rosea*** had no macroscopic symptoms at 4º or 20ºC. In contrast berries inoculated with ***P. expansum*** and stored at 20ºC were visible decomposed and

covered with green spores. As expected, ***Botryti***s inoculated berries were strongly decomposed (see Figure 29).

Table 20. Percent germinated conidia of *C. rosea, P. expansum* and *B. cinerea* on mature strawberries stored at 4ºC and 20ºC.

Fungal species	Conidia germination (%)		
	20ºC	4ºC	
	1 day	2 days	4 days
C. rosea, IK726*d11*	35.9 a[1)]	0.9 a	2.1 a
P. expansum, IBT 21525*gfp4*	42.6 a	7.9 b	29.8 b
B. cinerea, IK2020*gfp5*	87.4 b	74.8 c	86.8 c

[1)] Mean values within columns followed by different letters are significantly different according to Duncan´s multiple range test ($P < 0.05$)

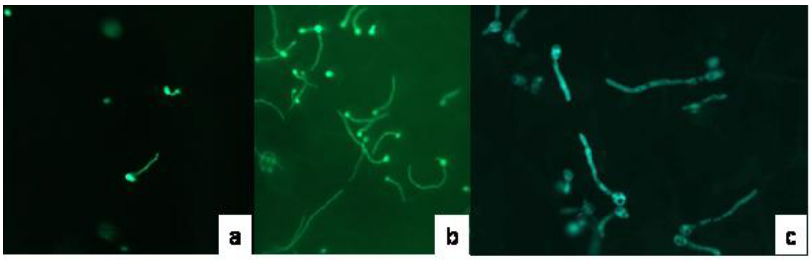

Figure 28. Germination of fungal conidia of *gfp* marked fungal visualized by fuorescence microscopy on the surface strawberries stored at 4ºC (a) germination of *C. rosea*, IK726*d11* four days after inoculation, (b) germination of *P. expansum* IBT 21525*gfp4* four days after inoculation, and (c) germination of *B. cinerea*, IK2020*gfp5* only two days after inoculation.

Table 21. Development in cfu/g berry of berries inoculated with *C. rosea* IK726*d11* and *P. expansum* IBT 21525*gfp4,* respectively and stored four days 4ºC and 20ºC.

Fungal species	Storage period					
	0 days		4 days			
	4ºC/20ºC		20ºC		4ºC	
	CFU/g berry	Log_{10} CFU/g berry	CFU/g berry	Log_{10} $_{CFU}$/berry	CFU/g berry	Log_{10} CFU/berry
C. rosea	1.4×10^4	4.1 a[1)]	2.1×10^4	4.3 a	4.0×10^3	3.6 b
P.expansum	1.8×10^3	3.2 a	3.8×10^7	7.6 b	9.8×10^2	2.9 a

[1)] Within rows, mean values followed by different letters are significantly different according to Duncan´s multiple range test ($P < 0.05$)

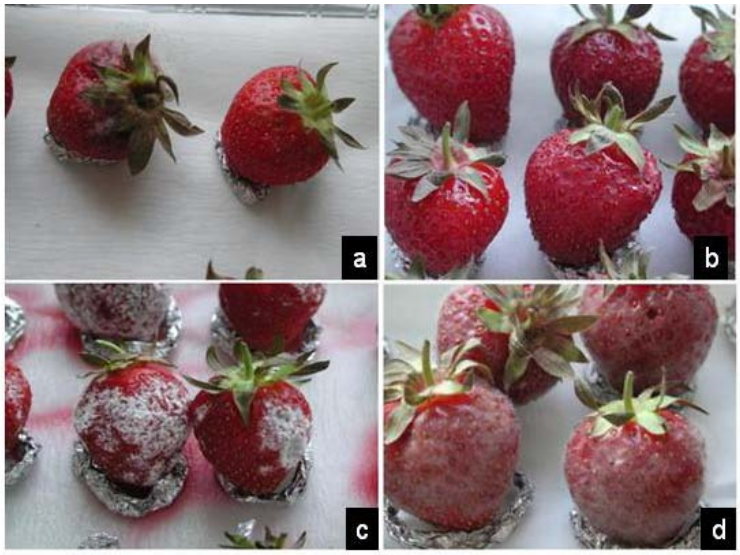

Figure 29. Growth of fungi on strawberries stored at 20ºC for 5 days. (a) Berries inoculated with water, (b) berries inoculated with *C. rosea*, (c) berries inoculated with *P. expansum* and (d) berries inoculated with *B. cinerea*.

3.6 Interaction between fungi on flowers and berries

3.6.1 Flower assay

Conidia germination of dual inoculated fungi – ***B. cinerea/C. rosea*** and ***T. harzianum/C. rosea*** was determined and compared with germination of the fungi applied singly (Figure 30).

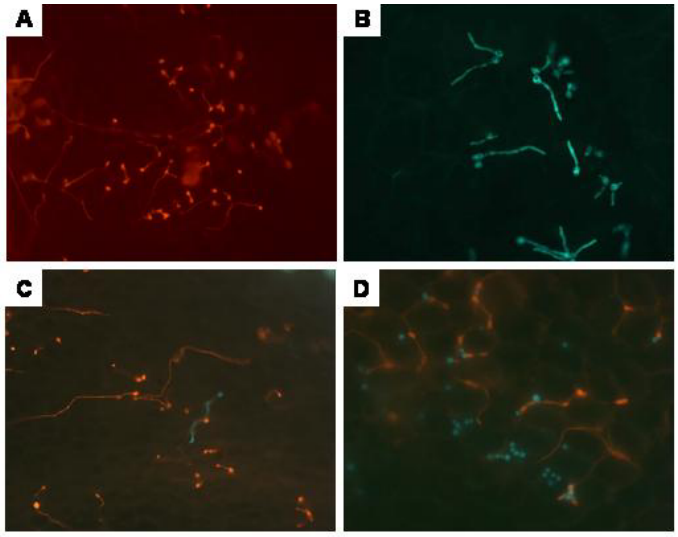

Figure 30. Germination of fungal strains marked either with the *gfp* or the *DsRed* gene and visualized by fluorescence microscopy on strawberry petals (A) Red fluorescent conidia of *C. rosea*, IK726*Dsred*. (B) Green fluorescent condia of *B. cinirea*, IK2018gfp. (C) Germinating conidia of *C.rosea* (red) and *B.cinerea* (green) on a petal.(D) Germinated *C. rosea* conidia (red) and non-germinated *T.harzianum*, 1295-22*gfp1* conidia (green) on a petal.

More than 70% of the ***Botrytis*** conidia germinated when the fungus was applied alone while germination was significantly reduced to <60% when co-inoculated with ***C. rosea*** (Figure 31). Germination of ***C. rosea*** conidia applied alone did not differ significantly from germination of conidia co-inoculated with either ***Botrytis*** or ***T. harzianum.*** In contrast ***Trichoderma*** germination was significantly inhibited by ***C. rosea*** co-inoculation (Figure 31).

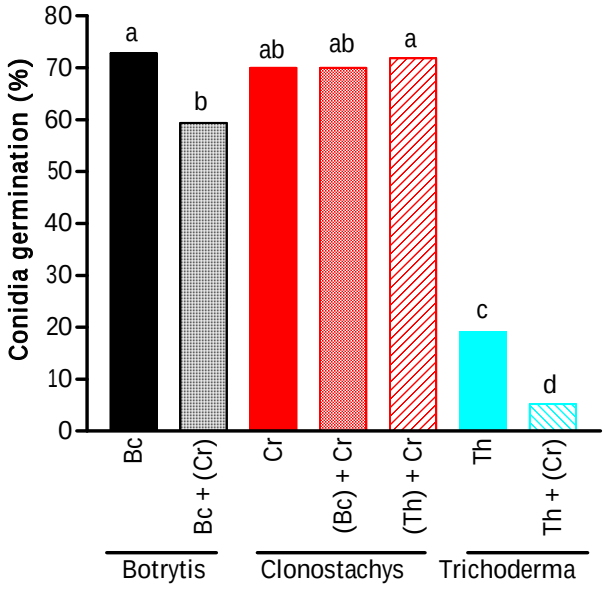

Figure 31. Germination of *B. cinerea* IK2018*gfp5* (Bc), *C. rosea* IK726*Dsred5* (Cr) and *T. harzianum* 1295-22*gfp1* (Th) on strawberry flower petals 24 hours after singly or dual inoculation of fungal conidia. Bc, Cr and Th = petals inoculated singly with the fungi. Bc +(Cr) = germination of *B. cinerea* determined on petals co-inoculated with *C. rosea*. (Bc) + Cr = germination of *C. rosea* on petals co-inoculated with *B. cinerea*, and so on. Mean values followed by different letters are significantly different according to Duncan´s multiple range test ($p < 0.05$).

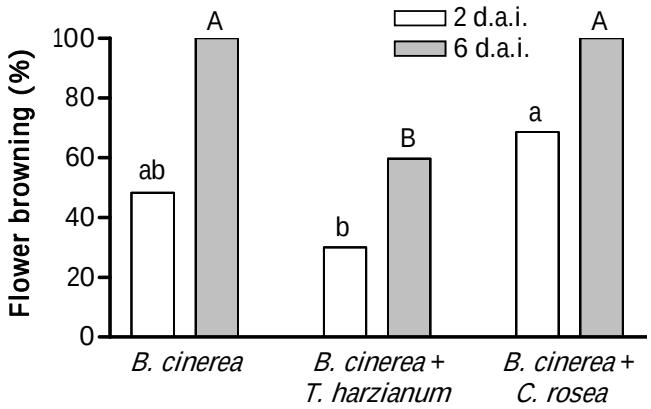

Figure 32. The effect on percentage browning of strawberry petals by dual inoculation of flowers with *B. cinerea* and the MPCAs *T. harzianum* and *C. rosea*, respectively. Mean values followed by different letters are significantly different according to Duncan´s multiple range test ($P < 0.05$).

Development of petal browning caused by ***Botrytis*** was recorded on the flowers 2 and 6 days after inoculation (see Figure 32 and 33). When ***Botrytis*** was co-inoculated with ***C. rosea*** the browning was reduced compared to the ***Botrytis*** control. The disease reduction was significant six days after inoculation. ***T. harzianum*** had no effect on symptom development (Figure 32). It was also noted that the ***C. rosea*** treatment inhibited the infection of sepals as they were still green after six days compared to the ***Botrytis*** control flowers where all sepals were complete brown and infected (Figure 33).

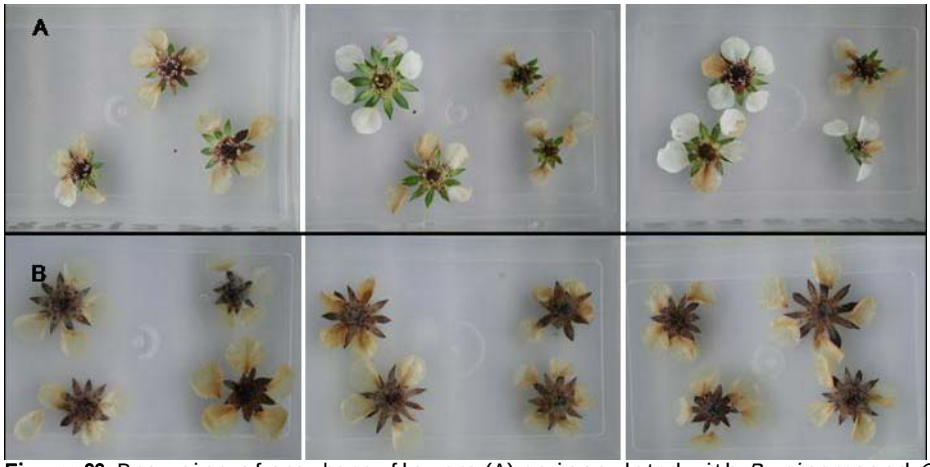

Figure 33. Browning of srawberry flowers (A) co-inoculated with *B. cinerea* and *C. rosea* and (B) flowers inoculated with *B. cinirea.* The inoculated flowers were incubated for 6 days at 20°C.

3.6.2 Berry assay

The CFU density of ***C. rosea*** and the amount of C. rosea DNA was significantly increased after 4 days incubation at 4˚C as compared to the control treatment (***C. rosea*** inoculation, t=0) (Figure 34). On the other hand

C. rosea CFU density was unchanged after 4 days incubation at 20˚C while the significantly highest amount of ***C. rosea*** DNA was detected from these berries (Figure 34). Relatively, the increase in ***C.rosea*** DNA was 60 times higher for ***C. rosea*** inoculated berries incubated at 20˚C as compard to the control while changes in CFU densities varied between 0.5-3 depending on the treatment (Figure 35). The results indicate that hyphal growth has taken place on ***C. rosea*** inoculated berries stored at 20˚C since CFU mainly quantifies spores. It also seems that ***B.cinerea*** restricts hyphal growth/sporulation of ***C.rosea*** on dual inoculated berries stored at 20˚C.

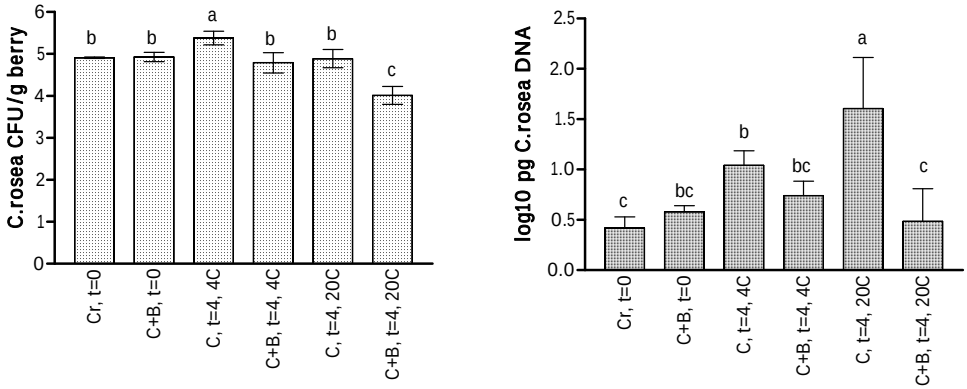

Figure 34. Effect of single inoculation of berries with *C. rosea* (C) and dual inoculation with C and *B. cinerea* (C+B) on CFU/g berry (left panel) and on pg DNA of *C. rosea* quantified by real-time PCr on DNA extracted from berries (right panel). Quantification was made at t=0 and after four days of incubation (t=4) of berries stored at 4ºC or 20ºC. Mean values followed by different letters are significantly different according to Duncan´s multiple range test ($P< 0.05$).

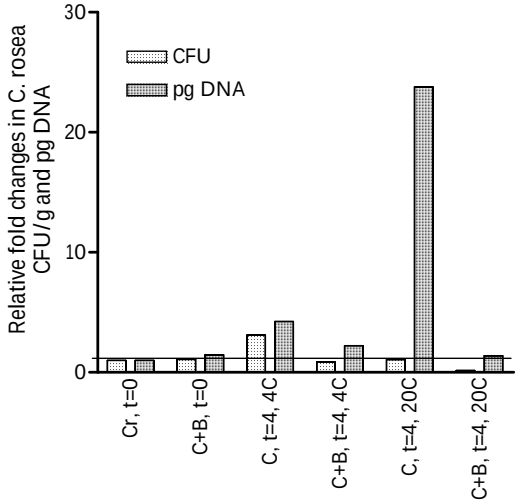

Figure 35. Fold change in *C. rosea* CFU/g berry and in picogram *C. rosea* DNA relative to the control (Cr, t=0) set to 1 on the graph.

3.7 *In vitro* Compatibility between BCAs and fungicides on agar

All the tested grey mould fungicides clearly inhibited growth of the target pathogen ***Botrytis cinerea***. However, Euparen and Scala only completely inhibited ***B. cinerea*** at x10 recommended dosage (Table 22).

Within the tested MPCPs both TRI003 and Supresivit were unaffected by all the fungicides included in this study. Binab-T was inhibited by Candit and

Signum at x10 recommended dosage, but was unaffected by the other four fungicides. ***C. rosea*** was inhibited by Euparen and Signum at x10 recommended dosage, but was unaffected by the other four fungicides.

Table 22. Sensitivity of the pathogen *B. cinerea* and four MPCAs against six fungicides conventionally used in strawberry production. Values refer to minimal inhibitory dosage relative to recommended dosage (see table 6). NS means that the fungus in insensitive to a fungicide within the tested dosages (x¼ to x10 of recommended dosage).

Fungi	Fungicides					
	Amistar	Candit	Euparen	Scala	Signum	Teldor
Target						
B. cinerea	NS	x10	x10	x10	X1	x¼
BCAs						
C. rosea	NS	NS	x10	NS	X10	NS
Binab-T	NS	x10	NS	NS	X10	NS
Supresivit	NS	NS	NS	NS	NS	NS
TRI 003	NS	NS	NS	NS	NS	NS

3.8 *In vitro* Compatibility between MPCAs and fungicides on flowers

All three grey mould fungicides Teldor, Scala and Euparen completely controlled grey mould development at recommended dosage (experiment 1). Teldor also efficiently controlled grey mould at a dosage corresponding to 50% of recommended dosage (data not presented). In experiment 2, none of the MPCAs affected grey mould development except ***U. atrum***, which reduced grey mould development by 10% (data not presented). Teldor only reduced disease development by 50% in experiment 2 (data not presented). In experiment 3, where the level of grey mould was reduced compared to experiment 1 and 2, both MPCAs and Teldor reduced disease development. The MBCA ***U. atrum*** efficiently controlled ***B. cinerea*** equivalent to the fungicide treatment (Figure 36).

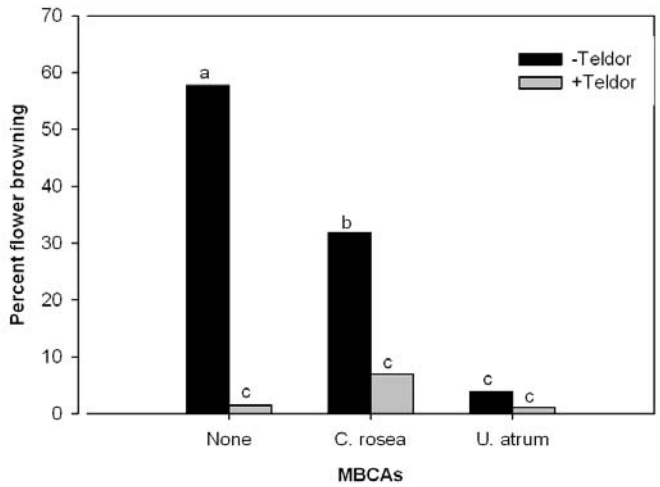

Figure 36. Effects of the MPCAs *C. rosea* and *U. atrum* alone and in combination with the fungicide Teldor in treatments artificially inoculated with *B. cinerea*. Different letters indicate significant differences between treatments.

3.9 *In* stablishment of MPCAs on strawberry plants with and without fungicide treatment

The three MPCPs TRI003, ***U. atrum*** and ***C. rosea*** were sprayed at the flowering stage singly or in combination with ½ x normal dosage of the

fungicide Teldor. The microbiota on red berries was examined four weeks after the applications. The mycobiota was dominated by ***Cladosporium*** spp. varying on average between approximately 1800 and approximately 3000 CFU/g berry while ***Penicillium*** spp. and ***Botrytis*** varied between 50 to700 CFU/g berry and 30-550 CFU/g berry, respectively (Figure 37 and 38). However, for ***Cladosporium, Penicillium*** and ***Botrytis*** CFU densities there was no significant difference between MPCPs and there was no significant effect of fungicide application on the CFU densities. ***Trichoderma*** spp. was the most frequently isolated MPCA as it was isolated from 5 out of 6 plots treated with TRI003 and with TRI003+Teldor, respectively. This corresponded to an average of 18 and 21 CFU/ g berry, respectively. ***Trichoderma*** spp was also isolated from 4 out of 12 ***U. atrum*** treated plots while it was only isolated from one out of 12 ***C. rosea*** and water sprayed plots, respectively. The density of ***Trichoderma*** on berries was significantly higher for ***T.harzianum*** sprayed plots than for the other treatments (***P***<0.0001).***C. rosea*** was only isolated from ***C. rosea*** sprayed plots and the CFU density of the ***C. rosea*** treatment differed significantly (***P***<0.0003) from the three other treatments. On average 7 and 2 CFU/g berry was isolated from ***C. rosea*** and of ***C. rosea***+Teldor sprayed plots, respectively. In contrast, ***U. atrum*** could not be detected from any of the plots, not even from ***U. atrum*** sprayed plots (Figure 37 and 38). Application of Teldor had no significant effect on the establishment of the three MPCPs.

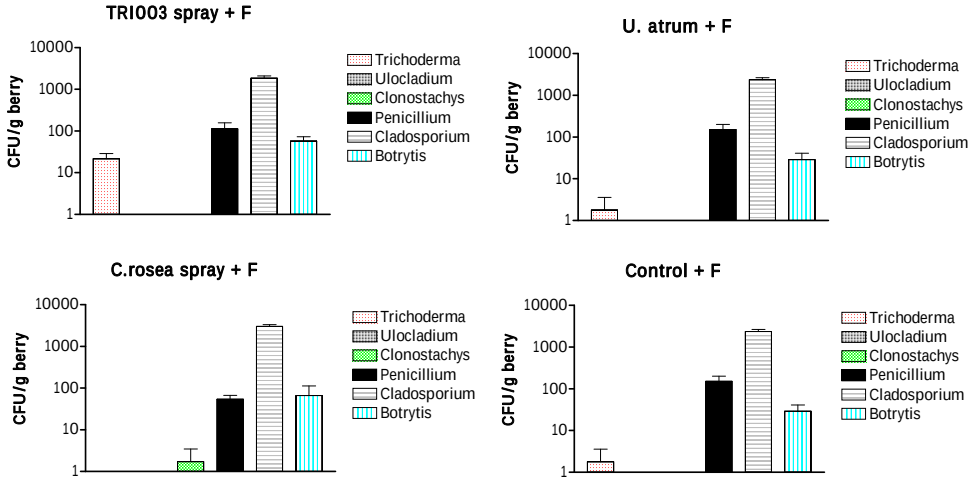

Figure 37. Quantification of fungi (CFU/g berry) on berries from semi-field plots sprayed with the MPCAs *T. harzianum* (TRI003), *U. atrum*, *C. rosea* and water, respectively, four weeks previously. (Data bars are mean values (n=6) with standard error bars).

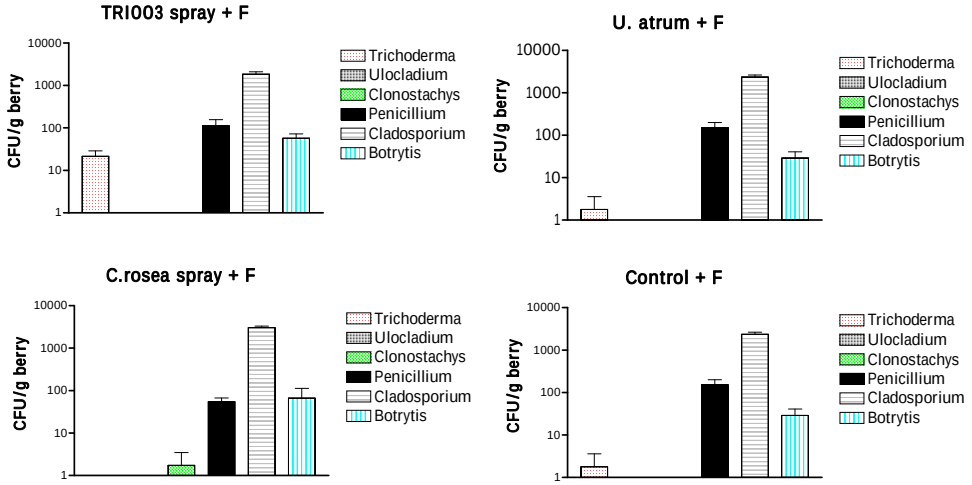

Figure 38. Quantification of fungi (CFU/g berry) on berries from semi-field plots treated with a combined application of the fungicide Teldor and the MPCAs *T. harzianum* (TRI003), *U. atrum*, *C. rosea* and water, respectively, four weeks previously. (Data bars are mean values (n=6) with standard error bars).

In order to determine the origin of ***Trichoderma*** strains, we reisolated and purified all isolates resembling ***Trichoderma*** spp. The pure isolates were firstly identified by morphology. Then 15 of 29 isolates from TRI sprayed plots and all twelve isolates of control plots were selected for UP-PCR fingerprinting and ITS1-ITS2 region sequencing (Figure 39, Table 23). Eleven of the15 isolates from TRI sprayed plots had a UP-PCR profile similar to TRI003 strain 1295-22 while 4 isolates had very different profiles indicating that these isolated were indigenous. Sequence polymorphism varied from 0 to 6 bases and all four isolates were identified as ***T. harzianum*** based on NCBI and TrichoKey blasting. Of the twelve isolates from plots not sprayed with TRI, only the isolates from the ***C rosea*** sprayed plot had a UP-PCR profile similar to TRI003. Blasting the seqences showed that 9 isolates belonged to ***T.harzianum*** while three isolates were identified ***T. asperellum*** and ***T. viride***, respectively (Table 23).

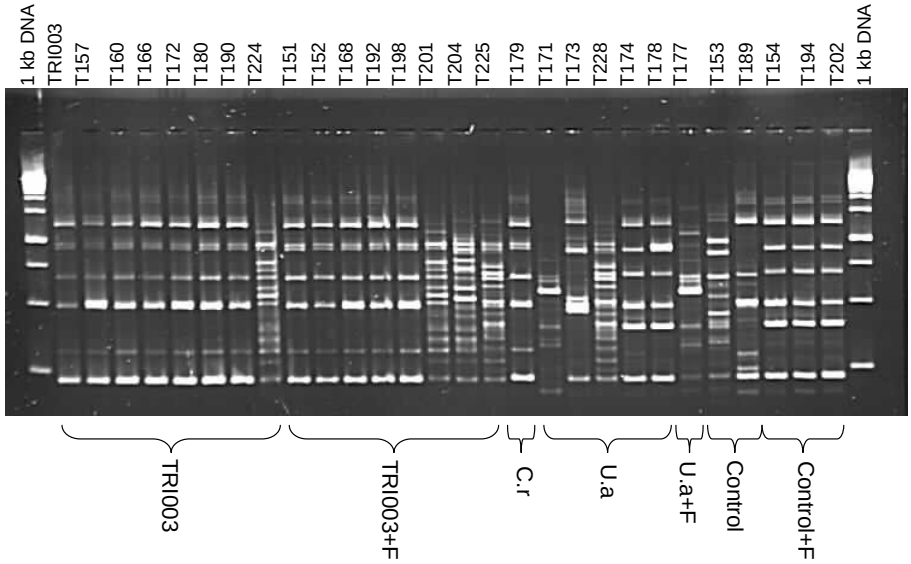

Figure 39. UP-PCR profile of *Trichoderma* strains isolated from strawberry in semi-field experiment.

Table 23. Identification of *Trichoderma* strains isolated from berries in semi-field experiment based on UP-PCR and sequence identity in the NCBI and TrichoKey databases.

Treatment in the field	Block	No. of isolate	UP-PCR profile	Similarity 0f sequence with 1295-22[1)]	Sequence identification	
					NCBI	TrichoKey
TRI	47	157	1295-22	1295-22	*T. harzianum*	*T. harzianum* -
TRI	39	160	1295-22	1295-22	*T. harzianum*	*T. harzianum*
TRI	15	166	1295-22	1295-22	*T. harzianum*	*T. harzianum*
TRI	16	172	1295-22	1295-22	*T. harzianum*	*T. harzianum*
TRI	7	180	1295-22	1295-22	*T. harzianum*	*T. harzianum*
TRI	7	190	1295-22	1295-22	*T. harzianum*	*T. harzianum*
TRI	31	224	other	1295-22	*T. harzianum*	*T. harzianum*
TRI+F	40	151	1295-22	1295-22	*T. harzianum* -	*T. harzianum*
TRI+F	40	152	1295-22	1295-22	*T. harzianum*	*T. harzianum*
TRI+F	24	168	1295-22	1295-22	*T. harzianum* -	*T. harzianum*
TRI+F	8	192	1295-22	1295-22	*T. harzianum*	*T. harzianum*
TRI+F	32	198	1295-22	1295-22	*T. harzianum*	*T. harzianum*
TRI+F	40	201	other	1295-22	*T. harzianum* -	*T. harzianum*

TRI+F	24	204	other	5	*T. harzianum*	*T. harzianum*
TRI+F	32	225	other	6	*T. harzianum* -	*T. harzianum*
C.rosea	11	179	1295-22	1295-22	*T. harzianum*	*T. harzianum*
U.atrum	21	171	Other	>40	*T. asperellum*	*T. koningiopsis/ T. asperellum*
U.atrum	14	173	Other	6	*T. harzianum*	*T. harzianum*
U.atrum	38	228	Other	7		
U.atrum	14	174	Other	6	*T. harzianum*	*T. harzianum*
U.atrum	5	178	other	6	*T. atroviride/ T. harzianum*	*T. harzianum*
U.atrum+F	6	177	other	>40	*T. asperellum*	*T. koningiopsis/ T. asperellum*
Control	41	153	other	>48	*T. viride*	*T. viride*
Control	1	189	other	5	*T. harzianum*	*T. harzianum*
Control+F	42	154	other	6	*T. citrinoviride/ T. harzianum*	*T. harzianum*
Control+F	18	194	other	5	*T. citrinoviride/ T. harzianum*	*T. harzianum*
Control+F	42	202	other	5	*T. harzianum*	*T. harzianum*

1) 1295-22 means complete similarity of sequence with the sequence from strain 1295-22. Other numbers indicate number of bases that differs from the 1295-22 sequence.

The total culturable population of bacteria and yeasts were unaffected by the MPCPs and fungicide treatments (Table 24).

Table 24. Effect of MPCAs and fungicide treatments on population density of total culturable bacteria and yeast on mature strawberries in semi-field experiment 2007.

MBCA	Teldor	Bacteria		Yeast	
	½ x dosis	(cfu/g berry)			
-	-	2320	A[1)]	1478	A
-	+	1786	A	1545	A
C. rosea	-	911	A	710	A
C.rosea	+	6033	A	2124	A
U. atrum	-	1431	A	2086	A
U. atrum	+	2108	A	1244	A
TRI003	-	2025	A	1740	A
TRI003	+	2150	A	1569	A

[1)] Within columns, mean values (n=6) followed by different letters are significantly different ($P < 0.05$)

In addition, overall both factors had no effects on the microbial community as measured using biomarker fatty acids from both culturable and unculturable microorganisms (Table 25). However, the amount of total microbial fatty acids tended to be lower from berries obtained from the fungicide treated plants than the corresponding berries obtained from plants which did not receive Teldor ($\boldsymbol{P}$=0.053).
Examination of all the individual biomarker fatty acid data using principal component analysis (PCA) also did not result in clear groupings (data not shown).

Table 25. Treatment means with different letters are significantly different.

Treatments		Amount of microbial biomarker fatty acids (nmole)		
MPCA	Teldor	Bacterial[x]	Fungal[z]	Total microbial[y]
-	-	0.6A[1)]	30.4A	111.3A
-	+	5.4A	24.2A	102.3A
C. rosea	-	0.5A	29.8A	81.1A
C. rosea	+	2.6A	17.2A	59.1A
U.atrum	-	1.3A	44.5A	197.9A
U.atrum	+	0.7A	30.1A	91.0A
TRI003	-	2.1A	24.6A	89.2A
TRI003	+	4.0A	21.3A	59.7A

[x]16:0 2OH, 16:0 3OH, 18:0 2OH, 17:1w8, 17:0 cyclo, 19:0 cyclo
[z]18:2w6,9
[y]Sum of x and z plus 16:0, 16:1w7, 18:0

Plant growth data and numbers of berries were unaffected by the MPCA and fungicide treatments except that TRI003 reduced the number of runners (Table 26).

Table 26. Effect of MPCA and fungicide spray treatments on plant growth, fruit number and fruit quality in semi-field experiment 2007.

MPCA	Teldor ½ x dosis	Shoot dry weight		Runners		Red berries		Green berries		Rotted berries	
		(g)		Number							
-	-	51.9	A	4.3	B	9.0	A	46.6	A	1.0	A
-	+	51.1	A	3.3	AB	9.3	A	42.2	A	0.5	A
C. rosea	-	47.1	A	3.0	AB	6.8	A	39.0	A	0.7	A
C.rosea	+	47.1	A	4.2	B	9.0	A	36.0	A	0	A
U. atrum	-	46.9	A	2.5	AB	8.0	A	42.7	A	0.3	A
U. atrum	+	47.5	A	1.3	A	9.8	A	41.7	A	0.5	A
TRI003	-	45.8	A	1.8	A	8.5	A	43.5	A	0.5	A
TRI003	+	46.8	A	1.7	A	6.5	A	43.0	A	0	A

[1)]Within columns, mean values (n=6) followed by different letters are significantly different ($P < 0.05$)

3.10 Metabolite analysis

3.10.1 Metabolite analysis of fungal pure

Culture extracts of purified fungal isolates from the survey of the naturally occurring microbiota on conventional and organic grown berries were analyzed by HPLC-UV. The production of known metabolites from representative fungal isolates is listed in Table 14. As seen in Figures 7, 11 and 14 ***Cladosporium***, ***Penicillium***, ***Alternaria*** and ***Botrytis*** species were the most dominating fungal genera in berries from both conventional and organic growers. ***Cladosporium*** and ***Botrytis*** are not known to produce any mycotoxins or other biologically active secondary metabolites, whereas ***Alternaria*** and especially ***Penicillium*** species are known for their production of biologically active metabolites. The analyses show that no mycotoxins sensu stricto were detected in any of the pure culture extracts except for ***P. expansum*** producing patulin. However, many of the fungal species isolated from the berries can produce metabolites with some known biological activity on laboratory substrates.

Chemical identification and metabolite profiling of the ***Penicillium*** isolates showed that ***P. bialowiazense*** and the closely related ***P. brevicompactum*** were the most common ones. Both ***Penicillium*** species are able to produce Raistrick phenols (Rai) and mycophenolic acid. Chemical identification of the ***Alternaria*** isolates, mostly found in berries from conventional growers, were belonging to both the ***A. tenuissima*** and the ***A. infectoria*** species-groups. The

A. tenuissima species-group is able to produce alternariols (AOHs), altertoxins (Alx) and tenuazonic acid (TeA) and the ***A. infectoria*** species-group can produce novo-zealandins (N-Z), altertoxin derivatives (alx) and infectopyrones (Infec).

The knowledge on the toxicity of most fungal metabolites is limited. Mycophenolic acid is known to be immunosuppressive, while alternariols are believed to be cytotoxic and tenuazonic acid to be a protein inhibitor (Cole and Cox, 1981). The effects of raistrick phenols, viridicatol, brevianamide or quinolactacin on animal or human are not known.

3.10.2 Patulin analysis of worst case inoculated berries

Patulin analysis was conducted for berry samples worst case inoculated with ***Penicillium expansum*** IBT 21525***gfp4, C. rosea*** and ***B. cinerea.*** Inoculated berries had been incubated at 4° and 20°C before sampling (see Table 21 for CFU data of analysed berries). Berries inoculated with ***P. expansum*** contained the carcinogenic mycotoxin patulin at levels of about 20 µg/g (Figure 40). This is 400 times higher than the maximum allowed level in human food, and 2000 times higher than the maximum level in baby food. Patulin was not detected in the water inoculated controls or in berries inoculated with ***C. rosea*** and ***B. cinerea***, respectively. Patulin was not detected in berries stored at 4°C.

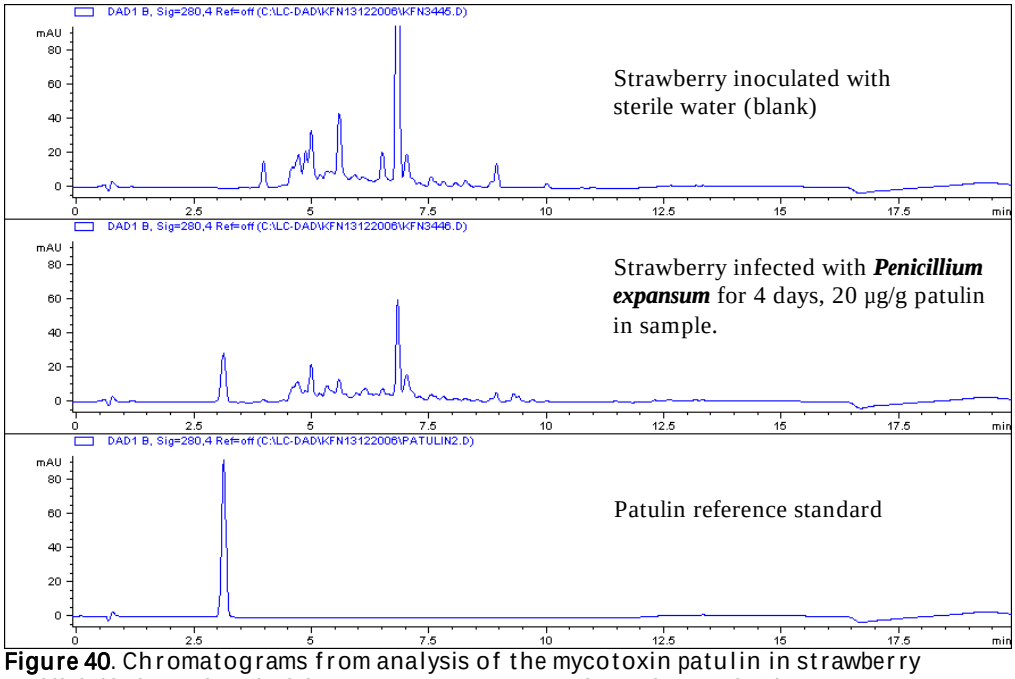

Figure 40. Chromatograms from analysis of the mycotoxin patulin in strawberry artificially inoculated with *P. expansum* IBT 21525*gfp4* and stored 4 days at 20ºC. Control berries were inoculated with sterile water.

3.10.3 Metabolite analysis of berries worst case inoculated with MPCAs

Growth of the MPCPs Supresivit, TRI003, Binab-T and IK726 was evaluated after 4 days storage at 4°C and 20°C. The density of the fungi (CFU/g berry) did not increase during storage at both temperatures except for Binab-T where CFU/g berry had increased significantly at 20°C (Table 27).

Table 27. Growth of MPCAs on strawberries stored at 4ºC and 20ºC for 4 days.

Product	Microorganisms	Storage period		
		0 days	4 days	
		4ºC/20ºC	4ºC	20ºC
		Cfu/g berry	Cfu/g berry	Cfu/g berry
Supresivit	*T. harzianum*	1.6 x 104 A[1)]	3.0 x 104 A	2.6 x 104 A
TRI003	*T. harzianum*	3.4 x 103 A	4.5 x 103 A	2.7 x 103 A
Binab-T	*T. harzianum* + *T. polysporum*	1.8 x 103 A	2.8 x 103 A	7.2 x 103 B
IK726 (peat-bran)	*C. rosea*	3.6 x 103 A	2.3 x 103 A	1.4 x 103 A
Ulocladium atrum	*U.atrum*	-nd	-nd	-nd

[1)] Within rows, mean values followed by different letters are significantly different according to Duncan´s multiple range test (***P***< 0.05)

Metabolites were analysed in selected samples of inoculated berries stored for 4 days. It should be noted that the samples consisted of 2 half berries where CFU/g berry (Table 27) was quantified on the other halves.

Analysis of the samples listed in Table 28 by LC-HR-MS could not detect any mycotoxins. Only one unknown metabolite with a molecular weight at 338 Da, which was not found in the blank strawberries, was detected in sample KFN03765. However, many unknown metabolites were detected in much higher amounts in the infected strawberries (across fungal species), than in the fresh ones (see Figure 41) indicating that these could be degradation products from the strawberries.

The third replicat of each treatment of the experiment was then analysed by LC-MS/MS. The ***T. harzianum*** peptaibols were detected in the two strawberry samples inoculated with Tri003 at 4°C and 20°C (Sample JMO00867 = KVL no. 9 and JMO00872 = KVL no. 27, respectively) and Binab-T at 4°C and 20°C (Sample JMO00868 = KVL no.12 and JMO00873 = KVL no. 30, respectively). An example of peptaibol detection in TRI003 inoculated berries is shown in figure 42. The peptaibols could not be quantified as they were tuned on ***Trichoderma*** culture extracts, since reference standards were not avaliable. Other ***Trichoderma*** metabolites were not detected.

No fungal metabolites were detected in JMO00869 (KVL no. 18, IK726, 4°C), JMO00870 (KVL no. 21, water, 20°C), JMO00871 (KVL no. 24, Supresivit, 20°C) and a control uninoculated berry at 4°C (JMO00874 = KVL no. 50).

In conclusion, the results shows that ***Trichoderma*** is capable of producing peptaibols on the strawberries, but it still remains to be shown if they are they are responsible for suppressing the pathogenic fungi

Table 28. Results from LC-HR-MS analysis of the strawberries inoculated with the MPCAs and incubated for 4 days at 20˚C and 4˚C.

KFN no.	KVL no.	Temp. (ºC)	Application	Metabolites identified
03754	19	20	Water	ND
03755	20	20	Water	ND
03756	22	20	Supresivit	Trace
03757	23	20	Supresivitt	ND
03758	25	20	Tri003	Linoleic acid
03759	26	20	Tri003	ND
03760	28	20	Binab-T	ND

03761	29	20	Binab-T	Linoleic acid
03762	31	20	Ulocladium	Linoleic acid
03763	32	20	Ulocladium	Linoleic acid
03764	34	20	Clonostachys rosea, IK726	ND
03765	35	20	Clonostachys rosea, IK726	Linoleic acid, unidentified MW 338
03766	1	4	Water	ND
03767	4	4	Supresivit	ND
03768	7	4	Tri003	ND
03769	8	4	Tri003	ND
03770	10	4	Binab-T	ND
03771	11	4	Binab-T	ND
03772	13	4	Ulocladium	ND
03773	14	4	Ulocladium	ND
03774	16	4	Clonostachys rosea, IK726	ND
03775	17	4	Clonostachys rosea, IK726	ND
03776		4	Blank can	ND

ND not detected.

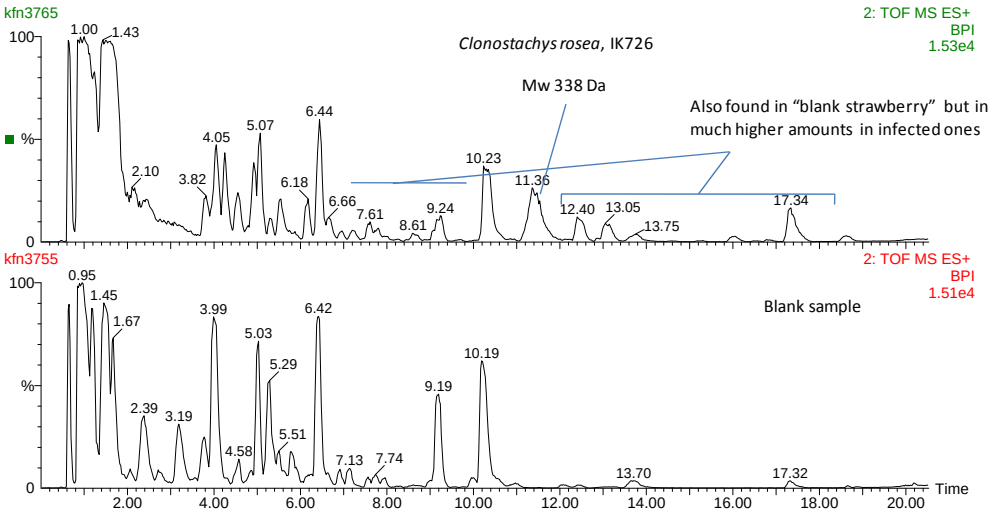

Figure41. Chromatograms from LC-HR-MS metabolite analysis of strawberries. Comparison of inoculated berries (upper chromatogram) and non inculated samples(lower chromatogram) showing peaks increasing due to spoilage.

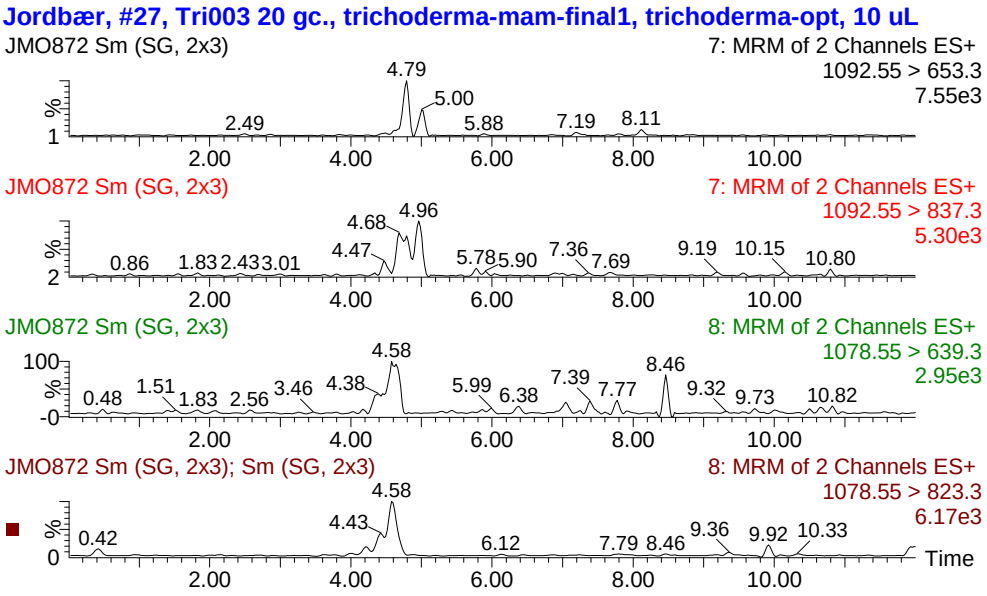

Figure 42. Multiple Reaction Monitorings (two for each) for two peptiabols detected in Tri003 (20°C) inoculated strawberries. Peaks are detected at 4.68, 4.96 and 4.58 min, based on comparison to crude *Trichodrma* extracts.

3.10.4 Metabolite analysis of worst case berries artificially inoculated with selected species isolated from the field experiments

Strawberries of two cultivars were inoculated with the fungal strains ***Aspergillus niger, Penicillium verrucosum, P. polonicum, P. brevicompactum, P. bialowiezense, Alternaria arborescens*** and ***Trichoderma*** sp. as described in table (Table 29). All eight strains were previously isolated from the bachground microbiota of field grown strawberries.

Table 29. Strawberries artificially inoculated with selected fungal spoilers and incubated for 4-8 days at 25 °C. LKN no. is an internal sample number; IBT refers to the culture collection at CMB/DTU Systems Biology.

LKN Sample no.	Strain IBT no.	Genus	Species	Strawberry cultivar
02648	28144	*Aspergillus*	*niger*	Unknown cultivar
02649	28144	*Aspergillus*	*niger*	Unknown cultivar
02650	28162	*Penicillium*	*verrucosum*	Unknown cultivar
02651	28162	*Penicillium*	*verrucosum*	Unknown cultivar
02652	28415	*Penicillium*	*polonicum*	Unknown cultivar
02653	28415	*Penicillium*	*polonicum*	Unknown cultivar
02654	28473	*Penicillium*	*brevicompactum*	Unknown cultivar
02655	28473	*Penicillium*	*brevicompactum*	Unknown cultivar
02656[a]	25262	*Penicillium*	*bialowiezense*	Unknown cultivar
02657[a]	25262	*Penicillium*	*bialowiezense*	Unknown cultivar
02658	41139	*Trichoderma*	sp	Unknown cultivar
02659	41139	*Trichoderma*	sp	Unknown cultivar
02660	41065	*Alternaria*	*arborescens*	Unknown cultivar
02661	41065	*Alternaria*	*arborescens*	Unknown cultivar
02662	41145	*Alternaria*	*tenuissima*	Unknown cultivar
02663	41145	*Alternaria*	*tenuissima*	Unknown cultivar
02664	28144	*Aspergillus*	*niger*	Unknown cultivar
02665	28144	*Aspergillus*	*niger*	Unknown cultivar
02666	28162	*Penicillium*	*verrucosum*	Unknown cultivar
02667	28162	*Penicillium*	*verrucosum*	Unknown cultivar
02668	28415	*Penicillium*	*polonicum*	Unknown cultivar
02669	28415	*Penicillium*	*polonicum*	Unknown cultivar
02670	28473	*Penicillium*	*brevicompactum*	Unknown cultivar
02671	28473	*Penicillium*	*brevicompactum*	Unknown cultivar
02672[a]	25262	*Penicillium*	*bialowiezense*	Unknown cultivar
02673[a]	25262	*Penicillium*	*bialowiezense*	Unknown cultivar
02674	41139	*Trichoderma*	sp	Unknown cultivar

02675	41139	*Trichoderma*	sp	Unknown cultivar
02676	41065	*Alternaria*	*arborescens*	Unknown cultivar
02677	41065	*Alternaria*	*arborescens*	Unknown cultivar
02678	41145	*Alternaria*	*tenuissima*	Unknown cultivar
02679 [b]	41145	*Alternaria*	*tenuissima*	Unknown cultivar
02680	28144	*Aspergillus*	*niger*	Honeoye
02681	28144	*Aspergillus*	*niger*	Honeoye
02682	28162	*Penicillium*	*verrucosum*	Honeoye
02683	28162	*Penicillium*	*verrucosum*	Honeoye
02684	28415	*Penicillium*	*polonicum*	Honeoye
02685	28415	*Penicillium*	*polonicum*	Honeoye
02686	28473	*Penicillium*	*brevicompactum*	Honeoye
02687	28473	*Penicillium*	*brevicompactum*	Honeoye
02688 [a]	25262	*Penicillium*	*bialowiezense*	Honeoye
02689 [a]	25262	*Penicillium*	*bialowiezense*	Honeoye
02690	41139	*Trichoderma*	sp	Honeoye
02691	41139	*Trichoderma*	sp	Honeoye
02692 [b]	41065	*Alternaria*	*arborescens*	Honeoye
02693	41065	*Alternaria*	*arborescens*	Honeoye
02694	41145	*Alternaria*	*tenuissima*	Honeoye
02695	41145	*Alternaria*	*tenuissima*	Honeoye
02696	28144	*Aspergillus*	*niger*	Honeoye
02697 [b]	28144	*Aspergillus*	*niger*	Honeoye
02698	28162	*Penicillium*	*verrucosum*	Honeoye
02699	28162	*Penicillium*	*verrucosum*	Honeoye
02700	28415	*Penicillium*	*polonicum*	Honeoye
02701	28415	*Penicillium*	*polonicum*	Honeoye
02702	28473	*Penicillium*	*brevicompactum*	Honeoye
02703	28473	*Penicillium*	*brevicompactum*	Honeoye
02704 [a]	25262	*Penicillium*	*bialowiezense*	Honeoye
02705 [a]	25262	*Penicillium*	*bialowiezense*	Honeoye
02706	41139	*Trichoderma*	sp	Honeoye
02707	41139	*Trichoderma*	sp	Honeoye
02708 [b]	41065	*Alternaria*	*arborescens*	Honeoye
02709	41065	*Alternaria*	*arborescens*	Honeoye
02710	41145	***Alternaria***	***tenuissima***	Honeoye
02711	41145	***Alternaria***	***tenuissima***	Honeoye

[a] No fungal growth, not analysed. [b] Contaminated with multiple fungal species, not analysed

3.10.4.1 Aspergillus niger

A. niger grew very well on the strawberries (figure 43), which was no surprise since it has a preference for both high amounts of sugars, can grow at low pH, as well as tolerates organic acids well (e.g. used for production of citric acid). As seen in Table 30 very high level of fumonisins and ochratoxin A was detected in the artificially inoculated strawberries. For ochratoxin A the lowest regulatory limit is 0.5 ppb (baby products), and the observed levels are thus up to 2000 higher than the regulatory limit. For fumonisins (sum of B_2 and B_4) the levels are also very high with levels up to 140 times higher than the lowest than the regulatory limit of fumonisins (200 ppb in baby products).

Figure 43. Strawberry overgrown with *A. niger* during 5 days.

Table 30. Determination of fumonisins and ochratoxin A (ppb) in strawberries inoculated with *Aspergillus niger*. Sample numbers refer to Table 29.

Sample number	FB2	FB4	OTA
02648, A. Niger	1285	102	107
02649, A. Niger	25578	2058	346
02664, A. Niger	14738	1212	1076
02665, A. Niger	8526	356	29
02680, A. Niger	5311	469	63
02681, A. Niger	129	38	ND
02696, A. Niger	5914	1519	137

Since nobody will eat ***A. niger*** infected strawberries the risks of exposure to fumonisins and ochratoxin A may come from processed strawberries in jam etc., and here it is clear that a few bad berries can contaminate a whole batch of product.

3.10.4.2 Penicillium

The amounts of biomass produced by the penicillia were much more modest (Figure 44) than for ***A. niger*** (Figure 43) and the amounts of metabolites were very low. This is presumable due to the stressing conditions of a live berry with low pH and high amounts of organic acids.

Cyclopenol, cyclopenin, viridicatin were found in the 8 ***P. polonicum*** infected samples (LKN 02652, 02653, 02668, 02669, 02684, 02685, 02700 and 02701 in Table 29), but could not be quantified due to very low amounts of the reference standard (not commercially available). Brevianamides A and B were not detected.

Interestingly only ***P. brevicompactum*** grew on the strawberries whereas the closely related ***P. bialowiezense*** did not (Table 29). Even more surprisingly ***P. brevicompactum*** only produced mycophenolic acid as trace quantities in one of four samples (LKN 02671, Table 29). This is almost the first growth condition where we do not see high amounts (high ppm) of mycophenolic acid production by ***P. brevicompactum***. The detection limit for mycophenolic acid is ca. 2-5 ppb (µg/kg).

Figure 44. *P. brevicompactum* (grey-bluish patches) growth on strawberry.

The ***P. verrucosum*** isolate did not produce ochratoxin A or viridicatic acid on the strawberries (Table 29). This was no major surprise since it generally produces ochratoxin A on dry cereal grains.

3.10.4.3 Alternaria

Due to contamination with other fungi in three samples, only 13 ***Alternaria*** infected samples were analysed (Table 29). Neither AAL toxins nor tentoxin were detected in any of the ***Alternaria*** infected samples**.**

In samples LKN02676 and 02677 tenuazonic acid was found in high amounts (ca. 1 ppm) and furthermore in three samples at 5-50 ppb levels (LKN02660, 02661, 02709). In the 7 ***A. tenuissima*** infected strawberries traces of altertoxin 1 was found in one sample (LKN02695), whereas altenuene was found in two samples (LKN02662 and 02663), although in trace amounts (ppb level). In ***A. tenuissima*** samples LKN02678, 02694, 02710, 02711 no known metabolites were detected.

3.10.4.4 Trichoderma

In all samples of berries infected with ***Trichoderma*** IBT 41139 listed in Table 29, the metabolites of the peptaibol family were detected qualitatively. The peptaibols could not be quantified as sufficient reference standards were not available. Other ***Trichoderma*** metabolites were not detected.

3.10.5 Metabolite analysis from field berries

The 24 samples from the field survey (8 growers x 3 replicats) and 30 worst case samples (berries with disease symptoms or otherwise low quality berries) were all tested negative for fumonisins B1 and B2; ochratoxins A, B, and α; alternariol, AAL toxin, mycophenolic acid, tenuazonic acid, altertoxin, infectopyrones, novo-zealandin, cyclopenol, viridicatol, viridicatin, brevianamides A and B, as well as ***Trichoderma*** peptaibols.

3.10.6 Metabolite analysis of MPCA sprayed field grown berries

The 24 semi-field berry samples (±MPCA and ±fungicide, 3 replicats) and the 18 samples from the TRI003 field experiments (berries developed from flowers/green berries sprayed with TRI003, 3 replicats) were all tested negative for fumonisins B1 and B2; ochratoxins A, B, and α; alternariol, AAL toxin, mycophenolic acid, tenuazonic acid, altertoxin, infectopyrones, novo-zealandin, cyclopenol, viridicatol, viridicatin, brevianamides A and B, as well as ***Trichoderma*** peptaibols.

4 Discussion

4.1 Pre experiment

A three step procedure with 12 different semi-selective agar substrates was used for isolation of the microbiota from strawberry. Firstly the strawberries were washed and several dilutions of washing water were plated on all media in order to isolate mircrorganisms mainly originating from loosely attached spores. Secondly the washed berries were homogenized in sterile water and dilutions of the homogenate were plated on the various media to isolate organisms more strongly attached or even internally present microorganisms. Thirdly pieces of washed berries were incubated on the various media for isolation of weekly sporulating organisms or sterile fungi. The main results were as follows: 1) concerning washing water in general, fungi were countable at dilutions 10^{-1} to 10^{-3}, yeast at 10^{-2} to 10^{-4} and bacteria at 10^{-3} to 10^{-4}. ***Cladosporium*** was the predominant fungal genus followed by ***Penicillium***. TSBA and NA media gave similar bacterial counts, 2) concerning homogenized berries similar results were obtained, and 3) for incubated berry pieces DG18, SNA and V8 media gave the highest isolation frequency of fungal genera of interest for the project (***Penicilliun, Alternaria, Aspergillus, Fusarium*** and ***Botrytis***. Based on results from the pre-experiment the most optimal media and dilutions for microbiota isolation were selected. Five media for fungal isolation (DG18, SNA, V8; DRYES and PDA-C) and dilutions within a range from 10^{-1} to 10^{-4} expected to be countable were chosen (see Table 3 for more details). Although identification of fungi generally was difficult on PDA-C the substrate was included anyway, because it is suitable for isolation of ***Trichoderma*** spp. and ***Clonostachys*** spp. occurring in low numbers. TSBA and SDBA were selected for isolation of yeast and bacteria, respectively, as they are both used as standard media in the MIDI system used for identification of yeast and bacteria by signature fatty acid.

It was expected that 20 min washing in an ultrasonic bath would remove the majority of the microorganisms from the berry. However, the microbial counts following 20 min washing were almost of the same magnitude from the washing water and from the washed homogenized berries. Therefore it was decided to increase the washing time from 20 to 30 min. in the following microbiota survey from 8 growers. In addition the washing water in the ultrasonic bath was cooled by ice-cubes to minimize heating of the samples.

4.2 Survey of strawberry microbiota

4.2.1 Bacteria

The population density of culturable heterotrophic aerobic bacteria was significantly higher in organically grown strawberries obtained from strawberry washing, whereas the bacterial population obtained from homogenates of washed berries did not differ between conventional and organic growers. This may reflect that the isolates from washing are mainly epiphytic, which during strawberry flowering were exposed directly to

pesticide application, whereas the isolates from the strawberry homogenate are endophytes protected from pesticide exposure. Similarly, Jager ***et al.*** (2001) showed that pesticide applications reduced the bacterial population density in a mango phyllosphere.

Among the 34 different bacterial species from 23 genera isolated from strawberries in the present project several have been reported as plant pathogens (Bradbury, 1983; Janse, 2005), human pathogens (e.g. Ramos and Dámaso, 2000; Falagas ***et al.***, 2006) and biocontrol agents (e.g. Francés ***et al***., 2006; Essghaier ***et al***., 2009; Enya ***et al.***, 2009). In a study of phyllosphere bacterial communities in field-grown sugar beets 73 species from 37 named and 12 unnamed genera were obtained (Thomson ***et al.***, 1993). Similarly, Kotan ***et al.*** (2006) retrieved 76 species from 36 genera on pome fruit trees, though focusing on pome pathogens.

It is generally believed that the bacterial phyllosphere community are dominated by a few species (Lindow and Brandl, 2003), which was confirmed in our study. However, Yang ***et al.*** (2001) demonstrated that culture-independent molecular methods revealed higher community complexity, than reported using culture dependent methods.

Bacteria from the genera ***Curtobacterium***, ***Serratia***, ***Pseudomonas***, ***Enterobacter*** and ***Rahnella*** were most frequently isolated. Krimm ***et al.*** (2005) reported ***Pseudomonas***, ***Stenotrophomas***, ***Bacillus*** and ***Arthrobacter*** as the dominating epiphytic bacteria of strawberry plants (leaves, flowers and berries). Methods used to isolate, quantify and identify bacterial phyllsophere populations differed between the two studies, which have been shown to play an important role in recovering of bacterial communities (Jacques and Morris, 1995). Krimm ***et al.*** (2005) isolated bacteria from agar imprints and washing water and the bacterial isolates were identified employing morphotyping and DNA based PCR methods. The method employed in the present project included sonication, washing and stomaching, which has been reported to harbor the highest number of phyllosphere epiphytic and endophytic bacteria (Jacques and Morris, 1995).

The bacterial communities on strawberries in the present project were dominated by relatively few species commonly encountered from the phyllsophere. Some of these are known as plant pathogens, human pathogens and others as biocontrol agents.

Plant pathogens

Bacterial plant pathogens known from potato (***Clavibacter michiganense*** and ***Erwinia carotovora***), bean (***Curtobacterium flaccumfaciens***) and tomato (***Pseudomonas syringae***) were isolated from mature strawberries, but none of these bacteria have been reported as strawberry pathogens. The frequency of the three different ***C. flaccumfaciens*** pathovars ranged between 7-50% in all four conventional growers, whereas ***C. flaccumfaciens*** was only recorded in one out of four organic growers and only in low frequency (4-8%). The experiments performed in the present project do not provide any explanation for this difference in occurrence of ***C. flaccumfaciens***, but may be related to crop rotation practice. The three other pathogens were only recovered from 1-3 growers in low frequency (3-8%) with no clear difference between organic and conventional growers.

Human pathogens

Six different well documented potential human pathogenic bacterial species were isolated from strawberry reported to be causing different infectious human diseases mainly related to imunodeficient hospitalized patients. In most cases their frequency were low (3-8%) and they were recovered only from 1-3 nurseries. One of the isolates (***Rahnella aquatilis***), was recovered in one nursery at a higher frequency (23%). Among the potential bacterial human pathogens, ***Staphylococcus*** isolates can cause severe human infections**.** Interestingly, ***Staphylococcus*** isolates were only recovered from the organic nurseries, though in low frequency (6-8%). ***Staphylococcus*** isolates have also been reported from partially processed conventional lettuce (Magnuson ***et al***., 1990). The experiments performed in the present project do not provide any explanation for this difference in occurrence of ***Staphylococcus*** isolates between conventional and organic strawberry growers. Also, it is important to note that also the skin of healthy humans contain a high diversity of opportunistic potential pathogenic bacteria.

Biocontrol agents

Six well documented bacterial biocontrol agents against plant pathogens were recovered from strawberries. Among these ***Bacillus megaterium***, ***Paenibacillus macerans*** and ***Pantoea agglomerans*** were exclusively recovered from organic nurseries, though only in 1-3 out of the four nurseries and in a moderate frequency (4-27%). The three other biocontrol agents; ***Pseudomonas chlororaphis***, ***Pseudomonas putida*** and ***Serratia liquefaciens*** were recorded in 5-6 of the 8 nurseries in a moderate frequency (3-33%). Interestingly, in two of the conventional growers with high pesticide application levels none of the mentioned biocontrol agents were recovered. In general, it seems that organically produced strawberries harbor a higher amount of bacterial biocontrol agents against plant pathogens, than conventionally produced strawberries, which may be related to the high input of pesticides and/or mineral fertilizers in conventional production systems, but this hypothesis needs to be further tested.

Some bacteria both possess pathogenic and biocontrol features. These are so-called opportunistic bacteria. ***Pantoea agglomerans*** and ***Serratia liquefaciens*** are such examples among the bacteria isolated from strawberry in the present project. Opportunistic pathogens have been suggested for biocontrol of several plant diseases and some non-pathogenic isolates such as ***Burkholderia cepacia*** have been registered and marketed as MPCAs in the USA. Among the plant pathogens ***P. syringae*** are also known as a MPCA against post harvest diseases. It is important to note that the above-mentioned functional traits in the bacterial isolates from the present study remains to be examined.

When examining the bacterial communities from the different strawberry nurseries PCA analysis revealed a separation between organic and conventional production systems, except that one of the organic nurseries were closer associated with the conventional growers and closest to a conventional nursery from the same local geographical area (Klippinge, Sealand). Interestingly, the bacterial communities from two other growers from another local geographical area (Skælskør, Sealand) were also closely associated, suggesting that not only the production system, but also the geographical location may be an important factor in phyllopsphere bacterial communities. In addition, seasonality, plant surface, age and aspect, pesticide spray and fertilization have been reported as factors influencing bacterial phyllosphere communities (Jager ***et al.***, 2001).

4.2.2 Yeasts

The population density of culturable aerobic heterotrophic epiphytic yeasts did not differ between strawberries from organic and conventional production. However, differences were observed between growers. In a study of the mango phyllosphere microbial community Jager ***et al.*** (2001) reported a reduction in the population density of yeast due to fungicide applications. However, in the study of Jager ***et al.*** (2001) leaves directly exposed to fungicides were included, whereas in the present study mature strawberries were included, which may explain the contrasting results.

To our knowledge the present study is the first extensive characterization of the yeast community on strawberries. In total 22 species from 9 genera were identified of which species from the genera ***Candida***, ***Cryptococcus*** and ***Rhodotorula*** were dominating. Among these several biocontrol agents (Sharma ***et al***., 2009) and human pathogens (e.g. Krcmery ***et al.***, 1999) were recorded.

Principal component analysis did not result in any clear separation neither between organic and conventional growers nor concerning geographic areas. Hence, it seems that either the yeast community was not affected by the fungicide application employed by the conventional growers or the yeast communities recovered during fruit development. Another explanation could be that the yeast community on mature strawberries differs from the yeast community on strawberry flowers, but this remains to be explored.

Biocontrol agents

Several yeasts have been reported as biocontrol agents mainly of post harvest diseases, such as blue, green and grey molds (Sharma ***et al.***, 2009). In the present study 8 of the 22 species recovered have been reported to have biocontrol activity of which the species ***C. famata*** (Arras, 1996), ***Rhodotorula minuta*** (Patiño-Vera ***et al***., 2005) and ***R. rubra*** (Dal Bello ***et al***., 2008) were most frequently recovered. Another yeast ***Cryptococcus albidus*** has been reported to be antagonistic against grey mold in strawberry (Helbig, 2002), but this species was not recovered in the present study. No clear difference in the presence of yeast with potential biocontrol features were observed between organic and conventional growers.

Human pathogens

Several of the yeast species isolated are known as opportunistic pathogens in humans causing infectious diseases. In total 6 of the 22 species recovered have been reported as human pathogens. Among the most frequently recovered species ***Crytococcus neoformans*** has been associated with fungual menigoencephalitis (Lix, 2009) and the Candida species ***C. famata*** (Pfaller and Dikema, 2004) and ***C. inconspicua*** (Loeffler ***et al***., 2000) have been associated with infectious diseases. However, in general yeast other than ***Candida albicans*** are uncommon human pathogens and most commonly associated with immuno-compromised humans. No clear difference in the presence of yeast with potential human pathogenic features was observed between organic and conventional growers, except that ***C. neoformans*** were found in all four conventional growers and only in one of the organic growers.

It is important to note that the functional traits (plant disease biocontrol and human pathogen) of the yeasts isolated from mature strawberries in the present study have not been examined.

4.2.3 Filamentous fungi

The survey of the mycobiota on strawberries sampled from four organic and four conventional growers, respectively, showed that ***Cladosporium*** was the dominating genus in both systems. However, of more interest for the present project potential mycotoxin producing genera such as ***Penicillium*** and in lower number ***Alternaria*** and ***Fusarium*** also appeared rather frequently. Several ***Aspergillus*** spp. was also isolated from both washing and berry pieces. This was a surprise since the ***Aspergillus*** genus has not been mentioned in the literature among fungal genera being isolated from high quality strawberries (Dennis, 1976; Tronsmo, 1986; McLean & Sutton, 1992; Tournas and Katsoudas, 2005).

Only few significant differences between organic and conventional berries were identified. Higher ***Cladosporium*** CFU/g berry was found in washing water and homogenate of organic berries compared to conventional berries. Since species from this genus are not known as mycotoxin producers, the consumer risk is probably not increased. On the other hand the isolation frequency of ***Alternaria*** and ***Fusarium*** was higher on conventional than on organic berries. Thus, the results of the current survey do not reveal increased risk of mycotoxin contamination in organic strawberry production. This is in accordance with the findings of literature reviews by Olsen (2009) and Magkos ***et al.*** (2006) comparing mycotoxin production in organic and conventional foods.

Mycotoxin producing species

A wide range of ***Penicillium*** species, more than 15 species, were identified both from wash water and berry pieces. Several of these species are known as mycotoxin producers e.g. the predominant species ***P. brevicompactum*** as well as ***P. verrucosum*** and ***P.expansum.*** We also isolated and identified other potential mycotoxin producers namely two species groups of ***Alternaria*** spp: ***Alt. tenuissima*** sp-grp and ***Alt. infectoria*** sp-grp, as well as ***Aspergillus niger*** and ***Aspergillus flavus***. To our knowledge the present study is the most thorough characterization of ***Penicillium***, ***Alternaria*** and ***Aspergillus*** species on marketable berries.

Biocontrol agents

Trichoderma and ***Clonostachys*** strains were only isolated from few berry samples of the present field experiments. The isolated MBCA strains occurring in the background mycobiota were characterized as ***T. harzianum, T.asperellum, T. viridescens, T.viride*** and ***Clonostachys rosea***. This is in accordance with investigations from Norway (Tronsmo, 1986) and McLean & Sutton (1992) finding none of the genera occurring naturally on berries while Tournas & Katsoudas (2005) reported ***Trichoderma*** spp. in 3% of strawberry samples purchased from supermarkets in USA. In contrast, Parikka ***et al***. (2009) reported abundant isolation of ***T. viride*** from Finish strawberries.

The results of the survey show that only a limited number of fungal species can be found on the surface or in the interior of the berries. Less than 30 fungal species from 20 fungal genera in total have been found on the berries compared to the 100s of known food-borne fungal species. Analysis of the location of the different fungal species on the berries (wash water = surface contamination with spores; berry pieces = mainly mycelium growth) suggests

that most of the ***Alternaria*** and ***Trichoderma*** species are located on the surface, while ***Penicillium*** species and ***Aspergillus*** species are found on the surface as well as in the interior of the berries. ***Penicillium bialowiezense*** and ***P. brevicompactum*** are the most common ones in berry pieces.

4.3 Establishment of *Trichoderma* in the field

After a single spray treatment where flowers were exposed to the Trichoderma MBCA, TRI003, only few strains were reisolated from berries developed from exposed flowers. The ***Trichoderma*** CFU/g berry was 7-100 CFU/g berry, which was close to the detection limit. However, it was justified by UP-PCR and sequencing of the IST1 and IST2 regions of isolates, that the active ingredient of TRI003, strain 1295-22, indeed was the strain isolated from berries 20-30 days after spraying. The ability of ***Trichoderma*** to establish under field conditions was confirmed in the semifield experiment as approximately 20 CFU/g berry was recovered 28 days after spraying and the majority of reisolated ***Trichoderma*** was strain 1295-22. A more intensive spraying scheme e.g. with weekly TRI003 sprayes and/or treatments close to the harvesting date would probably increase the density of ***Trichoderma*** on marketable berries. Tronsmo (1986) sprayed 4 to 6 times with ***Trichoderma*** during flowering and at least 7 days before fruit harvest. This resulted in ***Trichoderma*** levels on freshly harvested berries varying between 10^2 to 10^4 CFU/g berry and it was concluded that ***Trichoderma*** was able to survive under field conditions.

4.4 Biocontrol effects of MPCAs and compatibility with fungicides

Results from the flower-assays showed that the MPCAs ***U. atrum*** and ***C. rosea*** have strong potential to control grey mould in strawberries, which are in agreement with previous findings on ***U. atrum*** (Boff ***et al***., 2002) and ***C. rosea*** (Peng ***et al.***, 1992; Sutton ***et al.***, 1997).

In contrast, all of the ***Triochoderma***-based MPCPs included in the present project were ineffective against ***B. cinerea*** in the flower assays. Shafir ***et al***. (2006) reported promising biocontrol effects of ***T. harzianum*** T 39, the active ingredient of the Israeli product TRICHODEX, against grey mould in field grown strawberry when applied with pollinating humblebees. These contrasting results may be due to difference in pathogen inoculum level. Indeed, Shafir ***et al***. (2006) reported that ***T. harzianum*** efficiently controlled grey mould in situations with low to moderate disease pressure, but not with high level of disease pressure. In our studies ***Trichoderma*** MPCAs were applied in 10-fold higher concentrations than ***B. cinerea***, but this may still represent a high disease pressure.

The results of the ***in vitro*** compatibility tests clearly show that it is possible to combine different MPCAs and fungicides in an integrated control strategy against grey mould. These results are in agreement with the findings of Roberti ***et al***. (2006) that ***C. rosea*** and ***Trichoderma*** spp. are largely insensitive to fungicides and further suggest that MPCAs such as ***C. rosea*** may be included in integrated plant disease management strategies. Indeed, ***C. rosea*** has been shown to play a key role in integrated management of strawberry grey mould when combined with fungicide spray and debris removal in Brazil (Cota ***et al***., 2009).

4.5 Establishment of MPCA ± fungicide application Semi-field

The present results do not support the hypothesis that fungicide application can help establishment of MPCAs on strawberries. Generally fungicide treatment had no effect on MPCA density on berries nor had the fungicide treatment any effect on density of the natural occurring microbiota (fungi, yeast, bacteria).

The overall very weak effects of the MPCA and fungicide treatment on plant parameters were expected, as the disease pressure from ***B. cinerea*** was low. However, the observed reduction in numbers of runners from plants treated with TRI003 is interesting and indicates that ***T. harzianum*** is affecting plant growth.

4.6 Activity of MPCAs and other naturally occurring fungi on strawberry and mycotoxin production

GFP and DsRed transformed strains were used to evaluate the activity of the MPCAs ***C.*** rosea (IK726) and ***T. harzianum*** (1295-22) and the background mycobiota represented by ***P. expansum*** and ***B. cinerea.*** Activity of ***C. rosea*** was studied both on berries and leaves of whole plants and on detached flowers and berries. ***C***. ***rosea*** was able to germinate on green leaves, flowers and berries. Not surprisingly, flowers seem to be better substrate for the fungus than berries as the fungus germinated and grew faster on the flowers. On the other hand the whole plant assay showed that germination and growth of ***C. rosea*** was lower on green leaves than on berries. The ecological abilities strengthen the potential of ***C.rosea*** IK726 as a biocontrol agent against strawberry diseases. Thus, other ***C. rosea*** strains have proven to be effective MPCAs against strawberry gray mould (Sutton ***et al.,*** 1997, Cota ***et al***., 2008; Cota ***et al.,*** 2009). The ability of IK726 to germinate on berries also opens the possibility of metabolite production on the berry.

The flower is an important infection site for ***Botrytis*** in strawberry (Bristow ***et al.,*** 1986). The ability of ***C.rosea*** and ***T. harzianum*** to control ***B. cinerea*** was investigated following dual inoculation of the fungi to detached flowers. Spore germination of ***Botrytis cinerea*** was strongly inhibited by the MPCA ***C. rosea*** on flowers whereas the pathogen had no effect on spore germination of ***C. rosea***. Supression of ***Botrytis*** germination has also been shown as a mode of action for antagonism of ***Botrytis*** by ***C.rosea*** on strawberry leaves (Sutton ***et al.,*** 1997). Spore germination of ***T.harzianum*** was strongly inhibited by ***C. rosea*** when mutually inoculated on flower, whereas ***T.harzianum*** had no effect on spore germination of ***C. rosea***. This was probably a consequence of ***C.rosea*** K726 being less dependent on exogenous nutrients (e.g. pollen) for germination than often demonstrated for ***T. harzianum*** strains (Hjeljord ***et al***. 2001; Hjeljord & Tronsmo, 2003).

Conidial germination and growth of ***P. expansum***, ***C. rosea*** and ***B. cinerea*** was compared on inoculated berries stored at 4ºC and 20ºC. At 4ºC ***C. rosea*** conidia had germinated after 4 days compared to 30% for ***P.expansum*** and almost 90% for ***B. cinerea***. In the same period CFU/g berry decreased for ***C. rosea*** while the CFU density of ***P. expansum*** was unchanged. This indicates that optimal storage of strawberries at 4ºC result in very little ***C. rosea*** activity. Suboptimal storage of strawberries at 20ºC resulted in a 2 x 10^4-fold increase in ***P. expansum*** CFU density while no increase was measured for ***C. rosea*** after four days. Furthermore berries inoculated with ***C. rosea*** had no macroscopic

symptoms whereas ***P. expansum*** inoculated berries were visibly decomposed and covered with green spores. Nevertheless, confocal laser scanning microscopy of ***C. rosea*** inoculated berries incubated at 20ºC for 6 days disclosed hyphal growth of the fungus especially in the cavities around the seed of the berry. Growth of ***C. rosea*** on berries was also confirmed by the 60 fold increase in ***C. rosea*** DNA following 4 days incubation at 20ºC. However, laser scanning of the uppermost cell layers indicate that the hyphal growth is superficial. Despite growth of ***C. rosea*** on berries at 20ºC no fungal metabolites were detected. On the other hand metabolite analysis of ***P. expansum*** inoculated berries showed high levels of the carcinogenic mycotoxin, patulin. This mycotoxin is regulated within Europe (EEC, 2003) as many batches of apple juice are discarded due to the presence of patulin from ***P. expansum*** contaminated apples.

4.7 Chemical analysis of fungal pure cultures

There are few mycotoxin producing fungi growing in/on fresh fruit (stored and dried fruit products is a totally different situation with lots of problems), presumably due to the low pH and high contents of organic acids. The results of the chemical analyses show that only a limited number of metabolites can be produced by the fungi found on the surface or in the interior of the berries and that none of these are mycotoxins ***sensu stricto*** except patulin produced by ***P. expansum.*** However, this species was seldom isolated. Therefore the ***Penicillium*** species, ***P. bialowiezense*** and ***P. brevicompactum,*** constitute the biggest mycotoxigenic potential amongst the fungal species detected in this survey, due to their potential production of mycophenolic acid and their frequent occurrence in berries from all growers. Mycophenolic acid is an immunosuppressive agent and acts by inhibiting lymphocyte proliferation and antibody production (Samson and Frisvad, 2004). The experience at DTU is that ***P. brevicompactum*** is very common on many fruit, berries and soil and is able to produce mycophenolic acid on most substrates, including ginger, building materials and all agar substrates tested (Frisvad ***et al***., 2007a). The other ***Penicillium*** species isolated are not known to be producers of regulated mycotoxins (Samson and Frisvad, 2004), which were confirmed by the analyses.

Two ***Alternaria*** species were found in strawberries, the surface growing ***A. tenuissima*** producing the mutagenic alternariols, tenuazonic acid and altertoxins. The other species is ***A. infectoria***, which may infect the berries and is known to produce infectopyrones, novo-zealandins and many more undescribed compounds of unknown toxicity (Andersen ***et al***., 2002).

A few strains of ***Aspergillus niger*** were found and this species and the closely related ***A. carbonarius*** are very common contaminant of grapes where they produce the carcinogenic compound, ochratoxin A. This mycotoxin is a significant problem in both wine and raisins and may other products. Recently it has been shown that ***A. niger*** also produces the carcinogenic compound fumonisin B2 (Frisvad ***et al***., 2007b). With ***A. niger*** being a very common in soli and capable of growing at low pH and media with high concentrations of organic acids, it is a potential problem in strawberries. However, the production of mycotoxins is known to be regulated by environmental factors and is strain dependent.

The genus of ***Trichoderma*** is generally not known for many mycotoxins, and the 3 known mycotoxins: harzianum A, trichodermin and gliotoxin are produced by ***Trichoderma*** species phylogenitically far away from the species used as biocontrol agents (Nielsen ***et al.***, 2005; Degenkolb ***et al***., 2008a). Many species in ***Trichoderma*** produces peptaibols (cyclic peptides where some of the amino acids have been substituted by hydroxy-organic acids) (Degenkolb ***et al***., 2008b). These peptaibols can make channels in membranes and thereby kill other microorganisms. So far, peptaibols have not been shown to be toxic to higher animals when introduced via a natural route.

4.8 Chemical analysis of worst case inoculated berries

Since mouldy strawberries presumably are not consumed directly but discarded during cleaning, the risk of exposure to fumonisins and ochratoxin A is minimal. However, strawberries for processing, e.g. jams, may contain mouldy berries as the cleaning step is not by hand on a single berry level. In this case it is obvious that a few bad berries can contaminate a whole batch.

Our results show that ***Aspergillus niger*** can produce high amounts of fumonisins and ochratoxin A on strawberries – up to 2000 times the regulatory limit – highlighting that ***A. niger*** infected strawberries should be avoided.

For all the other artificially worst case scenarios, very low levels of known fungal metabolites were detected. As a surprise, even mycophenolic acid was produced in very low levels by ***P. brevicompactum*** that normally produces high amounts on all growth media (Samson and Frisvad, 2004). The other mycophenolic acid producer, ***P. bialowiezense,*** was unable to grow on strawberries, minimizing the risk for mycophenolic acid contamination of strawberries and products hereof.

Inoculation of berries with high dosages of the Trichoderma MPCAs, TRI003 and Binab-T, resulted in production of peptaibols following 4 days incubation at both 4ºCand 20ºC. The peptaibol production was generally not correlated with an increase in CFU/g berry indicating that mycelial growth but not sporulation had occurred. Such a growth pattern was seen for the MPCA ***C.rosea***, in the present project. Peptaibol production was also detected in berries inoculated with a ***Trichoderma*** strain from the naturally occurring strawberry mycobiota.Thus the potential of peptaibol production is not restricted to MPCA strains.

4.9 Chemical analysis of berries from field experiments

No fungal metabolites were detected in fresh berries of both poor and good quality or from berries from test fields where MPCAs had been applied. It is concluded that from our studies only growth by ***A. niger*** in strawberries can generate a risk for mycotoxin contamination, as growth by ***Alternaria***, ***Trichoderma*** and ***Penicillium*** species only will result in very low levels of fungal metabolites, and only in berries with a very intensive fungal growth. However, consumers will never eat mouldy strawberries that are completely degenerated by fungal growth.

5 Conclusions

Natural strawberry microbiota
Indigenous communities of culturable bacteria, yeasts and filamentous fungi on field grown mature strawberries were complex including potential plant pathogens, human pathogens, plant disease biocontrol agents and mycotoxin producers.

Bacteria were the most abundant and diverse group of strawberry microbiota followed by yeasts and filamentous fungi. Bacterial communities were dominated by the genera ***Curtobacterium***, ***Pseudomonas*** and ***Serratia***. Yeast communities were dominated by the genera ***Candida***, ***Cryptococcus*** and ***Rhodotorula***. Communities of filamentous fungi were dominated by ***Penicillium*** and ***Cladosporium.*** Fungi belonging to ***Trichoderma*** and ***Clonostachys***, well known MPCA genera, were seldomly isolated.

Grower practice did not seem to correlate with the strawberry microbiota. Limited difference between microbial communities on strawberries from conventional and organic production systems were observed, except that the population density of bacteria was higher on strawberries from organic growers than on conventional strawberries. In addition, organic strawberries harbored less potential plant pathogens and more potential plant disease biocontrol agents, than conventional strawberries. Organic strawberries also harbored higher levels of ***Cladosporium*** than conventional strawberries. However, lower incidence of the potential mycotoxin producers, ***Alternaria*** and ***Fusarium,*** was growing from washed berries. There was a difference in bacterial communities from strawberries with different geographic origin, whereas fungal communities on strawberries were unaffected by geographic location.

Mycobiota metabolites
Mycotoxins were not detected in field or semi-field grown berries neither from organic nor from conventional growers including both high quality berries and berries with disease symptoms or otherwise low quality berries.

None of the fungal MPCAs produced mycotoxins when artificially inoculated on strawberries, except that strawberries inoculated with ***Trichoderma***-based MPCPs, TRI003 and Binab-T, contained biologically active fungal metabolites of the peptaibol family. Peptaibol production was detected after 4 days incubation at both 4ºC and 20ºC although no increase in CFU density of the berry samples was detected. However, peptaibols were also detected on berries artificially inoculated with a naturally occurring ***Trichoderma*** species. In contrast, no peptaibols were detected in semi-field or field grown strawberries inoculated with a ***Trichoderma***-based MPCA once at flowering and furthermore peptaibols have not been shown to be toxic to higher animals via a natural exposure route.

Strawberries artificially inoculated with naturally occurring species of ***Penicillium*** and ***Aspergillus***, respectively contained high levels of the mycotoxins patulin, ochratoxin and fumonisins, suggesting that the natural

strawberry mycobiota can potentially produce high levels of mycotoxins with health risks to both human and animal populations.

Target effects of MPCAs

None of the commercial ***Trichoderma***-based MPCPs tested provided grey mold control in ***in vitro*** flower assays, whereas the laboratory isolates ***C. rosea*** and ***U. atrum*** both showed high biocontrol efficacy, the later similar to the control obtained with the fungicide Teldor.

Spore germination of ***Botrytis cinerea*** was strongly inhibited by the MPCA ***C. rosea*** when mutually inoculated on flowers, whereas the pathogen had no effect on spore germination of ***C. rosea***.

Non-target effects of MPCAs on other microbiota

None of the MPCAs ***C. rosea*** and ***U. atrum*** or the ***Trichoderma***-based commercial MBCP TRI003 had any effect on the natural microbial communities on mature strawberries, when applied once during flowering.

Spore germination of ***T.harzianum*** was strongly inhibited by ***C. rosea*** when mutually inoculated on flowers, whereas ***T.harzianum*** had no effect on spore germination of ***C. rosea***.

Establishment and fate of MPCAs

The population density of the ***Trichoderma***-based commercial MBCP TRI003,when applied at the recommended dosage to strawberry flowers or green berries in semi-field and field experiments, strongly diminished during the four week period from flower/green berry application to berry ripening from 10^4 cfu to <100 CFU. The recovery of strain 1295-22, the active ingredient of TRI003, from berries was demonstrated by UP-PCR profiling and ITS1-ITS2 sequencing. The field data suggest that the ability of ***Trichoderma*** to establish and survive on strawberry flowers/green berries is limited although laboratory experiments demonstrated the ability of 1295-22 to germinate on strawberry flowers.

Applying fungicides prior to application of the MPCA did not result in improved MPCA establishment.

IPM strategy

Fungicides employed in conventional strawberry production (Amistar, Candit, Euparen, Scala, Signum and Teldor) had no ***in vitro*** fungicidal effect on any of the tested fungal MPCAs (Binab-T, Supresivit, TRI003 and ***C. rosea***) except that some of the MPCAs were inhibited by the fungicides Candit, Euparen and Signum when applied at a dosage equivalent to x10 recommended dosage. Two ***Trichoderma***-based MPCPs (Supresivit and TRI003) were unaffected by all fungicides also when exposed to 10 times of recommended dosages.

Risk assessment of MPCAs

The relative importance of deliberately released fungal MPCAs and their metabolites in relation to that of the natural strawberry microbiota seem to be limited when considering the potential risk from the natural microbiota including mycotoxin producers and opportunistic human pathogens. In addition, none of the tested MPCAs produced mycotoxins when applied to strawberries and also their survival after application was very low.

6 Perspectives

The relative importance of MPCAs in relation to the natural microbiota and their metabolites were thoroughly examined in the present project covering a broad range of strawberry nurseries with different production practice and geographic distribution. However, it is important to note that phyllosphere microbial communities differ between plant genotypes why this topic should be further explored in more crops and different varieties within crops.

Culture-dependent methods were mainly employed in the present project. It is well-known that culture independent methods can provide more detailed information about phyllopshere microbial communities why future studies on phyllsophere microbiology should combine both culture- dependent and independent methods.

It is also important to note that in the present project MPCA application was only performed once during the strawberry production cycle in order to study the fate of introduced MPCAs. However, under practical conditions several applications would be made and in that way ripe berries would also be exposed to the MPCA, which depending on the subsequent handling and storage conditions would develop population densities higher than reported in the the present project.

Mycotoxins such as fumonisins, ochratoxin and patulin were detected in strawberries ***in vitro*** inoculated with respectively ***A. niger*** and ***P. expantum*** but fortunately not in field-grown berries. However, strawberries for processing, e.g. jams, may contain mouldy berries. This scenario should be addressed as few bad berries infected with these fungi might contaminate a whole batch.

Strawberries inoculated with ***Trichoderma***-based MPCAs contained peptaibols, which are biologically active compunds, with toxic effects on other microbiota and lower animals. Improved performance of ***Trichoderma***-based MPCPs may result in a higher level of peptaibols in the phytosphere with potential adverse effects on non-target organisms, calling for further studies on this matter.

Opportunistic human pathogens were part of the strawberry microbiota and may serve as a source of inoculum for infectious human diseases. The potential threat of plant associated human pathogens on strawberries and other fresh fruit and vegtables should be further addressed.

The natural strawberry microbiota also included several potential MPCAs. The role of these indigenous MPCAs in plant health should be explored. Also their possible management should be examined including the possible adverse effects of fungicide applications on these plant beneficial microbial populations.

The two laboratory isolates, ***C. rosea*** and ***U. atrum,*** demonstrated strong biocontrol potential against strawberry grey mould, which should be further explored under practical field conditions. The capability of ***C. rosea*** to germinate and grow on both leaves, flowers and berries was also shown which

strengthens its potential for protecting strawberries against e.g. ***B. cinerea*** as this pathogen can infect all above ground parts of the plant. In addition, since both ***C. rosea*** and ***U. atrum*** were unaffected by fungicides employed in conventional strawberry production an integrated strategy also looks promising including these MPCAs. In general, fungal MPCAs may prove to be more efficient when applied as one component in an IPM strategy, than when applied as a single disease control measure.

7 References

Andersen B., Smedsgaard J., Frisvad J.C. 2004. ***Penicillium expansum***: Consistent Production of Patulin, Chaetoglobosins, and Other Secondary Metabolites in Culture and Their Natural Occurrence in Fruit Products. Journal of Agricultural Food Chemistry 52: 2421-2428.

Andersen B., Krøger E., Roberts R.G. 2002. Chemical and morphological segregation of ***Alternaria arborescens, A. infectoria*** and ***A. tenuissima*** species-groups. Mycological Research 106: 170-182.

Andersen B., Hollensted M. 2008. Metabolite production by different ***Ulocladium*** species. International Journal of Food Microbiology 126: 172-179.

Andrews J.H. 1992. Biological control in the phyllosphere. Annual Review of Phytopathology 30: 603–635.

Andrews J.H., Harris R.F. 2000. The ecology and biogeography of microorganisms on plant surfaces. Annual Review of Phytopathology 38: 145-180.

Arras G. 1996. Mode of action of an isolate af ***Candida famata*** in biological controil of ***Penicillium digitatum*** in orange fruits. Postharvest Biology and Technology 8: 191-198.

Aziz N.H., Moussa L.A.A. 2002. Influence of gamma-radiation on mycotoxin producing moulds and mycotoxins in fruits. Food Control 13: 281-288.

Boff P., Köhl J., Gerlagh M., de Kraker J. 2002. Biocontrol of grey mould by ***Ulocladium*** atrum applied at different flower and fruit stages of strawberry. Biocontrol 47: 193-206.

Bradbury J.F. 1986. Guide to Plant Pathogenic Bacteria**.** Farnham Royal, England: CAB International, 1986.

Brimner T.A., Boland G.J. 2003. A review of the non-target effects of fungi used to biologically control plant diseases. Agriculture, Ecosystems & Environment 100, 3-16.

Bristow, P.R., McNicol, R.J. and Williamson, B., 1986. Infection of strawberry flowers by Botrytis cinerea and its relevance to grey mould development. Annals of . Applied Biology 109, 545–554.

Buck J.W. 2004. Combinations of fungicides with Phylloplane yeasts for improved control of ***Botrytis cinerea*** on geranium seedlings. Phytopathology 94: 196-202.

Christensen K.B., van Klink J.W., Weavers R.T., Larsen T.O., Andersen B., Phipps R.K. 2005. Novel chemotaxonomic markers for the ***Alternaria infectoria*** species-group. Journal of Agricultural and Food Chemistry 53: 9431-9435.

Cole R.J., Cox R.H. 1981. Handbook of toxic fungal metabolites. Academic Press, New York, NY.

Cordier C., Alabouvette C. 2009. Effects of the introduction of a biocontrol strain of Trichoderma atroviride on non-target micro-organisms. European Journal of Soil Biology 45, 267-274.

Cook R.J., Baker K.F. 1983. The Nature and Practice of Biological Control of Plant Pathogens. Amer. Phytopathol. Soc., St. Paul, Minnesota. 539 pp.

Cole R.J, Cox R.H. 1981. Handbook of toxic fungal metabolites. Academic Press, New York.

Cooney J.M., Lauren D.R., di Menna M.E. 2001. Impact of competitive fungi on trichothecene production by ***Fusarium graminearum*** Journal of Agricultural Food Chemistry 49: 522-526.

Correa O.S., Montecchia M.S., Berti M.F., Fernández Ferrari M.C., Pucheu N.L., Kerber N.L., García A.F. 2009. Bacillus amyloliquefaciens BNM122, a potential microbial biocontrol agent applied on soybean seeds, causes a minor impact on rhizosphere and soil microbial communities. Applied Soil Ecology 41, 185-194.

Cota L.V., Maffia L.A., Mizubuti E.S.G., Macedo P.F.F. 2009. Biological control by ***Clonostachys rosea*** as a key component in the integrated management of strawberry gray mold 50: 222-230.

Dal Bello G., Mónaco C., Rollan M.C., Lampugnani G., Arteta N., Abramoff C., Ronco L., Stocco M. 2008. Biocontrol of postharvest grey mould tomato by yeasts. Journal of Phytopathology 156: 257-263.

Degenkolb T., Dieckmann R., Nielsen K.F., Gräfenhan T., Theis C., Zafari D., Chaverri P., Ismaiel A., Brückner H., von Döhren H., Thrane U., Petrini O., Samuels G.J. 2008a. The ***Trichoderma brevicompactum*** clade: a separate lineage with new species, new peptaibiotics, and mycotoxins. Mycological Progress 7:177-219.

Degenkolb T., von Döhren H., Nielsen K.F., Samuels G.I., Brückner H. 2008b. Recent advances and future prospects in peptaibiotics, hydrophobin, and mycotoxin research, and their importance for chemotaxonomy of ***Trichoderma*** and ***Hypocrea***. Chemistry & Biodiversity 5: 671-680.

Dennis C. 1976. The microflora of the surface of soft fruits. In: Microbiology of Aerial plant surfaces. Eds. C.H. Dickinson & T.F. Preece. Academic Press. 419-439.

Doyle J.J., Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin 19: 11–15.

EEC 2003. Commission recommendation of 11 August 2003 on the prevention and reduction of patulin contamination in apple juice and apple juiceingredients in other beverages (2003/598/EC). Official Journal of the European Union L203: 54-59.

Elad Y., Kirshner B. 1993. Survival in the phylloplane of an introduced biocontrol agent (***Trichoderma harzianum***) and populations of the plant pathogen ***Botrytis cinera*** as modified by abiotic conditions. Phytoparasitica 21: 303-313.

Enya J., Shinohara H.,Yoshida S., Tsukiboshi T., Negishi H., Suyama K., Tsushima S. 2007. Culturable leaf-associated bacteria on tomato plants and their potential as biological bontrol agents. Microbial Ecology 53: 524–536.

Essghaier B., Fardeau M.L., Cayol J.L., Hajlaoui M. R., Boudabous A., Jijakli H. 2009. Biological control of grey mould in strawberry fruits by halophilic bacteria. Journal of Applied Microbiology 106: 833-846.

Falagas M.E., Kavvadi P.K., Mantadakis E., Kofteridis D.P., Bliziotis I.A., Saloustros E., Maraki S., Samonis G. 2006. Morganella morganii infections in a general tertiary hospital. Infection 34: 315-321.

Folker-Hansen P., Pedersen J.C., Kroer N., Hendriksen N.B., Hansen B.M. 1993. Miljømæssig vurdering af mikrobiologiske plantebeskyttelsesmidler. Miljøprojekt nr. 224, Miljøstyrelsen.

Francés J., Bonaterra A., Moreno M.C., Cabrefiga J., Badosa E., Montesinos E. 2006. Pathogen agressiveness and postharvest biocontrol efficiency in Pantoea agglomerans. Postharvest Biology and Technology 39: 299-307.

Freeman S., Minz D., Kolesnik I., Barbul O., Zveibil A., Maymon M., Nitzani Y., Kirshner B., Rav-David D., Bilu A., Dag A., Shafir, S., Elad,Y. 2004. ***Trichoderma*** biocontrol of ***Colletotrichum acutatum*** and ***Botrytis cinerea*** and survival in strawberry. European Journal of Plant Pathology 110: 361-370.

Frisvad J.C., Thrane U. 2002. Mycotoxin production by common filamentous fungi, p. 321-330. In R. A. Samson, E. S. Hoekstra, J. C. Frisvad, and O. Filtenborg (eds.), Introduction to Food- and Air Borne Fungi. 6th ed. Centraalbureau voor Schimmelcultures, Utrecht.

Frisvad J.C., Thrane U. 1987. Standardised High-Performance Liquid Chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (Diode Array Detection). Journal of Chromatography 404:195-214.

Frisvad J.C., Thrane U., Samson R.A. 2007a. Mycotoxin producers. In: Food mycology. A multifaceted approach to fungi and food, edited by J. Dijksterhuis and R. A. Samson, Boca Raton:CRC Press, p. 135-159.

Frisvad J.C., Smedsgaard J., Samson R.A., Larsen T.O., Thrane O. 2007b. Fumonisin B2 production by ***Aspergillus niger***. Journal of Agriculturtal Food Chemistry 55:9727-9732.

Gachon C., Saindrenan P. 2004. Real-time PCR monitoring of fungal development in ***Arabidopsis thaliana*** infected by ***Alternaria brassicicola*** and ***Botrytis cinerea***. Plant Physiology and Biochemistry 42, 367-371.

Green H. 2001. Effektivitetsvurdering af mikrobiologiske plantebeskyttelsesmidler. Miljøstyrelsen.

Harman G.E, Latorre B., Agosin E., San Martin R., Riegel P.A., Nielsen P.A., Tronsmo A., Pearson R.C. 1996. Biological and intergrated control of ***Botrytis*** bunch rot of grape using ***Trichoderma*** spp. Biological Control 7: 259-266.

Helbig J. 2002. Ability of the antagonistic yeast ***Cryptococcus albidus*** to control ***Botrytis cinerea*** in strawberry. BioControl 47: 85-99.

Hjeljord, L.G., Stensvand, A. and Tronsmo, A. 2001. Antagonism of nutrient-activated conidia of ***Trichoderma harzianum*** P1 against ***Botrytis cinerea***. Phytopathology. 91:1172-1180

Hjeljord, L.G. and Tronsmo A. 2003. Effect of germination initiation on competitive capacity of ***Trichoderma atroviride*** P1 conidia. Phytopathology. 93:1593-1598.

Inacio J., Pereira P., de Carvalho M., Fonseca A., Amaral-Collaco M.T., Spencer-Martins I. 2002. Estimation and diversity of phylloplane mycobiota on selected plants in a Mediterranean-type ecosystem in Portugal. Microbial Ecology 44: 344-353.

Jacques M-A., Morris C.E. 1995. A review of issues related to the quantification of bacteria from the phyllosphere. FEMS Microbiology Ecology 18: 1-14.

Jager E.S., Wehner F.C., Korsten L. 2001. Microbial ecology of the mango phylloplane. Microbial Ecology 42, 201-207.

Janse J.D. 2005. Phytobacteriology: Principles and practise, CABI Publishing.

Jennessen J., Nielsen K.F., Houbraken J., Schnürer J., Lyhne E.K., Frisvad J.C., Samson R.A. 2005. Secondary Metabolite and Mycotoxin Production by the ***Rhizopus microsporus*** Group. Journal of Agricultural Food Chemistry 53: 1833-1840.

Jensen D.F., Jensen B., Larsen J. 2008. Status og nye udfordringer: Biololgisk bekæmpelse af plantesygdomme. MoMentum 2, 8-9.

Jensen B. 1998. Mikrobiologiske bekæmpelsesmidler og toksiske metabolitter. Miljø- og Energiministeriet, Miljøstyrelsen. Miljøprojekt nr. 436. 95 sider.

Jensen B. 1999. Udvikling af et biologisk bejdsemiddel til frø. Miljø-og Energiministeriet Miljøstyrelsen, Miljøprojekt nr 463 1999, 118 sider.

Jensen B., Knudsen I.M.B., Jensen D.F. 2002. Survival of Clonostachys rosea on stored barley seeds and their biocontrol efficacy against seed-borne ***Bipolaris sorokiniana***. Biocontrol Science and Technology 12: 427-441.

Jensen B., Knudsen I.M.B, Madsen M, Jensen D.F. 2004. Biopriming of infected carrot seed with an antagonist selected for control of seedborne ***Alternaria*** spp. Phytopathology 94: 551-560.

Jensen D.F., Schulz A. 2004. Exploitation of GFP-technology with filamentous fungi. In. Handbook of Fungal Biotechnology (Arora, D.A, Bridge, P.D. & Bhatnagar, D. eds) Marcel Dekker.Inc., New York.

Johansen A., Knudsen I.M.B.; Binnerup S.J, Winding A., Johansen J.E., Jensen L.E., Andersen K.S., Svenning M.M., Bonde T.A. 2005. A greenhouse study of non target effects of the microbial control agents ***Pseudomonas fluorescens*** DR54 and ***Clonostachys rosea*** IK726 in field soil grown with barley followed by sugar beets. Soil Biology and Biochemistry 37: 2225-2239.

Kalogiannis S., Tjamos S.E., Stergiou A., Antoniou P.P., Ziogas B.N., Tjamos E.C. 2006. Selection and evaluation of phyllosphere yeasts as biocontrol agents against grey mould of tomato. European Journal of Plant Pathology 116: 69-76.

Kinkel L.L. 1997. Microbial population dynamics on leaves. Annual Review in Plant Pathology 35: 327-47.

Knudsen I.M.B., Hockenhull J., Jensen, D.F. 1995. Biocontrol of seedling diseases of barley and wheat caused by ***Fusarium culmorum*** and ***Bipolaris sorokiniana***: effects of selected fungal antagonists on growths and yield components. Plant Pathology 44: 467-477.

Knudsen I.M.B., Thomsen K.A., Jensen B., Poulsen K.M. 2004. Effects of hot water treatment, biocontrol agents, disinfectants and a fungicide on storability and control of the pathogen ***Ciboria batschiana*** on English oak acorns. Forest Pathology 33: 1-18.

Kotan R., Sahin F., Ala A. 2006. Identification and pathogenicity of bacteria isolated from pome fruit trees in the Eastern Anatolia region of Turkey. Journal of Plant Diseases and Protection 113: 5-8.

Krcmery V., Krupova I., Denning D.W. 1999. Invasive yeast infections other than ***Candia*** spp. in acute leukaemia. Journal of Hospital Infection 41: 181-194.

Krimm U., Abanda-Nkpwatt D., Schwab W., Schreiber L. 2005. Epiphytic microorganisms on strawberry plants (***Fragaria ananassa*** cv. Elsanta): Identification of bacterial isolates and analysis of their interaction with leaf surfaces. FEMS Microbiology Ecology 53, 483-492.

Kvistgård M. 1994. Mikrobiologiske bekæmpelsesmidler. Anvendelsesmuligheder og udviklingstendenser. Miljøprojekt nr. 256 Miljøstyrelsen.

Latorre B.A., Agosin E. San Martin R., Vasques G.S. 1997. Effectiveness of conidia of ***Trichoderma harzianum*** produced by liquid fermentation against Botrytis bunch rot of table grape in Chile. Crop Protection 16: 209-214.

Lindhardt H. 2003. Vurdering af muligheder for forebyggelse og alternativ bekæmpelse i frugt og bær. Bilag 4 til rapporten "Muligheder for forebyggelse og alternativ bekæmpelse inden for gartneri og frugtavl" Arbejdsrapport nr. 38, Miljøstyrelsen.

Lindow S.E., Brandl M.T. 2003. Microbiology of the phyllosphere. Applied and Environmental Microbiology 69, 1875-1883.

Lix X.R. 2009. Cryptococcus neoformans: Morphogenesis, infection and evolution. Infecection Genetics and Evolution 9, 401-416.

Loeffler J., Hebart H., Magga S., Schmidt D., Klingspor L., Tollemar J., Schumacher U., Einsele H. 2000. Identification of rare ***Candida*** species and other yeasts by polymerase chain reaction and slot blot hybridization Diagnostic Microbiology and Infectious Diseases 38: 207-212.

Lübeck M. 1994. Sekundære metabolitter fra udvalgte mikroorganismer. Mikrobiologiske plantebeskyttelsesmidler. Arbejdsrapport fra Miljøstyrelsen nr. 18. Miljøstyrelsen.

Lübeck M., Knudsen I.M.B., Jensen B., Thrane U., Janvier C., Jensen D.F. 2002. GUS and GFP transformation of the biocontrol strain ***Clonostachys rosea*** IK726 and the use of these marker genes in ecological studies. Mycological Research 106: 1-10.

Lübeck M., Poulsen S.K., Lübeck P.S., Jensen D.F., Thane U. 2000. Identification of ***Trichoderma*** strains from building materials by ITS1 ribotyping, UP-PCR fingerprinting and UP-PCR cross hybridization. FEMS Microbiology Letters 185: 129-134.

Lübeck M., Poulsen H. 2001. UP-PCR and UP-PCR cross blot hybridization as a tool for studying anastomosis group relationship in the ***Rhizoctonia solan***i complex. FEMS Microbiology Letters 201: 83-89.

Magkos F., Fotini A., Antonis Z. 2006. Organic Food: Buying More Safety or Just Peace of Mind? A Critical Review of the Literature',Critical Reviews in Food Science and Nutrition,46: 23-56.

Magnuson J.A., King Jr. A.D., Türük T. 1990. Microflora of partially processed lettuce. Applied and Environmental Microbiology 56: 3851-3854.

Mamarabadi, M, Jensen, B.,Jensen, DF and Lübeck, M. 2008. Real time RT-PCR expression analysis of chitinase and endoglucanase genes in the three way interaction between the biocontrol strain Clonostachys rosea IK726, Botrytis cinerea and strawberry. FEMS Microbiology Letters 285, 101-110.

Mansfeld-Giese K., Larsen J., Bødker L. 2002. Bacterial populations associated with mycelium of the arbuscular mycorhrhizal fungus ***Glomus intraradices***. FEMS Microbiology Ecology 41: 133-140.

McLean, M.A., Sutton J.C. 1992. Mycoflora on strawberry in Ontario. Canadian Journal of Botany 79: 846-852.

Mehli L., Kjellsen T.D., Dewey F.M., Hietala A.M. YEARA case study from the interaction of strawberry and ***Botrytis cinerea*** highlights the benefits of comonitoring both partners at genomic and mRNA level. New Phytologist 168: 465-474.

Mikkelsen L., Sarroco S., Lübeck M., Jensen D.F. 2003. Expression of the red fluorescent protein DsRed-Express in filamentous ascomycete fungi. FEMS Microbiology Letters 223: 135-139.

Møller K. 1999. Studies of the infecton biology and biocontrol of leaf and head rot of Chinese cabbage (causal agent: ***Pythium tracheiphilum***) and some taxonomical aspects of the genus ***Pythium***. Ph.D. thesis. Section for Plant Pathology. The Royal Veterinary and Agricultural University of Copenhagen.

Møller K., Jensen B., Andersen H.P., Stryhn H., Hockenhull J. 2003. Biocontrol of ***Pythium tracheiphilum*** in Chinese Cabbage by ***Clonostachys rosea*** under Field Conditions. Biocontrol Science and Tehnology 13: 171-182.

Nielsen K.F., Gräfenhan T., Zafari D., Thrane U. 2005. Trichothecene production by ***Trichoderma brevicompactum***. Journal of Agricultural Food Chemistry 53:8190-8196.

Nielsen K. F., Smedsgaard J. 2003. Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for de-replication by standardised liquid chromatography-UV-mass spectrometry methodology. J ournal of Chromatography A 1002: 111-136.

Olsen M. 2009. Mycotoxins in organic and Conventional Foods and Effects of the Environment. ***In*** : Health Benefits of organic Food : Effects of Environment. Eds. D. I. Givens, Samantha Baxter, A. M. Minihane, CAB International 2008. p 145-159.

Parikka, Kivijärvi, P., Prokkola, S. & Kemppainen, R. 2009. Microbiological quality of organic strawberry fruit. Acta Hort. 842. 377-380.

Parsley R. 1996. Operation manual, version 6. Microbial Identification System, MIDI Inc., Newark, Delaware, USA, 219 pages.

Patiño-Vera M., Jiménez B., Ortiz M., Carillo A, Galindo E. 2005. Pilot-scale production and liquid formulation of Rhodotorula minuta, a potencial biocontrol agent of mango anthracnose. Journal of Applied Microbiology 99: 540-550.

Peng G., Sutton J.C., Kevan P.G. 1992. Effectiveness of honey bees for applying the biocontrol agent ***Gliocladium roseum*** to strawberry flowers to suppress ***Botrytis cinerea***. Canadian Journal of Plant Pathology 14: 117-188.

Pfaller M.A., Diekema D.J. 2004. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of ***Candida***. Clinical Microbiology and Infection 10: 11-23.

Ramos A., Dámaso D. 2000. Extraintestinal infection due to Hafnia alvei. European Journal of Clinical Infection Diseases 19: 708-710.

Roberti R., Badiali F., Pisi A., Veronesi A., Pancaldi D., Cesari A. 2006. Sensitivity of ***Clonostachys rosea*** and ***Trichoderma*** spp. as potential biocontrol agents to pesticides. Journal of Phytopathology 154: 100–109.

Samonis G., Anatoliotaki M., Apostolakou H., Maraki D., Mavroudis D., Georgoulias V. 2001. Transinet Fungemia due to ***Rhodotorula rubra*** in a cancer patient: Case report and review of the literature. Infection 29:173-176.

Samson R.A., Hoekstra E.S., Frisvad J.C., Filtenborg O. 2002. Introduction to Food- and Airborne Fungi. 6th Edition, 2nd Print. Centraalbureau voor Schimmelcultures, Utrecht.

Samson R.A., Frisvad J.C. 2004. Penicillium subgenus Penicillium: new taxonomic schemes, mycotoxins and other extrolites. Studies in Mycology 49: 1-257.

Sasser M. 1990. Identification of bacteria through fatty acid analysis. In: (Clement, Z., Rudolph, K., Sands, D.C., (eds.), Methods in Phytobacteriology, Akadémiai Kiadó, Budapest, pp. 199-204.

Scherwinski K., Wof A., Berg G. 2007. Assessing the risk of biological control agents on the indigenous microbial communities: Serratia plymuthica HRO-C48 and Steptromyces sp. HRO-71 as model bacteria. BioControl 52, 87-112.

Shafir S., Dag A., Bilu A., Abu-Toamy M., Elad Y. 2006. Honey bee dispersal of the biocontrol agent ***Trichoderma harzianum*** T39: effectiveness in suppressing ***Botrytis cinerea*** on strawberry under field conditions. European Journal of Plant Pathology 116, 119–128.

Sharma R.R., Singh D., Singh R. 2009. Biological Control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. Biological Control 50: 205-211.

Smedsgaard J. 1997. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. Journal of Chromatography A 1997; 760:264-270.

Sutton J.C, De-Wei L., Peng G., Yu H., Zang P., Valdebenito-Sanhueza R.M. 1997. Gliocladium roseum: a versatile adversary of ***Botrytis cinerea*** in crops. Plant Disease 81: 316-328.

Takahashi-Ando N., Kimura M., Kakeya H., Osada H., Yamaguchi I. 2002. A novel lactonohydrolase responsible for the detoxification of zearalenone: enzyme purification and gene cloning. Biochemical Journal 365: 1-6.

Thrane U., Poulsen S.B, Nirenberg H.I., Lieckfelt E. 2001. Identfication of ***Trichoderma*** strains by image analysis of HPLC chromatograms. FEMS Microbiology Letters 203: 249-255.

Thompson I.P., Bailey M.J., Fenlon J.S., Fermor T.R., Lilley A.K., Lynch J.M., McCormack P.J., McQuilken M.P., Purdy K.J., Rainey P.B., Whipps J.M. 1993. Quantitative and qualitative seasonal changes in the microbial community from the phyllosphere of sugar beet (***Beta vulgaris***). Plant and Soil 150: 177–191.

Tronsmo A. 1986. Trichoderma used as a biocontrol agent against ***Botrytis cinerea*** rots on strawberry and apple. Meldinger fra Norges Landbrukshøgskole 65, 1-22.

Tournas V.H., Katsoudas E. 2005. Mould and yeast flora in fresh berries, grapes and citrus fruits. International Journal of Food Microbiology 105, 11-17.

Van den Boogert P.H.J.F., Luttikholt A.J.G. 2004. Compatible biological and chemical control systems for ***Rhizoctonia solani*** in potato European Journal of Plant Pathology 110: 111-118.

Weidenboerner M., Wieczorek C., Kunz B. 1995. Mold spectra of various foods in relation to plating medium. Journal of Food Protection 58: 661-665.

Yang C.H., Crowley D.E., Borneman J., Keen N.T. 2001. Microbial phyllosphere populations are more complex than previously realized. Proceedings of the National Science Academy USA 98: 3889-3894.

Yohalem D.S. 2004. Evaluation of fungal antagonists for grey mould management in early growth of pot roses. Annals of Applied Biology 144: 9-15.

Yohalem D.S., Kristensen K. 2004. Optimization of timing and frequency of application of the antagonist ***Ulocladium atrum*** for management of gray mold in potted rose under high disease pressure. Biological Control 29: 256-259.

Ørum J.E., Christensen J. 2001 Produktionsøkonomiske analyser af mulighederne for en reduceret pesticidanvendelse i dansk gartneri. Statens Jordbrugs- og Fiskeriøkonomiske Institut Rapport 128.

Zhang B., Bai Z., Hoefel D., Tang L., Yang Z., Zhuang G., Yang J., Zhang H. 2008. Assessing the impact of the biological control agent ***Bacillus thuringiensis*** on the indigenous microbial community within the pepper plant phyllosphere. FEMS Microbiology Letters 284, 102-108.

Abbreviations

Abbreviation	
ANOVA	Analysis of variance
CFU	Colony forming unit
CYA	Czapek yeast autolysate
CWDE	Cell wall degrading enzymes
DG18	Dichloran glycerol 18% agar
DNA	Deoxyribonucleic acid
DRYES	Dichloran Rose Bengal Yeast Sucrose agar
DsRed	Red fluorescent protein
DTU	Danmarks Tekniske Universitet
ESI	Electrospray ionization
GFP	Green Fluorescent Protein
GM	Glucose media
IPM	Integrated pest management
ITS	Internal transcribed spacer
HPLC	High Pressure Liquid Chromatography
HPLC-MS/MS	High Pressure Liquid Chromatography-Mass spectrometer
HPLC-UV	High Pressure Liquid Chromatography-Ultraviolet
LC-DAD-MS	Liquid chromatography-Diode array detector- Mass spectrometer
LSD	Least significant difference
MPCA	Microbial pest control agent. The term is used by EFSA and OECD (ENV/JM/MONO(2008)36.).
MPCP	Microbial pest control product. The term is used by EFSA and OECD (ENV/JM/MONO(2008)36.).
MWG	DNA sequencing company
NA	Nutrient agar
NCBI	National Center for Biotechnology Information
OM	Oat Meal agar
PDA	Potato dextrose agar
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
RB	Rose Bengal
SDBA	Saboraud Dextrose Broth Agar
SNA	Spezieller nährstoffarmer agar
SYBR green	Nucleic acid stain
THSM	Trichoderma selective media
TSBA	Tryptocase soy broth agar
UP-PCR	Universally Primed PCR
UV	Ultraviolet
V8	V8-juice agar
YES	Yeast extract sucrose

Growth media

DG18 Dichloran glycerol 18% agar	**1000 ml**
Dichloran Glycerol (DG 18) agar base1 (OXOID, cm729)	25,8 g
Glycerol (anhydrous) (J.T Baker, 7044)	180,3 g
Spor metals opløsning (SM)	0,82 ml
Chloramphenicol solution (CA)	0,82 ml
Water, dobbelt, dest.	820 ml

Adjust pH to 5.6±0.1
Mix ingredients. Autoclave 15 min. at 121°C and cool to 59C.
Add: Chlortetracycline solution (CT) 8,2 ml/L

CT Chlortetracyline solution	
Chlortetracycline hydrochloride 0,50 g (Sigma, C-4881) Keep at -20ºC	0.50 g
Water, dest. 100 ml	100 ml

Mix the solution in a blue cap bottle. Sterile filtrate the solution through 0.20 µm filter before adding it to media. Don't autoclave the solution. Keep the CT-solution at 4ºC

PCA Potato-carrot agar	
Potatoes	10 g
Carrots	10 g
Water	500 ml

Peel the vegetables and cut them into pieces. Add 10 g of each into a 500 ml blue cap bottle and add 400 ml distilled water, autoclave at 121°C for 15 min. Cool and homogenize the mixture and press it through a tea sieve.
Add 12,5 g Agar (Bie & Berntsen, BBB 10030, SO-BI-Gel, Agar-Agar) and fill up to 500 ml, autoclave at 121°C for 15 min. (pH approximately 6,25)

PDA Potato dextrose agar	
Potatoes	10 g
Carrots	10 g
Water	500 ml

SNA Spezieller nährstoffarmer agar	
KH_2PO_4 (MERCK, 4873)	1,0 g
KNO_3 (MERCK, 5063)	1,0 g
$MgSO_4,7H_2O$ (MERCK, 5886)	0,5 g
KCL (MERCK, 4936)	0,5 g
Glucose (D+) (BHD, 10117)	0,2 g
Sucrose (BHD,10274)	0,2 g
Agar (Bie & Berntsen,BBB10030,SO-BI-Gel,Agar-Agar)	20,0 g
Water, dest.	1000 ml

Mix the substrate and autoclave at 121°C, cool and pure into plates
Når substratet er størknet, eller dagen efter, placeres et sterilt stykke filterpapir (10x30 mm) i hver petriskål.

SM Trace metal solution	
$ZnSO_4,7H_2O$ (MERCK, 8883)	1.0 g
$CuSO_4$, $5H_2O$ (MERCK,2790)	0.50 g
Water, dest.	100 ml

V8-juice agar with **antibiotics**	**BATCH**
Campbell's V8-juice (American Company, Peder Hvitfeldts Str.13, 1173 Kbh K)	175 ml
$CaCO_3$ (Merck, 2066)	3,0 g
Chloramphenicol opløsning (CA)	1 ml
Water, dest.	825 ml
Agar (Bie & Berntsen,BBB10030,SO-BI-Gel,Agar-Agar)	20 g
Chlortetracycline opløsning (CT)	10 ml

Mix V8-juice, $CaCO_3$, water and CA and adjust pH to 6,4$\pm$ 0,1.
Add agar mix and autoclave at 121°C for 15 min. Cool to 59°C and add CT

CA Chloramphenicol solution	
Chloramphenicol (Sigma, c-0378)	0.05 g
Ethanol (96%)	10 ml

CHLORAMPHENECOL IS CANCEROGENIC

DS Dichloran solution	
Dichloran (Fluka, 36010) **OPBEVARES I GIFTSKAB**	0.20g
Ethanol (96%)	100

Dichloran=2,6-Dichloro-4-Nitroaniline=Botran=Dicloran

RB Rose bengal opløsning	
Rose bengal (BHD, 26172)	2.5 g
Water, dest.	100 ml

TSBA Tryptocase soy broth agar	
trypticase soy broth	15.0 g
Bacto agar	15.0 g
Water, dest.	1000 ml

SDBA Saboraud Dextrose Broth Agar	
Special low nutrient agar	23.4 g
Sabouraud dextrose agar	65.0 g
Water, dest.	1000 ml

Amend with Novobiocin (25 mg/l)

$THSM_{mod.}$ Trichoderma selective media	
$MgSO_4 \cdot 7H_2O$	0.2 g
K_2HPO_4	0.9 g
NH_4NO_3	1.0 g
KCl	0.15 g
Rose bengal	0.15 g
Glucose	3.0 g
Chloramphenicol	0.1 g
Streptomycin	0.09 g
Propamocarb (640 g a.i./L)	1.2 ml
Agar	20 g
Water, dest.	1000 ml