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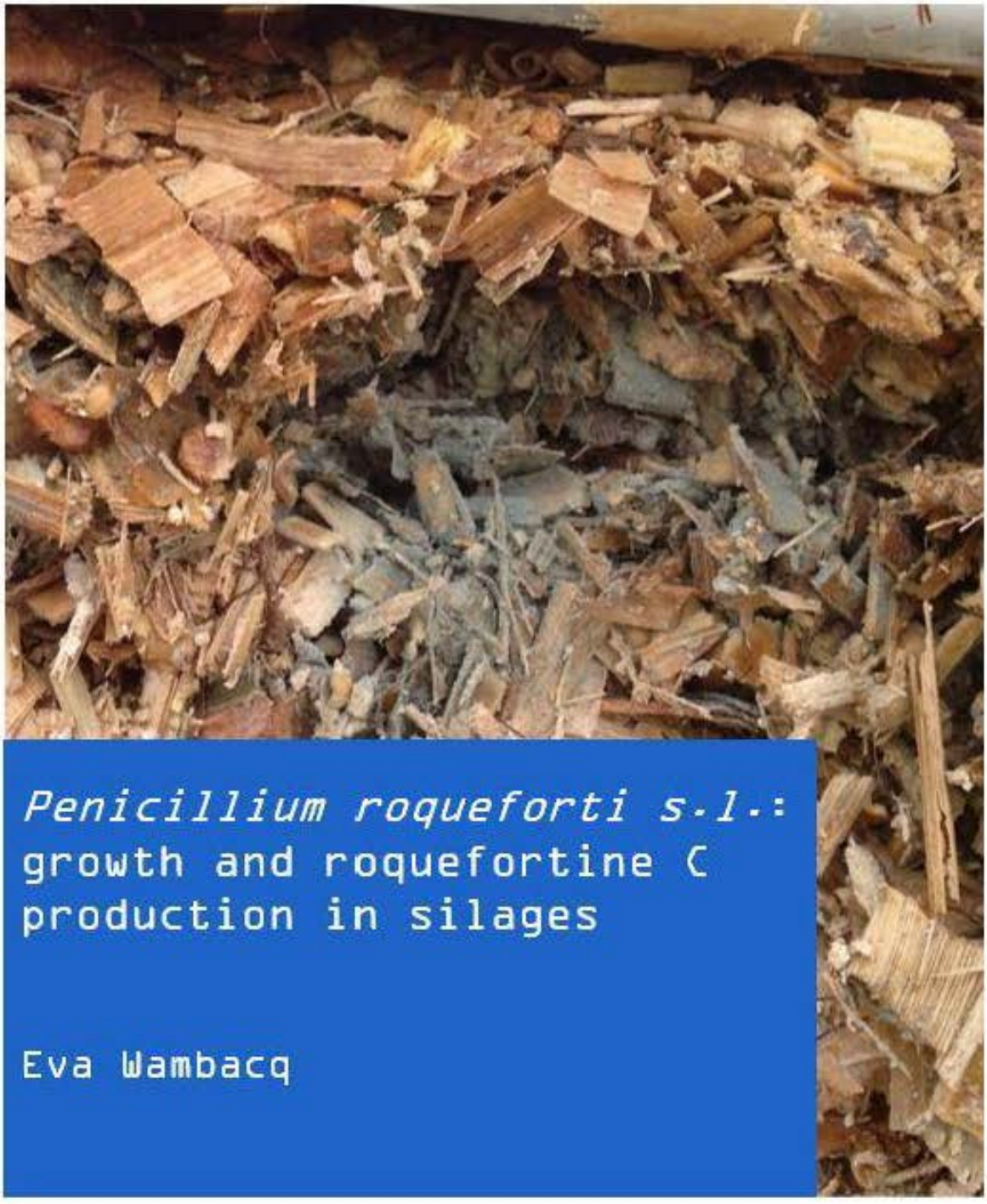

Penicillium roqueforti s.l.: growth and roquefortine C production in silages

Eva Wambacq

GHENT
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“The art and science of asking questions
is the source of all knowledge”
Thomas Berger

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Penicillium roqueforti s.l.: growth and roquefortine C production in silages

Eva Wambacq

Thesis submitted in fulfillment of the requirements for the degree of
Doctor (PhD) in Applied Biological Sciences: Agricultural Sciences

Dutch translation of the title:

Penicillium roqueforti s.l.: groei en roquefortine C productie in kuilvoeders

Cover illustrations and illustration on the first page of each chapter:

P. roqueforti s.l. infected whole-crop maize silage (photographs taken by Eva Wambacq)

Acknowledgments to financial institutions

This research was supported by the Research Fund of University College Ghent and Ghent University.

Cite as:

Wambacq E. (2017). *Penicillium roqueforti s.l.:* growth and roquefortine C production in silages. PhD thesis, Ghent University, Belgium.

Printed by University Press, Zelzate

ISBN number: 978-94-6357-046-6

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Dankwoord

Nooit gedacht dat ik dit ooit zou schrijven! Doctoreren was echt niet mijn ambitie toen ik afstudeerde als bio-ingenieur, maar door gedurende enkele jaren praktijkgericht onderzoek te doen naar schimmels in kuilvoeders is het er toch van gekomen: ik ben met veel plezier begonnen aan een doctoraatsonderzoek over *Penicillium roqueforti s.l.* in kuilvoeders, en nu sluit ik deze periode af met dit proefschrift!

Tot dit proefschrift komen was een werk van lange adem waarbij verschillende fasen doorlopen werden, een beetje vergelijkbaar met een inkuilproces …
Het in te kuilen gewas werd grondig bekeken: de problematiek van schimmelgroei in kuilvoeders op landbouwbedrijven werd in kaart gebracht.
De ondergrond van de silo werd gereinigd: via drie jaar van praktijkgericht onderzoek voorafgaand aan mijn doctoraatsonderzoek werd expertise opgebouwd inzake schimmelisolatie en –identificatie, kuilfermentatie en uitvoering van proeven met microkuilen.
Ervaren loonwerkers werden ingeschakeld als hulp voor de landbouwer om het materiaal op een goede manier in te kuilen: samen met mijn promotoren Geert Haesaert, Kris Audenaert en Monica Höfte werden de onderzoeksvragen geformuleerd, alsook de strategie om ze zo goed mogelijk te beantwoorden.
Vervolgens werd het materiaal luchtdicht afgedekt en kon het inkuilproces zich in al zijn facetten ten volle voltrekken: de onderzoeksvragen vertaalden zich naar een heel scala aan experimenten, finaal resulterend in het proefschrift dat u nu aan het lezen bent.

Inkuilen is teamwerk! Ik heb dan ook een heel team te bedanken …
Ik denk hierbij in de eerste plaats aan mijn promotoren:

> **Geert**, heel erg bedankt om mij gedurende de hele weg naar dit proefschrift bij te staan met raad en daad! Ondanks je drukke agenda kon ik altijd bij jou terecht met vragen, met ideeën voor proefopzetten, voor bespreking van de soms logische, maar vaak ook onverwachte bevindingen, voor het nalezen van teksten, voor een kritische kijk op het onderzoek in een breder perspectief, … We hebben vele knopen doorgehakt over wat wel en wat niet verder te onderzoeken op het nog grotendeels onontgonnen terrein van toxigene schimmels en hun mycotoxinen in kuilvoeders, maar samen zijn we er in geslaagd om van dit onderzoek een mooi geheel te maken, waarvoor zeer veel dank !!!

Kris, je werd pas laat mede-promotor, maar gedurende alle jaren van mijn doctoraatsonderzoek hadden we regelmatig overlegmomenten waarbij je mij geïnspireerd en geholpen hebt bij het uitwerken van nieuwe proeven. Dankjewel hiervoor, en ook enorm bedankt voor je hulp bij de statistische gegevensverwerking en voor het kritisch nalezen van mijn teksten!

Monica, hartelijk dank om promotor te willen zijn van dit proefschrift! Bij aanvang van dit onderzoek kon Geert als docent aan de HoGent niet de enige promotor zijn. We waren dan ook zeer blij dat je bereid was om mijn onderzoek te “adopteren”, ook al valt het onderwerp van *P. roqueforti s.l.* in kuilvoeders ietwat buiten het vakgebied Fytopathologie. Achteraf gezien bleek dit een voordeel aangezien je vanuit een andere invalshoek zeer waardevolle input gaf, waarvoor oprechte dank!

Er is iemand die geen promotor is van dit onderzoek, maar die hier zeker een bijzondere vermelding verdient: **Joos**, dankjewel om mij via het PWO-project i.v.m. kuilschimmels in te wijden in de wereld van het wetenschappelijk onderzoek. Dit project maakte mij vertrouwd met kuilvoeders en hun problemen qua schimmelontwikkeling. Ik vond het heel fijn om de boer op te gaan, waardoor de zin kwam in een praktijk-georiënteerd doctoraatsonderzoek rond deze complexe problematiek. Bedankt ook voor jouw frisse kijk en voor alle boeiende discussies!

Vanzelfsprekend ook een gigantische dankjewel aan alle (ex-)**collega’s** op de proefhoeve Bottelare naast Geert en Joos, met name Anneleen, Anneline, Bart, Betty, Bram, Chantal, Dieter, Elias, Elien, Etienne, Evelyn, Greet, Jasper, Jonas, Jos, Jules, Katrien, Kevin, Luc, Sarah, Stijn, Szanne, Veerle, Wouter, ... Bedankt voor alle goeie ideeën, alle hulp, alle ontspanningsmomenten enz. die ervoor zorgen dat Bottelare een tweede thuis is voor mij! Verder waardeer ik ook enorm de hulp van collega’s op de campus Schoonmeersen en het FFW-labo Bromatologie, met een speciale vermelding voor Boris, Christ’l, Ellen, Fré, Griet, Ilse, Maarten, Marijke, Mario, Marthe, Peter, Pieter, Sarah en Sofie!
Uiteraard ook een woord van dank aan ILVO Dier 68, de Hooibeekhoeve en alle landbouwers waar ik stalen mocht komen nemen van hun kuilvoeders. Kortom, aan allen die rechtstreeks of onrechtstreeks bij dit onderzoek betrokken waren: een korte en krachtige, maar welgemeende DANKUWEL !!!

Einddoel van het inkuilproces is kuilvoeder van zo hoog mogelijke kwaliteit, bestemd voor vervoedering. In het geval van dit proefschrift is er een dubbele doelgroep:
De eerste groep waarvoor het bestemd is zijn uiteraard degenen die dit proefschrift lezen. Ik wens elke lezer dan ook een fijne, boeiende leeservaring en ik bedank jullie nu al voor de aandacht!

De tweede groep voor wie en met wiens hulp dit proefschrift tot stand gekomen is, is mijn familie - in het bijzonder **mijn ouders, mijn ventje Luc en mijn kindjes Jenne en Stan**. Zeker de laatste tijd zag ik jullie bijna meer op de bureaubladachtergrond van mijn laptop dan “live”. Een gigantische dank-jullie-wel voor alle steun, voor het hart onder de riem en voor de knuffels op moeilijke momenten, voor jullie enthousiasme, … Zonder jullie zou het mij niet gelukt zijn om nu mijn derde “kindje”, dit doctoraatsonderzoek, voor te stellen !!!

Eva

Samenvatting

Kuilvoeders op landbouwbedrijven in België vertonen vaak visueel zichtbare schimmelontwikkeling. De toxigene schimmelspecies *Penicillium roqueforti sensu stricto* (*s.s.*) en *P. paneum*, samen aangeduid in dit proefschrift als *P. roqueforti sensu lato* (*s.l.*), zijn de meest frequent voorkomende schimmelspecies in kuilvoeders. Aangezien zowel het inademen van schimmelsporen als de consumptie van met mycotoxinen gecontamineerd voeder ernstige gezondheidsrisico's inhoudt, is het zeer belangrijk om schimmelontwikkeling in kuilvoeders zoveel mogelijk te vermijden. In dit proefschrift worden algemene preventieve maatregelen op een rijtje gezet. Het finale doel van dit doctoraatsonderzoek is evenwel om een bijdrage te leveren aan de preventie van specifiek *P. roqueforti s.l.* in kuilvoeders. In dat kader werden *in vitro* labo experimenten alsook *in vivo* proeven met miniatuurkuilen uitgevoerd om de invloed van verscheidene abiotische en biotische factoren op schimmelgroei en mycotoxineproductie te onderzoeken. Roquefortine C, een mycotoxine dat zowel door *P. roqueforti s.s.* als door *P. paneum* kan geproduceerd worden, wordt beschouwd als een indicator voor mycotoxineproductie door *P. roqueforti s.l.* in kuilvoeders. Daarom werd bij de uitgevoerde proeven gefocust op dit mycotoxine.

Tijdens het inkuilproces zetten melkzuurbacteriën suikers om naar voornamelijk melkzuur, maar ook naar azijnzuur, mannitol, ethanol, enz. Deze componenten kunnen gebruikt worden door *P. roqueforti s.l.* als koolstofbron. Melkzuur als enige koolstofbron liet echter maar beperkte schimmelgroei toe, terwijl bij azijnzuur (dat een remmende werking heeft naar aeroob bederf en daaropvolgende schimmelontwikkeling toe) als enige koolstofbron wel goede groei vastgesteld werd. Dit illustreert dat *P. roqueforti s.l.* zeer goed aangepast is aan zijn kuilvoeder-habitat, hetgeen preventie bemoeilijkt.

De bacterie *Bacillus velezensis* bleek *in vitro* antagonistische eigenschappen te hebben t.o.v. *P. roqueforti s.l.*: zowel cultuursupernatans als celsuspensie reduceerde de kieming en de overleving van conidiosporen en had een inhiberend effect op schimmelgroei, zonder te leiden tot een verhoogde roquefortine C productie. Dit leek veelbelovend ter preventie van *P. roqueforti s.l.* in kuilvoeders, maar via een *in vivo* proef met miniatuurkuilen konden de hoge verwachtingen qua antagonisme niet ingelost worden. Verder onderzoek is nodig om het potentieel van *B. velezensis* als kuiladditief in te schatten.

Zuurstof blijkt een cruciale rol te spelen in de ontwikkeling van *P. roqueforti s.l.*: in anaerobe omstandigheden is geen schimmelgroei mogelijk. Een *in vivo* experiment met kunstmatig geïnfecteerde maïs (@ 1500 conidia per gram verse stof) in miniatuurkuilen die uitgekuild werden na 50, 100 of 150 dagen heeft aangetoond dat na 50 dagen nog enkele *P. roqueforti s.l.* propagulen (66 per gram verse stof) overleefden, terwijl na een inkuilduur van 100 dagen geen actieve *P. roqueforti s.l.* meer aangetroffen werd. Dit experiment toonde het belang aan van een voldoende lange inkuilduur voor kuilvoeders in praktijk, tijdens dewelke de integriteit van de kuilafdekking dient gewaarborgd te worden. Ter preventie van *P. roqueforti s.l.* in kuilvoeders is het strikt navolgen van goede landbouwpraktijken bij in- en uitkuilen, leidend tot minimale blootstelling van kuilvoeder aan zuurstof, de sleutel tot succes.

Abstract

On Belgian farms, visible fungal growth is regularly encountered in ensiled feed commodities. The toxigenic fungal species *Penicillium roqueforti sensu strictu* (*s.s.*) and *P. paneum*, designated together as *P. roqueforti sensu lato* (*s.l.*) in this dissertation, are the most frequently isolated fungi in silages. Since the inhalation of fungal spores as well as the consumption of mycotoxin contaminated feed comprise serious health risks, it is of the outmost importance to prevent fungal contamination of silages. In this dissertation, general preventory measures are described. The final goal of this PhD research was to contribute to the prevention of specifically *P. roqueforti s.l.* development in silage. To achieve this goal, multiple *in vitro* lab experiments and *in vivo* trials with microsilos have been conducted, evaluating the effect of several abiotic and biotic factors on *P. roqueforti s.l.* growth and mycotoxin production. Roquefortine C, a mycotoxin that can be produced by both *P. roqueforti s.s.* and *P. paneum*, is considered to be an indicator of mycotoxin production by *P. roqueforti s.l.* in silages. Therefore, this particular mycotoxin has been determined to evaluate mycotoxin production.

During the ensiling process, lactic acid bacteria convert sugars to mainly lactic acid, but also some acetic acid, methanol, ethanol, etc. These compounds can be used by *P. roqueforti s.l.* as a carbon source. Lactic acid as sole carbon source was not very conducive for fungal growth, while acetic acid (inhibiting aerobic deterioration and subsequent fungal development in silages) as sole carbon source facilitated good fungal growth. This illustrates that *P. roqueforti s.l.* is very well adapted to its silage-habitat, rendering prevention difficult.

The bacterium *Bacillus velezensis* displayed antagonistic properties towards *P. roqueforti s.l.* in an *in vitro* experiment: both culture supernatant as cell suspension reduced spore germination and spore survival and inhibited fungal growth, without triggering an increased roquefortine C production. These observations seemed promising towards the capability of *B. velezensis* to prevent *P. roqueforti s.l.* development in silages, but the applied cell suspension could not live up to the great expectations regarding antagonism in an *in vivo* microsilo trial. Future research is required to investigate the potential of *B. velezensis* as a silage additive to counter *P. roqueforti s.l.* in silage.

Oxygen appears to play a crucial role in the development of *P. roqueforti s.l.*: in anaerobic conditions, no fungal growth can occur. An *in vivo* microsilo trial with artificially infected whole-crop maize (@ 1500 conidia per gram fresh matter) desiled after 50, 100 or 150 days demonstrated that at desiling after 50 days some *P. roqueforti s.l.* propagules (66 per gram verse stof) had survived, while after an ensiled period of 100 days no active *P. roqueforti s.l.* propagules were detected. This experiment emphasizes the importance of a sufficiently long ensiled period, during which the integrity of the silo coverage needs to be maintained. In order to prevent the development of *P. roqueforti s.l.* in silages, the strict application of good agricultural practices regarding ensiling and desiling, limiting air ingress into silages, is the key factor to success.

List of abbreviations

AA	acetic acid ($C_2H_4O_2$)
ATP	adenosine triphosphate
BAB	butyric acid bacteria
BHI	brain-heart infusion
Bv	*Bacillus velezensis*
CBS	Centraalbureau voor Schimmelcultures
cDM	dry matter content corrected for volatile compounds
cfu	colony forming units
CIA	corn infusion agar
CO_2	carbon dioxide
CSI	corn silage infusion
CYA	Czapek-Dox yeast extract agar
DM	dry matter
DNA	deoxyribonucleic acid
FM	fresh matter
HeLAB	heterofermentative lactic acid bacteria
HGT	horizontal gene transfer
HoLAB	homofermentative lactic acid bacteria
LA	lactic acid ($C_3H_6O_3$)
LAB	lactic acid bacteria
LC-MS/MS	liquid chromatography - tandem mass spectrometry
MEA	malt extract agar

MGC	metabolic gene cluster
MinM	mineral medium
MUCL	Mycothèque de l'Université Catholique de Louvain
O_2	oxygen
OD	optical density
OTA	ochratoxin A
PCA	plate count agar
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDAA	potato dextrose agar with 5 ml/l of acetic acid added
PDB	potato dextrose broth
PP	*Penicillium paneum*
PR	*Penicillium roqueforti s.s.*
RIP	repeat-induced point mutation
RNA	ribonucleic acid
RNAi	RNA interference
ROC	roquefortine C
s.l.	*sensu lato*
SM	secondary metabolite
s.s.	*sensu stricto*
TE	transposable element
TF	transcription factor
UV	ultraviolet
WSC	water-soluble carbohydrates
YES	yeast extract sucrose agar
ZAN	zearalanone

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Chapter 1

Introduction:

Problem statement

and research outline

1. Introduction

This first chapter kicks off with a literature review on the ensiling process and its main influencing factors. The different phases of the ensiling process are described, along with the micro-organisms playing a role in each particular phase. As for the influencing factors, some plant-related factors (*i.e.* epiphytic microflora, dry matter (DM) content, buffer capacity and sugar content) will be discussed along with other factors like silo type, silage additives, temperature and silo management.

In practice, the obtained silages do not always live up to the farmer's expectations and the livestock's needs, often due to fungal hot-spots in the silage mass. To illustrate this problem, the second section of this chapter describes on-farm sampling of mouldy whole-crop maize and grass silages during the feed-out phase. On the one hand, identification of the fungi contaminating the silages was executed. On the other hand, the fermentation characteristics of the fungal hot-spots were compared to those of visibly non-mouldy core samples and top layer samples obtained from the same silos, to determine the effect on fungal growth on silage fermentation. In order to find out the cause of fungal infestation of on-farm silages, farm surveys about silage making practices have been performed.

The insights obtained from sampling on-farm silages and from monitoring the ensiling and desiling practices of farmers resulted in the topic for this PhD research, *i.e. Penicillium roqueforti s.l.* growth and mycotoxin production in silages. The research outline and the research questions are presented at the end of this chapter.

2. The ensiling process and its main influencing factors

Ensiling is over 3000 years old: the terms "silage" and "silo" originate from the Greek word "σιρός" (siros), referring to the storage of grains and forage crops underground in pits. Today, the word "silo" is used to describe any container used for the storage of a broad range of agricultural products: dry cereal grains on the one hand, and wetter materials like forage crops (*i.e.* all parts of the crop above the stubble are harvested: e.g. maize, grasses, small-grain cereals and legumes), moist cereal grains and industrial by-products on the other hand (Alonso *et al.* 2013; Auerbach *et al.* 1998; Filya *et al.* 2000; Hargreaves *et al.* 2009; Lin *et al.* 1992; Monbaliu *et al.* (2010); Mustafa and Seguin 2003; Nout *et al.* 1993; Schwarz and Preissinger 2000; Weinberg and Ashbell 2003).

Wet materials, like forage crops (e.g. whole-crop maize, grasses and legumes), moist cereal grains and industrial by-products (e.g. sugar beet press pulp, beer draff and wet distiller's grains), require sealed silos and are preserved through spontaneous anaerobic fermentation of carbohydrates to mainly lactic acid but also to acetic acid by epiphytic lactic acid bacteria, after which the obtained feed is referred to as "silage" (Bolsen *et al.* 1996; Fulgueira *et al.* 2007; Oude Elferink *et al.* 2000; Wilkinson 2005).

The aim of ensiling is to preserve feed commodities during a prolonged period of time, maintaining the nutritional quality as well as the hygienic quality as high as possible. Ensiling has various reasons: to preserve forage for use when fresh forage is not available or when there is a surplus, to preserve forage that cannot be grazed, to provide to the livestock a ration with a consistent composition throughout the year, etc. (Driehuis and Oude Elferink 2000; Gonzalez Pereyra *et al.* 2008; Wilkinson and Davies 2012). In short, silage is made by collecting the fresh feed commodity in a silo, followed by compaction and airtight sealing. A well-executed ensiling process, based on a spontaneous fermentation by lactic acid bacteria (LAB) under anaerobic conditions, inactivates many but not all undesirable microorganisms which had contaminated the feed commodity (Dogi *et al.* 2013; Mansfield and Kuldau 2007; Wilkinson 2005).

The ensiling process can be divided into four phases: an aerobic phase, a fermentation phase, a stable phase and a feed-out phase (Driehuis and Oude Elferink 2000; McDonald *et al.* 1991). The course of the silage-pH through the different phases of the ensiling process of wetter feedstuffs is summarized in Figure 1.1, which also mentions the key elements of successful ensiling and some influencing factors. Compared to industrial fermentations, the silage fermentation process is less manageable. Feed commodities for ensiling contain a complex mixture of epiphytic micro-organisms, and the course of the fermentation process can vary according to the circumstances (*i.e.* crop, DM content, buffer capacity, epiphytic micro-organisms, etc.) (Wilkinson 2005). However, good management practices "from field to feed" play a key role in the production of good quality silage for livestock. Bad silo management impairs a desirable silage fermentation, resulting in elevated pH-values and aerobic deterioration (Bolsen *et al.* 1996; Pahlow *et al.* 2003; Wilkinson and Davies 2012).

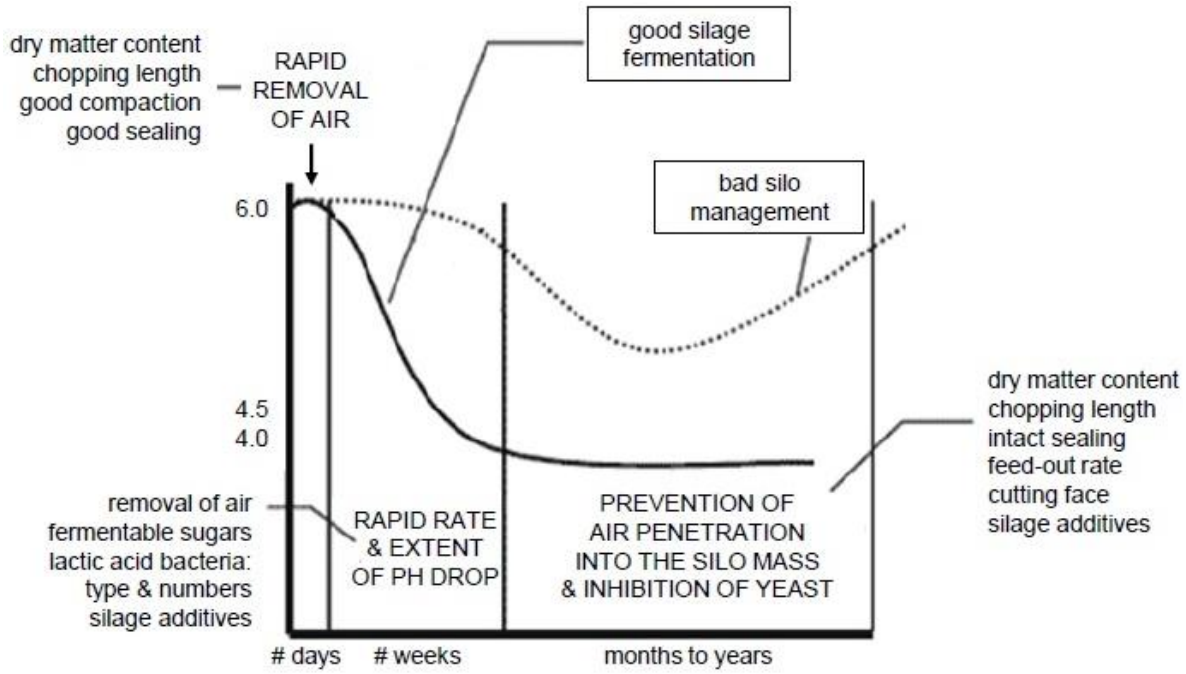


Figure 1.1. *Ensiling process: key success factors and influencing factors (adapted from Kung 1996).*

Ensiling is a complex process, requiring careful management at all stages of the process in order to obtain high-quality silage with minimal losses (Weinberg and Ashbell 2003). Several micro-organisms play a role in the ensiling process, which is a competition between aerobic and anaerobic processes. This is illustrated in Figure 1.2.

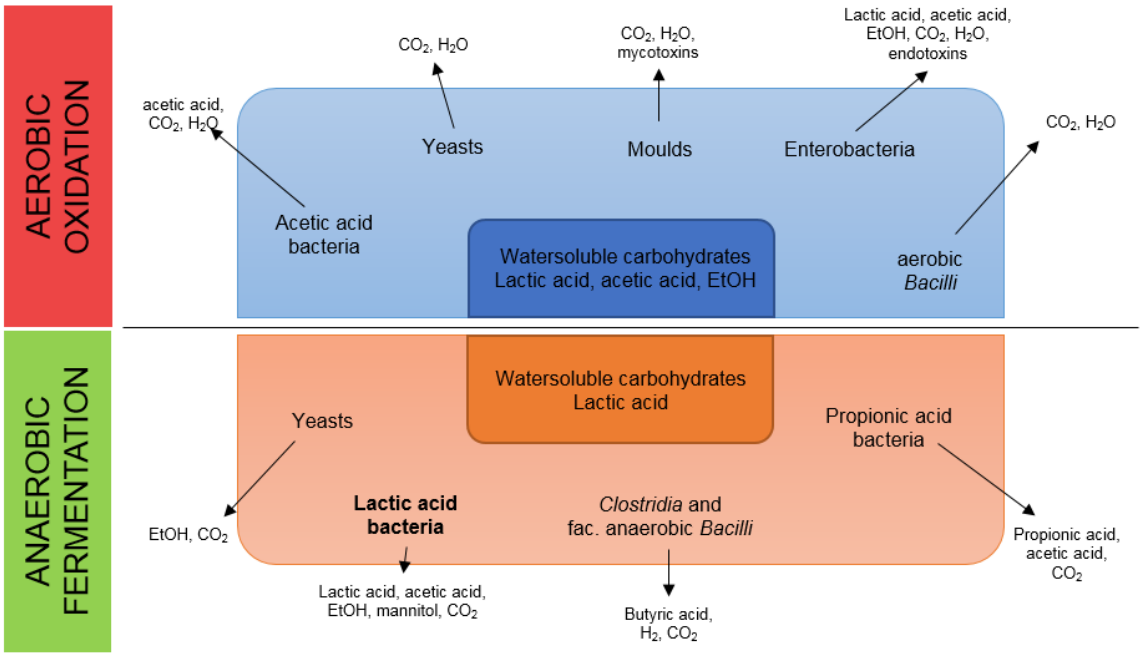


Figure 1.2. *Microbial metabolism during aerobic and anaerobic phases of the ensiling process (adapted from Merry and Davies 1999).*

In the following sections, the four distinct phases of the ensiling process - each with their own specificities - are discussed in detail, followed by the main factors influencing silage quality.

2.1. Aerobic phase

Immediately after sealing of the silo, atmospheric oxygen is still present between the silage particles. The amount of trapped oxygen is influenced by multiple factors, like the dry matter content of the ensiled feed commodity, the chopping length, the silo compaction, etc. (Kung 1996). Good silo filling techniques (*i.e.* unloading of plant material in layers, good compaction, quick and adequate sealing, etc.) can help in minimizing the amount of oxygen in silos (Oude Elferink *et al.* 2000; Weinberg and Ashbell 2003).

Ideally, the aerobic phase should be as short as possible (*i.e.* a few hours) in order to reduce the activity of plant enzymes and the growth of undesirable (facultative) aerobic micro-organisms (Bolsen *et al.* 1996; McDonald *et al.* 1991), as mentioned in Figure 1.1.
Plant carbohydrases and proteases remain active for some time after ensiling, at pH-values still around 6.0-6.5. Plant carbohydrases break down carbohydrates into simple sugars, increasing the amount of soluble carbohydrates available for fermentation. Plant proteases degrade proteins to amino acids, ammonia, peptides and amides (McDonald *et al.* 1991; Oude Elferink *et al.* 2000; Pahlow *et al.* 2003).
Residual respiration by plant cells and respiration of (facultative) aerobic micro-organisms use oxygen, resulting in the production of water and carbon dioxide gas, and a reduction of the carbohydrate content available for the LAB. Respiration processes release heat; excessive heating (> 65 °C) can result in Maillard reactions, reducing the digestibility of proteins and fibers (Bolsen *et al.* 1996; Dunière *et al.* 2013).

Enterobacteriaceae, like *Salmonella sp.* and *Escherichia coli*, are facultative anaerobic Gram-negative rod-shaped bacteria. They can oxidize sugars, forming acetic acid, formic acid, ethanol or butanediol. For anaerobic growth, they strictly depend on fermentable carbohydrates. They break down proteins into amino acids, ammonia, biogenic amines and branched fatty acids. Furthermore they can reduce nitrate to nitrite and nitric oxide, which selectively inhibit clostridial growth during the initial fermentation phase when the pH is still high enough for the germination of clostridial spores. In spite of this positive effect, *Enterobacteriaceae* are generally considered as undesirable because they compete with LAB for the available sugars and because they degrade proteins. *Enterobacteriaceae* have an optimum pH of 6-7 and usually don't grow below pH 5, so their numbers generally decline rapidly after ensiling. They are principally regarded as non-pathogenic, but their outer cell membrane contains endotoxins which can be associated with mastitis (Bolsen *et al.* 1996; McDonald *et al.* 1991; Pahlow *et al.* 2003).

Acetic acid bacteria, *Acetobacter* species in silage, are strictly aerobic Gram-negative bacteria that can produce acetic acid from sugars and ethanol, but they can also oxidize lactic acid. If their activity remains limited in time due to oxygen depletion, their effect on silage fermentation is rather beneficial in the initial phase of the ensiling process. They can however initiate aerobic spoilage during the feed-out phase (McDonald *et al.* 1991; Oude Elferink *et al.* 2000; Pahlow *et al.* 2003).

Bacilli are endospore-forming Gram-positive bacteria. The primary habitat of *Bacilli* is soil; they are scarce on fresh plant material. Aerobic as well as facultative anaerobic *Bacillus* species are known. The latter are able to ferment a wide range of carbohydrates to organic acids (lactic acid, acetic acid and butyric acid), ethanol, butanediol and glycerol, and are still able to grow at pH values of 4-4.5. Aerobic *Bacilli* usually don't grow below pH 5, so a steep pH decline eliminates these species. The proliferation of *Bacilli* should be discouraged in silages, since they are less efficient acidifiers than LAB and they can spoil milk (*B. cereus*) (McDonald *et al.* 1991; Pahlow *et al.* 2003; Smelt *et al.* 1982).

Yeasts are facultative anaerobic eukaryotes, able to oxidize sugars and organic acids. When oxygen gets depleted, they produce mainly ethanol. However, most yeasts require a certain level of oxygen to trigger alcohol fermentation: complete absence of oxygen will stop their growth. *Saccharomyces cerevisiae*, which is frequently isolated from silages, is an exception on this matter since it can produce ethanol under fully aerobic conditions and it can multiply under strictly anaerobic conditions. Other yeasts isolated from silage comprise the genera *Candida* and *Pichia* (McDonald *et al.* 1991; Pahlow *et al.* 2003). **Filamentous fungi** are strictly aerobic eukaryotes, but their development is undesirable (Bolsen *et al.* 1996; Dunière *et al.* 2013; McDonald *et al.* 1991; Oude Elferink *et al.* 2000). Some fungi can produce mycotoxins. The term "mycotoxin" originates from the Greek words "μύκης" (mykes = mould) and "τοξικόν" (toxikon = toxin). Mycotoxins are secondary metabolites produced by fungi that are toxic to other micro-organisms, plants, animals and humans. Therefore, fungi producing mycotoxins are called "toxigenic". Mycotoxins can play a role in communication as well as competition with other organisms, in fitness and in detoxification (Barug et al. 2006; Hymery et al. 2014; Pitt 2000).

2.2. Fermentation phase

Once the silage mass becomes more anaerobic, the fermentation phase starts and different groups of micro-organisms compete for the available nutrients. During the aerobic phase, the pH was around 6.0-6.5. As the oxygen level drops, lactic acid bacteria quickly become the most abundant micro-organisms, producing short-chain organic acids and thus lowering the

pH: a dynamic succession of lactic acid bacteria species, like *Enterococcus*, *Leuconostoc*, *Lactobacillus* and *Pediococcus,* forms mainly lactic acid, but also acetic acid (Dunière *et al.* 2013; Oude Elferink *et al.* 2000; Pahlow *et al.* 2003).

Two types of lactic acid bacteria are described: homofermentative lactic acid bacteria (HoLAB) and heterofermentative lactic acid bacteria (HeLAB). HoLAB produce lactic acid very efficiently from hexose sugars, whereas HeLAB produce acetic acid, ethanol and other compounds in addition to lactic acid from hexose and pentose sugars, as summarized in Table 1.1 (Dunière *et al.* 2013; McDonald *et al.* 1991; McEniry *et al.* 2008; Seale 1986).

Table 1.1. *Sugar metabolism by homofermentative lactic acid bacteria (HoLAB) and heterofermentative lactic acid bacteria (HeLAB).*

Lactic acid bacteria types	Sugar metabolism	
Homofermentative	glucose	to lactic acid*
lactic acid bacteria (HoLAB)	fructose	to lactic acid*
e.g. Lactobacillus plantarum		
Heterofermentative	glucose	to lactic acid, ethanol and CO_2
lactic acid bacteria (HeLAB)	fructose and water	to lactic acid, acetic acid, mannitol* and CO_2
e.g. Lactobacillus buchneri	pentose sugars	to lactic acid and acetic acid

* formation of two mol per mol sugar

A prompt and steep pH-decline inhibits the development of undesirable micro-organisms.

Butyric acid bacteria (BAB) convert sugars and lactic acid to butyric acid, which has an unpleasant smell and reduces palatability. BAB also break down proteins to amino acids, ammonia, biogenic amines and branched fatty acids. The main BAB found in silage are endospore-forming *Clostridium tyrobutyricum*, *C. butyricum* and *Bacillus cereus* (Dunière *et al.* 2013; Oude Elferink *et al.* 2000; Weinberg and Ashbell 2003).

Clostridia are strictly anaerobic Gram-positive rod-shaped bacteria, capable of forming endospores. Silages are usually contaminated with clostridial endospores through soil. Clostridia are sensitive to low pH and require wet conditions (DM < 30 %), so wilting of the crop and/or a quick pH-drop during fermentation can efficiently inhibit their growth (Dunière *et al.* 2013; Oude Elferink *et al.* 2000; Weinberg and Ashbell 2003). *Clostridium botulinum*, the causal agent of botulism, can gain access into silages by contaminated animal carcasses or by poultry manured crops. It can multiply in a pH range between 5.3 and 6.5, while the produced toxins are stable in a pH between 3.5-6.8 (Notermans *et al.* 1979; Pahlow *et al.* 2003).

The effects of *Bacilli* proliferation in silage have been discussed earlier in this section.

Listeria species are facultative anaerobic Gram-positive bacteria that are frequently found in surface layers of silage, with *L. monocytogenes* being pathogenic to animals and humans.

The degree of anaerobiosis and the pH are important factors determining the survival and growth of *Listeria* in silages: pH-values below 4.5 reduce the risk, but are not sufficiently low when oxygen is present (Bolsen *et al.* 1996; McDonald *et al.* 1991; Pahlow *et al.* 2003; Vilar *et al.* 2007).

Propionic acid bacteria are anaerobic, slow growing Gram-positive bacteria. They can ferment a wide range of sugars and lactic acid into mainly propionic acid, but also acetic acid and formic acid. Their growth is significantly reduced at pH-values below 4.5 (Higginbotham *et al.* 1998; Lind *et al.* 2005; McDonald *et al.* 1991; Pahlow *et al.* 2003). Their possible use as silage inoculant is discussed further in this section.

2.3. Stable phase

After a few weeks to months, the pH of the silage stabilizes. The end-point of the silage fermentation is reached when the supply of available substrates has been exhausted, when the pH of the crop mass has decreased to the point at which microbial growth is inhibited (around 3.8-4.0), or when the a_w-value reaches a minimum level for bacterial growth (Bolsen *et al.* 1996; Wilkinson and Davies 2012).

If anaerobic conditions are maintained, this stable phase with very little biological activity can last for several months or even more than a year. Numbers of LAB and other viable microorganisms decline, but the specialized HeLAB species *L. buchneri* can remain active during the fermentation phase as well as the stable phase: it can degrade lactic acid to acetic acid, 1,2-propanediol and traces of ethanol under anaerobic conditions. Acid-tolerant yeast species survive the stable phase in an almost inactive state, along with endospores of *Bacilli* and *Clostridia*. Acid-tolerant enzymes also remain active, causing a slow acid hydrolysis of structural and storage carbohydrates (Driehuis *et al.* 1999; Oude Elferink *et al.* 2000; Pahlow *et al.* 2003; Weinberg and Ashbell 2003).

2.4. Feed-out phase

Once the silo is opened for feed-out, the last phase starts: air can penetrate unrestrictedly into the silage mass via the cutting face. Inherently, yeasts and acetic acid bacteria start to grow rapidly, oxidizing the preservative acids and residual water-soluble carbohydrates. This causes aerobic deterioration: on the one hand, this causes a rise of the temperature; on the other hand lactic acid breakdown causes an increase of the pH. In combination with the release of water during aerobic deterioration, increasing the a_w of the silage, this creates a less inhibiting environment for microbial activity. So, *Bacilli* proliferation as well as fungal development can

be observed. Fungal growth results in a visible mycelial mass at the relatively low concentrations of lactic acid and the relatively high pH-values of deteriorated silage. If the silo coverage does not remain intact during the stable phase, aerobic deterioration can occur earlier than at feed-out. Eventually, all silage exposed to air deteriorates as a result of aerobic microbial activity (Bolsen *et al.* 1996; Borreani and Tabacco 2010; Johnson *et al.* 2002; Lindgren *et al.* 1985; McDonald *et al.* 1991; Pahlow *et al.* 2003).

A multitude of factors have an influence on the losses associated with aerobic deterioration of silage, as summarized in Figure 1.3. Many of these factors are discussed in section 1.5.

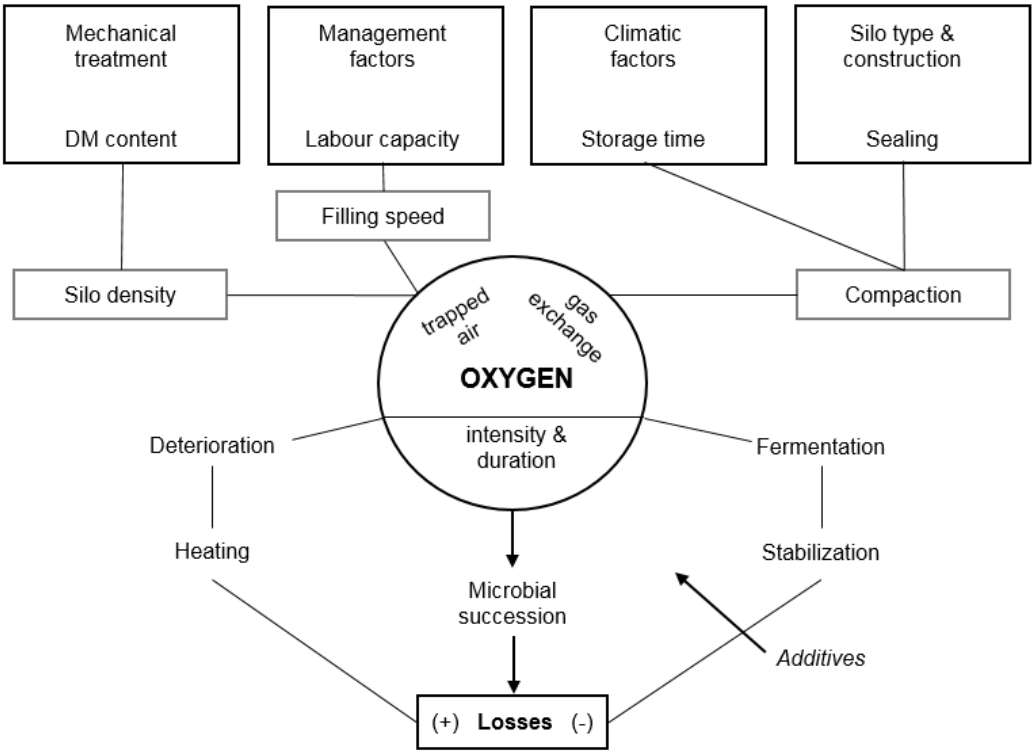


Figure 1.3. *Factors influencing the losses associated with aerobic deterioration (Pahlow and Muck 2009).*

Generally, well-preserved silage with a feed-out rate well adapted to the feed demand and the silo width should remain aerobically “stable” during a few days, so the silage can be fed to the livestock prior to aerobic deterioration and subsequent fungal development. The “aerobic stability” of silage is defined as the time required for heating to occur in silage exposed to air (e.g. 2 or 3 °C temperature rise above the surrounding temperature) (Honig 1991; Ranjit and Kung 2000; Spoelstra *et al.* 1988; Storm *et al.* 2010; Wilkinson and Davies 2012; Woolford 1990).

2.5. Factors influencing the ensiling process

Multiple factors have an influence on the ensiling process to a smaller or greater extent: plant species and stage of maturity, dry matter content, sugar content and buffer capacity of the crop, chopping length, epiphytic microflora, silo type, silage additives, temperature, etc.
Some of these factors can be controlled and/or managed by the farmer (e.g. chopping length and use of silage additives), others are largely beyond the farmer's control (e.g. temperature) (Bolsen *et al.* 1996; Muck 2013; Weinberg and Ashbell 2003). In the following paragraphs, a selection of influencing factors will be discussed more in-depth.

2.5.1. Epiphytic microflora

The species composition and quantity of the micro-organisms present on crops just prior to ensiling is variable and is affected by multiple factors, like forage species, stage of maturity, fertilization, weather conditions, mowing, wilting and chopping. Many species belong to the obligate aerobic bacteria and do not contribute to silage preservation.
The epiphytic LAB are the essential microflora for spontaneous silage fermentation, but their numbers are highly variable. A frequently observed phenomenon is "chopper inoculation": LAB numbers often increase dramatically after chopping of the plant material. Many LAB on plant surfaces are in a dormant state due to adverse environmental conditions and are viable but non-culturable. Chopping releases plant cell contents resuscitating the non-culturable LAB. The second most numeral group of epiphytic bacteria are the *Enterobacteriaceae*, competing with the LAB flora prior as well as after ensiling. They are well able to survive unfavorable weather conditions (Bolsen *et al.* 1996; Dunière *et al.* 2013; McDonald *et al.* 1991; Pahlow *et al.* 2003; Rammer *et al.* 1994).

2.5.2. Silo type

The most prevalent silo types include clamp silos, bunker silos, silobags and wrapped bales. Tower silos are a silo type well known by many people, but these are generally used for storage of dry cereal grains and not for ensiling of wet materials. In Belgium, bunker silos are the most frequently used silo type.

Clamp silos comprise a heap of crop, compacted on bare ground or on a concrete floor, sealed with plastic sheeting. On the contrary, bunker silos have concrete walls and floor of various widths and lengths.

Compaction of bunker silos and clamp silos is accomplished by tractors, rolling slowly back and forth on thin forage layers. The silo walls of bunker silos allow a higher compaction rate of the silage due to hollow filling compared to clamp silos. The seams between the concrete elements should be tightly sealed to prevent air penetration. The top layer and the lateral silo parts are the most susceptible to air penetration and aerobic deterioration. Deep silos have relatively less surface spoilage, while narrow silos are less sensitive to cutting face spoilage. The sealing of clamp silos and bunker silos has a great influence on the ensiling process and should be handled with care. Sealing is usually done by a double layer of plastic UV-resistant polyethylene sheeting, which is anchored to the silage with used tires, soil, straps, etc. (Buckmaster *et al.* 1989; Weinberg and Ashbell 2003; Wilkinson 2005).
Silobags are circular plastic bags, coming in a variety of diameters and lengths, but are not used as frequently as the other silo types since they need to be filled by a portable bagging machine. Wrapped bales are made by wrapping individual round or rectangular bales with 4-8 layers of pre-stretched polyethylene film and are generally used for wilted crops. Because of their high ratio of surface over volume, narrow silobags and baled silage are especially prone to aerobic deterioration (Forristal *et al.* 1999; Weinberg and Ashbell 2003; Wilkinson 2005).

No comparative studies on silage quality upon ensiling of the same feed commodity in different on-farm silo types were found. A few studies comparing on-farm silages with laboratory silos have been conducted, as will be discussed in chapter 6.

2.5.3. Dry matter content

Crops for ensiling should be harvested at the proper stage of maturity to maximize the nutritional value of the silage, but the dry matter (DM) content at ensiling is also very important. The DM content is a determinant factor for the pH at which LAB activity ceases. Silages with a high dry matter content are particularly sensitive to aerobic deterioration due to lower amounts of volatile fatty acids (e.g. acetic acid and propionic acid) produced during the fermentation phase, reduced silo density and increased porosity available to gas exchange at increasing DM content. Too low dry matter content at ensiling is not favorable either because this can allow *Clostridia* development, resulting in butyric acid production and compromising a good fermentation process. A dry matter content below 300 g per kg fresh matter (FM) can result in effluent production. The amount of effluent produced depends on the DM content, but also on the silo type, the chopping length, etc. Wilting in the field is thus strongly advisable for certain feed commodities (*i.e.* grasses and legumes) prior to ensiling. To enhance the moisture losses during wilting, they are preferentially harvested with a mower-conditioner that bruises their cuticule. Ensiling forage crops at optimal dry matter content (for whole-crop maize: 300-

350 g/kg FM, for grasses and legumes: 300-450 g/kg FM) is thus a very important determining factor for the outcome of the ensiling process (Bolsen *et al.* 1996; Muck 1988; Weinberg and Ashbell 2003).

2.5.4. Buffer capacity

The buffer capacity of a crop reflects its resistance to acidification during ensiling: at increasing buffer capacity, more acid production is required for a certain pH reduction (Giger-Reverdin *et al.* 2002; Smith 1962).

Buffering solutions contain weak acids and their salts, which only rarely react with water. The buffer capacity of a forage crop is influenced by the forage species and its composition, *i.e.* mainly the anion fraction (75-80 %). Buffering caused by plant proteins determines the total buffer capacity of crops only to a minor extent (10-20 %). Fresh forages contain malic acid, citric acid and glycerate as main buffers, while silages contain mainly lactic acid and acetic acid (Playne and McDonald 1966; Smith 1962). *Enterobacteriaceae* can breakdown proteins to ammonia and can also reduce nitrate, both increasing the buffering capacity of the ensiled crop and counteracting the intended rapid pH lowering in silage (Pahlow *et al.* 2003). Wilting of grasses in the field prior to ensiling reduces their buffer capacity, correlated with an increased osmotic pressure. Legumes are often ensiled at a relatively highly wilted stage in order to overcome problems associated with their high buffer capacity and the risk of butyric acid fermentation when ensiled at low dry matter content (Dinic *et al.* 2010; Driehuis and Oude Elferink 2000; Khan *et al.* 2015a; Wilkinson 2005).

2.5.5. Sugar content

Sugars are the substrate used by LAB for the spontaneous acidification of ensiled feed commodities. A so-called “complete” fermentation is stopped by pH-inhibition of bacterial growth and not by lack of substrate. The amount of sugars necessary to allow complete LAB fermentation depends on the buffer capacity and the dry matter content of the ensiled crop: the amount of substrate necessary for complete fermentation increases with the buffer capacity, while it is negatively correlated with the DM content of the crop. Additionally, the epiphytic microflora competing with the LAB for the available sugars can negatively influence silage fermentation (Muck 1988; Pahlow *et al.* 2003).

When no sugars or sugar releasing enzymes are used as silage additives, the sugar content of the ensiled feed commodity is somewhat out of the farmer’s hands. Whole-crop maize usually contains enough sugars to allow complete fermentation, while the sugar content of

other crops varies according to the weather conditions (e.g. a high sugar content can be expected when grass is mown in the later afternoon at sunny weather conditions with cold night temperatures) and the plant species (Wilkinson 2005; Winters *et al.* 2001).

2.5.6. Silage additives

Silages additives can serve various purposes and can be split-up in five categories: fermentation stimulating additives, fermentation inhibiting additives, aerobic deterioration inhibiting additives, nutrients or adsorbents, and combination products (McDonald *et al.* 1991; Oude Elferink *et al.* 2000; Weinberg and Ashbell 2003). A summary is presented in Table 1.2.

Table 1.2. *Categories of silage additives: when to use, and different options per category (McDonald et al. 1991; Oude Elferink et al. 2000).*

Category	When to use	Possible silage additives
Fermentation stimulating additives	* insufficient numbers of suitable LAB	Homofermentative lactic acid bacteria (HoLAB) → lactic acid
	* insufficient amounts of WSC according to the dry matter content and the buffer capacity	Enzymes releasing WSC (*e.g.* amylase, cellulase, glucanase)
		Direct addition of WSC (*e.g.* molasses)
Fermentation inhibiting additives	* to inhibit clostridial fermentation, particularly in low DM silage	Acids and their salts (*e.g*. propionic acid, formic acid)
		Nitrite
Aerobic deterioration inhibiting additives	* silages prone to aerobic spoilage, due to high DM content, low feeding rate, *etc.*	Acid-based chemical additives - acetic acid, propionic acid,benzoic acid, *etc.* and their salts
		- sulphuric acid, phosphoric acid, *etc.* and their salts
		Heterofermentative lactic acid bacteria (HeLAB) → acetic acid
		Propionic acid bacteria → acetic acid and propionic acid
		Antagonistic lactic acid bacteria, yeasts or *Bacilli*
		Plant extracts and essential oils
Nutrients and adsorbants	* to increase the crude protein content	Urea, ammonia
	* to supply minerals	Limestone, magnesium sulphate
	* to absorb effluent in low DM silage	Dried pulp of sugarbeet or citrus, straw
	* to reduce mycotoxin content	Mycotoxin adsorbing agents or degrading micro-organisms
Combination products, combining additives from the former categories		

In the context of this dissertation, only the aerobic deterioration inhibiting additives will be discussed (in Chapter 2, section 2.3.2), since they should help in preventing the development of toxigenic fungi in silages.

2.5.7. Temperature

Temperature influences silage fermentation: a moderate temperature (*i.e.* 20-30 °C) facilitates good silage fermentation, whereas high temperatures (> 37 °C) have a detrimental effect on silage quality. The effect of temperatures below 20 °C has not been extensively studied (Garcia *et al.* 1989; Kim and Adesogan 2006; McDonald *et al.* 1966; Weinberg *et al.* 2001).
High ensiling temperatures reduce the numbers of certain LAB, enhance proteolysis and make the fermentation less homolactic. A shift is observed from lactic acid fermentation to clostridial fermentation (Kim and Adesogan 2006, McDonald et al 1966, Muck and Dickerson 1988, Weinberg et al 2001).

Zhou *et al.* (2016) recently demonstrated that low temperatures also have a negative effect on silage fermentation. They have determined the epiphytic LAB population and fermentation characteristics of whole-crop maize ensiled in vacuum bags, which were incubated at 5, 10, 15, 20 and 25 °C during 0, 1, 2, 3, 7, 28 or 60 days. Silages fermented at 5 and 10 °C showed a higher pH and contained less lactic acid, less acetic acid, less ammonia and more residual water-soluble carbohydrates than those incubated at higher temperatures during 60 days, implying that low temperature inhibits bacterial metabolism. At 20 and 25 °C, the forage acidification started rapidly (*i.e.* no lag times were observed), whereas temperatures of 15, 10 and 5 °C delayed forage acidification by respectively 2, 3 and 7 days. However, all silage samples reached a sufficiently low stable pH. The LAB population varied significantly over the incubation temperatures as well as over the incubation periods. After an ensiled period of 60 days, the yeast numbers had dropped from 4.19 log colony forming units (cfu) per gram fresh forage to numbers below the limit of detection in case of incubation at 20 and 25 °C, while a similar number of yeasts persisted after incubation at 5, 10 and 15 °C. These lower ambient temperature could indirectly favor yeast survival by allowing a slower metabolism. Moreover, the membrane permeability to organic acids is reduced at lower temperatures, due to altered fatty acid composition of the phospholipid bilayer (Beales 2003).

2.5.8. Silo management

Of all influencing factors on the ensiling process, silo management is inherently the most farmer controlled one but also the most important one. During all the phases of the ensiling process, management decisions influence the outcome of the silage making process: the farmer decides on when to harvest, how long to wilt, which chopping length will be adopted, how the silo is filled, compacted and sealed, etc. in order to ensure a good start of the ensiling process. During the fermentation phase and stable phase, the farmer is responsible for maintaining anaerobic conditions by regularly checking the silo sealing. During the feed-out

phase, oxygen inevitably regains access to the silage and all the earlier efforts might be wasted due to aerobic spoilage if the feed-out management is not adequate.

During the feed-out period, the silage at the cutting face is prone to aerobic deterioration to a greater or lesser extent, depending on the duration of air exposure, the ambient temperature and the aerobic stability of the silage. The aerobic stability of silage depends e.g. on the numbers of aerobic micro-organisms present in the silage, on the time exposed to oxygen prior to unloading, on the silage fermentation characteristics and on the ambient temperature. So, sealing, compaction, silo density and feeding rate all have an influence on the aerobic stability of silage. Evidently, aerobic deterioration inhibiting silage additives should improve the aerobic stability of silages.

In order to maximize the feeding value of the silage, it is necessary to minimize aerobic deterioration by minimizing the ingress of oxygen. Therefore, it is advised to remove silage from the cutting face faster than oxygen can penetrate. The duration of air exposure of the silage at the cutting face is determined by the silo density and by the way this density is maintained during unloading on the one hand, and by the feed-out rate on the other hand, all depending hugely on farmers' management decisions throughout all phases of the ensiling process (Bernardes *et al.* 2012; Bolsen *et al.* 1996; Danner *et al.* 2003; Dolci *et al.* 2011; Johnson *et al.* 2002; McGechan and Williams 1994; Mohd-Setapar *et al.* 2012; Pahlow and Muck 2009; Parsons 1991; Weinberg and Ashbell 2003).

3. Problem statement by sampling mouldy on-farm silages

In Belgium, on-farm silages of whole-crop maize, grasses, legumes, sugar beet press pulp, etc. often harbor fungal hot-spots to a greater or to a lesser extent. It is essential to know which fungal species are the most frequent contaminants of silages, to be able to assess the risk of mycotoxin production by toxigenic fungi in silages. The results of sampling of mouldy on-farm silages are presented in this introductory chapter, illustrating the problem of fungal infestation in silages and its consequences on silage fermentation quality.

Sampling of silages with mouldy hot-spots on Belgian farms has been executed in the period 2006-2009 in the scope of two research projects:

- the practice-orientated research project “Identification and control of fungal development in conserved roughages” (financed by the University College Ghent, 2006-2009)
- the project “Characterization of fungal species and mycotoxins contaminating silage in Belgium” (financed by the Belgian federal science policy office (BELSPO), 2006-2008) aimed to make an inventory of the fungal species contaminating silages in Belgium.

In 2006 and 2007, joint expeditions in the scope of both research projects were undertaken to farms facing problems with fungal development in their on-farm silages. Additional samplings were performed in 2008 and 2009.

Farmers faced with mould contaminated silages were visited to collect samples. From whole-crop maize and grass silages with visible fungal hot-spots, three types of samples were taken:

1) a “healthy”, visually non-mouldy reference sample from the center of the silo
2) a sample from the top layer (which could be either healthy, either mouldy)
3) one or more samples from mouldy hot-spots

The silage samples were collected in labeled plastic bags. Air was removed as much as possible and the samples were transported in cool boxes.

On the visited farms, a detailed questionnaire was completed about numerous aspects associated with silage making. However, this did not reveal any significant correlation with the fungal species composition isolated from the silages (Tangni *et al.* 2017).

Occasionally, other silages than whole-crop maize and grass were sampled, but these are not considered here. Baled grass silage is also left out of the scope of these results.

3.1. Materials and methods

Upon arrival in the laboratory, different subsamples were taken for:

- direct plating to isolate the epiphytic fungi

  The visually non-mouldy silage samples taken from the center were not subjected to fungal isolation and identification. Top layer samples and mouldy hot-spot samples were stored at 4 °C for max. 48 hours. Subsequently silage particles were directly plated on Potato Dextrose Agar (PDA). Three standard 90-mm diameter Petri dishes per medium were used per sample. All plates were incubated aerobically in the dark at 20 °C for 2-7 days followed by 2-7 days of incubation at 25 °C, to avoid overgrowing of certain fungi by faster growing species.

  The different fungal isolates were separately subcultured in 45-mm diameter Petri dishes containing PDA. *Penicillium* isolates were also subcultured on PDA supplemented with 5 ml of acetic acid per liter medium (PDAA) to facilitate the isolation of *P. roqueforti s.l.* (Engel and Teuber 1978; O'Brien *et al.* 2008). Plates were aerobically incubated in the dark at 25 °C, followed by identification to genus or species level based on micro- and macromorphological features (Frisvad and Samson 2004; Samson *et al.* 2002; Samson and Frisvad 2004; Tangni *et al.* 2017). *Zygomycetes* were left out of the scope of the fungal identifications since they are very widespread and contamination of silage by airborne *Zygomycetes* cannot be excluded. Many but not all terverticillate *Penicillium* isolates' identity was checked to species level by beta-tubulin sequencing at the Université Catholique de Louvain-la-Neuve (Declerck *et al.* 2009; Tangni *et al.* 2017).

- analysis of fermentation characteristics

  From all three sample types, subsamples were stored at -20 °C prior to determination of the dry matter (DM) content and some fermentation characteristics (as described in Annex 1): ammonia and the ratio of ammonia nitrogen to total nitrogen, pH, lactic acid, acetic acid and butyric acid.

- mycotoxin analysis

  In the context of the BELSPO project (Declerck *et al.* 2009), the mycotoxin load of healthy references samples and mouldy samples was compared for a selected number of whole-crop maize and grass silages.

In this chapter, the results of the fungal identifications and of the fermentation characteristics determination are presented. The results of the mycotoxin analysis have been published by Tangni *et al.* (2013a) and are included in chapter 2 (Table 2.3).

3.2. Results and discussion

3.2.1. Identification of fungal species contaminating silages

The results of the identification of the fungi isolated from the silage samples analyzed in the context of both research projects are summarized in Figure 1.4. The results obtained in the context of solely the BELSPO-project have been published recently (Tangni *et al.* 2017, with Wambacq Eva as second author) and are highly similar to those mentioned here. In Figure 1.4, it is mentioned per fungal genus or species in how many percent of the sampled whole-crop maize and grass silages it was detected. *Penicillia* are indicated by a dot-pattern.

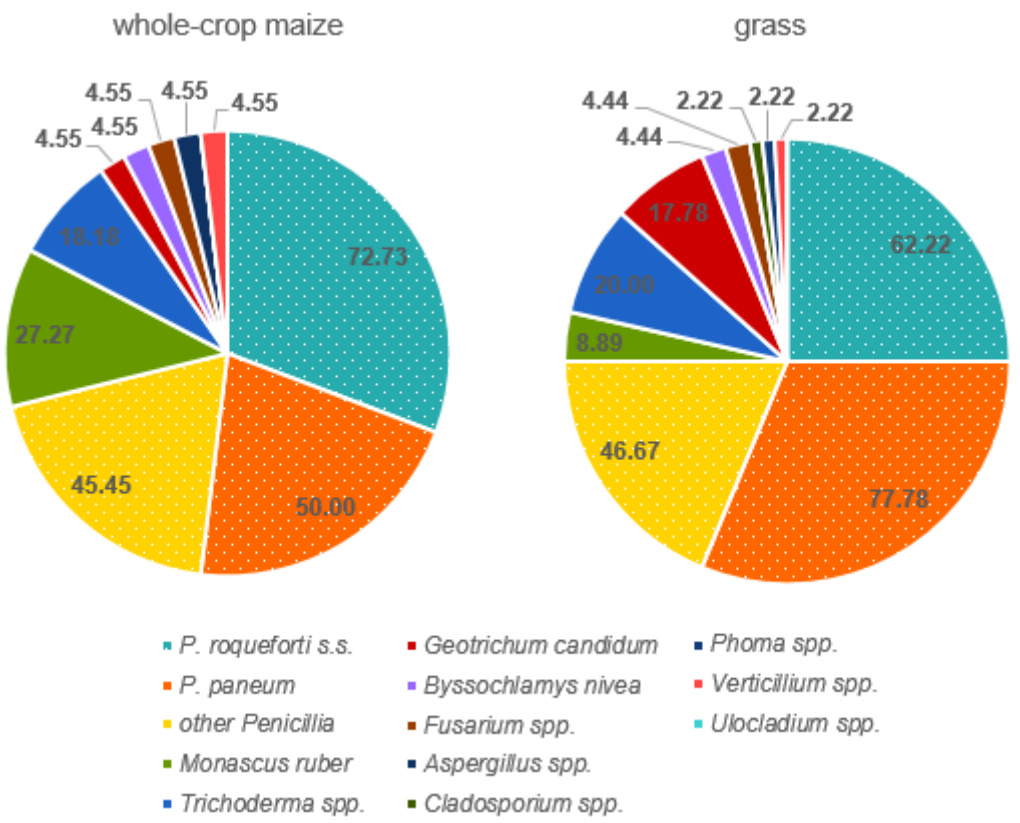


Figure 1.4. *Whole-crop maize and grass silages: percentage of isolation of fungal genera/species from silage samples collected in the period 2006-2009.*

In whole-crop maize silages, *P. roqueforti s.s.* was the predominant fungal species, followed by *P. paneum*. In grass silages, *P. paneum* was the most frequently isolated, followed by *P. roqueforti s.s.*. *P. roqueforti s.s.* and *P. paneum*, both members of the *P. roqueforti* series, were detected simultaneously in respectively 44 and 41 % of the sampled whole-crop maize and grass silos. In both silage types, other *Penicillium* species were isolated from about 45 % of the sampled silos. *P. crustosum* and *P. minioluteum* were isolated regularly, while *P. citreonigrum*, *P. expansum* and *P. palitans* were detected sporadically. *P. carneum*, also a member of the *P. roqueforti* series, was not found in any of the samples. These data confirm

the findings of numerous authors (Auerbach *et al.* 1998; Boysen *et al.* 2000; Garon *et al.* 2006; Mansfield and Kuldau 2007; Nout *et al.* 1993; O'Brien *et al.* 2007; Richard 2007).
Among other fungal genera than *Penicillium*, grass silages often contained *Monascus ruber* and *Trichoderma* species, while in whole-crop maize silages *M. ruber* and *Geotrichum candidum* were frequent contaminants. The other fungal genera/species mentioned were only occasionally isolated.

It should be noted that Figure 1.4 reflects only the presence of a fungal species or genus in a silage sample, but does not give an idea of the amount of fungal spores per species/genus, nor of the severity of the contamination by the different fungal species/genera (*i.e.* it is unclear if the silage contained dormant spores or actively growing mycelium).

3.2.2. Fermentation characteristics per sample type

Since the condition of the top layer varied between visually non-mouldy and mouldy, the mouldy top layer samples were considered as mouldy samples, while the non-mouldy top layer samples were considered separately.
The fermentation characteristics of the whole-crop maize silage samples are summarized in Table 1.3.

Table 1.3. *Fermentation characteristics of the three sample types for whole-crop maize silages.* Per parameter, the mean values per sample type are mentioned with their resp. standard deviation between brackets. Significant differences between the three samples types are indicated by lettercode, with horizontal interpretation.

WHOLE-CROP MAIZE SILAGES (140 samples)	**non-mouldy reference**		**N**	**non-mouldy top layer**		**N**	**mouldy hot-spots**		**N**
dry matter (DM) (g/kg fresh matter)	341 (30)	a	45	316 (30)	b	28	303 (73)	b	64
ammonia (g/kg DM)	0.86 (0.19)	b	44	0.56 (0.19)	c	28	1.09 (0.67)	a	63
ammonia-nitrogen / total nitrogen	5.80 (1.38)	a	32	3.38 (1.07)	b	18	5.90 (3.32)	a	63
pH	3.66 (0.12)	c	46	3.78 (0.20)	b	28	5.22 (0.98)	a	66
lactic acid (g/kg DM)	70.73 (21.10)	a	44	55.23 (26.88)	b	28	21.14 (14.53)	c	64
acetic acid (g/kg DM)	12.85 (4.62)	a	39	15.76 (9.58)	a	28	4.67 (3.26)	b	57
butyric acid (g/kg DM)	0.00 (0.00)	a	39	0.00 (0.00)	a	23	0.00 (0.00)	a	47

The "healthy" non-mouldy reference samples differed significantly from the top layer samples and the mouldy samples for most parameters. The non-mouldy reference samples were characterized by a significantly higher dry matter content compared to both other sample types. On the one hand, this can be explained by the inherently higher level of aerobic deterioration in the top layer since completely airtight silo coverage is utopic, and on the other hand fungi metabolize nutrients during their development.
The ammonia content and the ammonia fraction (*i.e.* the ratio of ammonia nitrogen over total nitrogen) was significantly lower in the non-mouldy top layer samples compared to the healthy

reference samples and the mouldy samples. Ammonia formation is due to protein degradation, attributable to plant enzymes, enterobacteria, facultative anaerobic *Bacilli* or *Clostridia* (as mentioned earlier in this chapter). In the top layer, no completely anaerobic conditions occur, which can explain the lower ammonia levels observed. However, in absolute value, average ammonia content as well as ammonia fraction was relatively low in all three sample types (Driehuis *et al.* 2001; Flieg 1938; Kung and Shaver 2001; McDonald *et al.* 1991).

As expected, the significantly lowest pH-values were detected in the non-mouldy reference samples. Micro-aerophilic conditions in the top layer result in a subtle but significant increase in pH, while mouldy hot-spots are characterized by dramatically increased pH-values. An increase in pH results from a decrease in acid amounts, which is confirmed nicely by the mean levels of lactic acid (which is the main conserving acid in silages) in the different sample types. Due to the logarithmic nature of the pH-scale, the mean pH-values have small standard deviations, while the standard deviation on the mean lactic acid and acetic acid content per sample type is high. There was no significant difference in acetic acid content between the non-mouldy reference samples and the top layer samples, while a significant decrease is observed in mouldy samples.

Table 1.4 presents the results of the fermentation characteristics obtained from the sampling of grass silages.

Table 1.4. *Fermentation characteristics of the three different sample types for grass silages.* Per parameter, the mean values per sample type are mentioned with their resp. standard deviation between brackets. Significant differences between the three samples types are indicated by lettercode, with horizontal interpretation.

GRASS SILAGES (52 samples)	**non-mouldy reference**			**non-mouldy top layer**			**mouldy hot-spots**			
dry matter (DM) (g/kg fresh matter)	390 (129)	a	18	400 (101)	a	10	347 (112)	a	22	
ammonia (g/kg DM)	1.97 (1.06)	a	18	1.73 (1.05)	a	10	2.00 (1.29)	a	23	
ammonia-nitrogen / total nitrogen	6.37 (2.83)	a	15	5.05 (3.14)	a	8	8.06 (5.25)	a	24	°
pH	4.41 (0.41)	b	18	4.64 (0.31)	b	10	5.87 (0.92)	a	24	
lactic acid (g/kg DM)	81.49 (59.51)	a	18	68.61 (49.47)	a	10	14.45 (18.61)	a	24	°
acetic acid (g/kg DM)	17.89 (13.93)	a	18	8.18 (4.70)	a	9	8.50 (9.84)	a	23	°
butyric acid (g/kg DM)	1.48 (1.52)	a	15	1.52 (1.89)	a	9	1.42 (1.88)	a	21	°

° Non-parametric test according to Kruskal-Wallis

Unlike for the whole-crop maize silages where most parameters differed significantly between the three sample types, only the pH differed significantly between the three sample types of grass silages: despite the broad range of the dry matter content of the non-mouldy reference samples, the pH-values remained in a relatively narrow range (3.64 - 5.05). The pH of the non-mouldy reference samples and top layer samples differed not significantly, while the mouldy samples had a significantly higher pH. Due to high standard deviations, this was not reflected in significant differences in lactic acid and acetic levels in the three sample types.

Butyric acid was not found in any of the whole-crop maize samples, but was detected in grass silage from all sample types in low amounts.

Making a comparison of the fermentation parameters between whole-crop maize silages and grass silages would be like comparing apples and oranges, so this suggestion has been definitely refrained from. However, one striking observation that can be made from comparing both tables is that the standard deviations of the mean values of nearly all parameters were higher for the grass silage samples than for the whole-crop maize silage samples. This is not surprising at all since whole-crop maize has a narrow window of harvest and is relatively alike among the different farms, while grass can be harvested from spring to fall and a multitude of factors is variable in the grass silage making: grass species and mixtures (e.g. different grasses and/or white clover in meadows), stage of maturity, number of cuts, wilting period and dry matter content, sugar and protein content, etc.

Baled grass silage samples were not considered in the earlier mentioned results, but O'Brien *et al.* (2007) have performed an extensive study on baled grass silage in Ireland in 2003-2004. They visited fifty farms and sampled two bales per farm. They observed fungal infestation on ninety of the one hundred sampled bales, often correlated with visible damage to the stretch film. They took samples for chemical analysis of visually non-mouldy silage from the outer layer, but did not chemically analyze the mouldy hot-spots. Therefore, their results cannot be compared with the results mentioned in Table 1.4. However, they could not find a significant correlation between the mouldy surface area of the bales and any of the fermentation characteristics, indicating that the fungi present (*i.e.* mainly *P. roqueforti s.s*. and *Schizophyllum commune*, but also *P. paneum, Geotrichum candidum, Trichoderma spp.* and *Fusarium spp.*) were tolerant to a wide range of conditions and therefore likely to be capable of colonizing any part of a bale upon oxygen ingress.

3.3. Conclusions of on-farm samplings

As for the fungi isolated from mouldy on-farm silages, it has been clearly demonstrated that *P. roqueforti s.s.* and *P. paneum* are the predominant fungi contaminating silages in Belgium. This finding was the trigger for this particular PhD research topic. In Chapter 2, it will be illustrated that these two fungal species, together referred to as *P. roqueforti s.l.* throughout this dissertation, are very well adapted to silage conditions and represent the main fungal silage contaminants in temperate climate regions.

The effect of fungal growth on silage fermentation characteristics has been assessed: the most striking observation, which was confirmed statistically for both whole-crop maize and grass silages, is that fungal infestation of silages is characterized by an increased pH in fungal hot-spots compared to non-mouldy reference samples. This increase results from a decrease in lactic acid and acetic acid levels. To point out the cause of this reduction of fermentation acids, a chicken-or-egg dilemma approach might be called upon:
On the one hand, it can be assumed that silage fermentation was well in the mouldy hot-spots prior to fungal infestation, which is correlated with a breakdown of lactic acid or acetic acid. However, this breakdown cannot be undoubtedly attributed to fungal growth: fungal spores like *Penicillium roqueforti s.s.* or *P. paneum* might have germinated at low pH-levels similar to those observed in non-mouldy reference samples upon contact with oxygen; another option is that yeasts and/or acetic acid bacteria have initiated aerobic deterioration, breaking down lactic acid and acetic acid and causing the pH to rise, facilitating fungal growth.
On the other hand, it might also be possible that in some specific locations within a silo, e.g. at air pockets, LAB could not perform a proper silage fermentation, locally resulting in silage with elevated pH-values due to low amounts of lactic acid and/or acetic acid.

The first hypothesis seems to be the most realistic (Borreani and Tabacco 2010), but the second hypothesis cannot be excluded and could hold truth specifically in poorly sealed silages. To check which hypothesis is the closest to the truth, *in vitro* as well as *in vivo* experiments have been executed on the direct and indirect effects of lactic acid and acetic acid on *P. roqueforti s.l.*, as will be described in chapter 4 and chapter 5 of this dissertation.

4. Farm surveys on whole-crop maize and grass silage making

Knowing which fungal species frequently contaminate silages is one thing, but determining the cause of fungal growth in silages is another. As a kick-off to this challenging question, surveys were performed among Flemish farmers in which they were questioned about whole-crop maize and grass silage making in a “from field to feed” approach.

Different stages were prospected:

- in the field: e.g. crop rotation, fungal infection, soil cultivation and manure application;
- harvest and ensiling: plant maturity and condition, weather conditions, chopping length, application of silage additives, silo type and dimensions, filling rate and mode of compaction and silo coverage, combination of different crops or grass cuts.
- desiling and feed-out: fermentation period, feed-out rate, fungal contamination in the past and in the present, removal of fungal hot-spots prior to feeding and animal health problems.

During the feed-out period of the silos concerned in the surveys, the farmers were contacted to find out if fungal contamination had occurred. Aim was to find a reasonable explanation for fungal infestation of silage. Therefore, only the answers relevant to solving this question will be discussed hereafter.

Field conditions differed only slightly, so these won’t be discussed.

As for harvest, it was striking that for both crops only a minority of the farmers applied a silage additive: 16 % applied an additive (*i.e.* sodium chloride) on whole-crop maize and 23 % used an additive on grass (one half used sodium chloride, one half applied a LAB inoculant).

For both maize and grass, two farmers out of three used a bunker silo while one out of three made a clamp silo.

Time between final filling and covering of the silos varied enormously for both crops, ranging from half an hour to 24 hours, with an average of 2.5 hours.

About 70 % of the questioned farmers cover their silos with two layers of plastic; 20 % used three layers for covering their maize silo compared to only 5 % for grass silos.

Silo height was highly variable for both crops, but of course this parameter should be evaluated relative to the feed-out rate. Both parameters are summarized in Table 1.5, along with data on the filling rate, the fermentation period and the percentage of farmers combining different crops or grass cuts into one silo. Non-optimal conditions for these parameters pose a certain risk of reduced silage quality, as mentioned earlier in this chapter and also in Table 1.5. However, most parameters were characterized by large differences between farms.

Table 1.5. *Results of farm surveys on whole-crop maize and grass silage making, including potential risk factors.*

Parameters	whole-crop maize (N=46)	grass (N=30)	Potential risk
filling rate	0.85 - 7 ha/hour median: 1.25 ha/hour	1 - 5.5 median: 3 ha/hour	high filling rate: insufficient silo compaction
silo height at center	1.2 - 3.5 meter median: 2 meter	0.5 - 3 meter median: 2 meter	high silos: low feed-out rate
fermentation period	0.5 - 8 months median: 1 month	1 - 8 months median: 5 months	short fermentation period: silage not stable yet
feed-out rate	0.3 - 2.5 meter/week median: 1.5 meter/week	0.6 - 2 meter/week median: 1.2 meter/week	below 1 meter/week: aerobic deterioration
combination in silo	13% grass, sugar beet press pulp	30% different grass cuts	re-opening of silo: re-ingress of oxygen

Two striking observations can be made from Table 1.5:

- The absolute minimum fermentation period comprises four weeks, but for whole-crop maize silages this period was also the median. This implies that many farmers feed sub-optimally fermented maize silage, which might favor aerobic deterioration.
- The feed-out rate should be around 1-1.5 meter per week (depending on the season and/or temperature). The median values for whole-crop maize and grass silages are within this range, but some farmers have a very low feed-out rate.

In conclusion, it can be stated that the surveys on various aspects of whole-crop maize and grass silage making have revealed that some basic principles are regularly sinned against, increasing the risk of fungal contamination of silages.
About one-half of the questioned farmers admitted to have had problems with fungal contamination of their whole-crop maize and grass silages in the past. Nearly all farmers removed the fungal hot-spots prior to feeding the silage to their livestock. As for the silos concerned in the surveys, one third suffered from fungal infestation during feed-out. However, no acute animal health problems associated with the consumption of contaminated silage occurred.

The results of these surveys confirm the hypothesis that fungal infestation of silages is not a rare phenomenon, but that farmers are regularly faced with fungal hot-spots in their ensiled feed commodities. Severe animal health problems associated with mould contaminated silage don’t occur frequently, but it is possible that chronic animal health problems could be partly attributed to silage-associated mycotoxins (which will be reviewed in chapter 2).

5. Research outline

On-farm sampling of silages in combination with questioning of farmers about their silage making practices clearly demonstrated that fungal infestation of silages is a rather large-scale problem on Belgian farms, not in the least because it is considered as quite a normal phenomenon by many farmers as long as it remains on a low scale. From a scientific point of view, however, even limited development of toxigenic fungi poses a certain risk for animal health and possibly even human health due to mycotoxin production. This will be discussed in chapter 2. This chapter subsequently delves into *P. roqueforti s.l.* as main fungal contaminant of silages.

The clear demonstration that *P. roqueforti s.s.* and *P. paneum* are the main toxigenic fungal species contaminating silages in Belgium was the trigger for this particular PhD research topic. In many cases, poor ensiling and/or desiling practices facilitating fungal infestation were detected by questioning farmers, but in even so many cases mouldy hot-spots were visible in well-preserved non-heating silages where no obvious explanation for the fungal development could be found.

To provide more insight into the phenomenon of *P. roqueforti s.l.* contamination of silages, this PhD research started off in 2009 with as ultimate goal attributing to a strategy for preventing *P. roqueforti s.l.* growth and mycotoxin production, even in well-managed, good quality silage. The different steps paving the way to this ambitious goal will be described in detail in the chapters 3 to 5 of this dissertation. *In vitro* laboratory experiments as well as *in vivo* microsilo trials have been executed to evaluate the effect of several abiotic and biotic factors on *P. roqueforti s.l.* growth and mycotoxin production, as listed in Table 1.6.

Table 1.6. *Research outline.*

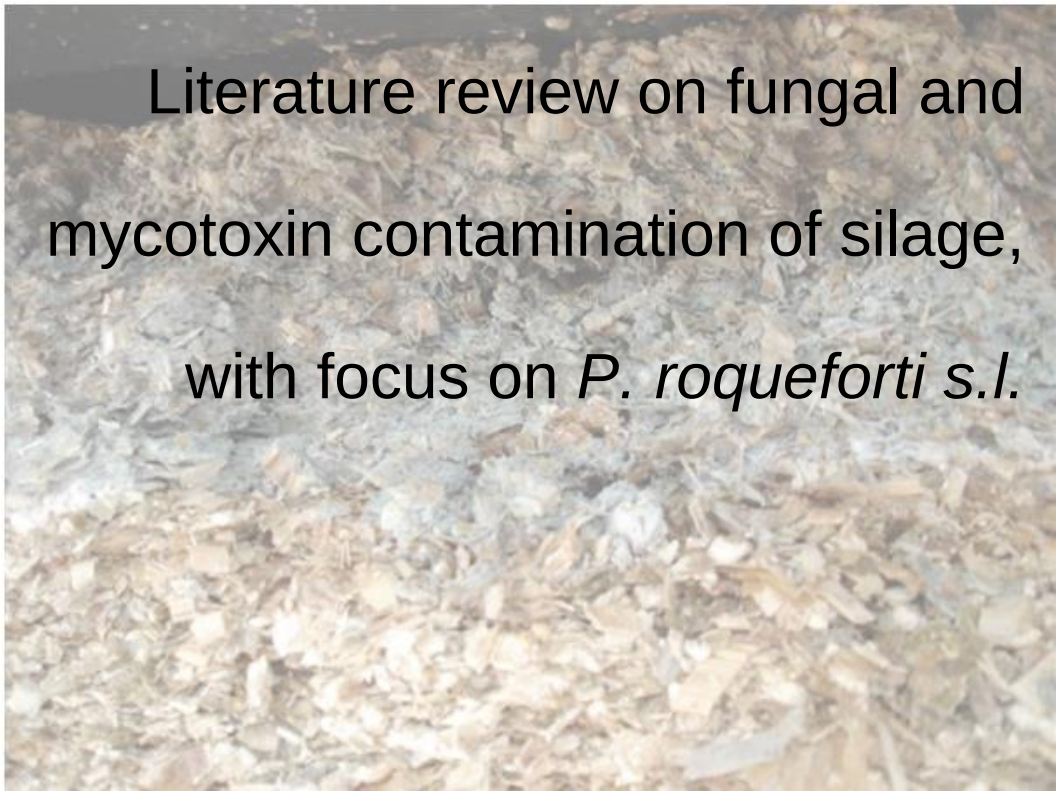
			PR MUCL 46746	PR CBS 116877	PR 2008-20	PR 2011-S1-G8	PP CBS 112295	PP 2011-S4-G8
Chapter 1. Introduction: Problem statement and research outline								
P. roqueforti s.s. **(PR) and** ***P. paneum*** **(PP) as main fungal contaminants of silages: growth and roquefortine C (ROC) production**								
Chapter 2. Literature review								
Chapter 3. Characterization of six selected ***P. roqueforti s.l.*** **isolates** ***in vitro***								
Partial sequencing of the beta tubulin gene	Growth and ROC production after 7 days on four agar media (PDA, YES, CYA, MinM)	Growth and ROC production after 15 days on eight different single carbon sources (MinM)						
No further molecular analyses		No further tests with single carbon sources						
Chapter 4. Effect of abiotic factors								
IN VITRO	Effect of variable amounts of inorganic and organic nitrogen on *P. roqueforti s.l.* growth and ROC production in a time-course experiment over 15 days (CYA)		x				x	
	Effect of temperature and oxygen on *P. roqueforti s.s.* growth after 7 days (PDA)		x					
IN VIVO	Effect of prolonged anaerobic conditions (50-100-150 days) on *P. roqueforti s.l.* numbers and fermentation characteristics of whole-crop maize silage		x				x	
	Effect of elevated temperature and oxygen supply on fungal counts and fermentation characteristics of grass silage and of whole-crop maize silage		x					
Chapter 5. Effect of biotic factors								
IN VITRO	*Bacillus velezensis* as antagonist towards *P. roqueforti s.l.* growth and ROC production (corn silage infusion)		x				x	
	Effect of HoLAB or HeLAB inoculant addition on *P. roqueforti s.l.* growth and ROC production (corn infusion agar)		x				x	
IN VIVO	Effect of HoLAB, HeLAB, *B. velezensis* and propionic acid on *P. roqueforti s.l.* numbers and fermentation characteristics of grass silage		x				x	
Chapter 6. Discussion and future perspectives								

The description of every trial starts with the experimental setup and the practical execution. General materials and methods are described in Annex 1, to whom will be referred to frequently. The results are presented and discussed per trial. Additionally, by combining the information obtained by different trials, the answers to the following research questions are sought:

- *P. roqueforti s.l.* is very well adapted to silage conditions, *i.e.* an acidic environment with high concentrations of organic acids produced by LAB (mainly lactic acid, but also some acetic acid) with an oxygen depleted and carbon dioxide enriched atmosphere.
    - How does *P. roqueforti s.l.* interact with LAB?
    - Can *P. roqueforti s.l.* use lactic acid and acetic acid as a carbon source, or do these acids have an inhibitory effect on *P. roqueforti s.l.* growth? If so, might this inhibition trigger mycotoxin production?
- In practice, fungal hot-spots of *P. roqueforti s.l.* appear to be more pronounced in whole-crop maize silages than in grass silages. On the one hand, this could be due to a better visibility of fungal hot-spots in maize silages. On the other hand, the carbon source composition of these two feed commodities differs before as well as after ensiling and from one silo to another, creating different nutritional circumstances for *P. roqueforti s.l.* development. So, could a difference in sensitivity to *P. roqueforti s.l.* infection between whole-crop maize and grass silages be explained based on the growth of *P. roqueforti s.l.* on different carbon sources?
- Can *P. roqueforti s.l.* growth and mycotoxin production be prevented by anaerobic conditions? *Vice versa*, does oxygen have a stimulatory effect on *P. roqueforti s.l.* in silages?
- Can *Bacillus velezensis* be a promising silage inoculant due to antagonistic activity towards *P. roqueforti s.l.*?

In the final chapter, the different insights obtained from the different *in vitro* and *in vivo* experiments are combined and subjected to critical consideration. Subsequently, the posed research questions are answered. Based on those answers, practical advice towards the prevention of *P. roqueforti s.l.* growth and mycotoxin production in silages is formulated.

Chapter 2



Literature review on fungal and mycotoxin contamination of silage, with focus on *P. roqueforti s.l.*

Parts of this chapter are redrafted from:

Wambacq E., Vanhoutte I., Audenaert K., De Gelder L. and Haesaert G. (2016) Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: a review. *Journal of the Science of Food and Agriculture* **96**: 2284-2302.

1. Introduction

The ensiling process has been reviewed in Chapter 1. The current chapter addresses fungal and mycotoxin contamination of ensiled feed commodities.

After a general introduction on the problems associated with mycotoxins, an overview is presented of the toxigenic fungi isolated from whole-crop maize and grass before and after ensiling, along with their mycotoxin production potential. On the one hand, a comparison is made of the mycotoxin load of these crops prior to and after ensiling. On the other hand, the mycotoxin load is compared between visibly non-mouldy samples and fungal hot-spots originating from the same silages. Different strategies to counteract fungal growth and mycotoxin production in silage are assessed. A logic first step is prevention of fungal growth and mycotoxin production in the field, during harvest and during ensiling. If prevention should fail, several remediation strategies are available. Finally, some remarks about problems associated with toxigenic fungi and mycotoxin contamination of silages will be formulated.

P. roqueforti s.s. and *P. paneum* (together referred to as *P. roqueforti s.l.*) are the most prevalent toxigenic fungi contaminating silages in Belgium, as demonstrated in chapter 1. In this literature review, the systematics of the *P. roqueforti* group is summarized, as well as the life cycle of the genus *Penicillium* and its implications on genome dynamics. Subsequently, the growth conditions of the *P. roqueforti* group are studied. Finally, mycotoxin production by the *P. roqueforti* group is addressed, focusing on roquefortine C (ROC) as indicator for mycotoxin production by *P. roqueforti s.l.* in silages.

2. Fungal and mycotoxin contamination of silages

Worldwide, a high proportion of the ruminant diet consists of silages made of forage crops (*i.e.* all parts of the crop above the stubble are harvested). In practice, silages are often contaminated with multiple mycotoxins. Exposure to a cocktail of mycotoxins can hamper animal production and have severe health consequences, possibly even affecting human health when mycotoxins enter the food chain.

In this section, the different aspects associated with mycotoxin contamination of silage are reviewed “from seed to feed”, with particular focus on *P. roqueforti s.l.* and its mycotoxins. Tüller *et al.* (1998) have detected trace amounts of ROC in the meat of sheep upon the ingestion of ROC at up to 25 mg per kg whole-crop maize silage, but *P. roqueforti s.l.*

mycotoxins are generally believed not to transfer into milk or meat (Fink-Gremmels 2007; Yiannikouris and Jouany 2002; Usleber *et al.* 2008). Food-related issues are therefore left out of the scope of this literature review, but have been described by several authors (Bhat *et al.* 2010; Bryden 2007; Da Rocha et al. 2014; Fink-Gremmels 1999; Hinton 2000; Sapkota et al. 2007).

2.1. General introduction on mycotoxin issues

The physiology of ruminants requires them to acquire a high proportion of roughages in their diet to maintain functional ruminal microbiota. Throughout the years, silages made of whole-crop maize, grasses, etc. have become an indispensable component in the diet of highly productive dairy cows and other ruminants in many parts of the world (De Brabander *et al.* 1999; Driehuis *et al.* 2008a; Fink-Gremmels 2008a; Tangni *et al.* 2013b). As will be reviewed thoroughly in section 2.2, fungal contamination of silages occurs frequently. The adverse effects of fungal infestation of silage on animals include allergic airway diseases due to spore inhalation and reduced palatability due to a “mouldy” scent, caused by the production of volatile organic compounds (Bui *et al.* 1994; May 1993; Pelhate 1976).

Moreover, some fungi can produce mycotoxins and are therefore called “toxigenic”. In the current agricultural and silage-making practices, it is difficult if not impossible to avoid mycotoxin contamination of forage crops. Ingestion is the most common route of mycotoxin exposure, followed by inhalation of airborne fungal spores possibly containing mycotoxins (Abbott 2002; Cho *et al.* 2005; de la Campa *et al.* 2007; Delmulle 2009; Norback 2009; Polizzi *et al.* 2009; Straus 2009). The importance of ensiled forage crops as sources of mycotoxins in the ruminant diet has been confirmed by numerous authors (Alonso *et al.* 2013; Bhat *et al.* 2010; Cavallarin *et al.* 2004; Cheeke 1998; Cheli *et al.* 2013; Driehuis *et al.* 2008b; Driehuis 2013; El-Shanawany *et al.* 2005; Fink-Gremmels and Diaz 2005; Fink-Gremmels 2008a; Gallo *et al.* 2015; Gonzalez Pereyra *et al.* 2008; Hussaini *et al.* 2009; McElhinney *et al.* 2015; Placinta *et al.* 1999; Scudamore and Livesey 1998; Storm *et al.* 2008; Tangni *et al.* 2013b; Yiannikouris and Jouany 2002; Zachariasova *et al.* 2014).

Mycotoxins may exert acute fatal intoxications upon ingestion, as well as sub-clinical disease conditions and suppression of the immune system. A reduction in weight gain, feed conversion and resistance to infectious diseases can be related to mycotoxin exposure of animals, but these symptoms are not always recognized as mycotoxin-related since animal sensitivity towards mycotoxins varies according to species, breed, age, sex, nutritional status, stress level, etc. (Barug *et al.* 2006; Bhat *et al.* 2010; Corrier 1991; Dell’Orto *et al.* 2015; DiCostanzo *et al.* 1995; Gonzalez Pereyra *et al.* 2008; McElhinney *et al.* 2015; Sharma 1993; Yiannikouris

and Jouany 2002;Binder 2007; Fink-Gremmels 2008b; Fink-Gremmels 1999; Morgavi and Riley 2007; Zain 2011).

In general, ruminants are considered to be less sensitive to the ingestion of many mycotoxins than monogastric animals, as the ruminal microbiota can successfully degrade and inactivate mycotoxins: rumen protozoa as well as rumen bacteria can metabolize various mycotoxins (Kiessling *et al.* 1984; Mobashar *et al.* 2010; Upadhaya *et al.* 2010; Vanhoutte *et al.* 2016; Westlake *et al.* 1989). However, the detoxifying capacity of the ruminal microflora is limited and varies with changes in the dietary composition or as a consequence of metabolic diseases like rumen acidosis (Fink-Gremmels 2008b; Jouany and Diaz 2005; Rodrigues 2014; Upadhaya *et al.* 2010). Furthermore, as multiple mycotoxins such as patulin, ROC, monacolins, beauvericin and enniatins exhibit antimicrobial properties, chronic exposure to such antimicrobial mycotoxins may disturb the ruminal microbiota and result in rumen acidosis, decreased feed efficiency and productivity, and even in clinical diseases like mastitis (Barug *et al.* 2006; Dennis *et al.* 1981; Fink-Gremmels 2008b; Jestoi *et al.* 2004; Schneweis *et al.* 2001). To date, research on toxicity associated with mycotoxins has focused mainly on the negative effects of single mycotoxin exposures on animal performance and health, however exposure to multiple mycotoxins can have additive, antagonistic or synergistic effects. In practice, silages often contain a cocktail of different mycotoxins at fairly low concentrations, resulting mostly in chronical health problems instead of acute mycotoxicosis (Murugesan *et al.* 2015; Pedrosa and Borutova 2011; Rodrigues 2014; Scudamore and Livesey 1998; Speijers and Speijers 2004; Streit *et al.* 2013; Yiannikouris and Jouany 2002).

2.2. Toxigenic fungi and mycotoxins associated with silages

Prior to ensiling, feed commodities can harbor a diverse mosaic of fungi which can infect growing plants in the field and produce mycotoxins. During harvesting and chopping, additional fungal contamination originating from air, soil and ambient dust can occur. These fungi as well as possibly produced mycotoxins are ensiled together with the plant material. A well-executed ensiling process, based on a spontaneous lactic acid fermentation under anaerobic conditions, inactivates many but not all undesirable microorganisms which had contaminated the feed commodity (Dogi *et al.* 2013; Mansfield and Kuldau 2007; Tangni *et al.* 2013b; Wilkinson 2005) Once conditions in the ensiled material are conducive for fungal growth (*i.e.* oxygen availability), fungal inoculum can proliferate in the silage and produce additional mycotoxins during storage or feed-out (Bouslimi *et al.* 2008; Dell'Orto *et al.* 2015; Garon *et al.* 2006; Grenier and Oswald 2011; Mansfield *et al.* 2008; Richard *et al.* 2003; Sharma 1993). Bad silo management can lead to badly preserved silage. For example, delayed and/or

inadequate sealing of the silo can allow field fungi like *Fusarium* to proliferate during the silage storage period because of high pH-values in silage exposed to oxygen. On the other hand, storage fungi like *Aspergillus* and *Penicillium* can develop in well-preserved silage in presence of oxygen (Honig 1991; Ranjit and Kung 2000; Spoelstra *et al.* 1988; Ström *et al.* 2002).

Table 2.1 gives an overview of toxigenic fungal species isolated from whole-crop maize and grasses, prior to ensiling and after ensiling. A non-limitative list of mycotoxins that can be produced by these fungal species is also mentioned.

Table 2.1. *Fungal species isolated from maize and grass before ensiling (BE) and after ensiling (AE), and possible mycotoxins produced (not limitative).*

Fungal species	Maize		Grass		Mycotoxins that can be produced (not limitative)
	BE	AE	BE	AE	
Aspergillus					
A. candidus		x			Candidulin, kojic acid, 3-nitropropionic acid, terphenyllins, xanthoascin
A. flavus	x	x			Aflatoxins, aspergillic acid, cyclopiazonic acid, kojic acid, 3-nitropropionic acid, sterigmatocystin
A. fumigatus	x	x	x	x	Agroclavine, fumigaclavines, fumagillin, fumitremorgins, gliotoxin, helvolic acid, tryptoquivalins, verruculogen
A. melleus		x			Ochratoxin A
A. niger	x	x		x	Aspergillin, malformins, ochratoxin A
A. ochraceus		x			Ochratoxin A, penicillic acid, viomellein, vioxanthin, xanthomegnin
A. parasiticus	x	x			Aflatoxins, aspergillic acid, kojic acid
A. terreus	x	x	x		Citreoviridin, citrinin, patulin, terrein, territrem
A. versicolor	x	x			Nidulotoxin, penicillin, sterigmatocystin
Fusarium					
F. avenaceum	x	x	x		Beauvericin, enniatins, fusarin C, moniliformin, trichothecenes type A
F. crookwellense		x			Chrysogine, culmorin, fusarin C, trichothecenes type B, zearalenone
F. commune				x	-
F. culmorum	x	x	x	x	Chrysogine, culmorin, fusarin C, trichothecenes type B, zearalenone
F. equiseti	x	x	x		Chrysogine, equisetin, fusarochromanones, trichothecenes type A and B, zearalenone
F. graminearum	x	x			Chrysogine, culmorin, fusarin C, trichothecenes type B, zearalenone
F. oxysporum		x	x	x	Fusaric acid, moniliformin, sambutoxin, wortmannin
F. poae		x	x		Fusarin C, trichothecenes type A and B
F. proliferatum	x	x			Beauvericin, fumonisins, fusaproliferin, fusapyrone, fusaric acid, fusarin C, moniliformin
F. pseudograminearum	x				Trichothecenes type B
F. redolens				x	Beauvericin
F. sambucinum		x			Beauvericin, enniatins, sambutoxin, trichothecenes type A
F. semitectum		x			Equisetin, fusapyrone, zearalenone
F. solani		x			Fusaric acid
F. sporotrichioides	x	x			Fusarin C, trichothecenes type A and B
F. subglutinans		x			Beauvericin, fumonisins, fusaproliferin, fusapyrone, fusaric acid, fusarin C, moniliformin
F. verticillioides	x	x			Beauvericin, fumonisins, fusaric acid, fusarin C, moniliformin

Table 2.1 (continued). *Fungal species isolated from maize and grass before ensiling (BE) and after ensiling (AE), and possible mycotoxins produced (not limitative).*

Penicillium					
P. aurantiogriseum		x			Nephrotoxic glycopeptides, ochratoxin A, penicillic acid, terrestric acid, verrucosidin
P. brevicompactum		x			Botryodiploidin, brevianamide A, mycophenolic acid
P. canescens		x			Griseofulvin, penitrem A
P. caseifulvum		x			Rugulovasine A
P. chrysogenum		x			Chrysogine, meleagrin, PR-toxin, roquefortine C
P. citreonigrum		x		x	Citreoviridin
P. citrinum	x	x			Citrinin, tanzawaic acid A
P. commune	x	x			cyclopaldic acid, cyclopiazonic acid, rugulovasins
P. corylophilum		x		x	Citrinin
P. coprophilum				x	Alternariol, griseofulvin, roqueforitne C
P. crustosum	x	x		x	Cyclopenin, cyclopenol, penitrem A, roquefortine C, terrestric acid
P. cyclopium		x			Penicillic acid, viomellein, vioxanthin, xanthomegnin
P. duclauxii		x			Duclauxin, xenoclauxin
P. expansum		x		x	Chaetoglobosin C, citrinin, patulin, roquefortine C,
P. glabrum	x	x			Citromycetin
P. griseofulvum		x			Cyclopiazonic acid, griseofulvn, patulin, roquefortine C
P. islandicum	x	x		x	Cyclochlorotine, emodin, erytroskyrin, islandi-toxin,
P. italicum		x			Italic acid, verrucolone
P. janczewskii		x			Griseofulvin, penitrem A
P. loliense		x			-
P. minioluteum		x		x	Miniolin A, B and C
P. olsonii		x			Verrucolone
P. oxalicum		x			Oxaline, roquefortine C, secalonic acid D and F
P. palitans		x		x	Cyclopiazonic acid, fumigaclavine A and B
P. paneum	x	x		x	Andrastatins, botryodiploidin, marcfortines, patulin, roquefortines
P. polonicum		x			Cyclopenin, cyclopenol, nephrotoxic glycopeptides, penicillic acid, verrucosidin
P. purpurogenum		x			-
P. roqueforti s.s.	x	x		x	Andrastatins, isofumigaclavine A and B, mycophenolic acid, PR-toxin, roquefortines
P. roseopurpureum				x	Rubratoxins
P. rubrum		x			Rubratoxins
P. variabile		x			Rugulosin
Trichoderma					
T. asperellum		x			Asperellines
T. atroviride		x		x	Gliotoxin
T. harzanium		x		x	Chrysophanol, koninginin A, trichorzianines A and B
T. viride			x	x	Alamethicins, emodin, suzukacillin, trichodermin, trichotoxin A

Table 2.1 (continued). *Fungal species isolated from maize and grass before ensiling (BE) and after ensiling (AE), and possible mycotoxins produced (not limitative).*

Other					
Acremonium spp.		x			Ergot alkaloids, lolitrem A
Alternaria spp.	x	x	x		Altenuene, alternariol, alernariol monomethyl ether, tentoxin
Byssochlamys nivea		x		x	Byssochlamic acid, malformins, patulin
Chrysosporium spp.		x			-
Cladosporium spp.	x	x	x	x	Cladosporic acid, emodin
Curvularia spp.	x				Cytochalasins
Eurotium spp.	x	x		x	-
Geotrichum candidum		x		x	-
Monascus ruber		x		x	Mevalonic acid, monacolins
Paecilomyces spp.		x		x	Patulin, mycophenolic acid, viriditoxin
Phoma spp.		x	x		Cytochalasins
Schizophyllum spp.				x	-
Scopulariopsis spp.		x			-
Talaromyces spp.		x	x		Duclauxin
Verticillium spp.		x			-
Zygomycetes	x	x		x	Rhizonin A

Trichothecenes type A: diacetoxyscirpenol, neosolaniol, T-2 toxin, HT-2 toxin

Trichothecenes type B: deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, fusarenon X

(Alonso *et al.* 2009; Auerbach *et al.* 1998; Boudra and Morgavi 2005; Christ *et al.* 2011; Cole *et al.* 1977; D'Mello *et al.* 1998; Declerck *et al.* 2009; Di Menna *et al.* 1997; Driehuis *et al.* 2010; Frisvad *et al.* 2009; Gonzalez Pereyra *et al.* 2008; Gonzalez Pereyra *et al.* 2011; Hussaini *et al.* 2009; Joffe 1986; Keller *et al.* 2013; Lattanzio *et al.* 2013; Mansfield and Kuldau 2007; McMullen and Stack 1983; Nitschke *et al.* 2009; Nout *et al.* 1993; O'Brien *et al.* 2006; O'Brien *et al.* 2007; O'Brien *et al.* 2008; Pitt 2000; Placinta *et al.* 1999; Rasmussen *et al.* 2010; Richard *et al.* 2007; Samson *et al.* 2002; Samson and Frisvad 2004; Sánchez Márquez *et al.* 2007; Smith and Henderson 1991; Storm *et al.* 2008; Van Asselt *et al.* 2012; Vanheule *et al.* 2014; Wilson *et al.* 1984)

Fusarium and *Aspergillus* species are frequently detected before and after ensiling, while *Penicillium* and *Trichoderma* species are most frequently isolated after ensiling. It is clearly demonstrated in Table 2.1 that a mosaic of fungal species is already present on forage crops prior to ensiling, and this aggregate broadens after ensiling. Another interesting observation is that maize harbors a broader range of fungal species than grasses. This could be attributed to the apparently more frequently performed research on maize than on grasses, or to differences in chemical composition between the two (Auerbach *et al.* 1998; Muck and Bolsen 1991; Petrovska *et al.* 2015; Pitt *et al.* 1991).

The subspecies *P. roqueforti sensu stricto* (*s.s.*) and *P. paneum* (Boysen *et al.* 1996; Boysen *et al.* 2000), together referred to as *P. roqueforti sensu lato* (*s.l.*) in this dissertation, are the main contaminating fungi in silages in temperate climates because they are very well adapted to conditions of high concentrations of organic acids and low pH, low oxygen and high carbon dioxide levels. *A. fumigatus* is also well adapted to silage conditions, but prefers higher temperatures than *Penicillia*. Other fungal species well adapted to ensilage conditions are *Monascus ruber* and *Byssochlamys nivea* (Auerbach *et al.* 1998; Cole *et al.* 1977; El-Shanawany *et al.* 2005; Gonzalez Pereyra *et al.* 2008; Ohmomo *et al.* 1994; Petersson and Schnürer 1999; Richard *et al.* 2007; Samson *et al.* 2002; Taniwaki *et al.* 2009).

Recently, Alonso *et al.* (2015) have studied toxigenic *A. fumigatus* strains isolated from maize silage and their gliotoxin production *in vitro* under environmental conditions as occurring in silage (*i.e.* water activity, temperature, pH and oxygen pressure). They observed that under reduced oxygen pressure, gliotoxin was only produced at water activities and pH-values similar to conditions occurring in actual maize silage, illustrating that *A. fumigatus* is indeed well adapted to silage conditions. Similar studies regarding mycotoxin production by *P. roqueforti s.l.* could not be found in literature.

Table 2.2 summarizes the mycotoxin load of whole-crop maize and grass before and after ensiling, for the same batches: the upper section of the table lists up mycotoxins produced by *Fusarium*, while the bottom section mentions mycotoxins produced by *Alternaria*, *Aspergillus* and *Penicillium*. If possible the incidence is also mentioned, but this information is often unavailable.

Table 2.2. *Mycotoxin levels (mg/kg fresh matter) in whole-crop maize and grass, before and after ensiling.*

Mycotoxin	Matrix	before ensiling				after ensiling				References
		N	incidence	mean	max	N	incidence	mean	max	
Deoxynivalenol	maize	17	12%	2.37 *	2.66	82	6%	1.63 *	2.97	Storm *et al.* 2014
	maize	-	21%	1.53	3.03	-	24%	1.61	3.42	Keller *et al.* 2013
	maize	-	-	0.15	0.23	-	-	0.28	0.87	Gonzalez-Pereyra *et al.* 2008
	grass	-	-	0.04	-	-	-	0.14	-	Skladanka *et al.* 2013
Enniatin B	maize	17	47%	0.13 *	0.37	82	24%	0.05 *	0.15	Storm *et al.* 2014
Fumonisin B1	maize	-	16%	1.59	2.99	-	16%	1.60	3.42	Keller *et al.* 2013
	maize	-	-	0.60	1.84	-	-	1.11	2.49	Gonzalez-Pereyra *et al.* 2008
Nivalenol	maize	17	29%	0.26 *	0.35	82	13%	0.27 *	0.76	Storm *et al.* 2014
T-2 toxin	grass	-	-	0.03	-	-	-	0.02	-	Skladanka *et al.* 2013
Zearalenone	maize	-	-	0.02	0.03	-	-	0.05	0.35	Gonzalez-Pereyra *et al.* 2008
	maize	17	65%	0.08 *	0.67	82	28%	0.07 *	0.31	Storm *et al.* 2014
	grass	-	-	0.02	-	-	-	0.07	-	Skladanka *et al.* 2013
Alternariol monomethyl ether	maize	1	6%	0.011 *	0.011	82	2%	0.008 *	0.009	Storm *et al.* 2014
Aflatoxin B1	maize	-	12%	0.007	0.011	-	12%	0.033	0.054	Keller *et al.* 2013
Ochratoxin A	maize	-	9%	0.008	0.028	-	10%	0.003	0.004	Keller *et al.* 2013
Cyclopiazonic acid	maize	60	30%	0.05	0.38	120	37%	0.12	1.43	Mansfield *et al.* 2008 **
Mycophenolic acid	maize	60	27%	0.06	0.60	120	42%	0.16	1.30	Mansfield *et al.* 2008 **
Patulin	maize	60	17%	0.05	0.91	120	23%	0.08	1.21	Mansfield *et al.* 2008 **
Roquefortine C	maize	60	50%	0.02	1.10	120	60%	0.38	5.71	Mansfield *et al. 2008* **

* Mean level in positive samples only

** Samples taken at dairy farms with a history of cattle health problems

\- Information not available

ROC, patulin, cyclopiazonic acid and mycophenolic acid, all produced by *Penicillium*, show increased levels and increased incidence after ensiling. Since *Penicillium* is a typical genus of storage fungi, this is not surprising.

A crucial fact that needs to be emphasized is that silages differ greatly according to the ensiled forage crop, the dry matter content, the epiphytic micro-organisms, the length of storage, the amount of air ingress, etc. All this together, including differences in sampling method and sample size, makes a general interpretation of mycotoxin levels before and after ensiling very difficult.

Ensiled forage crops are often contaminated with multiple mycotoxins, and the contamination is not homogeneously spread within a silo - especially when fungal growth has occurred in some parts of the silo. This is illustrated in Table 2.3, presenting an overview of multiple mycotoxins detected in maize and grass silage samples taken from visually non-mouldy parts as well as from fungal hot-spots within the same silo.

Table 2.3. *Mycotoxin load in visually non-mouldy samples and fungal hot-spots from whole-crop maize and grass silages: mean and maximum levels (mg/kg fresh matter).*

Matrix	Mycotoxins	visually non-mouldy samples				fungal hot-spots			References
		N	incidence	mean	max	incidence	mean	max	
Whole-crop maize silage	Deoxynivalenol	21	100%	4.9	30.3	100%	3.7	19.9	Declerck *et al.* 2009
	Enniatin B	21	86%	3.5	13.6	86%	2.2	13.8	Tangni *et al.* 2013
	HT-2 toxin	21	81%	0.005	0.015	86%	0.006	0.036	
	Zearalenone	21	90%	0.154	0.517	90%	0.104	0.408	
	Ochratoxin A	21	62%	0.001	0.002	72%	0.004	0.4	
	Gliotoxin	21	14%	0.2	1.5	0%	-	-	
	Citrinin	21	95%	0.037	0.137	95%	0.045	0.299	
	Mycophenolic acid	21	95%	6.323	20.801	86%	4.448	16.051	
	Patulin	21	29%	0.015	0.103	38%	0.041	0.551	
	Penitrem A	21	0%	-	-	19%	0.1	0.7	
	Roquefortine C	21	62%	0.459	0.743	100%	1.848	15.140	
	Mevalonic acid	21	29%	0.2	1.7	52%	0.5	1.8	
	Enniatin B	10	40%	0.044 *	0.063	30%	0.093 *	0.200	Rasmussen *et al.* 2010
	Zearalenone	10	40%	0.099 *	0.311	40%	0.071 *	0.156	
	Mycophenolic acid	10	10%	0.052 *	0.052	60%	0.507 *	1.646	
	Roquefortine C	10	10%	0.189 *	0.189	30%	11.83 *	33.66	
	Alternariol	10	10%	0.024 *	0.024	10%	0.236 *	0.236	
Grass silage	Ochratoxin A	20	85%	0.042	0.306	80%	0.017	0.087	Declerck *et al.* 2009
	Gliotoxin	20	80%	1.1	2.3	80%	1.0	3.8	Tangni *et al.* 2013
	Verrucologen	20	45%	2.6	21.7	50%	1.0	6.1	
	Citrinin	20	85%	0.050	0.283	95%	0.042	158	
	Mycophenolic acid	20	60%	1.268	14.026	85%	3.587	21.387	
	Patulin	20	60%	0.047	0.186	55%	0.040	0.166	
	Penitrem A	20	25%	0.3	2.3	20%	0.2	1.5	
	Roquefortine C	20	45%	0.319	1.114	75%	0.852	7.208	
	Mevalonic acid	20	35%	0.3	1.6	60%	0.6	2.3	

* Mean level in positive samples only

\- Information not available

In maize silages, the levels of the *Aspergillus* and *Penicillium* mycotoxins ochratoxin A, citrinin, patulin, penitrem A, ROC and mevalonic acid were higher compared to visually non-mouldy maize silage samples, suggesting that they were additionally produced during storage.

In grass silage, fungal hot-spots contained higher levels of gliotoxin, mycophenolic acid, ROC and mevalonic acid, and lower levels of ochratoxin A, verrucologen and citrinin than visually non-mouldy samples, illustrating that *Penicillium* and *Aspergillus* growth can occur before as well as after ensiling.

The same remark that has been made discussing Table 2.2, about great differences between silages, also holds true for the interpretation of the data in Table 2.3, especially since this table is based on three references only. Additionally, metabolism or reabsorption of mycotoxins by their producing fungi should also be considered in explaining differences in mycotoxin contents between visually non-mouldy silage samples and fungal hot-spots within the same silo (Barug *et al.* 2006; Kulakovskaya *et al.* 1997; Overy *et al.* 2005).

2.3. Prevention of fungal growth and mycotoxin production

2.3.1. Prevention in the field and at harvest

Prevention of fungal growth and mycotoxin production requires an integrated approach (Jard *et al.* 2011; Jouany and Diaz 2005; Montville and Matthews 2001; Rouse *et al.* 2008; Weinberg and Muck 1996). Development of fungi prior to ensiling should be prevented in the field, fungal contamination at harvest should be minimized, and the maintenance of anaerobic circumstances during silage storage and minimizing aerobic deterioration during feed-out should inhibit further fungal development and mycotoxin production in silage (Dunière *et al.* 2013; Miller 2008; Richard *et al.* 2007; Wilkinson and Davies 2012; Woolford 1990).

2.3.1.1. Field management and crop husbandry

Preventing the occurrence of mycotoxins produced by toxigenic fungi in forage crops starts in the field. Appropriate field management and crop husbandry seek to influence abiotic and biotic parameters in the crop environment towards inhibitory conditions for fungal infection and proliferation. This requires a multifactorial approach, taking at least the following aspects into consideration: crop rotation, choice of variety, tillage, irrigation, soil fertilizers, insecticide use, and of course also fungicide use (on seeds before sowing as well as in the field). Any crop

husbandry that results in the removal, destruction or burial of infected plant residues is likely to reduce the fungal inoculum for the following crop. Particular attention should also be paid to the harvest of the forage crops: growth stage, mowing height, wilting, ground contamination, etc. need to be taken into consideration. These aspects have been described thoroughly (Aldred and Magan 2004; Boudergue *et al.* 2009; Champeil *et al.* 2004; Cleveland *et al.* 2003; Codex Alimentarius Commission 2002; Cotten and Munkvold 1998; Edwards 2004; Goertz *et al.* 2010; Jard *et al.* 2011; Jouany 2007; Kabak *et al.* 2006; Maiorano *et al.* 2008; Meissle *et al.* 2010; Munkvold *et al.* 1999; Teller *et al.* 2012; Uegaki *et al.* 2013). Attention needs to be drawn to some aspects requiring extra consideration: 1) Concerning the choice of variety of maize and other cereal grains, significant differences were reported between hybrid varieties with regard to susceptibility towards *Penicillium* and *Aspergillus* species, which were linked to differences in kernel wax composition (Cantone *et al.* 1983; Friday *et al.* 1989; Russin *et al.* 1997). 2) Irrigation in general can be very useful in avoiding plant stress in particular plant growth stages, but during anthesis, excess irrigation may favour *Fusarium* infection (Codex Alimentarius Commission 2002). 3) Chemical or biological fungicides can be applied for fungal control in the field. However, regarding trichothecenes, it must be noted that fungicides should be applied with an adequate fungicide dose, since sub-lethal dosage can result in increased mycotoxin contents in crops showing no or low fungal infection. Also, timing of fungicide use is important (Audenaert *et al.* 2011; Kulik *et al.* 2012).

Besides field management and crop husbandry, weather conditions also play an important role in fungal development on crops in the field, as well as in mycotoxin production. However, weather conditions cannot be controlled by farmers, so farmers should anticipate to unfavorable weather conditions by the previously described field management and crop husbandry practices. Furthermore, it has been proven that field management influences *Fusarium* growth and mycotoxin production in the field more than climatic conditions do (Aldred and Magan 2004; Battilani *et al.* 2013; Landschoot *et al.* 2012; Langseth *et al.* 1995).

2.3.1.2. Antagonistic fungi

The introduction of non-toxigenic fungal strains in the field can establish competitive exclusion of toxigenic fungal strains. These non-toxigenic strains occupy the same ecological niche as toxin-producing strains, so they decrease the level of contamination by toxigenic fungi (Cotty and Bhatnagar 1994; Yiannikouris and Jouany 2002). For example, Dorner and Lamb (2006) describe the treatment of soils where peanuts are being grown with a competitive, non-toxigenic strain of *Aspergillus flavus*. The use of this biofungicide successfully diminished the amounts of toxigenic A. flavus in the soil and consistently decreased the aflatoxin levels in the harvested peanuts. Although the application of antagonistic fungi may be beneficial,

undesirable effects can arise unexpectedly depending on the circumstances. The results of Picco *et al.* (1999) of co-culturing the toxigenic *species A. flavus* and *Fusarium proliferatum* on maize seeds *in vitro* suggest that under optimal environmental conditions the interaction between the two fungal species leads to inhibition of aflatoxin B1 production by *A. flavus* but stimulation of fumonisin B1 production by *F. proliferatum*. On the other hand, some mycotoxins have an antifungal effect which could be beneficial in inhibiting the growth of other toxigenic fungal species (Karlovsky 1999; McCormick 2013).

2.3.2. Prevention during silage conservation

Multiple influencing factors on silage quality at the different phases of the ensiling process were summarized in Figure 1.1, emphasizing the need for good practices from filling to unloading (Kung 1996; O'Brien *et al.* 2007).

2.3.2.1. Silo management

Good silo management can dramatically reduce the numbers and types of fungi in silage samples. In well-preserved silage, the majority of fungal growth is limited by anaerobic or microaerophilic conditions and by the presence of organic acids. When oxygen gains access to ensiled material, due to inadequate sealing during storage or at feed-out, yeasts and acetic acid bacteria start to oxidize the preservative acids and residual water-soluble carbohydrates. The concomitant aerobic deterioration results in a rise of temperature and pH. These new silage conditions allow fungi to proliferate (Bernardes *et al.* 2012; Borreani and Tabacco 2010; Dolci *et al.* 2011; Lindgren *et al.* 1985; McDonald *et al.* 1991; Wilkinson and Davies 2012).

The dispersal of oxygen into a silage mass is strongly affected by the silo management regarding compaction, sealing and unloading (Auerbach *et al.* 1998; Dunière *et al.* 2013; Honig 1991; Kim and Adesogan 2006; Muck 2013; Munkvold 2003; O'Brien *et al.* 2007; Parsons 1991; Pitt *et al.* 1991; Woolford 1990). Compaction is closely related to the dry matter content of the ensiled material, so ensiling forage crops at optimal dry matter content is thus important (for grass: 350 - 450 g/kg fresh matter, but for baled silage a higher dry matter is allowed; for whole-crop maize: 300 - 350 g/kg fresh matter) (Khan *et al.* 2014; Wilkinson 2005; Wilkinson and Davies 2012). On the one hand, thorough compaction of the ensiled material is important, but on the other hand it is necessary to seal silages quickly and carefully. Furthermore, the sealing should remain intact during the whole ensiling period (Bernardes *et al.* 2012; Dolci *et al.* 2011; Kim and Adesogan 2006; Nelson 1993; Weinberg and Ashbell 2003). This is confirmed by Petersson (1998) and Richard-Molard *et al.* (1980), who observed that *P. roqueforti* spores stored under airtight conditions for three months had a strongly reduced

germination ability. So, leaving silos sealed during at least three months before starting with feeding the silage to the livestock should have a beneficial effect towards the prevention of fungal growth.

2.3.2.2. Aerobic deterioration inhibiting silage additives

Another prevention strategy is the use of aerobic deterioration inhibiting silage additives. Organic acids (acetic acid, propionic acid, formic acid, benzoic acid, sorbic acid, citric acid, etc.) and their salts, inorganic acids (sulphuric acid, phosphoric acid, sodium bicarbonate, etc.) and their salts on the one hand, and inoculants containing HeLAB on the other hand are the most widely used (Bal and Bal 2012; Bolsen *et al.* 1996; Brock and Buckel 2004; Conaghan *et al.* 2010; Mayne 1993; Nkosi *et al.* 2009; Richard *et al.* 2003; Stryszewska and Pys 2006; Wilkinson 2005).

Acids have a direct pH-lowering effect. When silage is treated with high amounts of acids, LAB fermentation is reduced. Low amounts of acid (e.g. 2-4 liter per ton fresh matter) hardly influence silage fermentation, but reduce aerobic spoilage by inhibiting yeasts and/or fungi. The precise inhibitory effect on micro-organisms varies considerably. E.g. propionic acid inhibits fungi and *Bacilli*, but not yeasts to the same extent. Yeasts are also relatively tolerant to formic acid, while acetic acid has strong yeast inhibiting properties (Bolsen *et al.* 1996; Suhr and Nielsen 2004; Yitbarek and Tamir 2014).

Salts of acids are used widely as safer alternatives to the acids themselves, since they are less corrosive to humans, animals and machinery. The effectiveness of acids and their salts as silage additives depends on their water solubility: the lower the solubility of the product, the less efficient in inhibiting microbial growth. Salts are usually more soluble in aqueous solutions than acids (Kung 1996; McDonald *et al.* 1991; Suhr and Nielsen 2004).

Non-dissociated acids have a much stronger antimicrobial effect than dissociated acids: undissociated molecules can penetrate cell membranes and accumulate in the cytoplasm, causing the loss of cell viability and cell destruction. At decreasing pH, the ratio of non-dissociated acid to dissociated acid increases, according to the pK_a value of the acid: when pK_a and pH are equal, the ratio of non-dissociated to dissociated acid is 1:1. The lower the pK_a value of an acid, the stronger the acid. E.g. acetic acid (pK_a 4.76) is a stronger acid than propionic acid (pK_a 4.87), but weaker than lactic acid (pK_a 3.86) (Eklund 1983; McDonald *et al.* 1991; Pahlow *et al.* 2003; Suhr and Nielsen 2004). However, fungi have developed mechanisms to counteract the effect of weak acids and maintain intracellular pH-homeostasis through proton pumps (Beales 2003).

Silage **inoculants** are biological additives containing micro-organisms at high numbers, overpowering the epiphytic microflora to guide the fermentation in the desired direction. Most silage inoculants contain LAB.

To reduce aerobic deterioration by yeast growth reduction, HeLAB like *L. buchneri* are used widely. *L. buchneri* converts sugars to lactic acid and acetic acid. Furthermore, lactic acid is converted into acetic acid and 1,2-propanediol under anaerobic circumstances, as a protective response mechanism to surrounding low pH: degradation of lactic acid into an alcohol and an acid with a higher pK_a value decreases the amount of non-dissociated acids. As described earlier, acetic acid inhibits yeasts initiating aerobic deterioration, but its inhibitory effect is not noticeably high compared to higher volatile fatty acids, so it may have a synergistic effect with other fermentation products. The 1,2-propanediol, which has little or no antifungal action, can be degraded by other anaerobic bacteria present in silage into propionic acid and 1-propanol, both having antimycotic activity (Carlsen *et al.* 1991; Danner *et al.* 2003; Driehuis *et al.* 1999; Driehuis *et al.* 2001; McDonald *et al.* 1991; Moon 1983; Oude Elferink *et al.* 2001; Suhr and Nielsen 2004). *L. buchneri* containing inoculants are widely used in silage production to improve the aerobic stability of silage, but this bacterium is not adapted to growth at temperatures below 15 °C. Other HeLAB species, e.g. *L. curvatus* and *L. sakei*, are the predominant HeLAB at lower temperatures (Zhou *et al.* 2016).

Propionic acid bacteria can ferment sugars and lactic acid to acetic acid and propionic acid and could be used as inoculants inhibiting aerobic deterioration. In practice, however, often no improvement of aerobic stability of silage is observed after addition of propionic acid bacteria as a silage inoculant. This lack of response is probably related to the acidic environment arising during the fermentation phase, which is not favorable for the growth of propionic acid bacteria. It appears that the success of the current strains depends on maintenance of a relatively high pH so that they can grow to significant levels (Dawson *et al.* 1998; Higginbotham *et al.* 1998; Lind *et al.* 2005; Pahlow and Honig 1994; Weinberg and Muck 1996).

Inoculants have benefits over chemical silage additives because they are easy to use and non-corrosive (Keles and Yazgan 2011; Schnürer and Magnusson 2005). However, chemical additives can be more powerful than inoculants. Their activity depends on the substrate, the water availability, the uniformity of their distribution over the forage at ensiling and the competitiveness of the epiphytic microflora (McAllister *et al.* 1995; Muck 2004). In silages made of various crops, inoculation with the HeLAB species *L. buchneri* has proven to increase aerobic stability (Adesogan *et al.* 2003; Driehuis *et al.* 1999; Filya *et al.* 2006; Hu *et al.* 2009; Kleinschmit and Kung 2006; Ranjit and Kung 2000; Wambacq *et al.* 2013).

Several **plant extracts and essential oils** can also prevent growth of toxigenic fungi and mycotoxin production upon aerobic deterioration of silage: onion, lemon, turmeric, mint, oregano, thyme, ginger, sea algae, etc. (Chavez-Quintal *et al.* 2011; Kabak *et al.* 2006; Kung *et al.* 2008; Pietri *et al.* 2009). For instance, citrus peels are an industrial waste product and therefore available in large quantities (Nam *et al.* 2009a). Physical conditions that improve the action of essential oils are low pH, low temperature and low oxygen levels; these conditions are fulfilled in well-preserved silages (Burt 2004).

In conclusion, aerobic deterioration of silages can be countered by several silage additives, but it must be kept in mind that silage additives cannot perform miracles: good ensiling and desiling techniques remain essential to obtain good silage quality for feed-out to livestock. A well-chosen silage additive, however, can dramatically reduce aerobic deterioration and the negative effects associated with it (Muck and Shinners 2001; Wilkinson 2005).

2.3.2.3. Antagonistic micro-organisms

LAB, yeasts and *Bacilli* have been discussed earlier in this dissertation, assessing their role during the ensiling process. Hereafter, their antagonistic properties towards toxigenic fungi during aerobic deterioration of silages are highlighted.

Besides the production of organic acids, the full antagonistic potential of LAB is not yet known, but it is widely believed that inhibition of mycotoxin production by fungi is due to microbial competition, depletion of nutrients, low pH and production of heat-stable low molecular weight metabolites (e.g. bacteriocins)..

The genus *Lactobacillus* is well known for its antifungal activity. Lavermicocca *et al.* (2000) found that a ten-fold concentrated culture filtrate of *L. plantarum* 21B possesses efficient antifungal activity against *P. roqueforti*, *Aspergillus niger*, *A. flavus* and *Fusarium graminearum*, while Ström *et al.* (2002) described *in vitro* broad-spectrum antifungal activity of *L. plantarum* MiLAB 393 isolated from grass silage against *F. sporotrichioides* and *A. fumigatus*, but not against *P. roqueforti*. Gourama and Bullerman (1997) have isolated a *L. casei pseudoplantarum* strain from a silage inoculant, inhibiting the *in vitro* biosynthesis of aflatoxins B1 and G1 by *A. flavus* subsp. *parasiticus*. On the other hand, Luchese and Harrigan (1990) found that co-culturing of *Lactococcus lactis* and *A. parasiticus* increased aflatoxin production compared to single culture of *A. parasiticus*. Karunaratne *et al.* (1990) have tested the effect of individual *Lactobacillus* strains and a silage inoculant comprising three strains of lactic acid bacteria on rice and maize: they observed decreased levels of aflatoxin G1 on maize grains, but increased levels of aflatoxin B1 on rice. Therefore, it can be concluded that the

effect of lactic acid bacteria in preventing growth of toxigenic fungi and production of mycotoxins is not always straightforward, not even in *in vitro* experiments, and should thus be thoroughly evaluated prior to practical application.

Yeasts are well-described as antagonists of spoilage fungi: Petersson and Schnürer (1995) have identified *Pichia anomala*, *P. guilliermondii* and *Saccharomyces cerevisiae* yeasts for the biocontrol of fungal growth in ensiled high-moisture cereals. Besides competition for nutrients, the antifungal activity of yeasts can be ascribed to the production of cell wall degrading enzymes (Droby *et al.* 1989; Jijakli and Lepoivre 1998). Some yeasts can additionally produce so-called “killer proteins” which are lethal to a wide spectrum of silage spoilage yeasts, thus preventing the initiation of aerobic deterioration and subsequent fungal growth in silages (Druvefors and Schnürer 2005; Kitamoto *et al.* 1999; Walker *et al.* 1995).

The production of compounds that display antifungal activity towards mycelial growth by *Bacillus* species is well documented (Munimbazi and Bullerman 1998; Pusey 1989; Zuber *et al.* 1993). Chitarra *et al.* (2003) have described a *Bacillus subtilis supsp. velezensis* strain isolated from pre-harvest maize that produces an antifungal compound inhibiting the germination of *P. roqueforti* conidiospores *in vitro*, which will be discussed in Chapter 5.

2.4. Remediation of mycotoxins in silages

When prevention was unsuccessful and mycotoxins are formed in a given feed commodity before or after ensiling, silage can be remediated prior to feeding to livestock. Adsorption is one strategy, biological degradation is another. Hereafter, both strategies will be discussed. Theoretically, chemical decontamination agents (e.g. ozone, ammonia and calcium hydroxide mono-ethylamine) would also be an option. However, mycotoxin remediating agents must fulfil certain requirements: they must destroy, inactivate or remove mycotoxins; they may not result in the formation of other toxic substances or leave harmful residues in the treated commodity; the nutritional value of the commodity should not be seriously decreased; they should not adversely affect the desirable physical and sensory properties of the commodity; they should be economically feasible and technically applicable in practice. Chemical decontamination does not live up to these requirements, especially towards practical applicability in silages (Doyle *et al.* 1982; Huwig *et al.* 2001; Jard *et al.* 2011; Kabak *et al.* 2006; Karlovsky 1999; Niderkorn 2007; Rustom 1997; Scott 1998).

Sequestration of mycotoxins by **adsorbing agents** reduces their bioavailability and can be accomplished by several types of mycotoxin binders:

Inorganic adsorbants comprise silica-based inorganic compounds (*i.e*. natural clays and synthetic polymers, like hydrated sodium and calcium aluminosilicates, bentonites, zeolites, etc.), activated carbon, micronized dietary fibers (*i.e.* thermally processed plant fibers obtained from cereal bran, carrot, bamboo, etc.), and other polymers like the resin cholestyramine, divinylbenzene-styrene polymers, polyvinylpyrrolidone, humic acid polymers, etc.

Microbial cell walls form an other category of mycotoxin adsorbing agents (Aoudia *et al.* 2009; Boudergue *et al.* 2009; Dalie *et al.* 2010; De Mil *et al.* 2015; Diaz and Smith 2005; Harris *et al.* 1998; Sabater-Vilar *et al.* 2007; Sera *et al.* 2005). Yeasts, like *Saccharomyces cerevisiae*, or their cell walls can be used as adsorbents. Yeast cell walls harbor polysaccharides (glucans, chitin and mannans), proteins and lipids and thus possess numerous different and easy accessible mycotoxin adsorption sites with different adsorption mechanisms (Devegowda *et al.* 1998; Freimund *et al.* 2003; Huwig *et al.* 2001; Jouany *et al.* 2005; Jouany 2007; Raju and Devegowda 2000; Shetty and Jespersen 2006). Some lactic acid bacteria, propionic acid bacteria and *Bifidobacteria* can also bind mycotoxins to their cell wall polysaccharides and peptidoglycan (Batish *et al.* 1997; Dalie *et al.* 2010; Dogi *et al.* 2013; El-Nezami *et al.* 2002; El-Nezami *et al.* 2004; Gourama and Bullerman 1995; Niderkorn *et al.* 2007; Rees 1997).

Most adsorbents need to be added at high levels, and some may have adverse nutritional effects due to binding of vitamins and minerals (Huwig *et al.* 2001; Yiannikouris and Jouany 2002). It is important to control the stability of the binder-mycotoxin complexes in the gastrointestinal tract. Additionally, many fecal excreted binder-mycotoxin complexes can accumulate in manure and harm soils and pastures upon their spreading in the field. Microbial cell walls are biodegradable and do not accumulate in the environment after being excreted by animals (Boudergue *et al.* 2009; De Mil *et al.* 2015; Galvano *et al.* 2001; Jouany 2007; Kolosova and Stroka 2012; Murugesan *et al.* 2015).

Since mycotoxin detoxification methods using adsorbents have their limits, **biological degradation** by microorganisms is a promising alternative. Either the microorganisms themselves or the mycotoxin degrading enzymes that they produce can be applied for the decontamination of feed commodities. Microbial detoxification is an efficient, specific and environmentally friendly method that does not decrease the nutritional value and palatability of the decontaminated feed commodities (Bata and Lásztity 1999; Wu *et al.* 2009). The biological decontamination strategy has been widely studied. It should be emphasized that actual detoxification only occurs when non-toxic degradation products are formed (Boudergue *et al.*

2009; Dolci *et al.* 2011; Kabak *et al.* 2006; Lindgren *et al.* 1985; McCormick 2013; McDonald *et al.* 1991; Vanhoutte *et al.* 2016; Yu *et al.* 2011).

Lactic acid bacteria are interesting candidates for successful implementation as detoxifying mycotoxins because of their essential role in silage. These bacteria are already being used as silage additives to improve silage quality and they can also function as organic mycotoxin binders (Niderkorn *et al.* 2006; Pahlow *et al.* 2003). Weinberg *et al.* (2003) demonstrated that lactic acid bacteria from silage additives can even survive in rumen fluid. The mycotoxin degradation potential of lactic acid bacteria has been investigated by multiple authors, but not concerning mycotoxins produced by *P. roqueforti s.l.*. Moreover, taking into account the low pH values of silages, only a few microorganisms with mycotoxin degrading capacity have been found in literature that can be active at such low pH (Brewer and Taylor 1967; Khan *et al.* 2015b; Motomura *et al.* 2003; Niderkorn *et al.* 2007; Tan *et al.* 2015). Detailed information on biological detoxification of mycotoxins falls beyond the scope of this literature review, but has been described in-depth in the review article “Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: a review” by Wambacq *et al.* (2016).

2.5. Critical remarks on mycotoxin contamination of silages

"Look and you will find it - what is unsought will go undetected." This quote from Sophocles also holds truth for the detection of mycotoxins in silage. Non-targeted studies determining all mycotoxins present in silage samples are rare, if not non-existing. Up to now, several hundreds of mycotoxins are known, but only five of them are regulated for animal feed by EU legislation: regulatory directives are currently only defined for aflatoxin B1, deoxynivalenol, fumonisins B1 and B2, ochratoxin A and zearalenone. For T-2 and HT-2 toxins in cereals and cereal products, indicative levels are determined by the European Commission. The Food and Drug Administration has defined advisory levels for deoxynivalenol and fumonisins, action levels for aflatoxins, but regulatory limits are not set for any mycotoxin in feed yet (FDA mycotoxin guidelines 2011; Devreese *et al.* 2013; European Commission 2006a, 2006b; European Food Safety Authority 2011; Streit *et al.* 2013; Zachariasova *et al.* 2014). So, a big legislative gap is present for mycotoxins in feed. In addition, legislation is hugely based on exposure data and toxicity of sole mycotoxins. Extensive knowledge on the effects of combined exposure of ruminants to multiple mycotoxins remains elusive, as well as more profound insights into the mechanisms by which mycotoxins present in feedstuffs are bound and/or metabolized in the rumen.

Silages are on-farm-produced feed commodities not routinely analyzed for mycotoxins. If routine mycotoxin analysis of silages would be considered in the future, this would be compromised because silages are heterogeneous matrices: fungi and mycotoxins are not homogeneously distributed in silages during the ensiled period and during feed-out. Therefore, variations in the time and area of sampling in the same silo stack can already lead to very different results (Driehuis *et al.* 2008b; Gonzalez Pereyra *et al.* 2011; Tangni *et al.* 2013a; Wilkinson and Davies 2012).

Furthermore, the magnitude of problems arising from mycotoxin contamination of silages is difficult to assess since reports on mould contaminated silages and subsequent mycotoxicosis in various animal species might be biased on multiple levels:

a) The visually non-mouldy or mouldy status of a silage sample has consequences towards which fungi will be isolated and which mycotoxins will be detected upon sampling (Auerbach *et al.* 1998; Declerck *et al.* 2009).

b) Background fungal load, which could potentially grow if air gains access into the silage, should be distinguished from fungal hot-spots in silages (O'Brien *et al.* 2007).

c) High propagule numbers of toxigenic fungi may be relatively harmless under certain storage conditions, but mycotoxins can be produced when the environmental conditions change (Joffe 1986; O'Brien *et al.* 2007).

d) There is not necessarily a direct relationship between the presence of certain toxigenic fungal species and certain mycotoxins in a silage sample taken at a given moment (Barug *et al.* 2006).

All these findings imply that sampling and analyzing silages for fungi and mycotoxins do not necessarily allow one to make a correct assessment of the risks associated with feeding these mycotoxin contaminated feeds to ruminants, given that studies investigating the effects of multi-mycotoxin contaminated feedstuffs on cattle are rare (Abeni *et al.* 2014; Coppock *et al.* 1990; Gallo *et al.* 2015; Kiyothong *et al.* 2012).

Lactic acid bacteria are already being used as silage additives to improve fermentation, but as described earlier these bacteria also have adsorption and degradation capabilities towards mycotoxins. Combining the knowledge gathered from several studies, silage additives could be developed to simultaneously improve the silage quality and eliminate mycotoxins from silages. More extended studies of microorganisms with mycotoxin detoxifying capabilities and their effect in fresh and ensiled forage crops containing a mycotoxin cocktail are also required.

There is an urgent need for in-depth studies thoroughly assessing the combinatory effect of different prevention and remediation strategies towards mycotoxins in forage crops for ensiling, since this can ultimately lead to a healthier diet for cattle and in the same time reduce economic losses. Keeping in mind that some mycotoxins (e.g. aflatoxin M1) could possibly ingress into the human food chain (Galvano *et al.* 1996), such efforts can certainly include benefits for the quality of human food and human health.

3. *P. roqueforti s.l.* as the main fungal silage contaminant

3.1. Systematics

The systematics of the *Penicillium roqueforti* series is summarized in Figure 2.1 (Houbraken *et al.* 2015; Samson *et al.* 2002; Samson *et al.* 2004).

Domain:	*Eukarya*
Kingdom:	*Fungi*
Phylum:	*Ascomycota*
Order:	*Eurotiales*
Genus:	*Penicillium*
Subgenus:	*Penicillium*
Section:	*Roquefortorum*
Series:	*Roqueforti*
Species:	*P. roqueforti s.s.* *P. paneum* *P. carneum* *P. psychrosexualis*

Figure 2.1. *Systematics of the Penicillium roqueforti series.*

Based on phenotypic, biochemical, and genotypic features, four species are defined within the series *Roqueforti*: *P. roqueforti s.s.*, *P. paneum*, *P. carneum* and *P. psychrosexualis*. The three species *P. roqueforti s.s.*, *P. paneum* and *P. carneum* are considered as separate species since 1996 (Boysen *et al.* 1996). A dark green reverse on Potato Dextrose Agar (PDA), Yeast Extract Sucrose agar (YES) and Czapek-Dox Yeast extract Agar (CYA) is distinctive for *P. roqueforti s.s.*, while the other two species have a pale brown colony reverse (Boysen *et al.* 1996; Frisvad and Samson 2004). In 2010, Houbraken *et al.* added a fourth member to the series *Roqueforti*: *P. psychrosexualis*. This species also has a pale colony reverse on YES and CYA. This is illustrated in Figure 2.2.

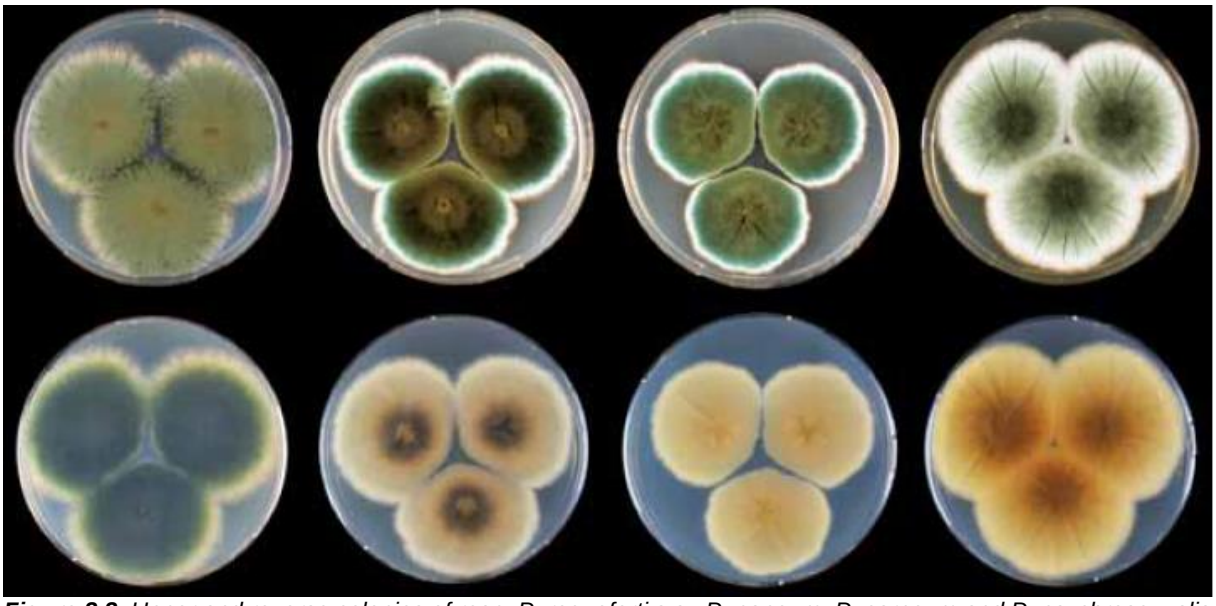


Figure 2.2. *Upper and reverse colonies of resp. P. roqueforti s.s., P. paneum, P. carneum and P. psychrosexualis on Czapek-Dox Yeast extract Agar (CYA) after 7 days at 24 °C (Houbraken et al. 2010).*

Most fungi reproduce asexually by conidia formation, while sexual reproduction involves interaction between individuals. The genus *Penicillium* has been thought for a long time to reproduce solely asexually (*i.e.* anamorph state), with *Eupenicillium* and *Talaromyces* as teleomorph states. Therefore, it was formerly placed into the artificial phylum *Deuteromycota*, for fungi lacking a known sexual reproductive state. However, by molecular phylogenetic analysis, the genus *Penicillium* could be assigned to the phylum *Ascomycota*, which's members produce haploid ascospores through meiosis of a diploid nucleus in an ascus. Asexual conidiospores are produced frequently on typically structured conidiophores, in a strategy to adapt to changes in the environment and to survive in time and/or space (Frisvad and Samson 2004; Huang and Hull 2017; Pitt 1979; Samson and Frisvad 2004).

Sexual reproduction has been observed recently in *Penicillium*, in the species *P. chrysogenum* (Bohm *et al.* 2013) and in *P. roqueforti s.l.* (Houbraken *et al.* 2010; Ropars *et al.* 2012), the latter being illustrated in-depth further in this section.

3.2. Life cycle of the genus *Penicillium*

The life cycle of *Penicillium* is summarized in Figure 2.3.

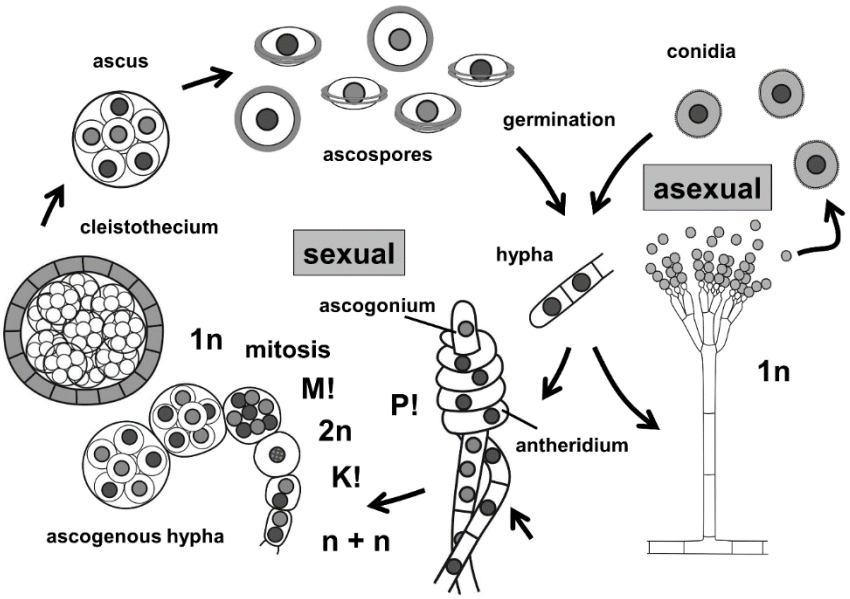


Figure 2.3. *Life cycle of Penicillium (Piepenbring, CC BY-SA).*

Among the *Eukarya*, fungi are unusual in the sense that many species lack a known sexual state and appear to rely solely on asexual reproduction methods.

In case of **asexual reproduction**, the full genome is transmitted from parent to progeny, but genome evolution can occur by horizontal gene transfer, which will be discussed in-depth further in this section.

Sexual reproduction at some point in a eukaryotic lifecycle seems essential for the long-term persistence of species, despite its costs. Two compatible mating types are required. In sexual populations, each parent transmits only one-half of their genes to the progeny. Favorable gene combinations built by past selection can be broken down by recombination. So, sexual reproduction must have multiple evolutionary advantages over strictly asexual reproduction, balancing its costs, e.g. reduction of deleterious mutations and production of recombinant progeny with an increased fitness, better suited to changing environments (Alexopoulos *et al.* 1996; Becks and Agrawal 2012; Dyer and O'Gorman 2011; Lee *et al.* 2010; Lehtonen and Jennions 2012; Ropars *et al.* 2012; Smith 1978).

Fungal sexual development is orchestrated by the *MAT* locus, involved in gamete recognition and mating. Sexual reproduction in heterothallic *Ascomycota*, like *P. roqueforti s.l.*, can only occur between two haploid individuals carrying alternate idiomorphs at the *MAT* locus (Coppin *et al.* 1997; Ropars *et al.* 2012).

Fungal sex comprises three steps, as also mentioned in Figure 2.3: 1) plasmogamy (P!): two compatible mating partners recognize each other and undergo cell fusion, 2) karyogamy (K!): the two parental nuclei fuse (immediately after cell fusion or delayed), and 3) meiosis (M!): haploid recombinant progeny is produced.

3.3. Implications of life cycle on genome dynamics

Horizontal gene transfer (HGT) or lateral gene transfer is defined as the nonsexual exchange and stable integration of genetic material between different strains or species (Doolittle 1999), whereas normal sexual reproduction is designated as vertical gene transfer. HGT is an important mechanism in eukaryotic genome evolution, but only little is known about the behavioral and ecological factors that enhance or discourage it. However, elevated rates of HGT have been coincident with the loss of typical eukaryotic traits, such as sexual reproduction (Boschetti *et al.* 2012; Fitzpatrick 2012; Keeling and Palmer 2008; Wisecaver and Rokas 2015).

HGT requires foreign genetic material to enter the host cell, to be incorporated into the host genome and to successfully express a functional protein, providing a selective advantage to the host species (Fitzpatrick 2012). HGT in fungi involves multiple mechanisms, e.g. transfer of genes, gene clusters or even whole chromosomes, plasmids, mycoviruses and transposable elements. Donors of fungal genetic material acquired through HGT comprise bacteria, plants and other fungi. Evidence suggests that rates of HGT into and between fungi are relatively low. This can be due to several possible barriers to HGT, like e.g. differential intron processing, incompatible gene promotors, an alternative genetic code or gene-silencing mechanisms. When HGT does actually takes place in fungi, it comprises most likely HGT from prokaryotes to fungi or between closely related fungal eukaryotes (Fitzpatrick 2012; Keeling and Palmer 2008; Rosewich and Kistler 2000; Stajich *et al.* 2007).

HGT can have significant impacts on niche specification, disease emergence or shifts in metabolic capabilities. E.g. transferred metabolic gene clusters (*i.e.* physically co-localized genes participating in the same metabolic pathway) can have three evolutionary fates: they can be maintained, they can be lost and they can undergo modification altering their function (Fitzpatrick 2012; Kidwell 1993; Rosewich and Kistler 2000; Wisecaver and Rokas 2015). Indications of HGT have been found for gliotoxin-like MGCs of *Penicillium roqueforti s.s.* and *P. expansum* (Ballester *et al.* 2015).

Horizontal transfer of selfish genetic elements (like mycoviruses, plasmids and transposable elements) has been observed, but the mechanisms of HGT in fungi are not fully understood yet (Fitzpatrick 2012).

Interspecies anastomosis is linked to HGT in fungal species: filamentous fungi frequently fuse conidia and conidial germlings using a specialized hypha known as a conidial anastomosis tube, allowing interconnected germlings to act as a single coordinated individual and also allowing genetic exchange (Fitzpatrick 2012; Read *et al.* 2009; Roca *et al.* 2004).

Transposable elements (TE), displaying autonomous mobility irrespective of their host, are considered as sensitive indicators of the potential for HGT by fungi (Keeling and Palmer 2008). TE are DNA-sequences capable to replicate autonomously and insert at new locations in a genome. TE have been found in every eukaryotic species studied so far. They can have beneficial effects for the host organism, but they can also have detrimental effects due to insertional mutagenesis, genome rearrangements and effects on gene expression. Their replication also increases the overall genome size, imposing an additional cost due to DNA replication. Therefore, they can be catalogued as "genomic parasites" (Horns *et al.* 2012; Wicker *et al.* 2007).

As a protection towards the negative effects of transposable elements, fungi have developed **Repeat-Induced Point (RIP) mutations** as a genome defense mechanism, besides DNA methylation and RNAi (Galagan and Selker 2004).

RIP is an irreversible homology-dependent genome silencing process, occurring in the sexual phase of the fungus' life cycle - after plasmogamy of two cells of opposite mating types, but prior to karyogamy and meiosis. RIP usually targets a specific dinucleotide site and protects the sexual progeny from the expression of duplicated genes (Hane and Oliver 2008; Selker 2002).

RIP specifically detects and mutates duplicated sequences in nuclear DNA and reliably destroys both copies of duplicated genes. All duplications that share more than ca. 80 % nucleotide identity are detected efficiently. RIP identifies duplications greater than ca. 400 bp and introduces C-to-T mutations in cytosines adjacent to RIP target sites, into both copies of the duplicated sequences. RIP-mutated sequences are frequent targets for subsequent DNA-methylation (causing epigenetic silencing) in vegetative cells. Only one enzyme has been identified as part of the RIP machinery, namely the RID protein: a DNA methyltransferase. RIP effectively suppresses the activity of transposons, but may also impair innovation by hampering the evolution of novel gene functions through gene duplication and gradual divergence of biological function (Cambareri *et al.* 1991; Freitag *et al.* 2002; Galagan and Selker 2004; Horns *et al.* 2012; Watters *et al.* 1999).

Until recently, *P. roqueforti s.s.*, *P. paneum* and *P. carneum* were considered to be asexual species, but the discovery of the sexually reproducing relative *P. psychrosexualis* has placed this assumption in another perspective. Currently, there is evidence that *P. roqueforti s.s.* can undergo sexual reproduction: Ropars *et al.* (2012) detected RIP-like footprints in the *P. roqueforti s.s.* genome, as well as the presence of the only gene known to be necessary for RIP, namely *rid*. Furthermore, both idiomorphs of the *MAT* locus have been detected in separate *P. roqueforti s.s.* individuals. Although the identification of mating type genes does not prove the presence of a sexual cycle, it strongly points in this direction. Finally, all the important genes involved in meiosis (which were identified in the heterothallic sexually reproducing fungus *Neurospora crassa*) are also present in the *P. roqueforti s.s.* genome sequence, in a functional state.

Cleistothecia production by *P. psychrosexualis* is induced by low temperatures (9-15 °C) on oatmeal-agar (Houbraken *et al.* 2010). The cleistothecia mature slowly (3-4 months of incubation) and finally produce ellipsoidal ascospores.

The sexual phase of *P. roqueforti s.s.* may be cryptic or may occur under exceptional conditions only. Since it is generally not induced under laboratory conditions, the sexual phase may have been overlooked for a long period of time (Braumann *et al.* 2008; Cheeseman *et al.* 2014; Ropars *et al.* 2012; Ropars *et al.* 2014). However, in 2014, Ropars *et al.* were able to induce sexual reproduction by *P. roqueforti s.s.* in laboratory conditions (Figure 2.4). From the sixteen crosses made, only one resulted in mature ascospores: both parents were isolated from non-cheese environments (*i.e.* wood and stewed fruit), suggesting that lower levels of fertility might occur in domesticated clonal *P. roqueforti s.s.* strains from the cheese environment.

The ability of *P. paneum* and *P. carneum* to reproduce sexually has not been investigated thoroughly, but since they are very closely related to *P. roqueforti s.s.* they probably had or still have this potential. Ropars *et al.* (2012) found that *P. paneum* is heterothallic, just like *P. roqueforti s.s..* Meiotic genes are also present in *P. paneum*, but they seem to evolve more rapidly in *P. paneum* than in *P. roqueforti s.s..*

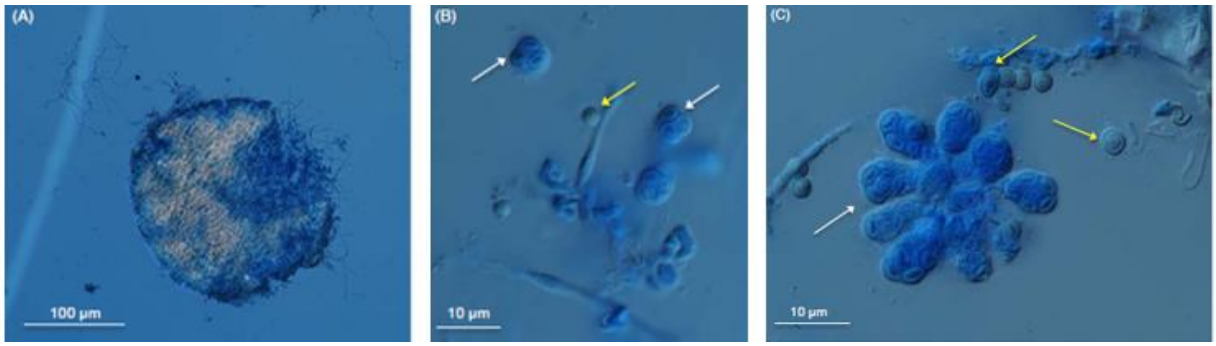


Figure 2.4. *Sexual structures in P. roqueforti s.s.: (A) a cleistothecium, (B-C) white arrows show asci containing ascospores (sexual spores), whereas yellow arrows show conidia (asexual spores) (Ropars et al. 2014).*

Furthermore, Ropars *et al.* (2014) studied the genetic diversity within 114 *P. roqueforti s.s.* isolates from different environments (mostly isolated from dairy environments, but also from silage, wood, bread, …) and were able to detect two clusters. The first cluster only contained cheese strains, most of which carrying the *Wallaby* region (recently shown to have been transferred horizontally between different *Penicillia* from the cheese environment, providing a competitive advantage against other cheese micro-organisms) (Cheeseman *et al.* 2014), while the second cluster comprised strains from diverse environments (silage, wood, cheese, …), none of which carrying the *Wallaby* region. Recombination no longer seems to occur between both clusters, which harbor different frequencies of the *MAT* types. The presence of cheese strains in both clusters indicates that there are major genetic differences between industrial strains, originating from before the domestication of this fungal species for cheese production. Furthermore, cheese strains in the different clusters may have different metabolic properties.

3.4. Growth conditions of the series *Roqueforti*

The series *Roqueforti* within the subgenus *Penicillium* is unique in its high tolerance towards organic acids (like propionic acid, acetic acid and lactic acid), low concentrations of oxygen and high concentrations of carbon dioxide. It probably developed this resistance by competition with lactic acid bacteria during evolution (Frisvad and Samson 2004; Taniwaki *et al.* 2009). Due to their resistance towards organic acids, isolation of isolates belonging to the series *Roqueforti* is facilitated by addition of 0.5 % of acetic acid to the culture medium (MEA or PDA) (Engel and Teuber 1978; Houbraken *et al.* 2010; O'Brien *et al.* 2008).

P. roqueforti s.s. is used industrially in the ripening of various blue cheeses, but is also a common spoilage organism in silages, spoiled bread and refrigerated stored foods and can be isolated from soil and wood. *P. paneum* is frequently isolated from spoiled bread and silages, while *P. carneum* is found mainly on fermented and dried meat products, spoiled bread and beer. All three species have been isolated from mouldy baker's yeast. The habitat of

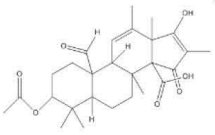
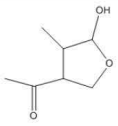
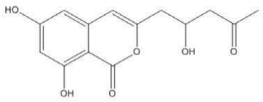
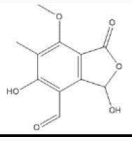
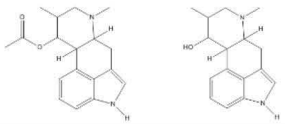
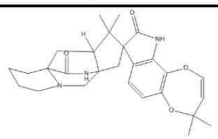
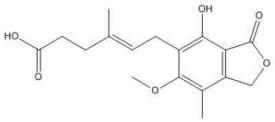
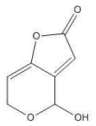
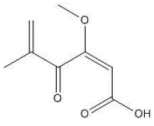
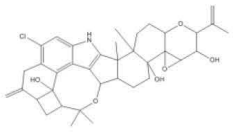
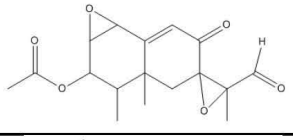
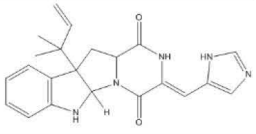
P. psychrosexualis is not well known, but it has been isolated from wooden apple crates (Houbraken *et al.* 2010; Samson and Frisvad 2004; Samson *et al.* 2004). It is noteworthy that these fungal species have all been isolated almost exclusively from human-associated habitats, so the wild ecological reservoirs might not have been identified yet (Gillot *et al.* 2015).

3.5. Mycotoxin production by the *P. roqueforti* series, with roquefortine C as indicator for mycotoxin production

The four *Penicillium* species belonging the series *Roqueforti* each have a unique mycotoxin production potential. Table 2.4 indicates per species which mycotoxins have been detected in fungal cultures, and also demonstrates the diverse structures of the potentially produced mycotoxins.

Andrastatin A, mycophenolic acid and ROC are produced by all four species of the *P. roqueforti* series. ROC is considered to be an indicator for mycotoxin production by *P. roqueforti s.l.* in silages since it is consistently produced, it is stable at low pH-values (as occurring in silage) and does not interact with other silage compounds, like amino acids and ammonia (Auerbach *et al.* 1998; Fernandez-Bodega *et al.* 2009; Gillot *et al.* 2017; O'Brien *et al.* 2006; Scott 2004). The role of ROC as an indicator for mycotoxin production in other matrices than silages has been confirmed by Tiwary *et al.* (2009). Therefore, in this dissertation, focus will be on this particular mycotoxin produced by *P. roqueforti s.l.*.

Table 2.4. *Mycotoxin production potential (not limitative) per species of the P. roqueforti series.*

Mycotoxins	production possible by …		Structure
andrastatin A	*P. roqueforti s.s.*	yes	
	P. paneum	yes	
	P. carneum	yes	
	P. psychrosexualis	yes	
botryodiploidin	*P. roqueforti s.s.*	no	
	P. paneum	yes	
	P. carneum	no	
	P. psychrosexualis	no	
citreoisocoumarin	*P. roqueforti s.s.*	yes	
	P. paneum	yes	
	P. carneum	yes	
	P. psychrosexualis	no	
cyclopaldic acid	*P. roqueforti s.s.*	no	
	P. paneum	no	
	P. carneum	yes	
	P. psychrosexualis	no	
isofumigaclavine A & B°	*P. roqueforti s.s.*	yes	
	P. paneum	no	
	P. carneum	yes	
	P. psychrosexualis	no	
marcfortin B	*P. roqueforti s.s.*	no	
	P. paneum	yes	
	P. carneum	no	
	P. psychrosexualis	no	
mycophenolic acid	*P. roqueforti s.s.*	yes	
	P. paneum	yes	
	P. carneum	yes	
	P. psychrosexualis	yes	
patulin	*P. roqueforti s.s.*	no	
	P. paneum	yes	
	P. carneum	yes	
	P. psychrosexualis	yes	
penicillic acid	*P. roqueforti s.s.*	no	
	P. paneum	no	
	P. carneum	yes	
	P. psychrosexualis	no	
penitrem A	*P. roqueforti s.s.*	no	
	P. paneum	yes	
	P. carneum	yes	
	P. psychrosexualis	no	
PR-toxin (& eremofortins)	*P. roqueforti s.s.*	yes	
	P. paneum	no	
	P. carneum	no	
	P. psychrosexualis	no	
roquefortine C (& roquefortine D)	*P. roqueforti s.s.*	yes	
	P. paneum	yes	
	P. carneum	yes	
	P. psychrosexualis	yes	

° Synonyms: roquefortine A & B

(Fontaine *et al.* 2015; Frisvad *et al.* 2004; Gallo *et al.* 2015; Houbraken *et al.* 2010; Martin and Liras 2017; Nielsen *et al.* 2006; O'Brien *et al.* 2006; Storm *et al.* 2014)

3.5.1. Introduction on genetic regulation of secondary metabolite production

Primary metabolites are directly required to ensure growth, in contrast to secondary metabolites. Secondary metabolites (SM) are not essential for normal growth and comprise a heterogeneous group of low molecular mass compounds playing a role in multiple cellular processes, like development and intercellular communication (Brakhage 2013; Fox and Howlett 2008; Keller *et al.* 2005). In fungi, secondary metabolism is commonly associated with sporulation processes and can be placed in three broad categories:
1) sporulation activating metabolites, influencing the development of the producing organism and neighboring members of the same species.
2) pigments required for sporulation structures, e.g. melanin as UV-protection.
3) toxic metabolites secreted by growing fungi, *i.e.* **mycotoxins**.
Mycotoxins play no obvious role in sporulation or spore protection, but are secreted into the environment at a time in the fungal life cycle corresponding to sporulation. They have a multiplicity of functions, e.g. they serve as chemical signals for intra-species and inter-species communications. The biological role of many mycotoxins remains elusive, but it is generally believed that mycotoxins are produced to protect their producers better against other organisms sharing the same ecological niche (Brakhage 2013; Calvo *et al.* 2002; Fox and Howlett 2008; Martin *et al.* 1999; Reverberi *et al.* 2010).

Generally, the biosynthesis genes for SM production by fungi are located in clusters, acting as single genetic loci. These metabolic gene clusters (MGCs) have evolved in individual fungal lineages in response to specific ecological needs (Brakhage 2013; Palmer and Keller 2010; Wisecaver and Rokas 2015; Yin and Keller 2011).

Biosynthesis of secondary metabolites is orchestrated by a complex regulatory network with hierarchical levels, generating distinctive gene expression profiles for different SM gene clusters. Chromatin structure and epigenetic regulation play a significant role in SM production, determining whether or not gene clusters are accessible by transcription factors. At a transcriptional level, global regulatory proteins play a significant role along with pathway-specific regulators. Post-transcriptionally, RNA can also influence SM biosynthesis (Brakhage 2013; Georgianna *et al.* 2010; Grewal and Moazed 2003; Keller *et al.* 2005).
Global regulation of SM biosynthesis is mediated by globally acting broad-domain transcription factors (TF's), encoded by genes that do not belong to any MGC and which also regulate a number of genes that are not involved in secondary metabolism. Several environmental conditions, e.g. carbon and nitrogen sources, trigger global TF's to positively or negatively regulate SM biosynthesis (Keller *et al.* 2005). Pathway-specific transcription factors are usually

embedded in SM biosynthesis gene clusters, positively regulating gene expression. The amount of nutrients available, the ambient temperature, water activity and oxygen are the most important factors governing the growth and mycotoxin production of fungi (Kokkonen *et al.* 2005a; Samson *et al.* 2002).

Fungi can use a diverse array of compounds as nitrogen sources and are capable of expressing the catabolic enzymes of many different pathways upon demand. Certain compounds are used preferentially and are therefore called primary nitrogen sources: ammonia, glutamine and glutamate. When these primary nitrogen sources are unavailable or are present at concentrations low enough to limit growth, many different compounds can be used as secondary nitrogen sources (e.g. nitrate, nitrite, most amino acids, peptides and proteins). Utilization of secondary nitrogen sources is highly regulated and nearly always requires the synthesis of a set of pathway-specific catabolic enzymes and permeases which are otherwise subject to nitrogen catabolite repression, preventing such gene activation in the presence of primary nitrogen sources. The de novo synthesis of pathway-specific catabolic enzymes and permeases is controlled at transcriptional level and generally requires two distinct positive signals: a global signal indicating nitrogen derepression, and a pathway-specific signal indicating the presence of a substrate or an intermediate of that pathway. This two-step requirement permits the selective expression of just the enzymes of a specific catabolic pathway from many potential candidates within the nitrogen regulator circuit. In several fungal genomes, G-protein coupled receptors with putative nitrogen-sensing function have been identified. Subsequent G-protein mediated signaling not only triggers the production of suitable catabolic enzymes, but also the production of secondary metabolites (Garcia-Rico *et al.* 2009; Li *et al.* 2007; Marzluf 1997).

In analogy, carbon source mediated regulation of secondary metabolite synthesis is strongly influenced by carbon catabolite repression. Via carbon catabolite repression, readily metabolizable carbohydrates (like glucose, maltose, fructose and galactose) repress the synthesis of enzymes related to the catabolism of alternative carbon sources, ensuring preferential utilization of the most favored carbon sources present (Jiao *et al.* 2008; Martin *et al.* 1999).

3.5.2. Structure and biosynthesis of roquefortine C

ROC is a **C-3 prenylated indole alkaloid** with a **diketopiperazine structure**, derived from the amino-acids tryptophan and histidine (Ohmomo *et al.* 1979; Scott *et al.* 1977). The pathway of ROC-biosynthesis by *P. roqueforti s.s.* has been described recently (Kosalkova *et al.* 2015) and is presented in Figure 2.5. Roquefortine D is an intermediate product in the biosynthesis pathway of ROC, often co-occurring with ROC in low concentrations. Some authors make

reference to the mycotoxins roquefortine A and B, but these compounds are structurally not related to ROC and roquefortine D. In fact, they are stereoisomers of fumigaclavine A and B (produced by *Aspergillus flavus*) and are therefore preferentially designated as isofumigaclavine A and B (Clark *et al.* 2005; Cole *et al.* 1983; Kozlovskii *et al.* 2012; Scott 1981).

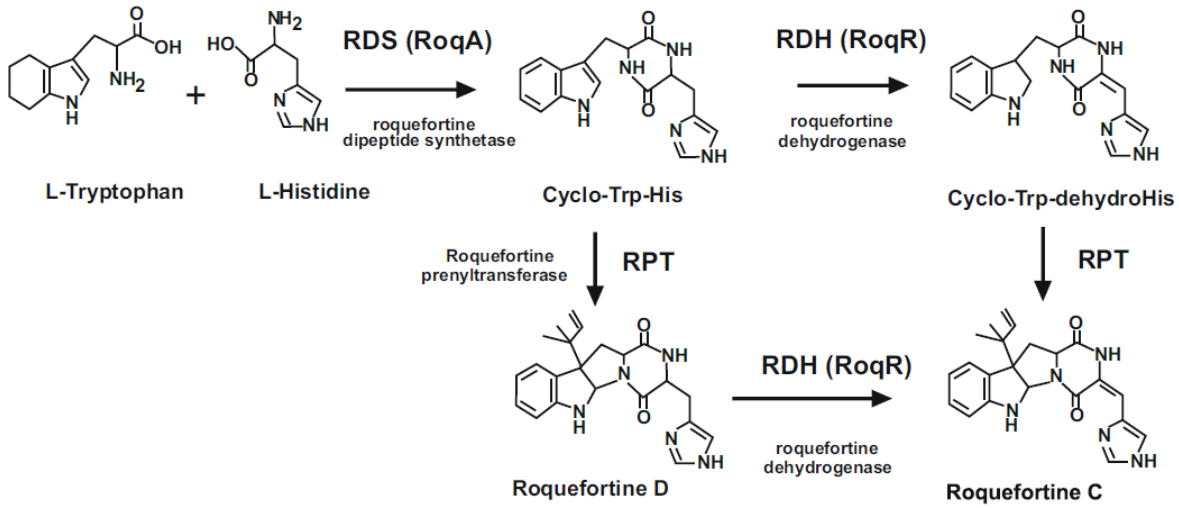


Figure 2.5. *Roquefortine C biosynthesis pathway in Penicillium roqueforti s.s. (Kosalkova et al. 2015).*

The *P. roqueforti s.s.* roquefortine gene cluster contains four genes encoding the enzymes roquefortine dipeptide synthetase (*rds*), roquefortine D hydrogenase (*rdh*), roquefortine prenyltransferase (*rpt*) and a methyltransferase (*gmt*) (Kosalkova *et al.* 2015).

Roquefortine-type mycotoxins are produced by several *Penicillium* species besides *P. roqueforti s.s., P. paneum* and *P. carneum*, like *P. chrysogenum*, *P. expansum*, *P. glandicola* and *P. griseofulvum*. It remains elusive wether the biosynthetic pathway for the production of roquefortine-type mycotoxins is identical or partially identical in these fungal species (Clark *et al.* 2005; Cole *et al.* 1983; Frisvad *et al.* 2004; Kosalkova *et al.* 2015; Kozlovskii *et al.* 2012; Scott 1981). However, the four-gene encoded pathway of ROC production by *P. roqueforti s.s.* appears to be a short version of the biosynthesis pathway of ROC and meleagrin in *P. chrysogenum*, where seven within-cluster genes are involved. In *P. chrysogenum*, these genes are usually poorly expressed since the ROC production by *P. chrysogenum* is low compared to *P. roqueforti s.s.*. The *P. roqueforti s.s.* species has evolved to this simpler ROC gene cluster under natural conditions, not determined by recent industrial cheese-making practices (Garcia-Rico *et al.* 2008; Garcia-Rico *et al.* 2009; Kosalkova *et al.* 2015).

3.5.3. Function of roquefortine C

The use of alkaloids as exclusive, transportable nitrogen source has been described in plants. Similarly, ROC can be produced by toxigenic fungi as a transportable **extracellular nitrogen reserve**. It is excreted by an energy-independent mechanism, and can be resorbed by both energy-dependent as energy-independent mechanisms. Energy-independent transport is based on passive diffusion, whereas the energy-dependent uptake mechanism is a succinate-dependent general amino-acid transport system. It remains elusive whether ROC is resorbed as a whole or after breakdown, but it is actually metabolized by growing mycelia as well as germinating spores (Boichenko *et al.* 2002a; Boichenko *et al.* 2002b; Kulakovskaya *et al.* 1997; Overy *et al.* 2005; Reshetilova *et al.* 1995). The role of ROC as extracellular nitrogen reserve will be assessed in Chapter 4.

3.5.4. Toxicity of roquefortine C

In eukaryotes, exogenous substances like mycotoxins can exert therapeutic and/or toxic effects, depending on their ability to cross the cytoplasmic membrane. If they succeed in crossing the cytoplasmic membrane (*i.e.* if they are rather hydrophobic), such xenobiotic compounds bind to their specific receptors in the cytoplasm or the nucleus, and they can be attacked by detoxification proteins: cytochrome P450s on the one hand and an active P-glycoprotein transport system on the other hand. Both are membrane-bound systems: P-glycoproteins are ATP-binding cassette transporters, whereas cytochrome P450 proteins are anchored in membranes and metabolize specific xenobiotics via reductase or oxidase enzymes. ROC **inhibits the activity of some cytochrome P450s** by binding to the haem-group with its imidazole moiety, but on the other hand ROC activates P-glycoproteins at concentrations exceeding 50 µM (Aninat *et al.* 2001; Aninat *et al.* 2005; Erhardt 2003).

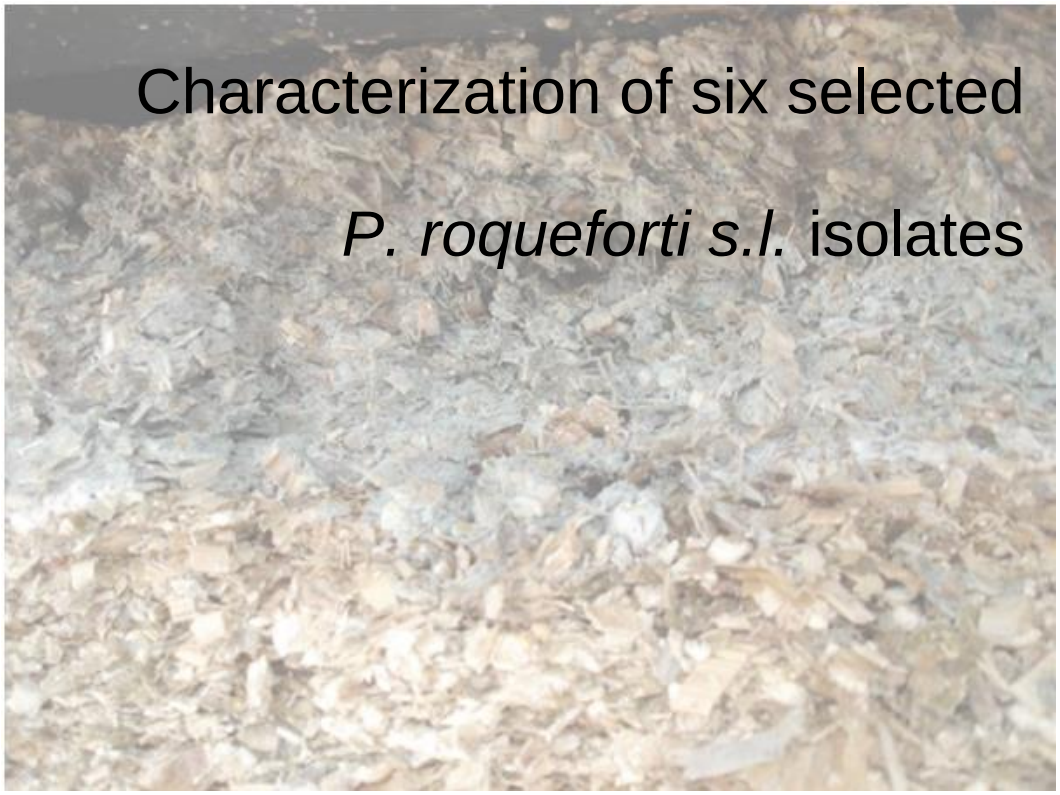
ROC has **bacteriostatic activity towards Gram positive bacteria**, protecting the fungus's accumulated extracellular nitrogen reserve against bacterial competitors (Kopp-Holtwiesche and Rehm 1990). As a consequence, consumption of ROC contaminated feed by ruminants might disturb the ruminal microflora (as already discussed in section 2.1). No publications were found ascribing antifungal properties to ROC.

ROC has **neurotoxic properties** upon ingestion, possibly causing paralysis (Arnold *et al.* 1978; Braselton and Rumler 1996; Tiwary *et al.* 2009; Wagener *et al.* 1980). Not much information is available on the toxicity of ROC upon ingestion. To date, no toxicity studies meeting good laboratory practice standards have been performed. Häggblom (1990) reported

that cows showed reversible paralytic effects upon ingestion of *P. roqueforti s.l.* contaminated feed grain containing 25.3 mg of ROC per kg dry matter. Additionally, other clinical symptoms included inappetence, ketosis, mastitis and abortions. The acute toxicity of ROC is rather limited, e.g. oral administration to sheep at concentrations up to 25 mg ROC per kg body weight for 16 - 18 days failed to trigger any negative effects on behavior, hematology or reproduction effects. *Post mortem* (histo-)pathological examination revealed no lesions which could be attributed to ROC administration. ROC was absent in the sheep's urine, while the highest amounts were found in the rumen fluid and the faeces. Residues of the unchanged compound were detected in the liver (1.15 mg/kg), kidneys (0.15 mg/kg) and bile (0.12 mg/kg), while traces were found in muscle, fat, lung and heart (Tüller *et al.* 1998).
The low toxicity of ROC compared to other mycotoxins is not surprising, since *P. roqueforti s.s.* is used in the production of blue cheese for human consumption. However, under normal cheese production conditions, ROC is not produced or only in minor concentrations (Häggblom 1990; Kokkonen *et al.* 2005b; Lopez-Diaz *et al.* 1996).

Ingestion is not the only route by which organisms can be intoxicated with ROC: inhalation of spores containing ROC is a different mode of exposure (Abbott 2002; Polizzi *et al.* 2009). Intratracheal exposure of mice to ROC provoked inflammatory lung responses (Rand *et al.* 2005). Despite that the toxicity of ROC upon inhalation is very poorly known, it can be linked to the "sick building syndrome" associated with mouldy building interiors due to fungal colonization of building materials: toxigenic moulds can produce non-volatile mycotoxins concentrated in mycelia and spores (Abbott 2002; Cho *et al.* 2005; de la Campa *et al.* 2007; Delmulle 2009; Norback 2009; Polizzi *et al.* 2009; Straus 2009).

Chapter 3



Characterization of six selected *P. roqueforti s.l.* isolates

1. Introduction

In the context of the practice-orientated research project “Identification and control of fungal development in conserved roughages” (financed by the University College Ghent, 2006-2009), a *P. roqueforti s.l.* collection was initiated at the Phytopathology lab of the Bottelare research station. During this PhD research, the collection was significantly extended with *P. roqueforti s.l.* isolates obtained at the ensiling and desiling of grass and whole-crop maize silages, isolated from air (using a MAS-100 Eco air sampler holding a 90-mm diameter Petri dish containing Potato Dextrose Agar supplemented with 5 ml acetic acid per liter (PDAA)) as well as from soil, fresh crop and silage samples (by dilution plating and direct plating on PDAA, as described in Annex 1).

Macromorphological observation of the more than 250 *P. roqueforti s.l.* isolates in collection resulted in a set of approx. 25 divergent isolates from various origins that have been used for in several experiments: fungal growth was monitored and ROC production was screened qualitatively by the Ehrlich reaction. Quantification of ROC by LC-MS/MS was not performed on all isolates in the context of these experiments.
For Ehrlich’s test, a piece of filter paper drenched in Ehrlich’s reagent (prepared as described in Annex 1) was gently pushed against the mycelial side of a 9-mm diameter mycelium plug. Purple coloration within 2-10 min indicates the presence of alkaloids: in *P. roqueforti s.l.* cultures these are very likely ROC and its derivatives, but isofumigaclavins may also be present in *P. roqueforti s.s.* cultures (cfr. table 2.4). This was kept in mind at the interpretation of the screening results, but no alternative screening technique for ROC was applicable. An image of Ehrlich’s test for alkaloids (four replicates per object) is presented in Figure 3.1.

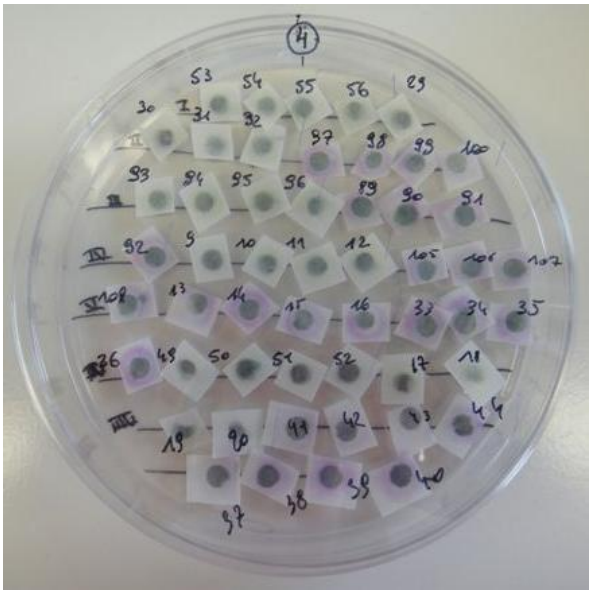


Figure 3.1. *Ehrlich’s test for alkaloid production.*

A selection of six *P. roqueforti s.l.* isolates out of the 25 divergent isolates was made based on the obtained growth and Ehrlich reaction data (Debruyne 2013; Lefebvre 2014; Vandekerkhove 2012). In the context of the current chapter, the six isolates were characterized in-depth by *in vitro* experiments: four *P. roqueforti s.s.* isolates and two *P. paneum* isolates, as summarized in Table 3.1. The reason to choose more *P. roqueforti s.s*. isolates than *P. paneum* isolates was that *P. roqueforti s.s.* is better characterized and more studied (e.g. in the context of its role in blue-cheese production).

Table 3.1. *Six Penicillium roqueforti s.l. isolates selected for characterization.*

Fungal species	Isolate	Description
P. roqueforti s.s.	MUCL 46746	reference, isolated from silage in Belgium
	CBS 116877	reference, isolated from grass silage in The Netherlands
	2008-20	isolated from grass silage in Belgium
	2011-S1-G8	isolated from grass prior to ensiling in Belgium
P. paneum	CBS 112295	reference, isolated from grass silage (pH 4.4) in Sweden
	2011-S4-G8	isolated from grass prior to ensiling in Belgium

At first, to determine/confirm *P. roqueforti s.s.* or *P. paneum* species identity of the six selected *P. roqueforti s.l.* isolates and to gain insight into sequence conservancy, partial sequencing of the beta-tubulin gene was executed.

Subsequently, to gain more information on the variation between and within the species *P. roqueforti s.s.* and *P. paneum*, the six selected isolates were grown on four different agar media: Potato Dextrose Agar (PDA), Yeast Extract Sucrose agar (YES), Czapek-Dox Yeast extract Agar (CYA) and a mineral medium (MinM). After an aerobic incubation period of 7 days at 25°C in the dark, macromorphology, growth and ROC production were evaluated.

Finally, to assess effect of different sole carbon sources on growth of and ROC production by the six selected *P. roqueforti s.l.* isolates, variations were made of mineral medium (MinM) by replacing sucrose as a single carbon source by other carbon sources present in silages: glucose, fructose, mannitol, lactic acid, acetic acid, succinic acid or ethanol.

2. Partial sequencing of the beta-tubulin gene

Partial beta-tubulin gene sequence analysis appears to be a good method to distinguish the members of the *P. roqueforti* series (Boysen *et al.* 1996; Boysen *et al.* 2000; O'Brien *et al.* 2008; Samson *et al.* 2004; Yin *et al.* 2017). Therefore, this particular approach was chosen for molecular identification of *P. roqueforti s.l.* species on the one hand, and to evaluate the conservancy of the beta-tubulin sequence of isolates belonging to the *P. roqueforti* series and some other *Penicillium* species on the other hand.

2.1. Materials and methods

Germinating monospores of the six selected *P. roqueforti s.l.* isolates (obtained by plating a dilution series of spore solution (Annex 1) on PDAA) were transferred to a standard 90-mm diameter Petri Dish containing approx. 20 ml of Potato Dextrose Broth (PDB). The Petri dishes were incubated statically in the dark at 25 °C during seven days. The mycelial mat formed on the PDB surface was transferred to a 2-ml Eppendorf tube. After centrifugation of the Eppendorf tubes at 12 500 rpm during 10 min, the PDB was removed and the Eppendorf tubes were stored at -80 °C prior to freeze-drying (as described in Annex 1). The freeze-dried mycelium was ground to powder with liquid nitrogen and subjected to genomic DNA-extraction by a modified CTAB-method (Saghaimaroof et al, 1984) as described by Audenaert *et al.* (2009).

The Polymerase Chain Reaction (PCR) was performed on the DNA-samples with beta-tubulin specific primers: *Bt2a* (5'-GGT AAC CAA ATC GGT GCT GCT TTC) and *Bt2b* (5'-ACC CTC AGT GTA GTG ACC CTT GGC) (Glass and Donaldson 1995). A 25 µl reaction mixture was made containing 1.5 units of GoTaq G2 DNA polymerase, 1 µM of both primers and 0.2 mM dNTP each in GoTaq reaction buffer (containing 1.5 mM $MgCl_2$). The PCR was carried out in the GeneAmp PCR 9700 system (Applied Biosystems) for 35 cycles of 30 sec at 95 °C (denaturation), 30 sec at 58 °C (annealing) and 30 sec at 72 °C (extension), with an initial denaturation step of 3 min at 95 °C and a final extension step of 5 min at 72 °C. The amplicons were visualized on 1.5 % (w/v) agarose gel using the GelDoc XR+ (Bio-Rad) and were sequenced in both directions by Macrogen (The Netherlands).

The forward sequences of the six selected *P. roqueforti s.l. i*solates were complemented with partial beta-tubulin sequences of two *P. roqueforti s.l.* isolates isolated from air and soil, and type strains of *P. roqueforti s.s.*, *P. paneum*, P. carneum, *P. psychrosexualis* (*i.e.* all members of the *P. roqueforti* series) and of *P. brevicompactum*, *P. camemberti*, *P. chrysogenum*,

P. expansum, *P. glandicola* and *P. griseofulvum* (http://www.ncbi.nlm.nih.gov/genbank). Sequence alignments were generated and manually refined in BioEdit 7.0.9. The phylogenetic relationships among the isolates were inferred using R-software, using maximum likelihood trees based on K2P distances (Kimura 2-parameter distance). Branch support of the groupings obtained in the unrooted tree was assessed by bootstrapping with 1000 replicates. Bootstrap values ≥ 75 % were considered as significant and are indicated in the maximum likelihood tree (Landschoot *et al.* 2017).

2.2. Results

The maximum likelihood tree generated based on partial beta-tubuline sequence information of 25 *Penicillium* isolates is presented in Figure 3.2. Representing the *P. roqueforti* series, nine *P. roqueforti s.s.*, five *P. paneum*, three *P. carneum* and two *P. psychrosexualis* isolates from different origins were included. Isolates of *P. chrysogenum*, *P. expansum*, *P. glandicola* and *P. griseofulvum* were added because these species can also produce roquefortine-type mycotoxins (Frisvad *et al.* 2004; Kosalkova *et al.* 2015), while *P. camemberti* was included for its role in cheese manufacturing. Finally, a *P. brevicompactum* isolate was included as a more distant *Penicillium* species.

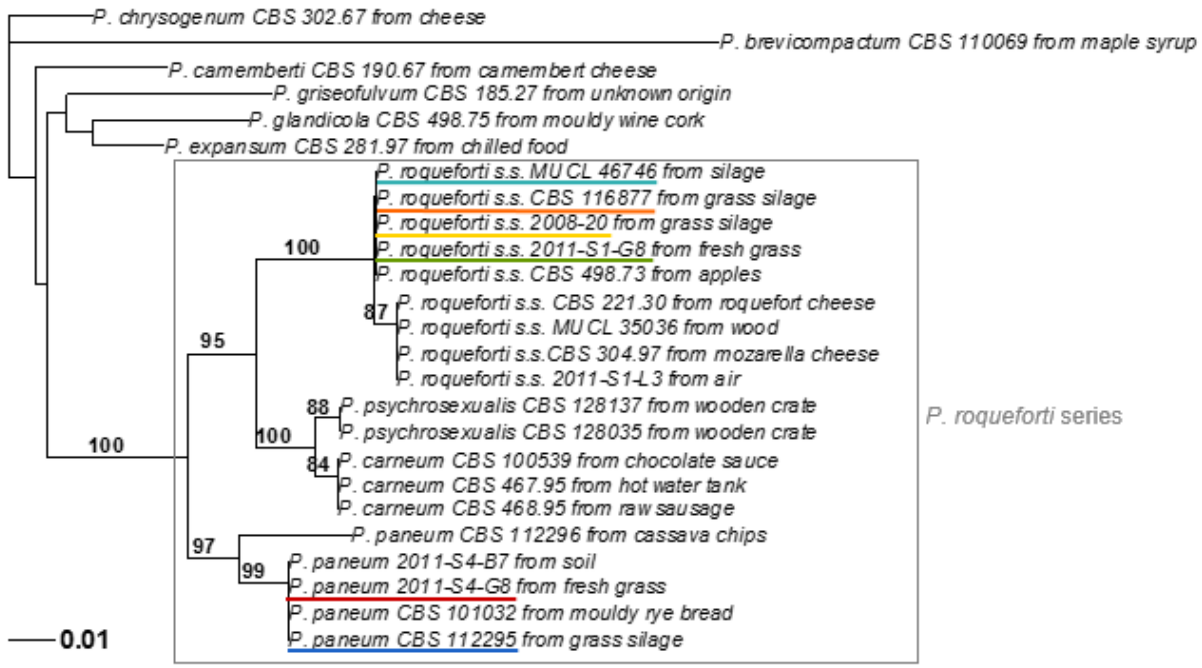


Figure 3.2. *Maximum likelihood tree for a 400 bp-fragment of the beta-tubulin gene: bootstrap values ≥ 75 % were considered as significant and are indicated.*

The close relationship between the four member species of the *P. roqueforti series* is nicely illustrated by a bootstrap value of 100 %. Within the *P. roqueforti* series, it is striking that *P. roqueforti s.s.* is more closely related to *P. psychrosexualis* and *P. carneum* than to *P. paneum*. Intra-species differences in beta-tubulin sequence are very limited.

The maximum likelihood tree confirms that *P. brevicompactum* is not so closely related to the *P. roqueforti* series as the other *Penicillium* species included, producing similar mycotoxins.

The obtained tree confirms the findings of Samson *et al.* 2004), that beta-tubulin sequences are excellent species markers for the subgenus *Penicillium*: the phylogeny for the *Roquefortorum* section (100 %) was robust, with excellent bootstrap support for all four included species. *P. expansum*, *P. glandicola* and *P. griseofulvum*, belonging to the section *Penicillium*, clustered together. *P. camemberti* is a member of the *Viridicata* section, while *P. brevicompactum* and *P. chrysogenum* respectively belong to the sections *Coronata* and *Chrysogena*.

Looking further than the beta-tubulin gene, it is interesting that the total genome size of *P. roqueforti s.s.* and *P. paneum*, resp. 29.0 and 26.6 Mbp, differs somewhat. The number of open reading frames is approximately 12 250 for both species (Nielsen *et al.* 2017).

3. Macromorphological characterization, growth and production of roquefortine C by *P. roqueforti s.l.* on four different agar media

To gain more insight into variations between the species *P. roqueforti s.s*. and *P. paneum* and within isolates per species, the six selected *P. roqueforti s.l.* isolates were grown on four distinctive agar media for monitoring of macromorphology, growth and ROC production.

PDA, YES and CYA are classical media for evaluating fungal growth and development, but their exact composition varies from batch to batch. E.g. it has been found that the use of different yeast extracts results in different amounts of secondary metabolites produced by *Fusarium* (Samson and Frisvad 2004; Sorensen and Sondergaard 2014), which may also be the case for *P. roqueforti s.l.*. Mineral media have the advantage that their composition is quasi stable over different batches, so a mineral medium was also included in this experiment.

PDA has been frequently used in several experiments in the context of this PhD research and was specifically included in this experiment because Gillot *et al.* (2015) found it to be a highly discriminative medium.
YES and CYA media both contain yeast extract (providing an organic nitrogen source, as well as sugars, minerals, trace elements and B vitamins) and are highly nutritious media, recommended for secondary metabolite analysis (Bridson and Brecker 1970; Fontaine *et al.* 2015; Gillot *et al.* 2017; Kokkonen *et al.* 2005a; Scott *et al.* 1977; Sumarah *et al.* 2005).
A liquid mineral medium inducing mycotoxin production in *Fusarium graminearum* was described by Gardiner *et al.* (2009). The composition of this mineral medium has been adapted through a preliminary experiment to better suit *P. roqueforti s.l.* growth (data not published): in the MinM, sodium nitrate as a nitrogen source was replaced by ammonium nitrate (Lawrence and Hawke 1968) and the pH of the medium was brought to 5.5 instead of 6.5, as summarized in Table 3.2.

3.1. Materials and methods

The agar media were prepared as described in Annex 1:

1. Potato Dextrose Agar (PDA) – pH 5.50 at 20 °C
2. Yeast Extract Sucrose agar (YES) – pH 5.60 at 20 °C
3. Czapek-Dox Yeast extract Agar (CYA) – pH 7.00 at 20 °C
4. Mineral medium adapted for *P. roqueforti s.l.* (MinM), containing sucrose as sole carbon source – pH 5.50 at 20 °C

Table 3.2. *Composition of the mineral medium (per liter).*

Mineral medium by Gardiner *et al.* (2009)	**MinM adapted for *P. roqueforti s.l.***
30 g sucrose	
1 g potassium phosphate (KH_2PO_4)	
0.5 g magnesium phosphate ($MgPO_4.7H_2O$)	
0.5 g potassium chloride	
0.010 g iron sulphate ($FeSO_4.7H_2O$)	
200 µl of trace element solution*	
2 g sodium nitrate	2 g ammonia nitrate
0,03% Phytagel	15 g agar
pH to 6.5	pH to 5.5

* trace element solution content per 100 ml: 5 g citric acid - 5 g $ZnSO_4.7H_2O$ - 0.25 g $CuSO_4.5H_2O$ - 0.05 g $MnSO_4.H_2O$ - 0.05 g H_3BO_3 - 0.05 g $NaMoO_4.2H_2O$

All media were poured into standard 90-mm diameter Petri dishes, each containing approx. 20 ml medium. After solidification of the agar medium, a 5-mm opening was made in the center of each plate with a cork borer. In this opening, 20 µl of spore solution (containing $0.5*10^6$ spores per ml, prepared as described in Annex 1) of one of the six selected *P. roqueforti s.l.* isolates was brought. For each of the four agar media and each of the six fungal isolates, four plates were prepared. After sealing the plates with parafilm, they were aerobically incubated upright in the dark at 25 °C during seven days.

After seven days, macromorphology was registered by taking photographs of the upper and reverse side of the plates.

Fungal growth was quantified per object (N = 3) by registration of the colony diameter at two orthogonal positions on the reverse side of the agar plates. The corresponding growth area was calculated.

Quantification of ROC by LC-MS/MS (as described in Annex 1) was executed per object (N = 3) on a pooled sample of three 9-mm diameter mycelium plugs taken near the center, at the outer mycelial growth and at an intermediate position. The plugs were collected in a 1.5-ml Eppendorf tube, which was stored at -20 °C prior to freeze-drying just before ROC analysis. The empty, filled and freeze-dried weight of each Eppendorf tube was noted. Frisvad *et al.* (2004) confirmed the findings of Smedsgaard 1997) that even three agar plugs are sufficient for mycotoxin detection, so this methodology for sampling agar media has been used systematically for *in vitro* experiments comprising ROC quantification. The ROC content detected in the three mycelium plugs (representing an area of 1.91 cm^2) was recalculated to the ROC content per cm^2 of growth area.

Data on fungal growth and ROC were statistically analyzed as described in Annex 1.

3.2. Results

3.2.1. Macromorphology

Macromorphology was highly variable across the six selected *P. roqueforti s.l.* isolates on the different agar media, as visualized in Figure 3.3.

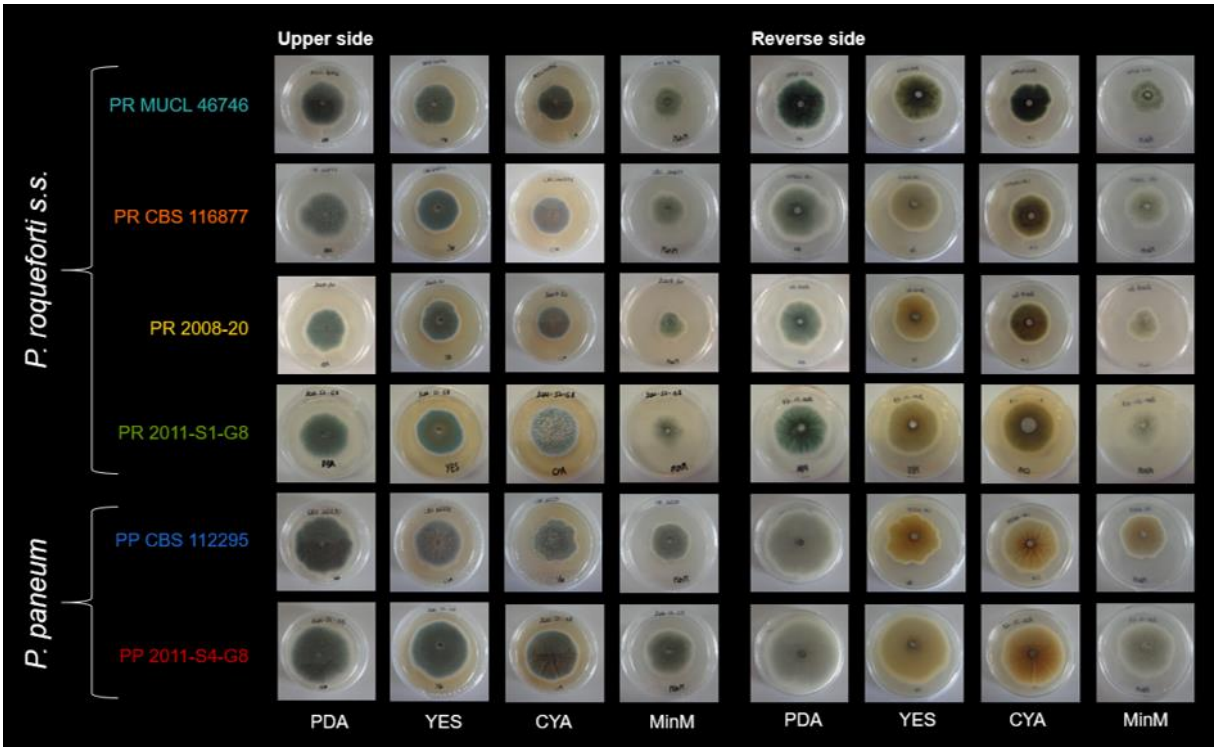


Figure 3.3. *Macromorphological characterization of six P. roqueforti s.l. isolates on four different agar media after seven days of aerobic incubation at 25°C.*

Visual observation suggests that the *P. paneum* isolates displayed stronger growth than *P. roqueforti s.s.* isolates during the seven-days incubation period.

Especially the reverse side of the plates showed large variation in colors between the six selected isolates on all four agar media. *P. paneum* isolates had a pale reverse on PDA, while *P. roqueforti s.s.* isolates should be characterized by a dark green reverse color, as described earlier in chapter 2. However, this property is not so clear for PR CBS 16877 and PR 2008-20, probably due to frequent sub-culturing (Samson and Frisvad 2004).

3.2.3. Fungal growth and roquefortine C production

The mean values per object for fungal growth, expressed as growth area, and ROC production are presented in Figure 3.4.

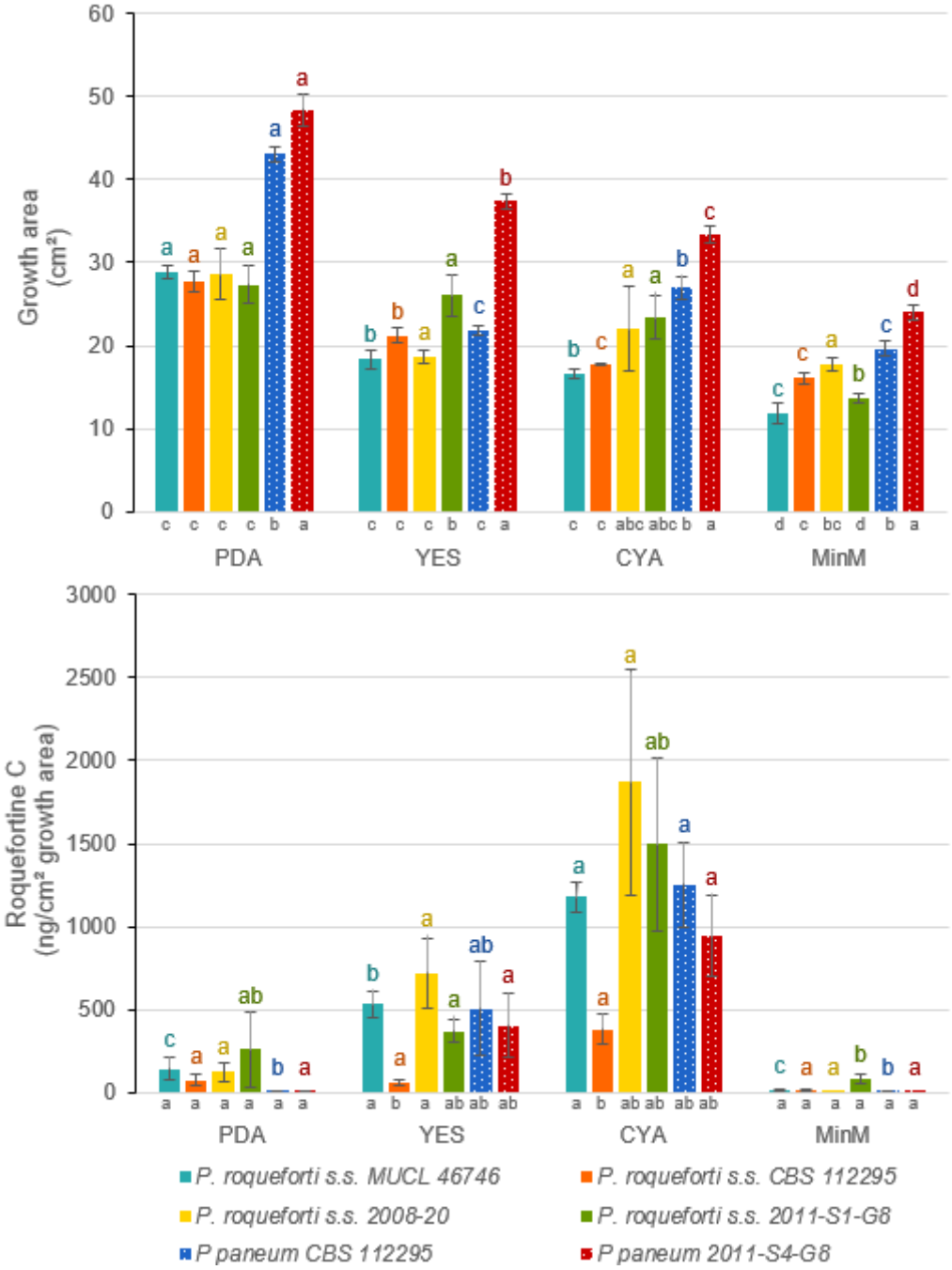


Figure 3.4. *Growth area (top) and roquefortine C production (bottom) of four selected P. roqueforti s.s. (PR) and two selected P. paneum (PP) isolates on Potato Dextrose Agar (PDA), Yeast Extract Sucrose agar (YES), Czapek-Dox Yeast extract Agar (CYA) and a mineral medium (MinM) after 7 days of aerobic incubation at 25 °C.* The mean values per object are presented in a bar chart with error bars indicating their resp. standard deviation. For both parameters, a significant interaction between infection and agar medium was observed, so the effect of one factor was assessed separately for each level of the other factor. Per *P. roqueforti s.l.* isolate, significant differences between the agar media are indicated by colored lettercodes above the bars. Vice versa, significant differences between the six isolates per agar medium are mentioned below the x-axis by lettercode.

Significant differences in growth as well as in ROC production were observed among the six selected *P. roqueforti s.l.* isolates on the different agar media. On all agar media, the strongest fungal growth during the 7-days incubation period was exhibited by the *P. paneum* (PP) 2011-S4-G8 isolate. The PDA medium facilitated the highest growth, whereas the MinM generally showed the least growth. PDA appeared to be the most discriminative medium regarding growth differences between the species *P. roqueforti s.s.* and *P. paneum*.
ROC production was highly variable among the *P. roqueforti s.l.* isolates and among the agar media. On PDA and MinM, no significant differences in ROC production between the six isolates were detected and ROC levels were rather low. In both yeast extract containing media, *i.e.* YES and CYA, elevated ROC levels were observed, varying significantly between the six isolates. However, large variation in ROC content among triplicates per object was also encountered, especially in isolates producing high ROC levels.

Not only differences between the six *P. roqueforti s.l. i*solates were evaluated. Also the effect of the fungal species on both growth and ROC production has been assessed, as demonstrated in Figure 3.5.

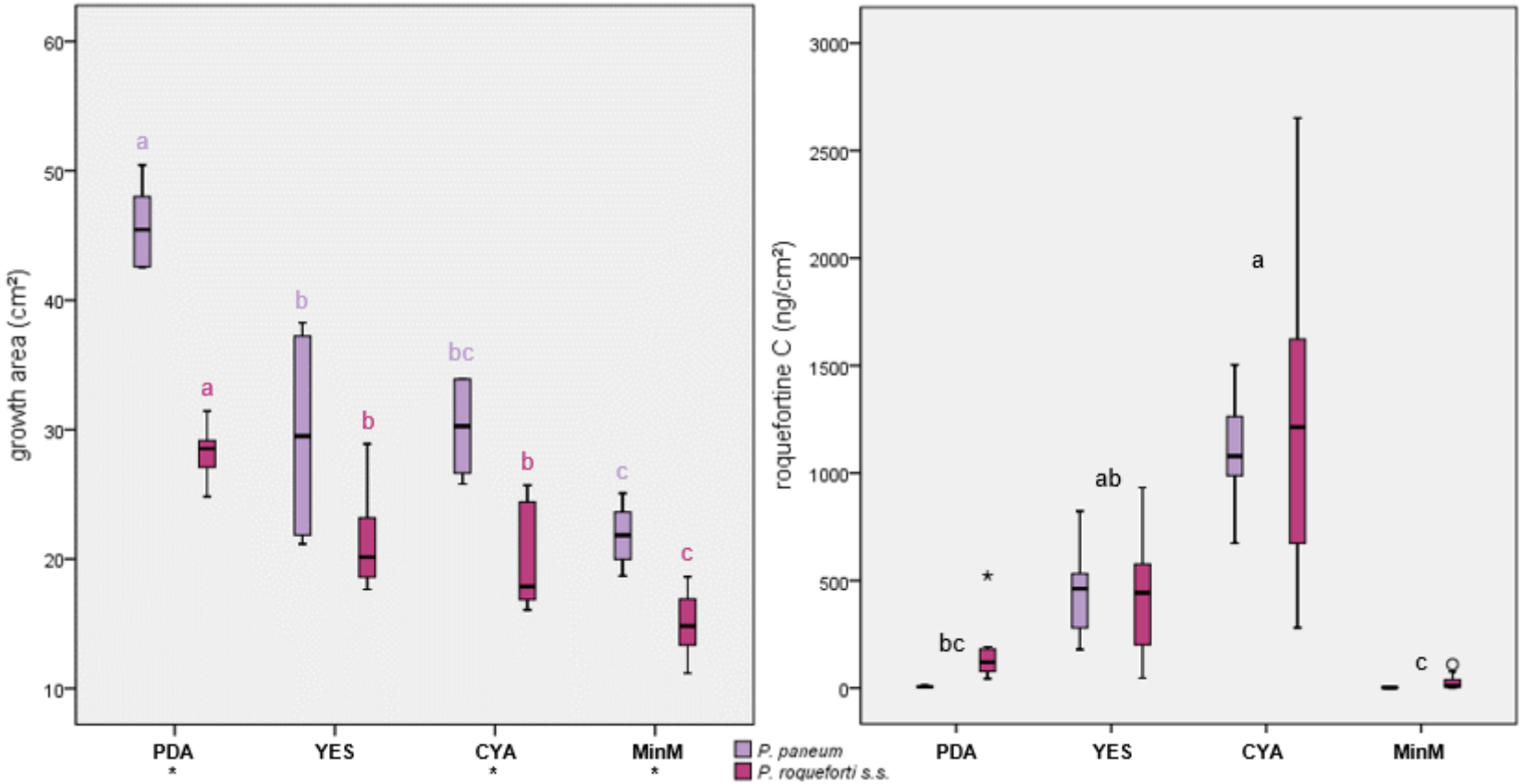


Figure 3.5. *Fungal growth (left) and roquefortine C production (right) by P. roqueforti s.s. and P. paneum on Potato Dextrose Agar (PDA), Yeast Extract Sucrose agar (YES), Czapek-Dox Yeast extract Agar (CYA) and a mineral medium (MinM) after seven days of aerobic incubation at 25 °C: boxplots per agar medium.* Outliers are indicated by a degree (°) symbol, but if their value exceeds three times the height of the box they are marked with an asterix (*) symbol.

Growth showed a significant interaction between the two fungal species and the four agar media. The effect of one factor was assessed per level of the other factor. Significant differences between the four media are indicated per fungal species in colored lettercodes, whereas significant differences between the two species are indicated per medium by asterix (*) symbols. ROC production did not show significant interaction between fungal species and agar media and was not significantly influenced by the fungal species. The agar medium did have a significant effect on ROC production, indicated by black lettercodes.

Both fungal species exhibited the strongest growth on PDA and the lowest growth on MinM, while intermediate growth was observed on YES and CYA. Significantly stronger growth of *P. paneum* compared to *P. roqueforti s.s.* was detected on all media except for YES, confirming the findings of Frisvad and Samson (2004).
Significant differences were detected in ROC production upon growth on the different agar media: the most abundant ROC production was registered on CYA, being significantly higher compared to PDA and MinM. No significant difference in ROC production between the species *P. roqueforti s.s.* and *P. paneum* was detected.

3.3. Discussion and conclusion

In the current *in vitro* experiment, macromorphology of the six selected *P. roqueforti s.l.* isolates was highly variable across the four agar media. Gillot *et al.* (2015) have studied the macromorphology of multiple *P. roqueforti s.s.* isolates (mainly isolated from cheese) grown on different agar media including PDA, CYA, YES and Malt Extract Agar (MEA) during aerobic incubation at 25 °C for seven days. They identified PDA as the most discriminative medium. Even within the species *P. roqueforti s.s*., huge differences were observed in colony color, texture and growth rate, confirming the findings of the current *in vitro* experiment.

In the currently described *in vitro* experiment, *P. roqueforti s.l.* isolates were grown to monitor fungal growth and ROC production on four agar media which's exact composition regarding carbon and nitrogen sources is unknown.

PDA was the agar medium exhibiting the highest growth, but this was not associated with the highest ROC production levels. As stated earlier, yeast extract containing culture media are highly recommended for secondary metabolite analysis (Bridson and Brecker 1970; Fontaine *et al.* 2015; Gillot *et al.* 2017; Kokkonen *et al.* 2005a; Scott *et al.* 1977; Sumarah *et al.* 2005). Frisvad and Filtenborg (1983, 1989) reported that *Penicillium* isolates consistently produce a large number of mycotoxins on CYA and YES, in species-specific profiles. However, the produced mycotoxin amounts varied according to the fungal strains.
On YES, Kokkonen *et al.* (2005a) detected rather moderate levels of ROC production, which was explained by primary metabolism prevailing during the 7-days incubation period at 25 °C. Since the incubation conditions were similar to those of the currently described *in vitro* experiment, it is possible that secondary metabolism and the production of ROC occur only later.

In the current *in vitro* experiment, the highest ROC levels were indeed observed upon growth on YES and CYA. CYA was the most conducive medium for ROC production by *P. roqueforti s.l.* out of the four tested agar media.

Boichenko *et al.* (2002a and 2002b) found a direct correlation between the ROC content in liquid Abe's medium and the amount of *P. roqueforti s.s.* inoculum. Furthermore, they suggested that variable ROC yield is related to the regulation of its biosynthesis, but also to transport and excretion, degradation and imprecise quantification.
Since the conidiospore suspensions of all six selected *P. roqueforti s.l.* isolates were prepared in a standardized way and equal conidiospore concentrations were applied, it is reasonable to assume that the encountered differences in ROC production on the different agar media can largely be ascribed to the effect of specific carbon and nitrogen sources present in the media on *P. roqueforti s.l.* growth on the one hand, and on the regulation of ROC biosynthesis on the other hand. However, the current knowledge on ROC yield variability does not allow evaluation of the contribution of regulatory processes.

The ultimate goal of this PhD dissertation is to gain more insight into the factors influencing the growth and mycotoxin production of *P. roqueforti s.l.* in silages. The four tested agar media are far from representative for a silage matrix, but have been used for *in vitro* studies with fungi by numerous authors. *In vitro* experiments described later in this manuscript will include the use of whole-crop maize based media to better mimic silage conditions.

Kokkonen *et al.* (2005) have cultured *P. crustosum* on YES and on bread and cheese analogues and determined the effect of substrate on mycotoxin production. Although cheese is generally regarded as a poor substrate for mycotoxin production (Engel and Teuber 1978; Scott 1998), ROC production levels were much higher in the cheese analogue than in the YES and bread analogue. This is most likely due to the similarity of this substrate to the natural habitat of *P. crustosum*. Since the natural habitat of *P. roqueforti s.l.* is silage, it might therefore very well be possible that silage is a conducive medium for ROC production by *P. roqueforti s.l.*. Driehuis *et al.* (2008b) detected much higher ROC levels in on-farm whole-crop maize silages compared to grass silages, probably related to increased fungal growth due to a larger supply of carbon sources in whole-crop maize silage (Pitt *et al.* 1991).

In a hereafter described *in vitro* experiment, the effect of eight different carbon sources added to MinM as single carbon sources on the growth and ROC production by the six selected *P. roqueforti s.l.* isolates is evaluated. However, it is important to keep in mind that culture media containing only one single carbon source as well as one sole nitrogen source are not representative at all for realistic *in vivo* growing conditions of *P. roqueforti s.l..* They are suitable to determine the potential of the six selected P*. roqueforti s.l.* isolates to metabolize certain carbon sources.

4. *P. roqueforti s.l.* growth and roquefortine C production in mineral medium containing one of eight single carbon sources

The mineral medium (MinM) described in the previous experiment contained sucrose as sole carbon source. To assess the effect of other single carbon sources on the growth and ROC production levels of the six selected *P. roqueforti s.l.* isolates, seven variations were made on this medium: sucrose was replaced by either glucose, fructose, mannitol, lactic acid, acetic acid, succinic acid or ethanol. These particular carbon sources were chosen because they are present in silages. Lactic acid bacteria convert sucrose, glucose and fructose into lactic acid, acetic acid, mannitol and ethanol during the fermentation process. Acetic acid can also be formed by anaerobic breakdown of lactic acid by *L. buchneri*, along with traces of ethanol. The levels of succinic acid increase during ensilage since it can be produced by a number of heterofermentative *Lactobacillus* strains (Danner *et al.* 2003; Driehuis *et al.* 2001; Kaneuchi *et al.* 1988; Nishino *et al.* 2003; Playne and McDonald 1966; Shao *et al.* 2005).

4.1. Materials and methods

Aim of the experiment was to provide the same amount of carbon in each of the eight media, namely 12.63 gram carbon per liter of mineral medium. This was successfully accomplished for sucrose, glucose, fructose, mannitol and ethanol. However, due to the possibility of acid hydrolysis of agar in the presence of high acid concentrations and sensitivity of *P. roqueforti s.l.* towards high levels of acids, the amount of carbon supplied with lactic acid was reduced to 20 % of the carbon amount in the other media, and to 10 % for acetic acid and succinic acid. This is summarized in Table 3.3.

All media were prepared similarly as described for the mineral medium containing sucrose (MinM) in Annex 1, but the pH was not brought to 5.50. Lactic acid, acetic acid, succinic acid and ethanol were added after autoclaving. Therefore, the media were prepared for autoclaving with a reduced amount of water, which was complemented by the appropriate volume of these particular carbon sources after autoclaving and cooling down to approximately 55 °C. Media were poured into standard 90-mm diameter Petri dishes, each containing approx. 20 ml medium.

Table 3.3. *Composition of the mineral media containing one of eight single carbon sources.*

Composition (per liter) of mineral media adapted for *P. roqueforti s.l.* containing one of eight different carbon sources	g	ml	
SINGLE CARBON SOURCES (12.63 gram of carbon per liter, 20 % or 10 %)			***pH***
sucrose	30.000		*4.25*
glucose	31.576		*4.13*
fructose	31.576		*3.78*
mannitol	31.930		*4.38*
lactic acid (20 %)		5.224	*2.65*
acetic acid (10 %)		3.010	*3.10*
succinic acid (10 %)	3.105		*3.01*
ethanol		25.740	*4.35*
ammonia nitrate (NH_4NO_3)	2.0		
potassium phosphate (KH_2PO_4)	1.0		
magnesium phosphate ($MgPO_4.7H_2O$)	0.5		
potassium chloride	0.5		
iron sulphate ($FeSO_4.7H_2O$)	0.010		
trace element solution°		0.200	

° Trace element solution content per 100 ml: 5 g citric acid - 5 g $ZnSO_4.7H_2O$ - 0.25 g $CuSO_4.5H_2O$ - 0.05 g $MnSO_4.H_2O$ - 0.05 g H_3BO_3 - 0.05 g $NaMoO_4.2H_2O$

After solidification of the agar medium, a 5-mm opening was made in the center of each plate with a cork borer. In this opening, 20 µl of spore solution (containing $0.5*10^6$ spores per ml, freshly prepared as described in Annex 1 without addition of glycerol) of one of the six selected *P. roqueforti s.l.* isolates was brought. For each of the eight agar media and each of the six fungal isolates, four plates were included as replicates. After sealing the plates with parafilm, all plates were aerobically incubated upright in the dark at a stress-inducing temperature regime of 12-hour periods of alternately 10 °C and 20 °C during 15 days. Compared to the earlier described *in vitro* experiment on four agar media, a longer incubation period has been adopted to facilitate secondary metabolism.

Fungal growth was registered per object (N = 3) by measurement of the colony diameter at two orthogonal positions on the reverse side of three plates per object after 15 days. The corresponding growth area was calculated.

Quantification of ROC production by LC-MS/MS (as described in Annex 1) after 15 days of incubation was executed (N = 3) on a pooled sample of one to three mycelium plugs (7.62 mm diameter each, taken near the center, at the outer mycelial growth and at an intermediate position). The mycelium plugs were collected in a 1.5-ml Eppendorf tube, which was stored at -20 °C prior to freeze-drying just before ROC analysis. The empty, filled and freeze-dried weight of each Eppendorf tube was noted, as well as the number of plugs per tube. The ROC content detected in the mycelium plugs was recalculated to the ROC content per cm^2 of growth

area. Due to limited growth on lactic acid, the ROC production of PR MUCL 46746, PR CBS 116877 and PP CBS 112295 on lactic acid has not been determined.

Fungal growth and ROC production data were statistically analyzed as described in Annex 1.

4.2. Results

The growth area for the six *P. roqueforti s.l.* isolates after seven days of incubation is presented per carbon source in Figure 3.6. The results of the ROC quantification are not mentioned separately for the six isolates on the eight carbon sources since standard deviations were very high.

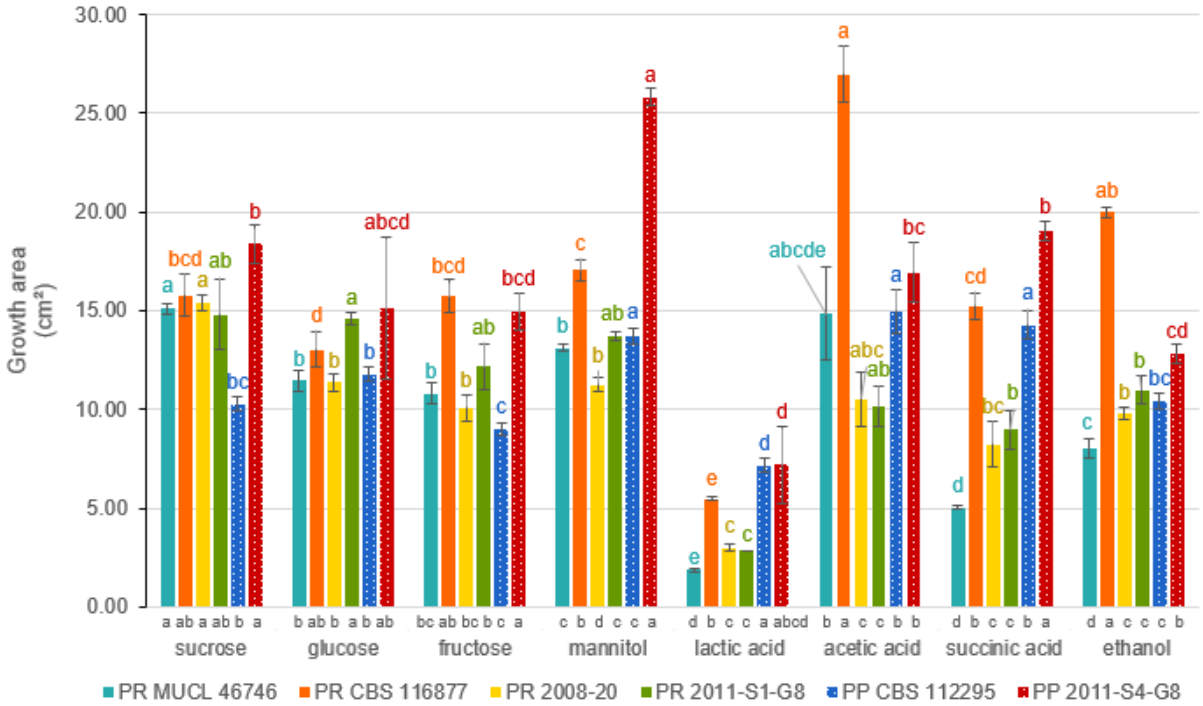


Figure 3.6. *Growth area (cm²) of four selected P. roqueforti s.s. (PR) and two selected P. paneum (PP) isolates on eight different single carbon sources in a mineral medium after fifteen days of incubation.* The mean growth area per object is represented in a bar chart, with error bars indicating their resp. standard deviation. A significant interaction between infection and carbon source was detected, so the effect of carbon source is indicated per *P. roqueforti s.l.* isolate by colored lettercodes above the bars. Vice versa, significant differences between fungal isolates are presented per medium by lettercode below the x-axis.

For all six isolates, significant differences were found in growth on the different carbon sources: PR MUCL 46746 exhibited the highest growth on sucrose. Somewhat lower growth was displayed on glucose, fructose and mannitol, followed by resp. ethanol, succinic acid and lactic acid. On ethanol, growth was highly variable. The PR CBS 116877 isolate grew maximally on acetic acid, followed by ethanol, mannitol, sucrose, fructose, succinic acid and glucose. Again, the lowest growth was observed on lactic acid. It is striking that acetic acid, ethanol and mannitol, which are all produced during silage fermentation, facilitate good growth of this

particular *P. roqueforti s.s.* isolate. Sucrose allowed maximum growth of PR 2008-20. Growth area did not vary very much on the other carbon sources, but again lactic acid was significantly the least conducive carbon source for fungal growth. Rather comparable growth rates were obtained by PR 2011-S1-G8 on all carbon sources except for lactic acid. PP CBS 112295 displayed the most abundant growth on mannitol, acetic acid and succinic acid. Intermediate growth levels were found on glucose, sucrose, ethanol and fructose, whereas the lowest growth area was observed on lactic acid. The PP 2011-S4-G8 isolate grew maximally on mannitol, which supported significantly stronger growth compared to all other carbon sources except glucose. Again, lactic acid was the least conducive carbon source.
Overall, it is clear that lactic acid as a single carbon source supports significantly lower growth of *P. roqueforti s.l.* compared to the other seven carbon sources tested. Still, all *P. roqueforti s.l.* isolates were capable of growing on lactic acid as sole carbon source. A possible explanation for the reduced growth on the lactic acid containing medium might be its low pH (2.65). The second most acidic medium contained succinic acid and had a pH of 3.01. Sucrose (pH 4.25) was usually a conducive carbon source for fungal growth, but some isolates grew maximally on mannitol (pH 4.38) or acetic acid (pH 3.10).
On the different culture media, significant differences in growth between the six *P. roqueforti s.l.* isolates were detected, as mentioned below the X-axis of Figure 3.6. No clear pattern of certain isolates exhibiting stronger growth on all media could be detected.

The effect of fungal species on growth and ROC production on the different sole carbon sources has been evaluated, as displayed in Figure 3.7. For both *P. roqueforti s.s.* and *P. paneum*, boxplots visualize the growth area and ROC production results on the eight single carbon sources.

For both fungal species, growth area was significantly influenced by the carbon source present in the mineral medium. *P. roqueforti s.s.* displayed significantly stronger growth on sucrose than on succinic acid or lactic acid. For *P. paneum*, growth on glucose, acetic acid, succinic acid or ethanol was significantly higher than on lactic acid. A significant difference in growth between both species was detected on mannitol, lactic acid and succinic acid. On all three carbon sources, *P. paneum* grew significantly stronger than *P. roqueforti s.s.*, confirming the findings of the previously described *in vitro* experiment using the media PDA, YES, CYA and MinM.

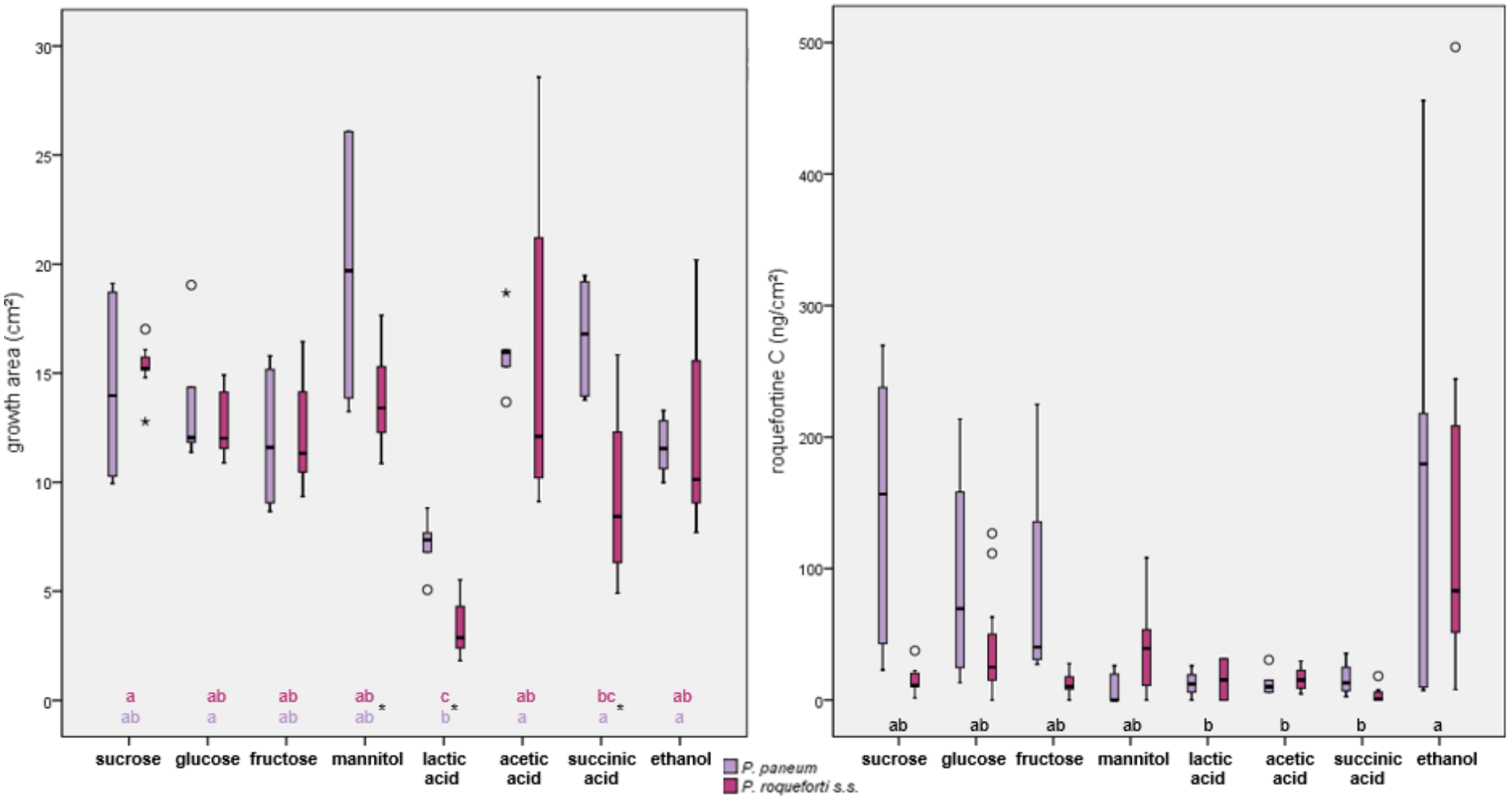


Figure 3.7. *Growth area (left) and roquefortine C production (right – extreme outliers > 500 ng/cm² removed) of P. roqueforti s.s. and P. paneum on eight different single carbon sources in a mineral medium after fifteen days of incubation: boxplots per carbon source*. Outliers are indicated by a degree (°) symbol, but if their value exceeds three times the height of the box they are marked with an asterix (*) symbol.

For growth, a significant interaction between fungal species and carbon source was detected, so the influence of one factor was evaluated per level of the other factor. Per fungal species, significant differences in growth between the different carbon sources are mentioned in colored lettercodes. Significant differences between both fungal species are indicated by asterix (*) symbols per carbon source. ROC production did not show significant interaction between fungal species and carbon source. The two species did not differ significantly in ROC production, while significant differences according to the carbon source in the culture medium are indicated by black-colored lettercode.

No significant difference in ROC production between *P. roqueforti s.s.* and *P. paneum* was detected over the eight carbon sources. ROC production by *P. paneum* appeared to be more variable than by *P. roqueforti s.s.* on most carbon sources. The carbon source did significantly influence ROC levels: ROC production upon growth on ethanol was significantly higher than on lactic acid, acetic acid and succinic acid. This suggests a reduced ROC production on acids as single carbon source or on media with a low pH, whereas carbohydrates resulted in intermediate ROC levels.

4.3. Discussion and conclusion

The six selected *P. roqueforti s.l.* display distinct growth and ROC production patterns on the eight sole carbon sources tested. Generally, growth was adequate on all carbon sources except for lactic acid. Since lactic acid is the main conservatory acid produced during the silage fermentation, this is a very interesting finding. However, in practice lactic acid is far from the sole carbon source present in an *in vivo* silage matrix. The current experiment demonstrated that lactic acid can be used as single carbon source by *P. roqueforti s.l.*, but did not support good growth at the given conditions. However, the low pH of the medium containing lactic acid may also be responsible for the reduced growth.

In order to separate the effect of carbon source and pH, it would be interesting to perform an additional experiment with the same eight single carbon sources containing media, all with the same carbon dose and all brought to the same pH.

Several authors have studied the effect of certain carbon sources on ROC production:
Wagener *et al.* (1980) observed a stimulatory effect of sucrose on ROC production by *P. commune* in a liquid medium containing also yeast extract.
Kulakovskaya *et al.* (1997) found that in *P. crustosum* the excretion of ROC is stimulated by glucose, whereas mannitol and succinic acid had no effect on ROC excretion. These findings were related to the transport of ROC: ROC uptake can occur by both energy-independent and energy-dependent mechanisms. The energy-dependent system proceeds via a general amino acid transport system in the presence of succinic acid and is repressed by ammonium.
Boichenko *et al.* (2002a) have studied ROC production by *P. roqueforti s.s.* in a liquid medium containing 50 g mannitol, 5.4 g succinic acid, 1 g KH_2PO_4 and 0.3 g $MgSO_4.7H_2O$ per liter adjusted to pH 5.2 with 25 % ammonium hydroxide, for submerged stir-culture at 24 °C during five days. Increasing the mannitol concentration from 50 to 65 g/l stimulated ROC accumulation 1.5-2 times.

The results obtained in the current *in vitro* experiment don't line up with the previously described findings regarding ROC production observed on media containing more than one carbon source. In the current experiment, the use of sucrose as single carbon source did not result in significantly higher ROC levels compared to the other carbon sources tested, nor did the use of mannitol. The significantly highest ROC production was detected on the MinM with ethanol as sole carbon source, being significantly higher than on the media containing lactic acid, acetic acid or succinic acid. The latter three media were the least conducive for ROC production by *P. roqueforti s.l.*, while fungal growth was only reduced on lactic acid. Ethanol has antifungal properties (Mills *et al.* 2002; Passoth and Schnürer 2003), but at the applied dose in this experiment acceptable growth of *P. roqueforti s.l.* was detected. However, the significantly higher ROC production on ethanol as single carbon source compared to lactic acid, acetic acid and succinic acid might be related to a certain degree of stress on *P. roqueforti s.l.* caused by ethanol.

The observation that ROC production was at a low level on the media containing lactic acid and acetic acid as sole carbon sources demonstrate that these individual compounds didn't trigger ROC production at the doses applied during this fifteen-days *in vitro* experiment. However, the combined effect of lactic acid and acetic acid on ROC production remains elusive. *In vivo* in silages, both organic acids are present together, along with other carbon sources.

Nishino *et al.* (2003) have analyzed the composition of whole-crop maize before and after ensiling. Prior to ensiling, up to 15 % of water-soluble carbohydrates are present in the fresh crop, mainly sucrose, glucose and fructose. After an ensiled period of 60 days, all water-soluble carbohydrates had been metabolized into lactic acid, acetic acid, propionic acid, mannitol and ethanol (not limitative). Shao *et al.* (2005) studied the composition of grass prior to and after ensiling: fructose was the main carbohydrate in fresh grass, followed by glucose and sucrose. During the ensiling process, these carbohydrates were largely converted to lactic acid, acetic acid, mannitol, succinic acid, ethanol and 1,2-propanediol. So, the carbon source composition of ensiled feed commodities is characterized by enormous variation: originating from huge differences in the carbon source composition of fresh crops, this composition gradually changes during the ensiling process and is not uniformly distributed throughout the entire silage mass. So, based on the results obtained in the current *in vitro* experiment with single carbon sources in a synthetic medium, it is impossible to draw conclusions on the effect of these individual carbon sources on ROC production by *P. roqueforti s.l.* in realistic silage conditions.

Repeating the experiment with a silage-mimicing medium would shed more light on this matter. In chapter 5, an *in vitro* experiment using Corn Silage Infusion as a liquid culture medium is described. By adding agar to the infusion prior to autoclaving, a solid whole-crop maize silage mimicing culture medium can be made. Furthermore, other ensiled feed commodities (e.g. grass silage) can also be used to prepare an infusion.

5. Conclusion

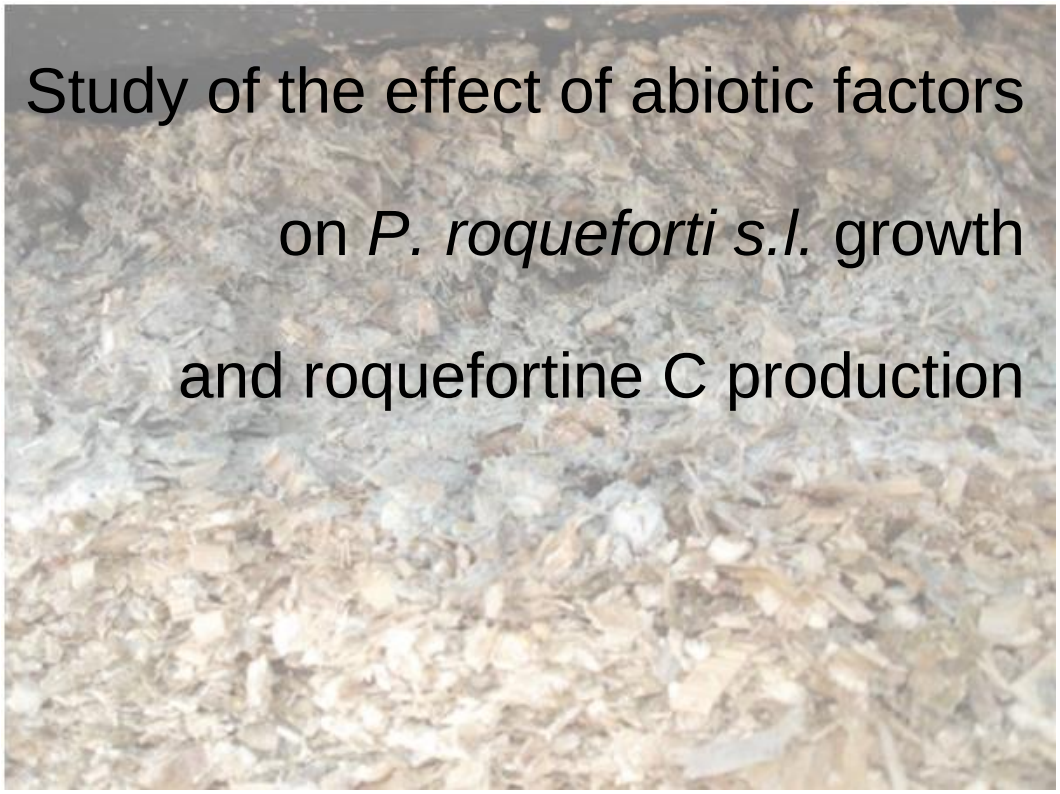
Four *P. roqueforti s.s.* and two *P. paneum* isolates have been selected from the approx. 250 *P. roqueforti s.l.* isolates in collection at the Bottelare research station.

First, the conservancy of the beta-tubulin gene sequence was evaluated and appeared to be very high both within and between the two species. Partial sequencing of the beta-tubulin gene proved to be an excellent tool for species determination.

Secondly, growth and ROC production by the six isolates was assessed by two *in vitro* experiments. For the first experiment, the four agar media PDA, YES, CYA and MinM were used. In the second experiment, variations on MinM were made by replacing sucrose as single carbon source by other carbon sources. However, silages contain several carbon sources in different amounts. Therefore, the *in vitro* experiments described in the following chapters have all been executed on culture media containing more than one carbon source.

Due to practical and budgetary considerations, it was impossible to perform all experiments with the six *P. roqueforti s.l.* isolates characterized in the current chapter. Depending on the experimental setup, one or two *P. roqueforti s.l.* isolates were used. *P. roqueforti s.s.* MUCL 46746 has been used in all experiments, whereas CBS 112295 has been included in some experiments as *P. paneum* isolate. These two particular isolates were chosen because they produced quasi the same amount of ROC upon seven days of aerobic incubation on CYA at 25 °C, facilitating the interpretation of the results of the first *in vitro* experiment described in Chapter 4: this experiment assesses the effect of variable amounts of inorganic and organic nitrogen in CYA-based media on *P. roqueforti s.l.* growth and ROC production. To provide consistency, these particular isolates have been used also for other *in vitro* and *in vivo* experiments. They will be indicated by the same colors throughout the manuscript, *i.e.* *P. roqueforti s.s.* MUCL 46746 and *P. paneum* CBS 112295. If an uninfected control object is included, this is designated in purple.

Chapter 4



Study of the effect of abiotic factors on *P. roqueforti s.l.* growth and roquefortine C production

1. Introduction

The effect of a selection of abiotic factors on *P. roqueforti s.l.* growth and ROC production has been evaluated by *in vitro* and *in vivo* experiments. *In vitro*, the effect of variable amounts of inorganic and organic nitrogen has been investigated, as well as the effect of different temperatures and oxygen concentrations. The combined effect of elevated temperature and oxygen supply during the ensiled period of grass and whole-crop maize was assessed *in vivo* using microsilos. In this chapter, a general introduction on microsilos is given. Additionally, a microsilo experiment evaluating the effect of prolonged anaerobic conditions on *P. roqueforti s.l.* numbers and fermentation characteristics in grass silage is described.

2. *In vitro* effect of abiotic factors on *P. roqueforti s.l.* growth and roquefortine C production

2.1. Effect of variable amounts of inorganic and organic nitrogen on *P. roqueforti s.l.* growth and roquefortine C production

As already mentioned in chapter 2, fungi can use a diverse array of compounds as nitrogen sources, and are capable of expressing the catabolic enzymes of many different pathways upon demand. When the primary nitrogen sources (*i.e*. ammonia, glutamine and glutamate) are unavailable or are present at concentrations low enough to limit growth, many different compounds can be used as secondary nitrogen sources (*i.e.* nitrate, nitrite, most amino acids, peptides and proteins) (Garcia-Rico *et al.* 2009; Li *et al.* 2007).

ROC is synthetized from the amino acids tryptophan and histidine. The breakdown of extracellular proteins generates free amino acids. To support this breakdown, several *P. roqueforti s.l.* strains possess extracellular protease activity, even at pH 3.0. Structural genes encoding extracellular proteases are expressed upon nitrogen limitation (Fernandez-Bodega *et al.* 2009; Gente *et al.* 1997; Larsen *et al.* 1998; Marzluf 1997; Modler *et al.* 1974; Pose *et al.* 2007). ROC is produced by certain toxigenic fungal species as an extracellular nitrogen reserve: it is excreted by an energy-independent mechanism on the one hand, and can be resorbed by both energy-dependent as energy-independent mechanisms. The resorbed ROC is metabolized by growing mycelia and germinating spores.
Since not all ROC producing fungal species exhibit extracellular protease activity, the use of ROC instead of amino acids/proteins as a nitrogen source might provide the advantage that nitrogen would not have to be actively pumped into mycelia (Boichenko *et al.* 2002a; Boichenko

et al. 2002b; Kulakovskaya *et al.* 1997; Overy *et al.* 2005; Reshetilova *et al.* 1995). Moreover, the antimicrobial properties of ROC may help to protect the accumulated nitrogen reserve against other micro-organisms (Kopp-Holtwiesche and Rehm 1990; Overy *et al.* 2005).

Sumarah *et al.* (2005) investigated the production of ROC by *Penicillium crustosum* in a 12-day time-course experiment on "standard" Czapek-Dox Yeast Extract broth and variations on it regarding nitrogen source (*i.e.* organic nitrogen (yeast extract) and inorganic nitrogen (sodium nitrate)). Every three days, the ROC content of the liquid medium and the mycelium was determined. The researchers found that organic nitrogen was required for growth, but also that the form of nitrogen *per se* was not material to the formation of ROC. Interestingly, negligible amounts of ROC were detected until depletion of the inorganic nitrogen. Maximum rate of ROC production coincided with a shift from inorganic nitrogen to amino acids as nitrogen source. In "standard" Czapek-Dox Yeast Extract broth, ROC was produced mainly between 6 and 9 days of aerobic incubation in the dark at 25 °C.

Triggered by the interesting observations of Sumarah *et al.* (2005), a similar experiment with *P. roqueforti s.s.* and *P. paneum* on solid CYA-based media was carried out, as described hereafter. However, Boichenko *et al.* (2002b) had observed that during the short-term storage of samples containing liquid culture medium as well as *P. roqueforti s.s.* mycelium, ROC was quickly metabolized by the mycelium prior to ROC analysis (depending on the oxygen supply and the temperature). Despite the fact that no stir-culturing in liquid medium is used in the current experiment, biased results are avoided with certitude by facilitating a quick and thorough separation of the agar medium and the fungal biomass at sampling: a cellophane sheet is placed on top of the agar medium surface prior to fungal inoculation. This cellophane sheet allows quick and effective separation of the agar medium and the fungal biomass at sampling. In the current *in vitro* experiment, the ROC content was determined in the total fungal biomass as well as in the conidiospores produced and in the culture medium, facilitating a partitioning study (Atoui *et al.* 2007).

2.1.1. Materials and methods

P. roqueforti s.s. MUCL 46746 and *P. paneum* CBS 112295 were grown on four agar media:

a) standard Czapek-Dox Yeast Extract agar (CYA)
b) CYA low on inorganic nitrogen (20 % of the inorganic nitrogen supplied in standard CYA)
c) CYA low on organic nitrogen (20 % of the organic nitrogen supplied in standard CYA)
d) CYA low on both inorganic and organic nitrogen (20 % of both the inorganic and organic nitrogen supplied in standard CYA).

The composition of the four agar media is summarized in Table 4.1. The media were prepared as described in Annex 1 for standard CYA. To avoid bacterial growth between the agar medium and the cellophane sheet, the media were supplemented with 100 mg of chloramphenicol per liter medium after autoclaving. All media were poored into standard 90-mm Petri dishes at approximately 20 ml per Petri dish.

Table 4.1. *Composition of Czapek-Dox Yeast extract Agar (CYA) based media used for the in vitro experiment with variable amounts of inorganic anc organic nitrogen.*

COMPOUNDS	**standard CYA**	**CYA low on inorganic nitrogen**	**CYA low on organic nitrogen**	**CYA low on both inorganic and organic nitrogen**
inorganic nitrogen				
sodium nitrate	3 gram	0.6 gram	3 gram	0.6 gram
organic nitrogen				
yeast extract	5 gram	5 gram	1 gram	1 gram
sucrose		30 gram		
dipotassium phosphate		1 gram		
magnesium sulphate		0.5 gram		
potassium chloride		0.5 gram		
iron sulfate		0.010 gram		
agar		15 gram		
chloramphenicol		0.100 gram		
pH	7.01	7.09	7.13	7.15

Cellophane sheets (uncoated Natureflex NP 28 µm, Innovia) were cut circularly to approx. 85 mm diameter and drenched into 70:30 ethanol/water at 55 °C during three hours for sterilization (Carmichael 1963; Marin *et al.* 2014). After solidification of the media, the agar surface was carefully covered with a cellophane sheet: a sheet was taken out of the 70 % ethanol solution with a sterile pincer, dipped to dryness on a sterile towel and placed on top of the agar surface. The plates were kept overnight in a laminar flow to allow any remaining ethanol to vaporize.

For both *P. roqueforti s.l.* isolates, *P. roqueforti s.s.* (PR) MUCL 46746 and *P. paneum* (PP) CBS 112295, thirty Petri dishes of each medium were centrally inoculated with 20 µl of spore solution (containing $0.5*10^6$ conidiospores per ml, prepared as described in Annex 1) and incubated aerobically in the dark at 22 °C.

To monitor the partitioning of ROC, samples were taken from the agar medium, the conidiospores and the total fungal biomass (N = 3) every three days during the 15-days incubation period (Atoui *et al.* 2007; Ramos *et al.* 1999). ROC was quantified by LC-MS/MS as described in Annex 1. However, since very high concentrations of ROC were found in some first replicate samples per object, extracts of the other two replicate samples were diluted before addition of the internal standard.

For sampling of the agar medium, the area underneath fungal growth was marked on the reverse side of the Petri dishes. Subsequently, the cellophane sheet was removed from the agar surface as a whole and transferred to a 50-ml centrifugation tube. Agar samples were taken with a cork borer of 9 mm diameter: 1 to 3 agar plugs (depending on the fungal growth rate) were taken from the marked area. If possible, one agar plug was taken near the center, one near the outer growth area and one at an intermediate position. These agar plugs were transferred into Eppendorf tubes (of which the empty weight as well as the filled weight was noted) and stored at -20 °C prior to freeze-drying (as described in Annex 1) just before ROC quantification.

Conidiospores were washed off from the cellophane sheet by adding 10 ml of sterile distilled water containing 0.01 % of Tween 80 to the 50-ml centrifugation tube and intensive shaking during 1 min. Spores were isolated by filtration through a double layer of miracloth into a 10-ml centrifugation tube (of which the empty weight was noted). The 10-ml centrifugation tubes were centrifuged at 6 000 rpm during 10 min and the supernatant was discarded (Garcia-Rico *et al.* 2008). The filled weight of the tubes containing the spore pellet was noted and the samples were stored at -20 °C until freeze-drying just before ROC quantification. At sampling after three days, no spores could be harvested.

Fungal biomass was obtained by transferring the cellophane sheet into a 50-ml centrifugation tube (of which the empty weight was noted), by adding 10 ml of sterile distilled water and gentle shaking during 1 min. The cellophane sheet was removed with a sterile pincer, followed by centrifugation of the 50-ml centrifugation tubes at 6 000 rpm during 10 min. The supernatant was discarded and the filled weight of the tubes containing the total fungal biomass was noted; subsequently the tubes were stored at -20 °C prior to freeze-drying just before ROC analysis.

The obtained data were statistically analyzed as described in Annex 1.

The setup of the experiment evaluating the effect of variable amounts of inorganic and organic nitrogen on *P. roqueforti s.l.* growth of and ROC production is summarized in Table 4.2.
For both media with a reduced level of organic nitrogen, initially one replicate was screened for ROC production by the fungal biomass. The levels observed were very low compared to the two media with a "normal" level of organic nitrogen supplied (as will be illustrated further in this section). Therefore it was decided not to perform further quantification of ROC on samples originating from the two media low on organic nitrogen.

Table 4.2. *Experimental setup of in vitro experiment assessing the effect of variable amounts of inorganic and organic nitrogen in Czapek-Dox Yeast extract Agar (CYA) on growth of and roquefortine C production by P. roqueforti s.l..*

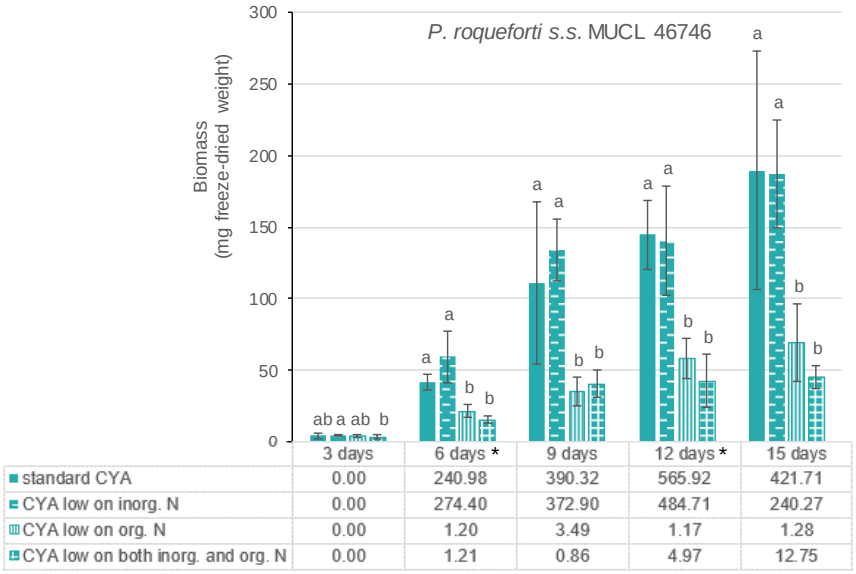
OBJECTS		**Monitoring**	
Infection	**Medium**	**3 days**	**6 - 9 - 12 - 15 days**
P. roqueforti s.s. MUCL 46746	standard CYA	Fungal biomass °	Fungal biomass (N=3) Roquefortine C in agar medium, conidiospores and fungal biomass (N=3)
	CYA low on inorganic nitrogen		
	CYA low on organic nitrogen	Fungal biomass °	Fungal biomass (N=3) Roquefortine C in agar medium, conidiospores and fungal biomass (N=1)
	CYA low on both inorganic nitrogen and organic nitrogen		
P. paneum CBS 112295	standard CYA	Fungal biomass °	Fungal biomass (N=3) Roquefortine C in agar medium, conidiospores and fungal biomass (N=3)
	CYA low on inorganic nitrogen		
	CYA low on organic nitrogen	Fungal biomass °	Fungal biomass (N=3) Roquefortine C in agar medium, conidiospores and fungal biomass (N=1)
	CYA low on both inorganic nitrogen and organic nitrogen		

° After 3 days of incubation, no sporulation was observed. On one replicate per object, the roquefortine C content of the agar medium and of the fungal biomass was determined, but the level was below the limit of detection in all samples.

2.1.3. Results

2.1.3.1. Fungal growth and screening of roquefortine C production upon growth on four CYA-based media with variable amounts of inorganic and organic nitrogen

Fungal growth, expressed as freeze-dried weight of the total biomass, has been registered in triplicate for both *P. roqueforti s.l.* isolates on the four CYA-based media. These results are presented in Figure 4.1. In the tables below the bar charts, the ROC content of the fungal biomass detected in the first replicate per object is mentioned.



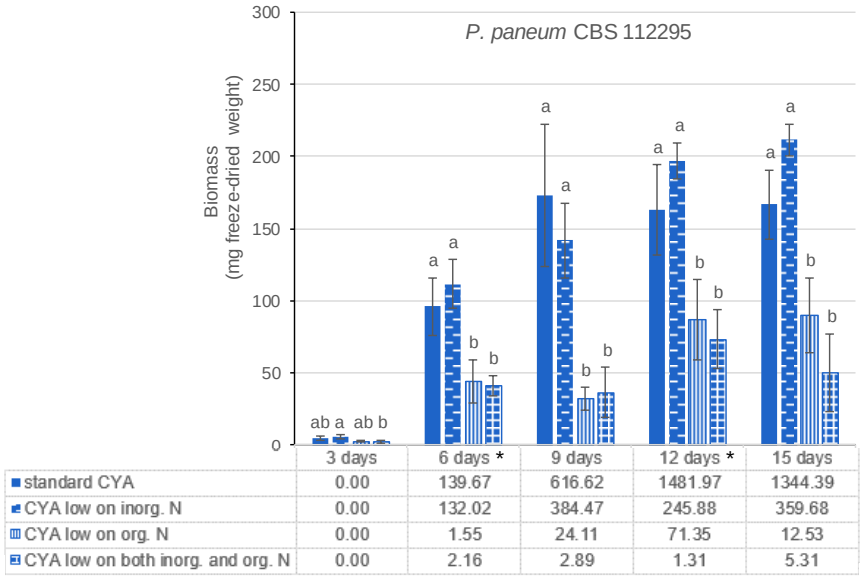


Figure 4.1. *P. roqueforti s.s. MUCL 46746 and P. paneum CBS 112295 biomass (bar graph, mg freeze-dried weight, N=3) and roquefortine C production (table, ng/mg freeze-dried weight, N=1) on four Czapek-Dox Yeast extract Agar (CYA) based media containing variable amounts of inorganic and organic nitrogen.* Per object, the mean biomass yield of three replicates is presented as a bar chart, with error bars representing their resp. standard deviation. No significant three-fold interaction between incubation time, medium and infection was detected, but significant two-fold interactions between time and the other two factors were found. Data were analyzed per time point: since no significant interaction between medium and infection was found at any time point, the effect of infection was evaluated over all levels of medium, and vice versa. Significant differences between both *P. roqueforti s.l.* isolates are indicated by asterix (*) symbols below the x-axis, while significant differences between the media are indicated by lettercode.

The two *P. roqueforti s.l.* isolates had a significantly different biomass yield after 6 and 12 days of incubation, being higher for PP CBS 112295 than for PR MUCL 46746. From 6 to 15 days of incubation, both media with “normal” levels of organic nitrogen facilitated significantly more biomass production compared to both media with a low level of organic nitrogen. On the latter two media, screening of the ROC content of the fungal biomass revealed that ROC production was very low compared to the CYA media with “normal” levels of organic nitrogen. Therefore, it was decided not to perform further analyses on samples originating from both media containing low levels of organic nitrogen.

2.1.3.2. Growth on standard CYA and CYA low on inorganic nitrogen: partitioning study of ROC in fungal biomass, conidiospores and agar medium

Fungal growth (registered as the freeze-dried weight of the fungal biomass) as well as ROC content of the fungal biomass at sampling after 6, 9, 12 and 15 days of incubation on the standard CYA and the CYA low on inorganic nitrogen (N = 3) are presented in Figure 4.2.

As described in Chapter 2, mycotoxin production by toxigenic moulds in indoor environments can be associated with the sick building syndrome due to inhalation of mycotoxin-containing conidiospores. Specifically for ROC, little or no information is available on the conidiospore-associated levels. In this *in vitro* experiment, the ROC content of conidiospores has been determined. These results are presented in Figure 4.3, along with the conidiospore freeze-dried weight data.

To assess ROC excretion, ROC was quantified in the agar medium. Not the entire agar medium has been extracted due to practical considerations, so total ROC excretion into the medium was not determined. A representative subset of 1, 2 or 3 nine-mm diameter plugs of agar medium underlying fungal growth was removed for ROC quantification by LC-MS/MS. The agar medium outside the fungal growth area was not sampled for ROC. Overy *et al.* (2005) have inoculated CYA with *P. tulipae* and did sample the agar from outside the fungal colonies for ROC, but this mycotoxin was not detected.
The ROC content of the agar plugs removed from standard CYA or CYA low on inorganic nitrogen upon growth of PR MUCL 46746 or PP CBS 11295 during 6, 9, 12 and 15 days is summarized in Figure 4.4.

In all three figures, the mean values per object are presented as a bar chart, with error bars representing their resp. standard deviations.

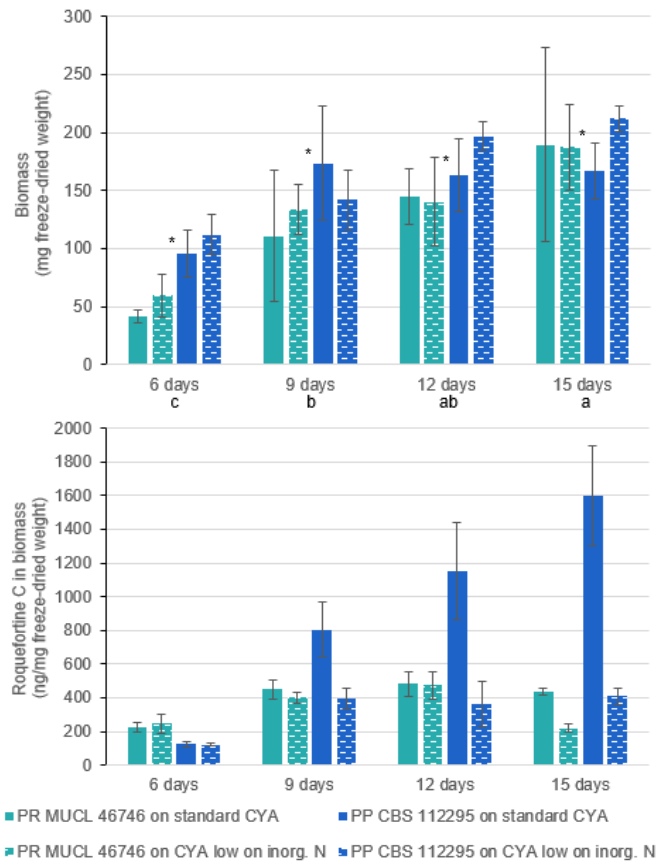


Figure 4.2. *Freeze-dried weight (top) and roquefortine C content (bottom) of PR MUCL 46746 and PP CBS 112295 biomass upon growth on standard CYA and CYA low on inorganic nitrogen.* For biomass weight, no significant interactions between time, medium and infection were found, nor a significant effect of medium. The effect of time is indicated by lettercode below the x-axis, whereas significant differences between both isolates are marked by asterix (*) symbols. For the roquefortine C content, a significant three-fold interaction was detected.

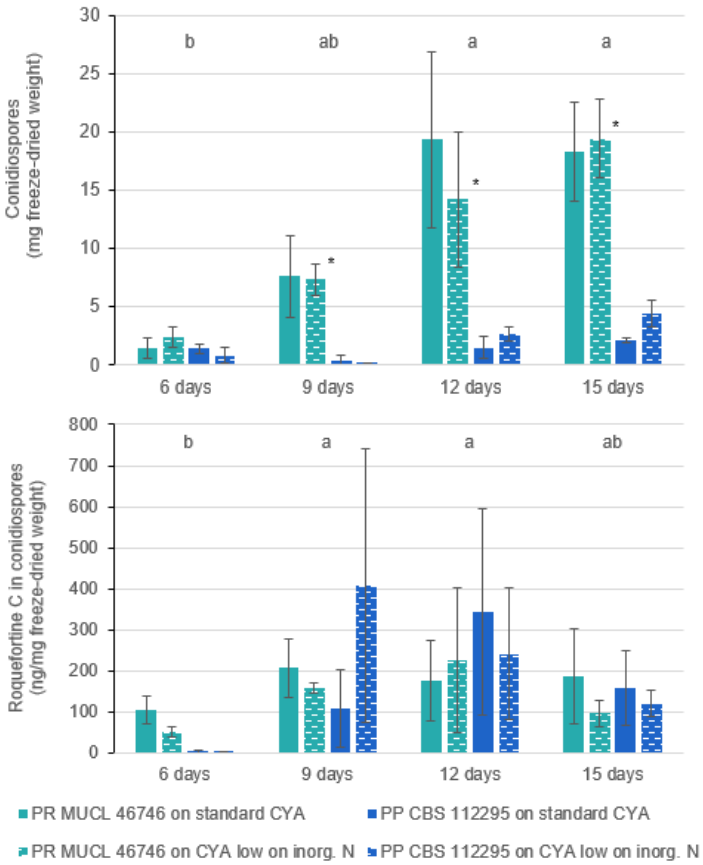


Figure 4.3. *Freeze-dried weight (top) and roquefortine C content (bottom) of PR MUCL 46746 and PP CBS 112295 conidiospores upon growth on standard CYA and CYA low on inorganic nitrogen.* No significant three-fold interaction between time, medium and infection was observed. For spore weight, infection and time interacted significantly, while medium had no significant effect. The effects of infection and time were assessed per factor for each level of the other factor, over both media. Lettercodes indicate significant differences between time points, while significant differences between both isolates are designated by asterix (*) symbols. For roquefortine C, no significant effect of medium or infection was observed. The effect of time is indicated by lettercode.

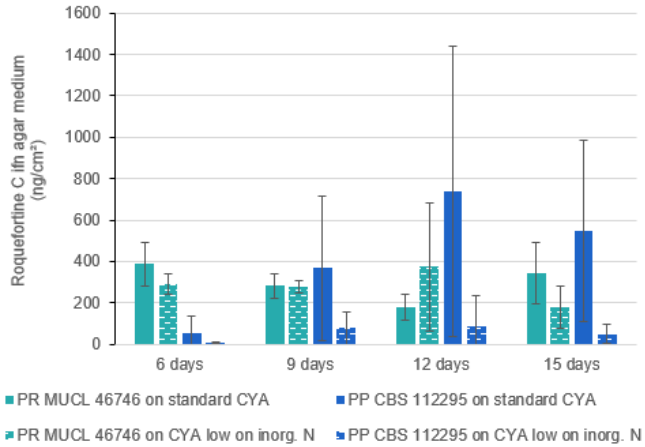


Figure 4.4. *Roquefortine C content of agar medium (ng/cm²) upon growth of PR MUCL 46746 and PP CBS 112295 on standard CYA and CYA low on inorganic nitrogen.* No significant three-fold interaction between time, medium and infection was detected. Infection and medium did interact significantly, while time had no significant effect. The effects of medium and infection were assessed per factor for each level of the other factor, taking all time points into account: for PP CBS 112295, roquefortine C content differed significantly between both media, whereas on CYA low on inorganic nitrogen a significant difference between both isolates was detected regarding roquefortine C levels in the agar medium.

- Fungal biomass

Evidently, the biomass weight increased significantly over time, as indicated by lettercode below the X-axis. Moreover, PP CBS 112295 produced significantly more biomass than PR MUCL 46746 throughout the experiment, confirming the findings of the *in vitro* experiment on four agar media (including standard CYA) described in chapter 3.

To facilitate the interpretation of the factors influencing ROC production, profile plots per medium are presented in Figure 4.5.

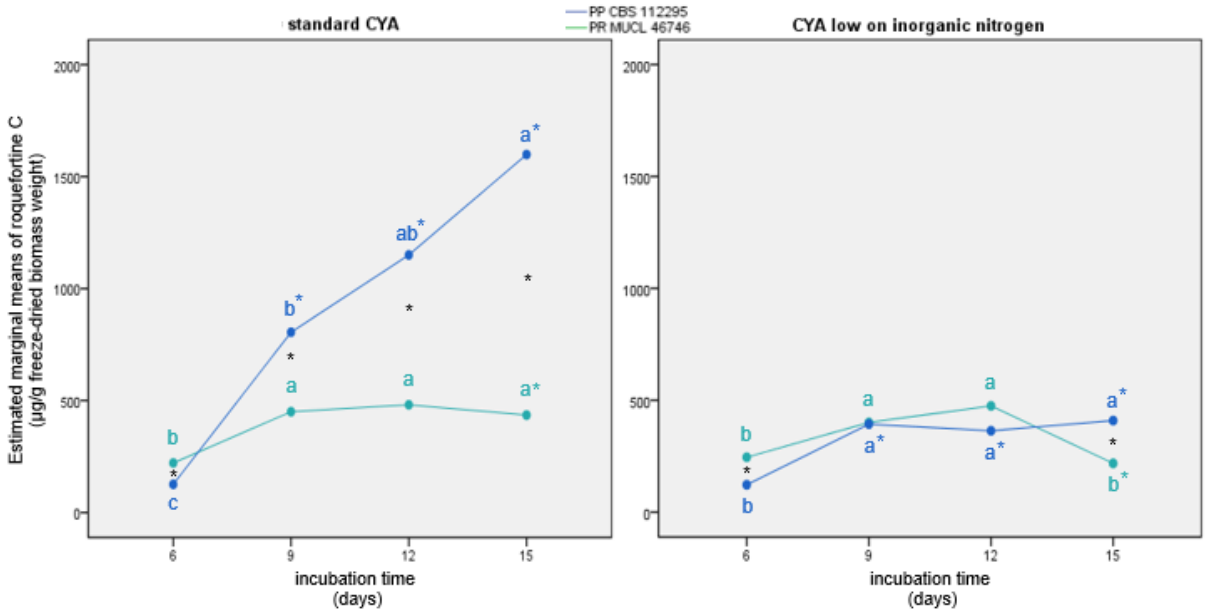


Figure 4.5. *Profile plots facilitating the interpretation of the significant interaction observed between time, medium and infection for the roquefortine C content of P. roqueforti s.s. (PR) MUCL 46746 and P. paneum (PP) CBS 112295 biomass.* Per medium, the effect of infection was determined per incubation time point, and vice versa. Significant differences between both *P. roqueforti s.l.* isolates at a given time point are indicated per medium by black asterix (*) symbols, while significant differences during the incubation period are indicated per isolate by colored lettercodes. Significant differences in ROC production on the two media are indicated for both isolates per time point by colored *-symbols.

The evolution of the ROC content of the fungal biomass showed distinct patterns according to the fungal isolates and the media tested.

On standard CYA, the ROC content of PP CBS 112295 biomass incremented throughout the entire 15-days incubation period, while the ROC content of PR MUCL 46746 biomass increased from 6 to 9 days of incubation and remained at a stable level until 15 days of incubation. After 6 days of incubation, the PR isolate biomass contained significantly more ROC compared to the PP isolate, but from 9 to 15 days of incubation the PP isolate produced significantly more ROC.

The *in vitro* experiment with four agar media described in Chapter 3 revealed no significant difference in ROC production on standard CYA between PR MUCL 46746 and PP CBS 112295 during a seven-day incubation period at 25 °C. In the currently described experiment, ROC levels after six days of incubation were relatively alike, while a clear increase of ROC production by PP CBS 112295 was found upon longer incubation.

On CYA low on inorganic nitrogen, differences in ROC content of the two *P. roqueforti s.l.* isolates were relatively low in absolute value and were only significant at sampling after 6 and 15 days. ROC levels were in the same range as those observed for the PR MUCL 46746 isolate on standard CYA. After 6 days of incubation, the PR biomass contained significantly more ROC than the PP biomass, while the opposite was observed after 15 days of incubation. Over time, the two isolates showed a slightly different pattern of increasing and decreasing of the ROC concentration in the biomass: a significant increase between 6 and 9 days of incubation followed by a stagnation at sampling after 12 days was observed for both isolates. After 15 days of incubation, however, the ROC level remained stable for the PP isolate and decreased significantly for the PR isolate.

For PR MUCL 46746, ROC content only differed upon growth on the two media at sampling after 15 days, being significantly higher on standard CYA than on CYA with reduced inorganic nitrogen content. PP CBS 112295 produced significantly more ROC on standard CYA than on CYA low on inorganic nitrogen from 9 days of incubation onwards.

- Conidiospores

Both *P. roqueforti s.l.* isolates significantly differed in conidiospore yield from 9 to 15 days of incubation: PR MUCL 46746 produced significantly more spores than PP CBS 112295. For the PR isolate, conidiospore freeze-dried weight clearly increased during the 15-days incubation period. This was not the case for the PP isolate, possibly due to difficult sampling of low quantities of conidiospores.

Despite the low conidiospore yield of PP CBS 112295, the ROC content of the spores did not differ significantly from the ROC content of PR MUCL 46746 spores throughout the incubation period. Taking into account the high standard deviations for ROC content, it might be concluded that the ROC content of *P. roqueforti s.l.* conidiospores was relatively constant over the 15-days incubation period.

- Agar medium

Upon growth on standard CYA, no significant difference between both *P. roqueforti s.l.* isolates in ROC content of the agar medium was detected. However, on CYA with reduced inorganic nitrogen the PR isolate excreted significantly more ROC to the medium than the PP isolate. For the PR isolate, no significant differences in ROC excretion between both media were found. As for the PP isolate, the ROC content of CYA low on inorganic nitrogen was significantly lower compared to the ROC content of standard CYA. This might suggest a reduced excretion of ROC by PP CBS 112295 in a culture medium low on inorganic nitrogen.

2.1.4. Discussion and conclusion

- Depletion of inorganic nitrogen

During fungal growth on CYA low on inorganic nitrogen, inorganic nitrogen gets depleted more quickly compared to standard CYA. Since ROC is an amino acid derived mycotoxin, this is expected to trigger an increase of ROC production (Miller 2008), but in this experiment the total fungal biomass did not produce ROC more quickly nor more intensely upon incubation on CYA with reduced inorganic nitrogen content compared to standard CYA. Taking the ROC concentrations in the agar medium into account, it cannot be said that a significant amount of ROC was excreted to the medium, available for re-uptake later on. The possibility that some of the produced ROC has been metabolized by mycelia or germinating spores does remain.

Sumarah *et al.* (2005) found in their 12-days time course experiment that *P. crustosum* produced negligible amounts of ROC until depletion of the inorganic nitrogen in the liquid culture medium. Maximum production was observed after 6 and 9 days, coinciding with a shift to amino acids as nitrogen source. The majority of the produced ROC was contained in the mycelium (70 %) and not in the liquid culture medium. However, it is unclear to what extent the findings about ROC production by *P. crustosum* can be extrapolated to *P. roqueforti s.l..* Boichenko *et al.* (2002a) studied ROC production by *P. roqueforti s.s.* in different media and at different cultivation conditions. They detected a significantly positive correlation between biomass yield and ROC content, indicating an increased ROC production associated with more intense fungal growth. In the currently described *in vitro* experiment, however, this finding was not supported by the data on biomass weight and ROC content.

Kulakovskaya *et al.* (1997) found that in *P. crustosum*, nitrogen starvation favored intracellular accumulation of ROC and decreased ROC excretion. A synthetic liquid medium containing

mannitol, ammonium succinate and ammonium hydroxide at pH 5.2 was used, and *P. crustosum* was grown in stirred culture at 24 °C for two days. After harvesting the mycelia, they were grown in a 20 mM Mops-Tris buffer (pH 6.0) for 24-48 hours at 24 °C, causing nitrogen starvation. Subsequently, a C^{14}-labelled ROC uptake assay was performed with starved *P. crustosum* cells. Additionally, a ROC excretion assay was executed with non-starved cells. They found that the optimum pH for ROC excretion is around 6-7, while ROC uptake is maximal at 4.5. Therefore, it was concluded that an acid medium facilitates uptake and subsequent metabolism into germinating conidia and developing hyphae.
For the current *in vitro* experiment, CYA based media were used with a pH of 7.00-7.15, which should facilitate ROC excretion more than ROC uptake, if ROC transport by *P. roqueforti s.l.* is influenced by pH likewise as by *P. crustosum*.

- Comparison of current experiment with other mycotoxin partitioning studies

In 1977, Scott *et al.* published the results of a 49-days time course experiment with presumably a *P. roqueforti s.s.* strain (isolated from Gorgonzola cheese). They monitored biomass yield and ROC content of a stationary culture, in a liquid medium containing 20 gram yeast extract and 150 gram sucrose per liter medium (N=3). ROC was determined in the mycelial mats and in the liquid medium, by thin-layer chromatography. Most of the ROC was present in the mycelium. Essentially, ROC production paralleled mycelial growth: yield was maximum after 16 days of incubation and remained quasi stable until 49 days. Furthermore, they observed a quite high variability of ROC production between isolates on the one hand and between triplicates per isolate on the other hand.
Compared to the currently described experiment, the duration of Scott *et al.*'s experiment was much longer than 15 days and moreover, a liquid medium was used instead of a solid agar medium. The liquid medium used contained sucrose and yeast extract at much higher quantities than CYA medium. In the current experiment, a clear increase of the ROC content of the biomass was observed for the PP CBS 112295 isolate over the 15-days incubation period on standard CYA, unlike for the PR MUCL 46746 isolate. Variability of ROC production by fungal biomass between triplicates was relatively small, except for the elevated ROC levels observed for the PP isolate on standard CYA after 9, 12 and 15 days of incubation.

Atoui *et al.* (2007) studied the partitioning of ochratoxin A (OTA) produced by four *Aspergillus carbonarius* isolates on an artificial grape juice agar medium during a 20-days incubation period at 20-25 °C. Sampling of agar medium, conidiospores and fungal biomass was similar to the current experiment. For three of the four isolates, OTA content was highest in the

conidiospores (60-70 % of total OTA produced), followed by the mycelium and finally the agar medium. OTA was present in the outer layer of the spores. They also compared the OTA partitioning between *A. carbonarius* and *A. ochraceus* and found that for the latter species, OTA was mainly present in the agar medium. Some OTA was detected in the mycelial biomass, but the conidiospores contained very little OTA.

Comparing the partitioning of ROC in PR MUCL 46746 and PP CBS 112295 with the partitioning of OTA in *A. carbonarius* and *A. ochraceus* is far from evident, especially since in the current *in vitro* experiment not the whole agar media was sampled for ROC determination. However, the findings of Atoui *et al.* (2007) might still put the results obtained for ROC production by *P. roqueforti s.l.* into a different perspective due to the following considerations: first, ROC is synthetized from tryptophan and histidine, while OTA is derived from phenylalanine. So, both mycotoxins are amino acid derived (Frisvad *et al.* 2004). Secondly, the *Aspergillus* species *A. carbonarius* and *A. ochraceus* are very closely related (Gallo *et al.* 2013), just like *P. roqueforti s.s.* and *P. paneum*. Since OTA partitioning differs in both species, it would not be surprising if ROC partitioning would also differ between *P. roqueforti s.s.* and *P. paneum*. The current experiment detected a significantly higher ROC content in PP CBS 112295 biomass compared to PR MUCL 46746 biomass when grown on standard CYA during 9, 12 and 15 days.

- Effect of inoculum on ROC production

Boichenko *et al.* (2002a) found that a direct correlation existed between the extracellular ROC concentration and the amount of *P. roqueforti s.s.* inoculum, probably related to a more or less intense mycelial growth. However, just one fungal isolate was used in the study. In the current experiment, the amount of inoculum per object and per replicate was theoretically equal for both *P. roqueforti s.l.* isolates. The *in vitro* experiment with four agar media described in chapter 3 did reveal a significantly stronger growth of PP CBS 112295 compared to PR MUCL 46746 on CYA, but this was not associated with a significant difference in ROC production.

- Correlations between parameters in relation to literature data

Pearson's correlations between the five parameters assessed in the current *in vitro* experiment (i.e. biomass weight and its ROC content, conidiospore weight and its ROC content, and ROC content of agar media) have been determined on the whole dataset (total N = 48). Significant positive correlations were detected between the ROC content of the biomass and its weight, as well as the ROC content of the agar medium, with resp. correlation coefficients 0.291 (p-value 0.045) and 0.409 (p-value 0.004). The first confirms that ROC production generally

parallels fungal growth. The latter indicates that increased ROC production by the biomass is associated with an increased excretion of ROC into the agar medium, which is in line with energy-independent excretion driven by a concentration gradient from cytosol to culture medium (Kulakovskaya *et al.* 1997).

The current *in vitro* experiment could not detect an increased production of ROC upon depletion of the inorganic nitrogen. Perhaps the inorganic nitrogen did not become depleted during the 15-days incubation period. Another option is that ROC production might have increased between two sampling moments, while ROC content of the biomass dropped subsequently due to metabolization of the produced ROC. In order to investigate this more profoundly, it would be interesting to perform a similar experiment in the future including also the monitoring of the nitrogen content of the medium, with short sampling intervals.

2.2. Effect of temperature and oxygen concentration on growth of *P. roqueforti s.s.*

Most *in vitro* experiments with fungi which are described in literature are performed in aerobic conditions, with “normal” atmospheric oxygen and carbon dioxide concentration (respectively 21 % and 0.04 %). In silages, however, oxygen depleted and carbon dioxide enriched conditions are created during the ensiling process. The effect of high carbon dioxide and low oxygen atmospheres on food spoilage fungi, including *P. roqueforti s.s.* growth and ROC production, has been investigated *in vitro* at 25 °C on Potato Dextrose Agar (PDA) and Czapek-Dox Yeast extract Agar (CYA) by Taniwaki *et al.* (2009): oxygen concentration was below 0.5 %, while carbon dioxide levels were 20, 40 and 60 %. *P. roqueforti s.s.* displayed growth during a 30-days incubation period at 20 % carbon dioxide, but not at 40 and 60 %. ROC production remained at a low level: maximum ROC production was observed on PDA after 15 days of incubation (*i.e.* 15 ng per ml agar medium).

In the current *in vitro* experiment, silage atmospheric conditions were mimicked using Anaerocult® C in anaerobic jars, which lowers the oxygen volume from 21 % to 5-7 % and increases the carbon dioxide volume from 0.04 % to 8-10 %. Literature data on the oxygen and carbon dioxide levels in sealed on-farm silages are scarce (Green *et al.* 2012, McGechan and Williams 1994; Parsons 1991; Williams 1994). Green *et al.* (2012) have detected oxygen concentrations up to 1.6% at 10 cm below the top layer in a silo with damaged coverage, whereas in a similar silo with intact cover no oxygen was detected from three days after ensiling onwards. It is difficult to determine whether an oxygen concentration of about 6 % in combination with about 9 % of carbon dioxide is representative for a silage environment, especially since the gas composition of silages is not homogeneous throughout the silo and throughout the ensiled period (Green *et al.* 2012; Williams 1994). Therefore, and also based on the available infrastructure, it was decided to use Anaerocult® C in anaerobic jars for modified atmosphere testing.
As a reference, normal atmospheric conditions were also included in the experimental setup. At both incubation regimes, the effect of different temperatures ranging from 5 to 35 °C on growth of *P. roqueforti s.s.* has been evaluated. To check if anaerobic conditions effectively inhibit fungal growth, *P. roqueforti s.s.* has also been incubated anaerobically at 25 °C.

2.2.1. Materials and methods

The PDA medium was prepared as described in Annex 1 and poured into standard 90-mm Petri dishes. After solidification of the medium, an agar plug of 5 mm diameter was removed from the center of each plate. In this central opening, 20 µl of conidiospore solution of *P. roqueforti s.s.* MUCL 46746 (containing $0.5*10^6$ spores/ml, prepared as described in Annex 1) was brought. Subsequently, plates were sealed with parafilm and incubated upright during seven days at the appropriate temperature and incubation regime. To create an O_2-depleted and CO_2-enriched environment, anaerobic jars with Anaerocult® C were used. Anaerobic conditions were created in anaerobic jars with Anaerocult® A.

Fungal growth was monitored by registration of the colony diameter at two transversal positions on the reverse side of the plates after seven days of incubation, and the corresponding growth area was calculated.

Data were statistically analyzed as described in Annex 1.

The setup of the experiment is summarized in Table 4.3.

Table 4.3. *Experimental setup of an in vitro study assessing the effect of temperature and incubation regime on growth of P. roqueforti s.s. MUCL 46746 on Potato Dextrose Agar (PDA).*

Infection	Incubation regime	Temperature	Monitoring
P. roqueforti s.s. MUCL 46746	Aerobic conditions	5 °C	Fungal growth after 7 days of incubation (N=3)
		10 °C	
		15 °C	
		20 °C	
		25°C	
		30 °C	
		35°C	
	Conditions of oxygen depletion and carbon dioxide enrichment	5 °C	
		10 °C	
		15 °C	
		20 °C	
		25°C	
		30 °C	
		35°C	
	Anaerobic conditions	25°C	

2.2.2. Results

The mean growth area per object after seven days of incubation is presented in Figure 4.6. Since no growth was observed at 35 °C in aerobic and oxygen depleted conditions nor during anaerobic incubation at 25 °C, these results are not mentioned.

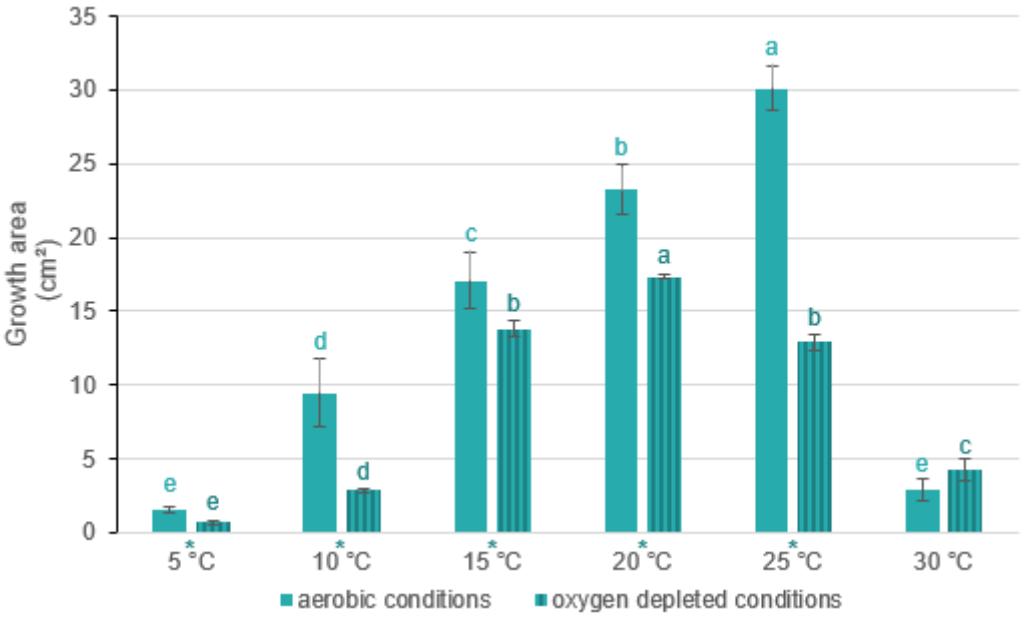


Figure 4.6. *Growth of P. roqueforti s.s. MUCL 46746 in aerobic conditions or oxygen depleted conditions, at different temperatures.* Mean growth area per object is presented as a bar chart, with error bars representing the resp. standard deviation. A significant interaction between temperature and incubation regime was detected, so the effect of each factor was assessed per level of the other factor. The effect of temperature is indicated by colored lettercode per incubation regime. Significant differences between the incubation regimes are indicated per temperature level by asterix (*) symbols.

At “normal” aerobic incubation conditions, the growth area of *P. roqueforti s.s.* MUCL 46746 significantly increased at rising temperatures, except for 30 °C. Growth area after aerobic incubation at 30 °C was at a comparable level as at 5 °C. Optimum growth temperature was 25 °C, corresponding to the findings of Samson and Frisvad (2004).

Incubation in oxygen depleted and carbon dioxide enriched conditions resulted in significantly lower growth compared to aerobic incubation at all tested temperatures except for 30 °C. The growth area increased significantly from 5 over 10 and 15 °C, until 20 °C which was the optimum temperature observed in oxygen depleted conditions. Growth at 25 °C was at a similar level as at 15 °C, while growth at 30 °C was intermediate between growth at 5 and 10 °C.

2.2.3. Discussion and conclusion

Walsh (1972) observed that colony diameter (and the growth area derived from it) may not be an effective way to evaluate fungal growth under reduced oxygen levels due to formation of large, sparse colonies with little biomass under such conditions. Taniwaki *et al.* (2009), however, did not observe such colony morphology for *P. roqueforti s.s.* in oxygen depleted conditions. It was not observed in the current *in vitro* experiment either.

In the same experiment as already described in section 2.1.4 of this chapter, Scott *et al.* (1977) have compared *P. roqueforti s.s.* growth and ROC production at 15 °C and 25 °C. Mycelial weight followed nearly the same trend during the 49-days incubation period at 15 °C as at 25 °C. ROC production during incubation at 15 °C was maximal after 49 days of incubation and was then at a level of 60-70 % of the maximum ROC yields obtained at 25 °C (which was already observed after 16 days, after which no subsequent degradation was detected). However, isofumigaclavine production was also evaluated and, contrary to ROC, incubation at 15 °C instead of 25 °C did not result in a reduction but in a severe augmentation of isofumigaclavine production (*i.e.* a triplication).
In the current *in vitro* experiment, ROC analyses have not been performed. Growth area of PR MUCL 46746 after 7 days did not differ significantly upon incubation at 15 or 25 °C in oxygen depleted conditions. However, at “normal” aerobic incubation, 25 °C significantly stimulated growth compared to 15 °C.

No detailed literature data on the effect of temperature on *P. roqueforti s.s.* growth and mycotoxin production in a non-cheese production context could be found, so hereafter *in vitro* experiments with other fungal species are discussed.

Wagener *et al.* (1980) have studied growth of *P. commune* as well as ROC production in a liquid medium (containing 20 gram of yeast extract and 40 gram of sucrose per liter) in a stationary culture during 84 days. As in the current *in vitro* experiment, the tested temperatures were 5, 10, 15, 20, 25, 30 and 35 °C. At 35 °C, no fungal growth was detected either. The strongest growth was observed at 25 and 30 °C, so the results obtained with *P. commune* are well in line with the current results for PR MUCL 46746. Wagener *et al.* also found that stationary cultures of *P. commune* generated higher levels of ROC than shake cultures, indicating an inhibitory effect of oxygen on ROC biosynthesis. Since ROC production has not been determined in the current *in vitro* experiment, this finding cannot be checked. Moreover, Wagener *et al.* expressed ROC production as mg per 100 ml culture medium, rendering it impossible to check the relationship between fungal growth and ROC production.

Baert *et al.* (2007) have studied the effect of storage conditions of apples on growth and patulin production by *P. expansum*. Apples are usually stored under controlled atmosphere conditions (*i.e.* temperature 0.5-3.5 °C, oxygen level 1-3 % and carbon dioxide 0.8-3 %). They evaluated the effect of different incubation temperatures (2, 4, 7, 10, 12, 16, 20, 25 and 30 °C) in a "normal" air atmosphere, using an apple puree agar medium. The growth observed at 30 °C was intermediate between growth observed at 12 and 16 °C. Optimum growth temperature was 25 °C, just as observed for PR MUCL 46746 in the current experiment. As for patulin production, an increase over time was observed, corresponding with an increasing fungal growth area. The lower the temperature, the longer the incubation time to reach a certain growth area and the higher the patulin level. The highest concentrations for a given growth area were observed at 4°C. Patulin production was characterized by a large variability between different *P. commune* strains, but also between replicates (N = 2, 3 or 5 according to the incubation temperature). Growth experiments performed at reduced oxygen levels (*i.e.* 1-3 %) revealed very little to no influence on *P. commune* growth. They concluded that generally the induction of limited stress, such as lowering temperature or oxygen levels, stimulates patulin production, whereas the combination of both factors reduced patulin formation.

It can be concluded from the current *in vitro* experiment that the optimum growth temperature of *P. roqueforti s.s.* is around 20-25 °C, while fungal growth is not observed at 35 °C. Decreasing the atmospheric oxygen content from 21 to approx. 6 % and increasing the carbon dioxide content from 0.04 % to approx. 9 % generally slowed down fungal growth, but did not inhibit it. Since ROC content has not been determined, no conclusions can be drawn about the effect of temperature and incubation regime on ROC production. Given the findings of other authors about the effect of oxygen levels and temperatures on fungal growth and mycotoxin production, it would be very interesting to repeat the experiment in the future, including more *P. roqueforti s.l.* isolates on the one hand and the analysis of not only ROC but also other mycotoxins produced by *P. roqueforti s.l.* on the other hand.

3. *In vivo* effect of abiotic factors on *P. roqueforti s.l.* growth, evaluated with microsilos

3.1. Introduction on microsilos

For microsilo experiments, small-scale silos are used with a volume of 2.75 liter, simulating the ensiling process in farm-scale silos. Such a microsilo is shown in Figure 4.7. Their small seize allows inclusion of multiple replicates per treatment, so statistical analysis can be performed.
A microsilo consists of a PVC tube sealed with rubber caps. The bottom cap is intact, while a central perforation is made in the upper cap. In this perforation, a CO_2-slot is placed. Such a CO_2-slot allows fermentation gasses to escape, but prevents air ingress. The microsilos are not equipped with an effluent discharge valve. Each PVC tube bears two holes (8 mm diameter) at 5.5 cm from the upper and lower edges of the tube. These holes are covered with Duct Tape, but can be opened during the ensiled period to provide “aerobic stress” to the silage.

Every microsilo has a unique number for identification. The empty weight of each microsilo is noted before filling. The microsilos are filled using attachment tubes and a hydro-pneumatic press, as visualized in Figure 4.7. The different objects are ensiled chronologically, starting with a negative control. Per object, the fresh feed commodity is spread evenly in a thin layer onto a polyethylene sheet and sprayed with an equal amount of treatment solution using handheld sprayers in a ratio of 10 ml of treatment solution per kg FM. The negative control is treated with the same amount of sterile physiological water. For each object, a new sprayer is used, as well as a new polyethylene sheet. After dosage of the treatment solution, the material is homogenized prior to filling of the microsilos. From the starting material of the negative control object, samples are taken for determination of the dry matter content or other analyses (e.g. fungal counts or nutritional value).

Figure 4.7. *Microsilo (left) and filling of a microsilo (right).*

The amount of fresh matter that is brought into a microsilo is calculated based on the desired silo density on the one hand and on the estimated dry matter content of the starting material on the other hand. The microsilos are filled in two stages, with intermediate and final pressing. Microsilos are filled by hand, wearing plastic gloves which are renewed per object. After filling and sealing of all the microsilos, their filled weight is noted. Weighing is repeated on a weekly basis to monitor the fermentation losses during the ensiled period.

After a certain period of time, the microsilos are desiled. After removal of the rubber caps, approx. 3 cm of silage is removed from the top and the bottom of the microsilos. The remaining silage is desiled, wearing plastic gloves which are renewed per object. In case of whole-crop maize silage, the desiled material is not homogenized prior to sampling to avoid de-mixing. In case of grass silage, the material is homogenized thoroughly before sampling (unless mentioned otherwise).

Several samples can be taken at desiling, for the following optional analyses (described in Annex 1):

- determination of the dry matter content of the desiled material by air drying at 65 °C.
- determination of the pH of the desiled material.
- mycotoxin quantification by LC-MS/MS.
- determination of the fermentation characteristics: ammonia, ratio of ammonia nitrogen over total nitrogen, pH, lactic acid, acetic acid and butyric acid.
- enumeration of fungi by streak-plating of a dilution series on Potato Dextrose Agar (PDA).
- enumeration of *P. roqueforti s.l.* by streak-plating of a dilution series on Potato Dextrose Agar supplemented with 5 ml of acetic acid per liter (PDAA).

- determination of the aerobic stability: the stability of desiled material upon exposure to oxygen is determined by the protocol described by Honig (1990). The equivalent of approximately 100 gram dry matter is placed loosely into aerated recipients with a volume of 1 liter. At the bottom of the recipients, a 1-cm diameter hole is present to allow gas exchange driven by differences in weight between carbon dioxide and air. Temperature data loggers, each bearing a unique reference number, are placed into the geometric center of each silage mass. The recipients are not sealed, but are covered with a double layer of miracloth to prevent drying. The desiled material is allowed to deteriorate aerobically at 20 °C in insulated boxes allowing gas exchange, as illustrated in Figure 4.8. Silages are not disturbed during testing over a 7-days period. Ambient temperature and silage temperature are recorded every 20 minutes and later on averaged over 2-hour periods.

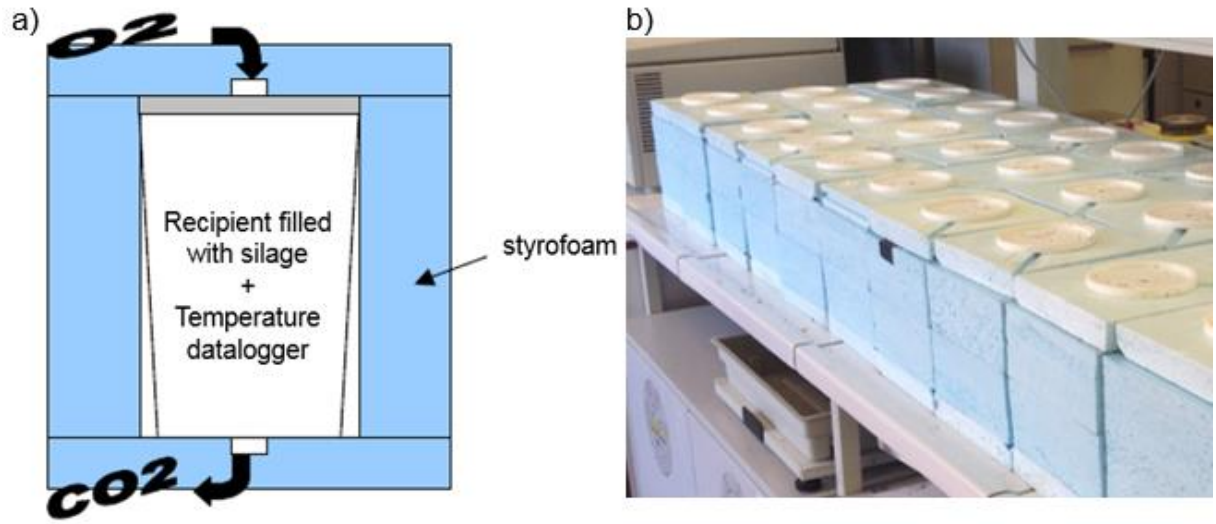


Figure 4.8. *Honig protocol: a) principle, and b) infrastructure for determination of the aerobic stability of silages.*

A temperature rise of 3 °C above ambient temperature is taken as an indicator of aerobic instability, so the calculated aerobic stability represents the number of hours it took for the temperature of the desiled material to rise up to 3 °C above the ambient temperature. If no 3 °C temperature difference is observed within 7 days, an arbitrary aerobic stability of 175 hours is adopted for statistical analysis. At the end of the Honig protocol, the DM content is determined again.

Depending on the experimental setup of each microsilo experiment, the analyses performed on the desiled material vary, but the dry matter content is systematically determined along with the pH.

3.2. Evaluation of the effect of prolonged anaerobic conditions in whole-crop maize silage

Petersson (1998) and Richard-Molard *et al.* (1980) observed that *P. roqueforti* spores stored under airtight conditions for three months had a strongly reduced germination ability. To assess the effect of prolonged anaerobic conditions on the survival of *P. roqueforti s.s.* (PR) in silage, a microsilo experiment was carried out with whole-crop maize artificially infected with PR MUCL 46746, comprising three desiling moments: 50, 100 and 150 days. Additionally, the effect of three frequently used silage additives on conidiospore survival in silage was examined: HoLAB, HeLAB and propionic acid. As stated in chapter 1, HoLAB inoculants stimulate silage fermentation by efficient lactic acid production. However, they might impair the aerobic stability of silage (Driehuis *et al.* 2001; Kung and Ranjit 2001).

Aerobic deterioration of silage is initiated by yeasts, which are the principal target of aerobic deterioration inhibiting additives like HeLAB inoculants and propionic acid. Since aerobic deterioration caused by yeast may provoke fungal growth, all three silage additives might also influence *P. roqueforti s.l.* in silages in an indirect manner.

Due to the inclusion of LAB-based inoculants, this ensiling trial might also be included in the next chapter, evaluating the effect of some biotic factors on *P. roqueforti s.s.* growth and ROC production. However, since the main goal of the trial was to determine the effect of prolonged anaerobic conditions, it suits well in the current chapter.

3.2.1. Materials and methods

Whole-crop maize was chopped to a theoretical particle length of 6 mm with a New Holland precision chopper prior to ensiling. For the negative control object, freshly chopped whole-crop maize was treated with 20 ml of sterile physiological water per kg FM. Subsequently, samples were taken prior to ensiling for determination of the DM content and for enumeration of the amount of *P. roqueforti s.l.* propagules: the DM content was 35 %, while no *P. roqueforti s.l.* propagules were found on the fresh crop prior to ensiling (N=3).

P. roqueforti s.s. MUCL 46746 spore solution was freshly prepared as described in Annex 1 (but no glycerol was added and the conidiospore concentration was not adjusted to $1*10^6$ spores/ml). Per kg FM, 10 ml of PR MUCL 46746 conidiospore solution was applied to artificially infect whole-crop maize, as well as 10 ml of the appropriate additive solution. For both Pioneer 1188 (HoLAB) and Pioneer 11A44 (HeLAB), a new commercial unit was used, containing resp. $1.25*10^{11}$ cfu/g and $1.0*10^{11}$ cfu/g of powder. The appropriate amount of

inoculant powder was dissolved in sterile physiological water. For propionic acid, a sterile 45/55 mixture (vol/vol) with distilled water was prepared.
From the object artificially infected with PR MUCL 46746 without additive application, the *P. roqueforti s.l.* propagules were enumerated (N=3): the whole-crop maize was infected with 1500 conidiospores per gram FM, representing a moderately low infection level.

Per object, twelve microsilos were filled with approximately 1.70 kg fresh matter. Mean silo density was 215 kg DM/m³, representing a very well compacted silo. The microsilos were stored at the barn of the Bottelare research center during 150 days. Barn temperature was not recorded, but was estimated to be in the range of 5-15 °C based on weather records.
The fermentation losses were monitored on a weekly basis. No aerobic stress was provided during the ensiled period, mimicking good quality silage. Per object, four microsilos were desiled after 50 days, 100 days and 150 days. At desiling, all microsilos were sampled for determination of the dry matter (DM) content and fermentation characteristics, and for enumeration of *P. roqueforti s.l.* propagules.
The obtained data were statistically analyzed as described in Annex 1.

The experimental setup of the current microsilo trial is summarized in Table 4.4.

Table 4.4. *Experimental setup of microsilo trial evaluating the effect of prolonged anaerobic conditions in whole-crop maize silage: negative control (no infection and no additive) versus additive applications after infection with P. roqueforti s.s. MUCL 46746.*

OBJECTS			
Infection	**Additive**	Additive solution	**N**
no infection	no additive	sterile physiological water	12
P. roqueforti s.s.	no additive	sterile physiological water	12
MUCL 46746	HoLAB	Pioneer 1188: *L. plantarum* and *E. faecium* @ 1.10^6 cfu/g FM	12
@ 1500 spores/g FM	HeLAB	Pioneer 11A44: *L. buchneri* @ 1.10^6 cfu/g FM	12
	propionic acid	99% propionic acid @ 4.5 liter/ton FM	12

Desiling after 50, 100 and 150 days (N=4 per object)
Fermentation losses, *P. roqueforti s.l.* enumeration, dry matter and fermentation characteristics

3.2.2. Results and discussion

3.2.2.1. Fermentation losses

The evolution of the fermentation losses per object over an ensiled period of 150 days is presented in Figure 4.9.

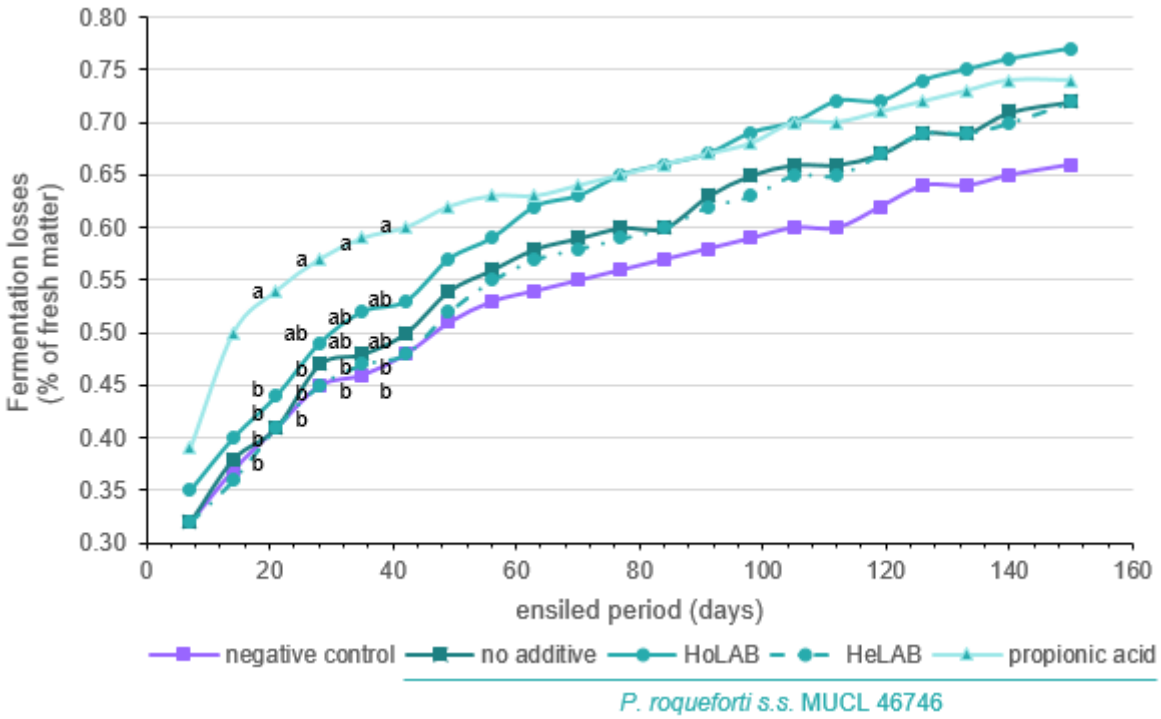


Figure 4.9. *Fermentation losses during the ensiled period (% of fresh matter) observed in the microsilo trial evaluating the effect of prolonged anaerobic conditions in whole-crop maize silage: negative control (no infection and no additive) versus additive applications after infection with P. roqueforti s.s. MUCL 46746.* The mean values per object are represented without error bars indicating their resp. standard deviation, but standard deviations were small for all objects (max 0.08 % of fresh matter). At some time points, significant differences between the objects were detected, as indicated by lettercode.

Fermentation losses were low for all objects during the 150-days ensiled period (McDonald *et al.* 1991). Only during the first 40 days after ensiling, some significant differences between the objects were detected. The highest fermentation losses were observed in PR-infected maize treated with propionic acid. This observation is opposite to the expectations: propionic acid is a fermentation inhibiting silage additive, so it is expected to reduce losses. Regarding HoLAB and HeLAB inoculants, HoLAB are more efficient fermenters than HeLAB, so HeLAB inoculation is expected to increase fermentation losses compared to HoLAB inoculation (McDonald *et al.* 1991; Oude Elferink *et al.* 2001; Pahlow *et al.* 2003).

Why the observed pattern of fermentation losses was divergent from the expected pattern remains unclear, but it must be noted that the differences in fermentation losses between the different objects were very small in absolute values.

3.2.2.2. Analyses at desiling

To examine if prolonged anaerobic conditions have a lethal effect on *P. roqueforti s.l.* propagules in whole-crop maize silage and to assess if *P. roqueforti s.l.* survival is affected by the application of a silage additive, *P. roqueforti s.l.* propagule enumeration was performed upon desiling after 50, 100 and 150 days on samples taken from the center of the microsilos.

No visible fungal growth was detected at desiling for any microsilo. The results of the *P. roqueforti s.l.* counts are presented in Figure 4.10.

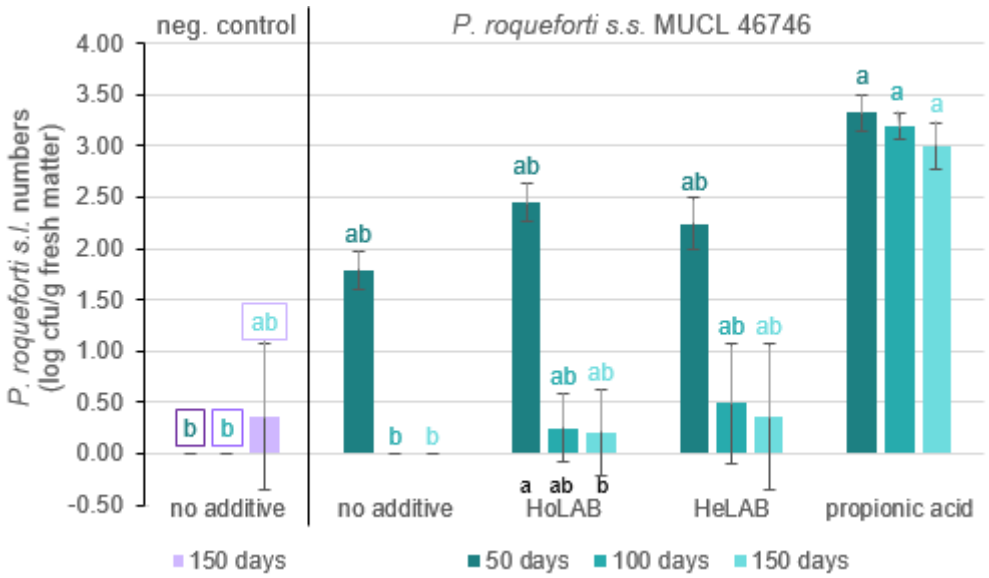


Figure 4.10. *P. roqueforti s.l. numbers (log cfu per gram fresh matter) at desiling after 50, 100 and 150 days for the microsilo trial evaluating the effect of prolonged anaerobic conditions in whole-crop maize silage: negative control versus additive applications after infection with P. roqueforti s.s. MUCL 46746.* The mean values per objects are presented as a bar chart with error bars indicating their resp. standard deviation. A significant interaction between object and ensiled period was detected. The effect of each factor was assessed per level of the other factor. Differences between objects are indicated by colored lettercodes per time point. A significant difference according to the ensiled period was only detected for the HoLAB additive (by non-parametric testing according to Kruskal-Wallis), indicated by lettercode below the x-axis.

Prolonged anaerobic conditions (*i.e.* 150 days) significantly lowered *P. roqueforti s.l.* numbers compared to an ensiled period of 50 days.

The negative control object contained no *P. roqueforti s.l.* propagules upon desiling after 50, 100 days. At desiling after 150 days, a high standard deviation was observed: three samples did not contain *P. roqueforti s.l.* propagules, while one sample contained 28 cfu/g FM.

For all PR MUCL 46746 infected objects, propagule counts at desiling after 50 days still outnumbered 60 cfu/g FM (corresponding to 1.81 log cfu/g FM). When no additive was applied, no more propagules were detected after an ensiled period of 100 or 150 days, confirming the findings of Petersson (1998) and Richard-Molard *et al.* (1980). Very low *P. roqueforti s.l.* numbers were detected in HoLAB and HeLAB inoculated silage, whereas propionic acid application was not successful in reducing *P. roqueforti s.l.* numbers: even after 150 days of ensiling, still $1*10^3$ cfu/g FM were detected. So, despite the fact that propionic acid has antifungal properties, the applied dosage seems to have been too low to exert this effect on the *P. roqueforti s.l.* spores present on the whole-crop maize.

The results of the determination of dry matter content and fermentation characteristics per object for the three ensiled periods are presented in Table 4.5.

Table 4.5. *Results of analyses at desiling of microsilo trial evaluating the effect of prolonged anaerobic conditions in whole-crop maize silage: corrected dry matter (DM), ammonia, ratio of ammonia nitrogen over total nitrogen, pH, lactic acid and acetic acid.* The mean values per object are mentioned with their resp. standard deviation between brackets. A significant interaction between the factors object and ensiled period was only observed for acetic acid. For this parameter, the effect of one factor is assessed per level of the other factor. The obtained lettercodes indicating significant differences are mentioned in grey. For the other parameters, significant main effects of object and ensiled period are indicated by black-colored lettercodes.

Ensiled period	OBJECTS		cDM		ammonia			NH3-N / total N			pH		lactic acid		acetic acid		
	Infection	Additive	(g/kg FM)	*obj.*	(g/kg cDM)	*obj.*	*ens.p.*		*obj.*	*ens.p.*		*ens.p.*°	(g/kg cDM)	*obj.*	(g/kg cDM)	*obj.*	*ens.p.*
50 days	negative control	no additive	362 (4)	ab	0.70 (0.03)	a		4.8 (0.3)	a		3.79 (0.01)		55 (1)	a	17 (1)	a	b
	P. roqueforti s.s. MUCL 46746	no additive	358 (2)	c	0.70 (0.02)	a		4.6 (0.2)	a		3.80 (0.02)		56 (2)	a	20 (2)	a	b
		HoLAB	365 (9)	a	0.67 (0.02)	a	c	4.5 (0.4)	a	c	3.78 (0.01)	b	55 (2)	a	19 (1)	a	c
		HeLAB	360 (1)	bc	0.69 (0.02)	ab		4.6 (0.2)	a		3.79 (0.00)		56 (2)	a	18 (1)	a	c
		propionic acid	356 (4)	c	0.62 (0.05)	b		3.9 (0.3)	b		3.80 (0.01)		52 (2)	b	13 (1)	b	b
100 days	negative control	no additive	364 (3)	ab	0.83 (0.01)	a		5.5 (0.2)	a		3.77 (0.01)		57 (1)	a	20 (1)	b	ab
	P. roqueforti s.s. MUCL 46746	no additive	357 (4)	c	0.82 (0.02)	a		5.4 (0.4)	a		3.78 (0.01)		58 (2)	a	23 (1)	a	a
		HoLAB	365 (2)	a	0.78 (0.02)	a	b	5.3 (0.2)	a	b	3.78 (0.01)	a	55 (1)	a	22 (1)	a	b
		HeLAB	358 (4)	bc	0.79 (0.03)	ab		5.3 (0.2)	a		3.78 (0.01)		57 (1)	a	22 (1)	a	b
		propionic acid	355 (0)	c	0.68 (0.05)	b		4.4 (0.3)	b		3.80 (0.01)		51 (2)	b	15 (1)	c	ab
150 days	negative control	no additive	362 (2)	ab	0.87 (0.01)	a		6.2 (0.6)	a		3.78 (0.01)		55 (3)	a	21 (1)	b	a
	P. roqueforti s.s. MUCL 46746	no additive	355 (4)	c	0.84 (0.01)	a		5.8 (0.4)	a		3.77 (0.01)		55 (2)	a	25 (1)	a	a
		HoLAB	361 (2)	a	0.90 (0.10)	a	a	5.9 (0.7)	a	a	3.78 (0.01)	a	56 (1)	a	26 (1)	a	a
		HeLAB	360 (2)	bc	0.85 (0.02)	ab		5.5 (0.2)	a		3.77 (0.01)		57 (1)	a	24 (1)	a	a
		propionic acid	360 (2)	c	0.78 (0.05)	b		5.0 (0.3)	b		3.78 (0.01)		52 (1)	b	17 (1)	c	a

° Non-parametric test according to Kruskal-Wallis

The dry matter content was determined as described in Annex 1 and has been corrected for volatile compounds according to Dulphy and Demarquilly (1981), therefore the corrected dry matter content (cDM) is mentioned. The ensiled period did not have a significant effect on the cDM, but a significant effect of object was detected: HoLAB inoculation of PR-infected silage resulted in a significantly higher cDM content compared to PR-infected silage without additive application, HeLAB inoculation and propionic acid treatment. However, the absolute values of cDM of the different objects were within a very narrow range, ascribing the observed significant differences quasi unimportant for on-farm practice.

The ammonia content did not differ much between objects on the three desiling moments in absolute values, but a significant effect of ensiled period as well as object was found. Propionic acid treatment reduced the ammonia content of the silage, as well as the ratio of ammonia-nitrogen over total nitrogen. This can be ascribed to its inhibiting properties towards protein degrading bacteria. However, this ratio was at a low level for all objects (*i.e.* below ten), indicating no severe protein degradation (McDonald *et al.* 1991; Oude Elferink *et al.* 2000; Oude Elferink *et al.* 2001; Shao *et al.* 2005). Both ammonia content and ammonia fraction increased significantly from an ensiled period of 50 to 100 and 150 days.

The pH at desiling was at a low level for all objects at all three desiling moments, indicating well-preserved whole-crop maize silage. No significant influence of object on pH was detected: pH-values were quasi the same for the different objects at a given ensiled period. The ensiled period did have a significant effect on pH: pH was significantly higher at desiling after 50 days compared to the pH observed at desiling after 100 and 150 days. However, in absolute value there was quasi no difference.

The amounts of lactic acid and acetic acid were more variable than the pH-values. HoLAB inoculation of silages usually results in elevated lactic acid levels and reduced acetic acid levels, while HeLAB inoculation usually augments the acetic acid amounts produced. This was not confirmed statistically in this microsilo experiment: only propionic acid treatment resulted in significantly lower lactic acid levels compared to the other objects, due to partial inhibition of the LAB fermentation. No significant difference in the production of acetic acid was found either between HoLAB and HeLAB. Propionic acid treatment resulted in the lowest acetic acid levels for all three ensiled periods, compliant with findings of Shao *et al.* (2005). Interestingly, the acetic acid content of the two objects without silage additive application differed significant at desiling after 100 and 150 days, being higher after artificial infection with PR MUCL 46746 than without. No reasonable explanation for this phenomenon could be found in literature. Butyric acid has been determined, but since the levels were below the limit of detection in all samples, this acid is not mentioned in Table 4.5.

3.2.2.3. Discussion

Propionic acid has been dosed at 4.5 ml/kg FM, corresponding to a propionic acid concentration of 94 mM in the plant moisture. An *in vitro* experiment with PR MUCL 46746 in double-concentrated Potato Dextrose Broth (PDB) supplemented with different propionic acid concentrations (unpublished data) revealed no detectable growth (registered spectrophotometrically by the optical density at 620 nm) and no spore germination (evaluated microscopically) during an aerobic incubation period at 25 °C for three days for propionic acid concentrations higher than 75 mM. The pH of the liquid medium containing 75 mM of propionic acid was 3.63.

In the same *in vitro* experiment, different concentrations of lactic acid or acetic acid were also added to double-concentrated PDB. The lactic acid and acetic acid contents of the silage samples obtained in the current microsilo trial can be related to the results of this *in vitro* experiment:
The average lactic acid content of all microsilos was 55 g/kg cDM, corresponding to a lactic acid concentration of nearly 400 mM in the plant moisture. At this lactic acid concentration (pH of 2.22 in the liquid medium), fungal growth as well as conidiospore germination was registered *in vitro* after 72 hours.
Over all microsilos, the average acetic acid content was 20 g/kg cDM. This corresponds to an acetic acid concentration of 216 mM in the plant moisture. *In vitro*, up to 210 mM of acetic acid was added to double-concentrated PDB (resulting in a pH of 3.10). Fungal growth was not detected at 200 mM, but spore germination was visible microscopically. At 210 mM, no fungal growth and no spore germination were detected.

When comparing these *in vitro* results with the observations *in vivo* in a silage matrix, the following considerations can be made:

1) Most importantly, absence of spore germination and subsequent fungal growth *in vitro* during a three-days incubation period do not imply a fungicidal effect. However, the *in vitro* experiment did not comprise a conidiospore survival assay.

2) *In vitro*, the liquid media were deliberately not buffered to allow an increase of the pH upon *P. roqueforti s.l.* growth, since Wagener *et al.* (1980) observed an increasing pH in media supporting high ROC production. On-farm sampling of silages also showed a significant rise of pH in fungal hot-spots, despite the fact that silages contain buffering agents. Therefore, relatively low pH values were registered *in vitro* compared to the pH of silage samples in the current *in vivo* experiment.

3) In silages, lactic acid and acetic acid are present together, possibly complemented with some propionic acid. Synergistic effects between these acids and other fermentation products may arise (Moon 1983), so the final effect on fungal growth is difficult to assess.

4) During the *in vitro* experiment, the *P. roqueforti s.s.* conidiospores were in direct contact with the liquid culture medium containing lactic acid, acetic acid or butyric acid. *In vivo* in silages, however, the conidiospores are not submerged in a liquid phase. They are contained in the silage mass, composed of 35 % dry matter and 65 % of moisture. This moisture is largely present within the plant cells, in indirect contact with the conidiospores.

Looking at the obtained data on *P. roqueforti s.l.* counts per object for the microsilo trial, it is clear that the applied dosage of propionic acid did not have a fungicidal effect on the conidiospores present on the whole-crop maize at ensiling.

No additive application on artificially infected silage did result in a significant reduction of *P. roqueforti s.l.* numbers at desiling after 100 and 150 days. The effectivity of the applied inoculants can be questioned, since their application did not result in a significantly different organic acid production pattern compared to the negative control. However, it is also possible that the epiphytic LAB composition was so well established that inoculated LAB could not overpower the epiphytic LAB at the applied dosage.

3.2.3. Conclusion

The conclusion of the microsilo trial evaluating the effect of prolonged anaerobic conditions on *P. roqueforti s.l.* numbers and fermentation characteristics in grass silage towards on-farm practices is that the application of silage additives on *P. roqueforti s.s.* infected maize did not imply an added value regarding the lowering of *P. roqueforti s.l.* numbers at desiling nor regarding the fermentation characteristics. Additive application did result in some significant differences in fermentation profile. However, these differences were of relatively minor importance in absolute value, since very good silage quality was obtained without additive application. The whole-crop maize was ensiled at a suitable dry matter content and at high silo density. It is very likely that the additives will have had an influence on the aerobic stability of the silage, if this parameter would have been determined. This microsilo trial confirms that when ensiled properly, taking good ensiling and desiling practices into account, whole-crop maize silage fermentation quality is expected to be good even without silage additive application.

3.3. Evaluation of the effect of elevated temperature and oxygen supply on silage

During the ensiling process, silage temperature rises in the initial phases due to plant respiration and enzyme activity as well as microbial activity. In on-farm silages in a warm climate, temperatures of around 40 °C have been registered (Weinberg *et al.* 2001). However, Belgium has a temperate climate. No accurate information could be found in literature on the maximum temperatures observed during silage fermentation. To evaluate the effect of elevated temperature *in vivo* with microsilos, a suitable incubation temperature was chosen considering the following matters:

- Temperatures above 37 °C have a detrimental effect on silage quality, as described in Chapter 2
- *P. roqueforti s.l.* still grows at 30 °C, even in oxygen depleted conditions, but no growth was observed at 35 °C (as described in section 1.2 of this chapter).
- In silages, the Maillard reaction already occurs at temperatures of 35-40 °C (Muck *et al.* 2003).
- Borreani and Tobacco (2010) found that temperature in fungal hot-spots of whole-crop maize silages rises often above 30 °C.

As a compromise to increase temperature sufficiently and in the meantime remain below some critical values, temperature was increased to **32 °C** in microsilo experiments with grass and whole-crop maize by placing the microsilos in a ventilated dryer. Borreani and Tobacco (2010) also demonstrated that the ambient temperature influences the core temperature of on-farm silages to a depth of 20 cm. Regarding the small scale of the microsilos used, it can be assumed that the incubation temperature rules throughout the entire volume of the microsilos.

For both microsilo experiments, the effect of ambient temperature without oxygen supply (mimicking optimal silage fermentation conditions) was compared with the effect of elevated temperature from 61 to 64 days after ensiling in combination with oxygen supply (mimicking not 100% airtight sealed silage suffering from local aerobic deterioration due to damaged silo coverage) by means of a plastic drain inserted into the upper hole of the microsilos: the drain reached till the center of the microsilos and the inner part was micro-perforated. The outer part of the drain wasn't micro-perforated and was sealed at the end. After an ensiling period of 60 days, the end of the drain was cut off, allowing air ingress for 24 hours. Subsequently, the drain was closed again by heat-sealing and the microsilos were placed at 32 °C during four days.

Prior to and during the oxygen supply as well as after the four-days elevated temperature period, the microsilos were also stored at ambient temperature in the barn of the Bottelare

research center. The ambient temperature varied between 10 and 25 °C for the grass trial and between 5 and 15 °C for the whole-crop maize trial.

For whole-crop maize, an additional level of incubation regime was included in the experimental setup: elevated temperature (32 °C) during the first four days after ensiling, without oxygen supply (mimicking on-farm silage fermentation, characterized by a temperature rise due to microbial activity).

3.3.1. In vivo experiment with grass silage evaluating the effect of elevated temperature and oxygen supply

3.3.1.1. Materials and methods

Two incubation regimes were included in the microsilo trial with grass, as mentioned before, both without and with artificial infection with *P. roqueforti s.s.* MUCL 46746. Artificial infection with *P. roqueforti s.s.* spores was intense: $1*10^6$ conidiospores per gram fresh matter were inoculated onto the fresh grass prior to ensiling. In combination with the choice for a moderate silo compaction rate (McDonald *et al.* 1991; Wilkinson 2005), this should facilitate fungal growth.

Perennial ryegrass (*Lolium perenne*) was prewilted in the field and chopped to a theoretical particle length of 8 cm with a New Holland precision chopper. The uninfected grass was treated with sterile distilled water. Subsequently, samples were taken for determination of the dry matter (DM) content: the grass was ensiled at 37 % DM.

P. roqueforti s.s. (PR) MUCL 46746 spore solution was freshly prepared as described in Annex 1 (however no glycerol was added) and adjusted to a concentration of $1*10^8$ cfu/ml.

Per level of infection, six microsilos were filled with approximately 1.2 kg fresh matter, corresponding to a mean silo density of 160 kg DM/m^3.

Per incubation regime, three microsilos were stored as described in the introduction to this *in vivo* microsilo trial. The fermentation losses were monitored on a weekly basis. All microsilos were desiled after 76 days. Visible fungal growth on the desiled material was noted.
From each individual microsilo, samples were taken for aerobic stability testing and for determination of the fungal counts, the DM content and fermentation characteristics (as described in Annex 1). The obtained data were statistically analyzed as described in Annex 1.
The experimental setup of the microsilo trial is summarized in Table 4.6.

Table 4.6. *Experimental setup of microsilo trial evaluating the effect of elevated temperature and oxygen supply on grass silage: uninfected control versus P. roqueforti s.s. (PR) MUCL 46746.*

OBJECTS		
Incubation regime	**Infection**	**N**
Ambient temperature during the entire ensiled period, without oxygen supply	no infection	3
	PR MUCL 46746 @ 1.10^6 spores/g FM	3
Temperature of 32 °C at 61-64 days after ensiling, in combination with oxygen supply by plastic drain	no infection	3
	PR MUCL 46746 @ 1.10^6 spores/g FM	3
Desiling after 76 days		
Fermentation losses, mould enumeration, aerobic stability, corrected dry matter° and fermentation characteristics		

° Dry matter content corrected for volatile compound loss according to Dulphy and Demarquilly (1981).

3.3.1.2. Results and discussion

- **Fermentation losses**

Fermentation losses occur during the fermentation process. However, in the current microsilo trial, some objects have been subjected to oxygen supply. Therefore, the registered fresh weight losses don't only comprise fermentation losses but also losses due to aerobic metabolism, but the term "fermentation losses" will be adopted.

The evolution of the fermentation losses during the ensiled period is visualized in Figure 4.11.

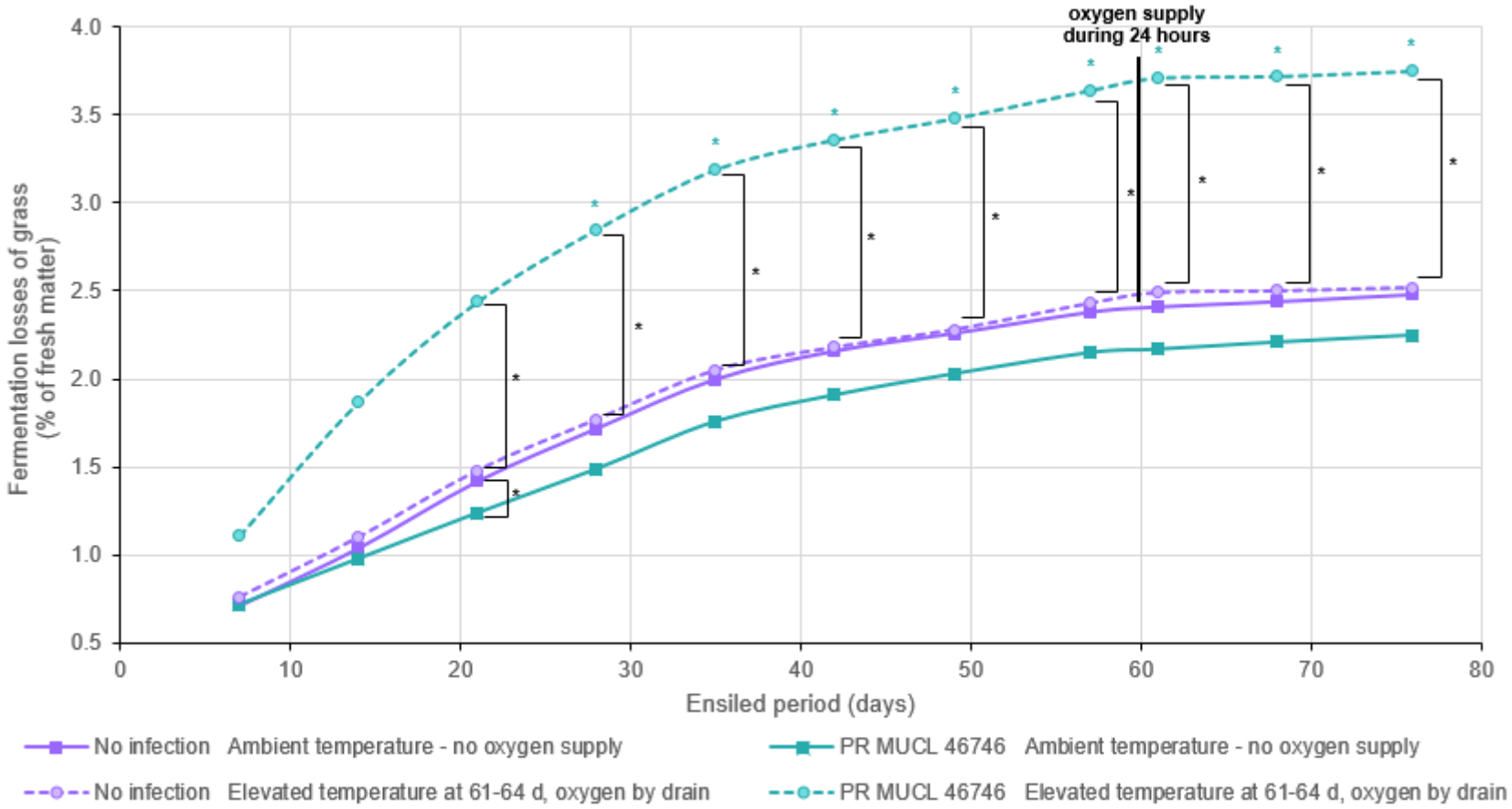


Figure 4.11. *Fermentation losses during the ensiled period (% of fresh matter) observed in the microsilo trial evaluating the effect of elevated temperature and oxygen supply on grass silage: uninfected control versus infection with P. roqueforti s.s. (PR) MUCL 46746.* The mean values per object are presented without error bars representing their resp. standard deviation. Standard deviations were in a range of 0.03-0.09, 0.02-0.23, 0.09-0.32 and 0.26-0.56 respectively for the uninfected and PR MUCL 46746 infected grass stored at ambient temperature and the uninfected and PR MUCL 46746 infected grass subjected to elevated temperature and oxygen supply. At all time points, a significant interaction between incubation regime and infection was observed, so the effect of each factor was assessed per level of the other factor. Significant differences between no infection and infection with *P. roqueforti s.s.* are indicated by black asterix (*) symbols per incubation regime. Significant differences between the incubation regimes are indicated by colored *- symbols.

Increased levels of fresh weight losses were observed for the object artificially infected with PR MUCL 46746 and subjected to elevated temperature in combination with oxygen supply compared to the other three objects throughout the entire ensiled period, but the losses still remained in a normal range (McDonald *et al.* 1991).

The fermentation losses for microsilos incubated at ambient temperature without oxygen supply did only differ significantly between uninfected and PR-infected silage at 21 days after ensiling. When elevated temperature in combination with oxygen supply was applied, the fermentation losses of the infected silage were significantly higher than those of uninfected silage from 21 days after ensiling until desiling after 76 days.

PR-infected silage showed significantly higher fermentation losses when subjected to elevated temperature and oxygen supply from 28 days after ensiling until desiling after 76 days. Since oxygen supply was provided at 60 days after ensiling followed by four days of elevated temperature, it is remarkable that the fermentation losses were significantly higher from 28 days after ensiling onward. This suggests that the plastic drain for oxygen supply did compromise the airtight sealing of the microsilos, resulting in significantly increased losses in PR-infected silage. However, uninfected silage receiving oxygen in a similar way did not exhibit increased fermentation losses throughout the 76-days ensiled period.

- **Analyses at desiling**

At desiling after 76 days, each microsilo was checked for visible fungal growth. Afterwards, the desiled material was homogenized prior to sampling. Fungal propagules were enumerated and the aerobic stability was determined by the Honig protocol. Dry matter content was determined at desiling and at the end of the Honig protocol. Some fermentation characteristics have been determined: ammonia and ammonia fraction, pH, lactic acid, acetic acid and butyric acid. The dry matter content at desiling has been corrected for volatile compounds according to Dulphy and Demarquilly (1981).

The results of the analyses at desiling are summarized in Table 4.7.

Table 4.7. *Results of analyses at desiling of microsilo trial evaluating the effect of elevated temperature and oxygen supply on grass silage: number of visibly mouldy microsilos, fungal counts, corrected dry matter (cDM), aerobic stability, dry matter after Honig protocol, ammonia, ratio of ammonia nitrogen over total nitrogen, pH, lactic acid, acetic acid and butyric acid.* Mean values per object are mentioned with their resp. standard deviation between brackets. For some parameters, a significant interaction between the factors object and ensiled period was observed. For these parameters, the effect of one factor is assessed per level of the other factor. The obtained lettercodes indicating significant differences are indicated in grey. For the other parameters, significant main effects of object and ensiled period are indicated by black-colored lettercodes.

Incubation regime	**Infection**	**visibly mouldy** (number)	*inc.*	**fungal counts** (log cfu/g FM)	*inf.*°		**corrected DM** (g/kg FM)	*inf.*	*inc.*	**aerobic stability** (hours)	*inc.*
Ambient temperature,	no infection	0 / 3	b	1.89 (0.40)	b	a	340 (5)	a	a	94 (10)	a
without oxygen supply	PR MUCL 46746	0 / 3		5.04 (0.48)			344 (5)	a	a	90 (13)	
Elevated temperature in	no infection	1 / 3	a	2.53 (0.89)			342 (5)	a	a	52 (15)	b
combination with oxygen supply	PR MUCL 46746	3 / 3		3.76 (1.82)			322 (5)	b	b	43 (11)	

Incubation regime	**Infection**	**DM after Honig** (g/kg FM)	*inc.*	**ammonia** (g/kg cDM)	**NH3-N / total N**	**pH**	*inf.*°	
Ambient temperature,	no infection	319 (2)	a	0.06 (0.00)	7.1 (0.1)	4.62 (0.01)	b	a
without oxygen supply	PR MUCL 46746	319 (6)		0.06 (0.00)	7.2 (0.0)	4.70 (0.02)		
Elevated temperature in	no infection	304 (10)	b	0.05 (0.01)	6.4 (1.0)	4.55 (0.13)		
combination with oxygen supply	PR MUCL 46746	295 (1)		0.06 (0.00)	6.7 (0.9)	4.71 (0.08)		

Incubation regime	**Infection**	**lactic acid** (g/kg cDM)	*inf.*	*inc.*	**acetic acid** (g/kg cDM)	**butyric acid** (g/kg cDM)	*inc.*
Ambient temperature,	no infection	22 (1)	a	a	15 (1)	3 (0)	a
without oxygen supply	PR MUCL 46746	22 (1)	a	b	13 (1)	9 (0)	a
Elevated temperature in	no infection	19 (2)	b	a	13 (3)	4 (5)	a
combination with oxygen supply	PR MUCL 46746	24 (1)	a	a	14 (1)	2 (1)	b

° Non-parametric test according to Kruskal-Wallis

Visible mould growth was not detected at incubation at ambient temperature. For elevated temperature in combination with oxygen supply, one uninfected microsilo showed fungal growth upon desiling and all three infected microsilos were visibly mouldy. Fungal counts, however, did not reveal a significant effect of elevated temperature in combination with oxygen supply. Artificial infection did significantly increase the number of fungal propagules.

The cDM content at desiling showed significant interaction between incubation regime and infection. For uninfected silage, no significant influence of incubation regime was detected. PR-infected silage showed a significantly reduced cDM content after subjection to elevated temperature and oxygen supply.

The aerobic stability of the desiled material was not significantly affected by infection, but elevated temperature in combination with oxygen supply had a significantly detrimental effect on aerobic stability. This is also reflected in a significantly lower dry matter content after Honig. Elevated temperature at 61-64 days after ensiling in combination with oxygen supply appears to have boosted the development of aerobic deterioration initiating yeasts, while fungal counts were not significantly affected.

Ammonia content and ammonia fraction were not significantly influenced by any of the factors infection and incubation regime, and were at a low level in all objects. The pH of the desiled material was not significantly affected by the incubation regime, but artificial infection with PR MUCL 46746 resulted in a significantly higher pH compared to uninfected silage. Lactic acid levels did not vary much in absolute value between the objects, but a significant interaction between incubation regime and infection was detected. In uninfected silage, the lactic acid content did not differ significantly according to the incubation regime. PR-infected silage surprisingly contained significantly more lactic acid upon subjection to elevated temperature and oxygen supply. No significant effect of infection was detected in case of incubation at ambient temperature. No plausible explanation for this phenomenon could be found in literature. Regarding the narrow range of lactic acid contents over the different objects, the observed differences are rather irrelevant from a practical point of view. The acetic acid levels did not differ much between the objects. No significant effects of infection and incubation were found. Butyric acid has been detected in the grass silage samples, showing a significant interaction between infection and incubation regime. In neither of the incubation regimes, a significant effect of infection was found. No effect of incubation was found in uninfected silages either. PR-infected silage, however, did contain significantly more butyric acid in case of ambient temperature without oxygen supply.

3.3.2. In vivo experiment with whole-crop maize silage

3.3.2.1. Materials and methods

For the maize trial, three incubation regimes were included, without or with artificial infection with *P. roqueforti s.s.*. Again, artificial infection with PR MUCL 46746 spores was intense (*i.e.* $1*10^6$ conidia per gram fresh matter) and the silos were moderately compacted.

Whole-crop maize was chopped to a theoretical particle length of 8 mm with a New Holland precision chopper. The uninfected maize was treated with sterile distilled water. Subsequently, samples were taken for determination of the DM content: the whole-crop maize was ensiled at 37 % DM. *P. roqueforti s.s.* (PR) MUCL 46746 spore solution was freshly prepared as described in Annex 1 (however no glycerol was added) and spore concentration was adjusted to $1*10^8$ spores/ml. Per level of infection, nine microsilos were filled with approx. 1.2 kg fresh matter, corresponding to a moderate silo density of 162 kg DM/m³ (McDonald *et al.* 1991).
The experimental setup of the microsilo trial is summarized in Table 4.8. Per incubation regime and infection level, three microsilos were stored as described in the introduction to this *in vivo* trial. The fermentation losses were monitored on a weekly basis. All microsilos were desiled after 76 days. Visible fungal growth on the desiled material was noted. From each individual microsilo, samples were taken for aerobic stability testing and for determination of the fungal counts, the DM content and some fermentation characteristics (as described in Annex 1). Multi-mycotoxin determination by LC-MS/MS was also performed on one sample per microsilo, as described by Monbaliu *et al.* (2010): 24 mycotoxins were determined per run, as listed in Table 4.9. The method was validated for all mycotoxins except for enniatin B, which was determined semi-quantitatively.

The obtained data were statistically analyzed as described in Annex 1.

Table 4.8. *Experimental setup of microsilo trial evaluating the effect of elevated temperature and oxygen supply on whole-crop maize silage: uninfected control versus P. roqueforti s.s. (PR) MUCL 46746.*

OBJECTS		
Incubation regime	**Infection**	**N**
Ambient temperature during the entire ensiled period, without oxygen supply	no infection	3
	PR MUCL 46746 @ 1.10^6 spores/g FM	3
Temperature of 32°C at 0-4 days after ensiling, without oxygen supply	no infection	3
	PR MUCL 46746 @ 1.10^6 spores/g FM	3
Temperature of 32°C at 61-64 days after ensiling, with oxygen by plastic drain after 61 days	no infection	3
	PR MUCL 46746 @ 1.10^6 spores/g FM	3
Desiling after 76 days		
Fermentation losses, mould enumeration, aerobic stability, corrected dry matter°, fermentation characteristics and multi-mycotoxin analysis by LC-MS/MS		

° Dry matter content corrected for volatile compounds loss according to Dulphy and Demarquilly (1981).

Table 4.9. *Multi-mycotoxin determination on silage samples by LC-MS/MS.*

Mycotoxins	**limit of detection** (µg/kg fresh matter)	**limit of quantification** (µg/kg fresh matter)
Detected in silage samples		
deoxynivalenol	111	222
3- & 15-acetyl deoxynivalenol	15	29
nivalenol	66	133
zearalenone	33	65
sterigmatocystin	9	17
enniatin B	semi-quantitative: < or > than 80 µg/ kg FM	
roquefortine C	2	4
Not detected in silage samples		
aflatoxin B1	3	6
aflatoxin B2	3	6
aflatoxin G1	4	7
aflatoxin G2	4	9
altenuene	8	17
alternariol	22	44
alternariol methyl ether	32	65
diacetoxyscirpenol	1	2
fumonisin B1	58	116
fumonisin B2	45	89
fumonisin B3	42	85
fusarenon-X	30	61
HT2-toxin	17	34
neosolaniol	16	31
ochratoxin A	6	13
T2-toxin	17	34

3.3.2.2. Results and discussion

- **Fermentation losses**

Similar to the grass trial, some microsilos were subjected to oxygen supply after an ensiled period of 60 days. Therefore, the registered fresh weight losses don't only comprise fermentation losses, but also losses due to aerobic metabolism. Still, the term "fermentation losses" will be adopted.

The evolution of the mean fermentation losses per object during the ensiled period is visualized in Figure 4.12.

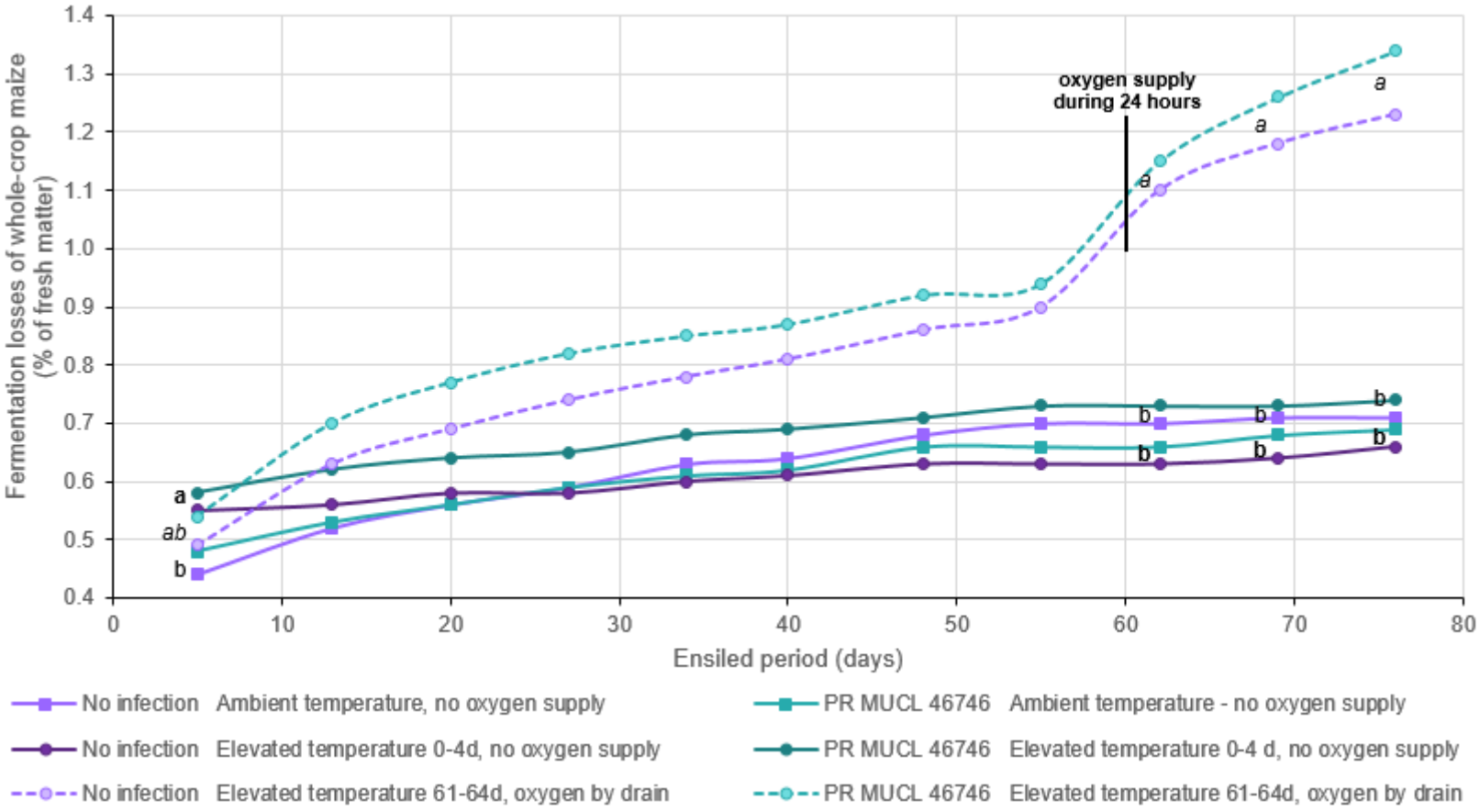


Figure 4.12. *Fermentation losses during the ensiled period (% of fresh matter) observed in the microsilo trial evaluating the effect of elevated temperature and oxygen supply on whole-crop maize silage: uninfected control versus infection with P. roqueforti s.s. (PR) MUCL 46746.* The mean values per object are presented without error bars representing their resp. standard deviation. Standard deviations were low for all objects (maximum 0.21 % of fresh matter), except for the uninfected grass subjected to elevated temperature at 61-64 days after ensiling in combination with oxygen supply (maximum 0.50 % of fresh matter). No significant interaction between incubation regime and infection was detected at any time point, and infection did not have a significant influence on fermentation losses. Significant differences according to the incubation regime are indicated by lettercodes: for ambient temperature normal letters are used, while for elevated temperature during the first four days after ensiling bold type is used, and for elevated temperature from 55 to 64 days after ensiling in combination with oxygen supply lettercodes are marked in italic.

At five days after ensiling, the microsilos that have been subjected to elevated temperature without oxygen supply during the first four days of the ensiled period showed significantly higher fermentation losses compared to the microsilos stored at ambient temperature during the entire ensiled period. After 60 days, some microsilos were subjected to oxygen supply in combination with elevated temperature from 61 to 64 days after ensiling. The fermentation losses of these microsilos after 62, 69 and 72 days were significantly higher compared to the other two incubation regimes, due to aerobic microbial metabolism.

- **Analyses at desiling**

Analogously to the grass trial, each microsilo was checked for visible fungal growth at desiling after 76 days. Afterwards, the desiled material was homogenized prior to sampling. Fungal propagules were enumerated and the aerobic stability was determined by the Honig protocol. Dry matter content was determined at desiling and at the end of the Honig protocol. Some fermentation characteristics have been determined: ammonia and ammonia fraction, pH, lactic acid, acetic acid and butyric acid. The dry matter content at desiling has been corrected for volatile compounds according to Dulphy and Demarquilly (1981).

The results of the analyses at desiling are summarized in Table 4.10. Butyric acid was absent in nearly all silage samples: only infected silages incubated at elevated temperature at 61-64 after ensiling in combination with oxygen supply contained trace amounts. Since no significant differences according to infection or incubation level were detected, this parameter is not mentioned in Table 4.10.

Table 4.10. *Results of analyses at desiling of microsilo trial evaluating the effect of elevated temperature and oxygen supply on whole-crop maize silage: number of visibly mouldy microsilos, fungal counts, corrected dry matter (cDM), aerobic stability, dry matter after Honig protocol, ammonia, ratio of ammonia nitrogen over total nitrogen, pH, lactic acid, acetic acid and butyric acid.* Mean values per object are mentioned with their resp. standard deviation between brackets. For none of the parameters, a significant interaction between infection and incubation regime was detected. Significant main effects of infection and incubation regime are indicated by lettercode.

Incubation regime	**Infection**	**visibly mouldy** (number)	**fungal counts** (log cfu/g FM)	*inc.*	**corrected DM** (g/kg FM)	*inf.*	*inc.*	**aerobic stability** (hours)	*inc.*
Ambient temperature, without oxygen supply	no infection	0 / 3	2.36 (0.39)	c	335 (2)	b	b	158 (13)	a
	PR MUCL 46746	0 / 3	2.37 (0.40)		344 (2)	a		112 (74)	
Elevated temperature at 0-4 days after ensiling, without oxygen supply	no infection	0 / 3	3.01 (0.10)	b	345 (3)		a	75 (5)	a
	PR MUCL 46746	0 / 3	3.01 (0.06)		346 (2)			85 (46)	
Elevated temperature at 61-64 days after ensiling, with oxygen supply by plastic drain	no infection	1 / 3	5.44 (1.99)	a	343 (7)		ab	21 (5)	b
	PR MUCL 46746	2 / 3	6.30 (1.29)		346 (4)			23 (8)	

Incubation regime	**Infection**	**DM after Honig** (g/kg FM)	*inc.*	**ammonia** (g/kg cDM)	*inc.*	**NH3-N / total N**	*inc.*	**pH**	*inc.*
Ambient temperature, without oxygen supply	no infection	339 (12)	a	0.66 (0.02)	a	5.2 (0.1)	a	3.78 (0.03)	b
	PR MUCL 46746	352 (3)		0.68 (0.00)		5.6 (0.2)		3.79 (0.01)	
Elevated temperature at 0-4 days after ensiling, without oxygen supply	no infection	343 (8)	a	0.57 (0.01)	b	4.6 (0.1)	b	3.83 (0.06)	ab
	PR MUCL 46746	340 (6)		0.52 (0.03)		4.5 (0.2)		3.78 (0.10)	
Elevated temperature at 61-64 days after ensiling, with oxygen supply by plastic drain	no infection	303 (24)	b	0.66 (0.11)	ab	5.3 (1.0)	ab	3.97 (0.14)	a
	PR MUCL 46746	319 (16)		0.58 (0.17)		4.8 (1.3)		3.96 (0.12)	

Incubation regime	**Infection**	**lactic acid** (g/kg cDM)	**acetic acid** (g/kg cDM)
Ambient temperature, without oxygen supply	no infection	49 (2)	16 (2)
	PR MUCL 46746	53 (4)	16 (2)
Elevated temperature at 0-4 days after ensiling, without oxygen supply	no infection	50 (1)	13 (2)
	PR MUCL 46746	45 (3)	14 (1)
Elevated temperature at 61-64 days after ensiling, with oxygen supply by plastic drain	no infection	39 (9)	19 (3)
	PR MUCL 46746	40 (10)	13 (3)

Microsilos incubated at ambient temperature or at elevated temperature immediately after ensiling did not show visible mould growth at desiling, while microsilos incubated at elevated temperature from 61-64 days after ensiling in combination with oxygen supply did: one of the three uninfected microsilos and two of the three PR-infected microsilos were visibly mouldy.
As for fungal counts, significant differences according to the incubation regimes were detected. Elevated temperature at 61-64 days after ensiling along with oxygen supply resulted in the significantly highest fungal counts, whereas incubation at ambient temperature without oxygen supply yielded the significantly lowest fungal counts.

The cDM content was significantly lower in uninfected silage than in PR-infected silage. The highest cDM was detected in microsilos incubated at elevated temperature during the first four days after ensiling, being significantly higher compared to silage incubated at ambient temperature. This suggests that the elevated temperature immediately after ensiling resulted in a more efficient fermentation, alignating with an optimal growth temperature of lactic acid bacteria around 30-40 °C (Machielsen *et al.* 2010; Wood and Holzapfel 1995).
Aerobic stability was significantly reduced in silages subjected to elevated temperature at 61-64 days after ensiling in combination with oxygen compared to the other two incubation regimes, due to boosting of the yeasts initiating aerobic deterioration. The dry matter content after Honig differed accordingly between the three incubation regimes.
Ammonia content and ammonia fraction were significantly higher after incubation at ambient temperature throughout the entire ensiled period than after incubation at elevated temperature during the first four days after ensiling, indicating less protein degradation at higher temperatures at the start of the ensiling process. This might be ascribed to reduced protein breakdown by *Enterobacteriaceae*, due to a more efficient LAB fermentation and subsequent acidification of the silage: *Enterobacteriaceae* have an optimum pH of 6-7 and usually don't grow below pH 5 (Bolsen *et al.* 1996; McDonald *et al.* 1991; Pahlow *et al.* 2003).
Incubation at ambient temperature during 76 days resulted in significantly lower pH values at desiling compared to elevated temperature at 61-64 days after ensiling in combination with oxygen supply. However, the lactic acid and acetic acid levels did not differ significantly between the incubation regimes.

Besides fungal enumeration, aerobic stability testing and determination of the fermentation characteristics, a multi-mycotoxin analysis was performed on the samples of this microsilo trial. From the 24 mycotoxins searched for, eight were retrieved from the silage samples: deoxynivalenol, 3- and 15-acetyl deoxynivalenol, nivalenol, zearalenone, sterigmatocystin, enniatin B and ROC. For none of these mycotoxins, a significant effect of infection or incubation regime on mycotoxin concentration was detected. Therefore, the data over all objects are summarized in Table 4.11.

Table 4.11. *Mycotoxin determination results for the microsilo trial evaluating the effect of elevated temperature and oxygen supply on whole-crop maize silage: minimum, median, maximum and mean levels (mg/kg dry matter) of positive samples.*

Mycotoxins	# positive samples	mg/kg dry matter min	median	max	mean
deoxynivalenol	18 / 18	0.609	0.933	1.735	1.022
3- and 5-acetyl deoxynivalenol	18 / 18	0.023	0.049	0.360	0.068
nivalenol	8 / 18	0.005	0.005	0.094	0.028
zearalenone	1 / 18		0.142		
sterigmatocystin	7 / 18	0.004	0.006	0.008	0.006
enniatin B	18 / 18	0.073	0.077	0.116	0.081
roquefortine C	5 / 18	0.004	0.006	0.448	0.142

Except for ROC, all mycotoxins are produced by *Fusarium* species and most likely originate from the field (as discussed in chapter 2). Mean deoxynivalenol concentration was around 1 mg per kg fresh matter, while the concentrations of the other mycotoxins were generally low. Furthermore, high variability of mycotoxin concentrations was observed among the three replicates per object and among the objects. This illustrates the difficulties associated with the sampling for and analysis of mycotoxins in silages (e.g. mycotoxins are not homogeneously distributed over the silage mass), as addressed in chapter 2 (section 2.5).

ROC may already be present in the field (Mansfield *et al.* 2008), but is usually formed in silages. Driehuis *et al.* (2008b) have sampled whole-crop maize and grass silages which were ensiled for at least three weeks and were not yet opened for feed-out. By LC-MS/MS, 140 whole-crop maize and 120 grass silages from The Netherlands were subjected to multi-mycotoxin analysis. Deoxynivalenol and zearalenone were frequently detected in both matrices, whereas ROC was only found in one grass silage sample. On the other hand, Auerbach *et al.* (1998) detected ROC in 21 of 24 visibly mouldy silage samples, in a concentration up to 36 mg/kg dry matter. ROC was also found in six of 24 visibly non-mouldy samples, but only trace amounts.
In the current microsilo trial, ROC was only found in two silages exhibiting *P. roqueforti s.l.* growth (one uninfected, one infected) and in three visibly non-mouldy silages (all infected), as summarized in Table 4.12.

The ROC levels were clearly higher in the silages displaying visible mould growth (which had all been subjected to elevated temperature at 61-64 days after ensiling in combination with oxygen supply) compared to the non-mouldy samples. This alignates with the findings of Auerbach *et al.* (1998) that non-mouldy silage samples don't contain ROC frequently, and when they do the levels are usually close to the limit of detection.

Table 4.12. *Roquefortine C content of five samples of the microsilo trial with whole-crop maize silage containing a detectable amount of roquefortine C (above the limit of quantification).*

Incubation regime	Infection	roquefortine C (mg/kg DM)	visibly mouldy?
Ambient temperature, without oxygen supply	no infection	-	
	PR MUCL 46746	0.004	no
Elevated temperature at 0-4 days after ensiling, without oxygen supply	no infection	-	
	PR MUCL 46746	0.005	no
		0.006	no
Elevated temperature at 61-64 days after ensiling, with oxygen supply by plastic drain	no infection	0.448	yes
	PR MUCL 46746	0.246	yes

3.3.3. Conclusion on the in vivo experiments assessing the effect of elevated temperature and oxygen on silages

In practice, completely airtight sealing of silages is utopic, therefore the fermentation losses on-farm are suspected to be higher than those observed *in vivo* for airtight microsilos. Depending on the numbers of fungal propagules and other aerobic undesirable micro-organisms on the ensiled plant material, the fermentation losses may rise well above the normal range of 1-4 % of the fresh matter (McDonald *et al.* 1991). In the currently described microsilo experiments, fermentation losses remained at a low level even in the air leaking microsilos equipped with a plastic drain.

In order to determine if the objects in the two microsilo trials evaluating the effect of elevated temperature in combination with oxygen supply were successful in mimicking good quality silage on the one hand and mould infested silage on the other hand, a comparison between the fermentation characteristics obtained for two objects in the currently described *in vivo* trials with microsilos and obtained by on-farm sampling of visibly non-mouldy reference samples and mouldy hot-spots within the same silos (Chapter 1) is presented in Table 4.13, for both whole-crop maize and grass silage. Three replicates were included per object in the microsilo trials, whereas the number of samples was much higher for on-farm samplings. Statistical analysis was performed as described in Annex 1. Data from on-farm collected samples are mentioned on the left part of the table, whereas microsilo data are presented on the right part of the table. To put Table 4.13 in the right perspective, it should be emphasized that the mouldy on-farm silage samples were very often contaminated with *P. roqueforti s.l.,* but not exclusively.

Table 4.13. *Comparison of fermentation characteristics of whole-crop maize and grass silages sampled on-farm and in the context of microsilo trials evaluating the effect of elevated temperature and oxygen supply, for uninfected samples (purple) and mould contaminated samples (green)*. Significant differences between the on-farm samples taken from mouldy silages and the samples from the microsilo trials are indicated by asterix (*) symbols in the central column of the table for both sample types (i.e. uninfected samples and mouldy samples).

WHOLE-CROP MAIZE SILAGES	**On-farm sampling of mouldy silages**				**Microsilo trial** (N = 3)	
Parameters	**non-mouldy reference** (N = 46)	**mouldy hot-spots** (N = 65)	**Sign.**		**ambient temperature, no infection**	**elevated temperature 61-64 d & oxygen supply, PR MUCL 46746**
dry matter (DM) (g/kg fresh matter)	341 (30)	303 (73)		*	335 (2)	346 (4)
ammonia (g/kg DM)	0.86 (0.19)	1.09 (0.67)	*		0.66 (0.02)	0.58 (0.17)
ammonia nitrogen / total nitrogen	5.8 (1.4)	5.9 (3.3)			5.2 (0.1)	4.8 (1.3)
pH	3.66 (0.12)	5.22 (0.98)	*	*	3.78 (0.03)	3.96 (0.12)
lactic acid (g/kg DM)	71 (211)	21 (15)	*	*	49 (2)	40 (10)
acetic acid (g/kg DM)	13 (5)	5 (3)		*	16 (2)	13 (3)
GRASS SILAGES	**On-farm sampling of mouldy silages**				**Microsilo trial** (N = 3)	
Parameters	**non-mouldy reference** (N = 18)	**mouldy hot-spots** (N = 24)	**Sign.**		**ambient temperature, no infection**	**elevated temperature 61-64 d & oxygen supply, PR MUCL 46746**
dry matter (DM) (g/kg fresh matter)	390 (129)	347 (112)			340 (5)	322 (5)
ammonia (g/kg DM)	1.97 (1.06)	2.00 (1.29)	*	*	0.06 (0.00)	0.06 (0.00)
ammonia nitrogen / total nitrogen	6.4 (3)	8 (5)			7.1 (0.1)	6.7 (0.9)
pH	4.41 (0.41)	5.87 (0.92)	*		4.62 (0.01)	4.71 (0.08)
lactic acid (g/kg DM)	81 (60)	14 (19)	*		22 (1)	24 (1)
acetic acid (g/kg DM)	18 (14)	9 (10)			15 (1)	14 (1)

For the whole-crop maize silages, the pH range of samples from the microsilo trial was much narrower compared to on-farm samples.
Uninfected samples from the microsilo trial had a significantly higher pH value than on-farm samples, attributable to the dry matter content of the starting material for the microsilo trial: whole-crop maize was ensiled at 37 % dry matter, corresponding to the upper limit of the dry matter range observed in on-farm samples. Associated with the higher pH, decreased lactic acid levels were observed in samples from the microsilo trial. These samples did contain significantly less ammonia compared to on-farm collected samples, but due to high variability of the ammonia fraction of on-farm samples this was not reflected in a significant difference in ammonia fraction.
Mouldy samples obtained from the microsilo trial were characterized by a significantly lower pH compared to on-farm collected fungal hot-spots. This is not surprising since the microsilo trial samples were not entirely visibly mouldy. The significantly lower pH values were associated with significantly higher levels of lactic acid and acetic acid. Spoilage losses were significantly lower in microsilo samples, reflected in a significantly higher dry matter recovery at desiling.

The fermentation characteristics of on-farm grass silages showed a high variability, very likely originating from a broad range of dry matter contents (McDonald *et al.* 1991; Oude Elferink *et al.* 2001). Evidently, pH values as well as levels of lactic acid and acetic acid varied accordingly. Therefore, less significant differences were found between on-farm collected samples and samples collected in the context of the microsilo trial with grass compared to whole-crop maize silages.
Both samples types varied significantly in ammonia content, being significantly lower in microsilo trial samples than in on-farm collected samples. Ammonia levels were very low in both samples types collected from the microsilo trial.
The pH of uninfected samples collected on-farm was significantly lower compared to microsilo samples, which is associated with significantly higher lactic acid levels. It must be noted that the production of lactic acid in the microsilo trial was quite low, despite the dry matter content of 37 % which was in the optimum range for grass silage.

In conclusion, it can be stated that the incubation regime for microsilos comprising of oxygen supply during 24 hours at 60 days after ensiling in combination with elevated temperature (*i.e.* 32 °C) during four days did alter the aerobic stability and fermentation characteristics of whole-crop maize and grass silages, but could not mimic fungal hot-spots during the feed-out phase of on-farm silages. However, this was not the goal of the microsilo trials since the feed-out phase was not mimicked.

If mimicking of fungal hot-spots during feed-out is aimed for, oxygen should be supplied at the end of the ensiled period and should be prolonged in time. Using microsilos, fungal hot-spots of *P. roqueforti s.s*. have been successfully created in whole-crop maize silages by providing oxygen through eight holes in the microsilos during several weeks prior to desiling in combination with slightly elevated temperatures (15-20 °C) (contractual research with confidential data).

4. Conclusions on the effect of abiotic factors on *P. roqueforti s.l.* growth and roquefortine C production

In the context of this chapter on abiotic factors, two *in vitro* experiments have been described.

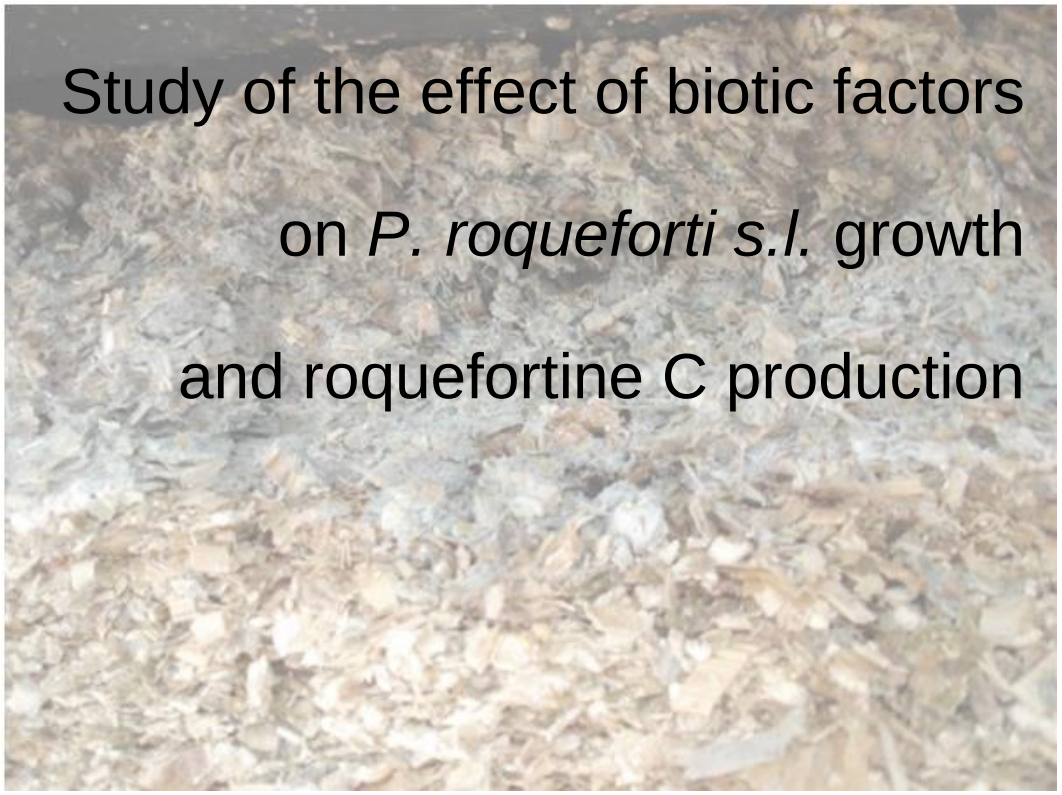
The first experiment evaluated the effect of variable amounts of inorganic and organic nitrogen in CYA-based media on growth and ROC production by PR MUCL 46746 and PP CBS 112295. Low levels of organic nitrogen (*i.e.* 1 gram yeast extract per liter medium) did not support good *P. roqueforti s.l.* growth, and ROC production was low. Normal levels of organic nitrogen (*i.e.* 5 gram yeast extract per liter medium) were conducive for *P. roqueforti s.l.* growth. By varying the level of inorganic nitrogen in CYA (*i.e.* 3 versus 0.6 gram sodium nitrate per liter medium), it has been determined if depletion of inorganic nitrogen triggered the production of ROC: total fungal biomass, conidiospores and culture medium were sampled every three days during 15 days of aerobic incubation at 22 °C. PR MUCL 46746 biomass contained similar levels of ROC throughout the 15-days incubation period on both media with normal organic nitrogen levels, whereas the ROC content of PP CBS 112295 biomass was significantly higher on the medium with a normal level of inorganic nitrogen compared to the medium low in inorganic nitrogen from nine days of incubation onwards. The ROC content of the conidiospores varied considerably between triplicates per object throughout the 15-days incubation period, as was the agar content of the medium underlying fungal growth. Overall, it can be concluded that ROC production by *P. roqueforti s.l.* biomass paralleled fungal growth. Moreover, increased ROC production by the biomass was associated with increased excretion of ROC into the medium, confirming energy-independent excretion of ROC.

The second *in vitro* experiment assessed the effect of temperature and oxygen concentration of growth of PR MUCL 46746 on PDA. No fungal growth was registered upon anaerobic incubation at 25 °C during seven days. Within a temperature range of 5 to 35 °C, fungal growth at two incubation regimes was determined: normal aerobic conditions (*i.e.* 21 % oxygen and 0.04 % carbon dioxide) were compared to oxygen depleted conditions (*i.e.* 6 % oxygen and 9 % carbon dioxide). At 35 °C, no growth could be detected in any of the two incubation regimes. In the range of 5 to 30 °C, PR MUCL 46746 did display growth, being maximum at 25 °C in aerobic conditions and at 20 °C in oxygen depleted conditions.

A microsilo experiment with whole-crop maize artificially infected with PR MUCL 46746 investigated the effect of prolonged anaerobic conditions on *P. roqueforti s.l.* numbers at desiling. In this context, the effect of HoLAB, HeLAB and propionic acid as silage additives was also evaluated. At desiling after 50 days, *P. roqueforti s.l.* numbers were still quite high in all artificially infected samples, but at desiling after 100 and 150 days nearly no viable *P. roqueforti s.l.* propagules were found in the absence of a silage additive and in silage inoculated with HoLAB or HeLAB. Interestingly, despite its antifungal properties, propionic acid was not able to significantly reduce *P. roqueforti s.l.* numbers at the dosage of 4.5 liter per ton fresh matter at an ensiled period of either 50, 100 and 150 days. Propionic acid did significantly reduce the amounts of lactic acid and acetic acid produced during silage fermentation, confirming its LAB fermentation inhibiting properties (discussed in Chapter 1).

Two microsilo trials were executed to study the effect of elevated temperature in combination with oxygen supply on fungal counts and fermentation characteristics of whole-crop maize and grass silage. For both feedstuffs, incubation at ambient temperature during 76 days was compared with an incubation regime including oxygen supply during 24 hours at 60 days after ensiling followed by four days of elevated temperature (*i.e.* 32 °C). The latter incubation regime, assumed to mimic local damage to the silo coverage during the ensiled period, had a significant influence on fermentation characteristics and aerobic stability of both silage types at desiling after 76 days. In whole-crop maize silage, a significant increase of fungal counts at desiling was also detected upon oxygen supply after 60 days in combination with elevated temperature at 61 to 64 days after ensiling. The microsilo experiment with whole-crop maize was a bit more extensive compared to the grass experiment an additional incubation regime of elevated temperature during the first four days after ensiling was included, as well as multi-mycotoxin determination on desiled material. For none of the detected mycotoxins a significant effect of artificial infection with *P. roqueforti s.s.* or incubation regime on mycotoxin concentration was detected. Elevated temperature at the start of the ensiled period had a significant effect on fermentation characteristics and resulted in intermediate mould numbers at desiling compared to the other two incubation regimes.

Chapter 5



Study of the effect of biotic factors on *P. roqueforti s.l.* growth and roquefortine C production

1. Introduction

A selection of biotic factors has been evaluated for its effect on *P. roqueforti s.l.* growth and ROC production, both *in vitro* by lab experiments as *in vivo* using microsilos. The antagonistic activity of a *Bacillus velezensis* strain against *P. roqueforti s.l.* has been assessed *in vitro* by monitoring the conidiospore survival, conidiospore germination and fungal growth in a whole-crop maize silage based liquid medium containing variable amounts of *B. velezensis* culture supernatant or cell suspension. A fresh whole-crop maize based agar medium was prepared to evaluate the effect of HoLAB or HeLAB inoculant addition on *P. roqueforti s.l.* growth and ROC production. The effect of both *B. velezensis* and LAB inoculants on *P. roqueforti s.l.* numbers in grass silage has been assessed *in vivo* by a microsilo experiment.

2. *In vitro* effect of biotic factors on *P. roqueforti s.l.* growth and roquefortine C production

2.1. *Bacillus velezensis* as antagonist towards *P. roqueforti s.l.*

In 2003, Chitarra *et al*. published experimental results revealing that an antifungal compound in the culture supernatant of *Bacillus velezensis* strain NRRL B-23189 (isolated from pre-harvest maize) inhibits the germination of *P. roqueforti s.s.* conidiospores. Inhibition of conidiospore germination is a beneficial characteristic of a biocontrol organism, since germination is the starting event of the fungus' asexual life cycle. Therefore, this particular *B. velezensis* strain was used in an *in vitro* experiment. Chitarra *et al.* (2003) used only *B. velezensis* culture supernatant, while in this experiment *B. velezensis* cell suspension is also evaluated since this is more representative for future application as a silage inoculant. Moreover, not only the antagonistic activity against *P. roqueforti s.s.* was assessed: *P. paneum* was also included in the experiment, as both species are the most frequent fungal contaminants of silage in Belgium. Chitarra *et al.* (2003) used Malt Extract Agar in an agar diffusion assay, but since corn silage is a better-suited matrix for testing a potential silage additive, Corn Silage Infusion (CSI) was chosen as liquid culture medium.

The objects included in the currently described *in vitro* experiment were defined based on:

- two *P. roqueforti s.l*. isolates:
  P. roqueforti s.s. MUCL 46746
  P. paneum CBS 112295
- *B. velezensis* (*Bv*) as antagonist: negative control vs. 5 treatment solutions
  negative control: 100% corn silage infusion (CSI)
  10% *Bv* supernatant + 90% CSI
  25% *Bv* supernatant + 75% CSI
  50% *Bv* supernatant + 50% CSI
  10% *Bv* cell suspension + 90% CSI
  25% *Bv* cell suspension + 75% CSI

2.1.1. Materials and methods

The choice for Corn Silage Infusion (CSI) as a liquid culture medium facilitated to include both a microtiter plate assay and a falcon tube assay.

On the one hand, a flat-bottomed 96-well microtiter plate experiment assessing the antagonistic activity of different ratios of *Bv* culture supernatant or cell suspension added to CSI against *P. roqueforti s.s.* and *P. paneum* growth was performed. Fungal growth was monitored spectrophotometrically.

On the other hand, one set of 15ml-falcon tubes was used to monitor spore germination and spore survival, analogous to Chitarra *et al.* (2003), while a second set was used for screening of ROC production. All falcon tubes were aerobically incubated in static conditions in the dark at 20 °C during five days.
Conidiospore survival and germination were determined after 24 hours of incubation. ROC production during the five-days incubation period was screened quantitatively.

- Preparation of the different solutions used

CSI was prepared as described in Annex 1, using whole-crop maize silage which had been ensiled for 50 days. Prior to filter sterilization, the corn silage infusion was brought to pH 3.79 with a ten-fold diluted mixture of 3.17/1 (vol/vol) of lactic acid and acetic acid (reflecting the pH and lactic acid over acetic acid ratio of the whole-crop maize silage used for making the CSI) and 0.01 % of Tween 80 was added.

Fungal spore solutions were prepared as described in Annex 1.

B. velezensis NRRL B-23189 was grown aerobically in the dark at 30 °C on Plate Count Agar (PCA), prepared as described in Annex 1. Four-day old cultures were subcultured *in duplo* in 15 ml of Brain-Heart Infusion broth (BHI – prepared as described in Annex 1) and aerobically stir-cultured in the dark on a magnetic shaker at 130 rpm and at 30°C. Additionally, an additional 15-ml portion of sterile BHI was not inoculated with *B. velezensis*. After 48 hours, one BHI-replicate inoculated with *B. velezensis* was centrifuged during 15 min at 9 500 rpm. The *Bv* supernatant was collected and sterilized through a syringe filter. The other inoculated BHI-replicate was used as such, as *Bv* cell suspension (containing $8*10^7$ cfu/ml, as determined by streak-plating a dilution series in physiological water on PCA). Sterile BHI was used as a negative control.

By combining different volumes of sterile BHI, *Bv* supernatant or *Bv* cell suspension with CSI, nine different liquid culture media were prepared:

1. 100 % CSI
2. 90 % CSI + 10 % sterile BHI
3. 90 % CSI + 10 % *Bv* supernatant
4. 90 % CSI + 10 % *Bv* cell suspension
5. 75 % CSI + 25 % sterile BHI
6. 75 % CSI + 25 % *Bv* supernatant
7. 75 % CSI + 25 % *Bv* cell suspension
8. 50 % CSI + 50 % sterile BHI
9. 50 % CSI + 50 % *Bv* supernatant

- Start of the experiment

The nine media were distributed *in duplo* into two sets of eighteen 15-ml falcon tubes, for infection with PR MUCL 46746 or PP CBS 112295 to a final conidiospore concentration of $1*10^4$ spores/ml medium. The first set of falcon tubes was filled with 3 ml of medium and was used for the microtiter plate assay, as well as for monitoring of spore germination and spore survival. The second set of falcons was used for screening of ROC production. The empty weight of each falcon was noted to allow calculation of the freeze-dried mycelium weight, and all falcons were filled with 1 ml of medium.

For the microtiter plate assay, 200 µl of culture medium was introduced per well (N=4 per object, except for 90 % CSI + 10 % sterile BHI and 75 % CSI + 25 % sterile BHI: N = 8). Two negative controls without *P. roqueforti s.l.* spores (*i.e.* 90 % CSI + 10 % *Bv* cell suspension and 75 % CSI + 25 % *Bv* cell suspension) were included to enable monitoring of solely *Bv* growth in CSI and to provide some insight into its possible use as a silage inoculant. The exact organization of the 96-well microtiter plate is presented in Figure 5.1. After sealing of the

microtiter plate with respiratory foil, the plate was statically incubated in aerobic conditions in the dark for five days at 20 °C. The optical density was determined spectrophotometrically at 620 nm immediately after filling.

Figure 5.1. *Microtiter plate assay to evaluate the antagonistic activity of Bacillus velezensis supernatant or cell suspension towards P. roqueforti s.l. in Corn Silage Infusion.*

	controls	10% supernatant	25% supernatant	50% supernatant	10% cell suspension	25% cell suspension	controls	10% supernatant	25% supernatant	50% supernatant	10% cell suspension	25% cell suspension
	1	2	3	4	5	6	7	8	9	10	11	12
A	100% CSI	90% CSI + 10% sterile BHI	75% CSI + 25% sterile BHI	50% CSI + 50% sterile BHI	90% CSI + 10% sterile BHI	75% CSI + 25% sterile BHI	100% CSI	90% CSI + 10% sterile BHI	75% CSI + 25% sterile BHI	50% CSI + 50% sterile BHI	90% CSI + 10% sterile BHI	75% CSI + 25% sterile BHI
B												
C												
D												
E	90% CSI + 10% *Bv* cell susp.	90% CSI + 10% *Bv* supern.	75% CSI + 25% *Bv* supern.	50% CSI + 50% *Bv* supern.	90% CSI + 10% *Bv* cell susp.	75% CSI + 25% *Bv* cell susp.	75% CSI + 25% *Bv* cell susp.	90% CSI + 10% *Bv* supern.	75% CSI + 25% *Bv* supern.	50% CSI + 50% *Bv* supern.	90% CSI + 10% *Bv* cell susp.	75% CSI + 25% *Bv* cell susp.
F												
G												
H												

P. roqueforti s.s. **MUCL 46746** | ***P. paneum*** **CBS 112295**

CSI: Corn Silage Infusion
BHI: Brain-Heart Infusion broth
Bv cell susp.: *Bacillus velezensis* cell suspension
Bv supern.: *Bacillus velezensis* supernatant

- Monitoring during the experiment

To monitor fungal growth, the optical density of the microtiter plate's wells at 620 nm was determined after 1, 2, 4, 8 and 24 hours and after five days (without shaking the plate prior to OD_{620}-measurement).

After 24 hours, conidiospore survival in the different culture media was monitored by streak-plating 100-µl samples from the first set of falcon tubes (N=3) on PDAA in standard 90-mm diameter Petri dishes. All plates were aerobically incubated bottom-up in the dark at 20 °C and fungal development was evaluated after four days by taking photographs.
Conidiospore germination was evaluated after 24 hours of incubation on 20-µl samples (N=4) taken from the first set of falcon tubes. Per replicate, a 20-µl sample was placed on a glass slide cleaned with 70 % ethanol, followed by covering with a clean cover slide and flame fixation. Randomly, 100 conidiospores were counted (evenly spread over the glass slide) and the percentage of germinated spores was determined, using a phase-contrast microscope at 400x magnification. Spores were considered to have germinated when the length of the germ tube exceeded one-half of the spore diameter.

The second set of falcon tubes was statically incubated in aerobic conditions during five days in the dark at 20 °C without shaking, and stored at -20 °C prior to freeze-drying. After registration of the freeze-dried weight, ROC was quantified by LC-MS/MS as described in Annex 1 (N = 1). ROC was not detectable in sterile CSI.

The obtained data were statistically analyzed as described in Annex 1.

2.1.2. Results

2.1.2.1. Conidiospore survival

Conidiospore survival in the different culture media was evaluated after 24 hours of incubation. After four days, photographs were made of the plates, as shown in Figure 5.2.

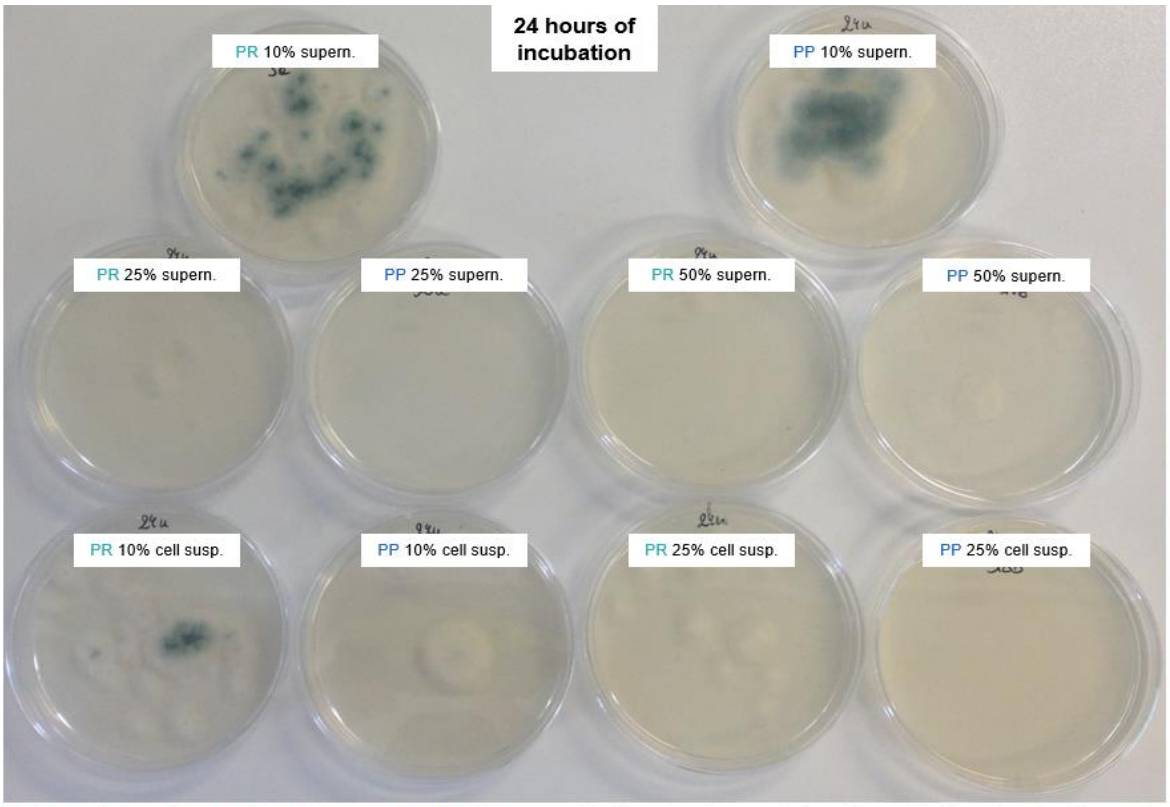


Figure 5.2. *In vitro experiment evaluating the antagonistic activity of Bacillus velezensis towards P. roqueforti s.l.: monitoring of P. roqueforti s.s. MUCL 46746 and P. paneum CBS 112295 conidiospore survival after 24 hours of incubation.*

Spore survival varied considerably among the culture media: 90 % CSI in combination with 10 % of Bv supernatant facilitated the highest conidiospore survival, followed by 90 % CSI and 10 % Bv cell suspension. Inclusion of 25 % or 50 % of Bv supernatant or cell suspension in the medium resulted in lower spore survival.

A striking observation that can be made is that the spores of PP CBS 112295 exhibited stronger growth over the four-days incubation period of the plates compared to spores of the PR isolate.

2.1.2.2. Conidiospore germination

The results of the conidiospore germination evaluation after 24 hours of incubation are presented in Table 5.1.

Table 5.1. *In vitro experiment evaluating the antagonistic activity of Bacillus velezensis towards P. roqueforti s.l.: conidiospore germination (%) for P. roqueforti s.s. (PR) MUCL 46746 and P. paneum (PP) CBS 112295 after 24 hours of incubation.* Mean values per object are given, with their resp. standard deviation between brackets. A significant interaction between infection and medium was found. Significant differences between the media are mentioned separately for both *P. roqueforti s.l.* isolates by grey-colored lettercode. Significant differences between the two isolates are indicated per culture medium by asterix (*) symbols. Additionally, black-colored lettercodes indicate differences between culture media with the same percentage of CSI.

Culture medium	PR MUCL 46746 germination (%)				PP CBS 112295 germination (%)			
100% corn silage infusion (CSI)	40.33 (2.08)	-	a		37.33 (2.52)	-	a
90% CSI + 10% sterile BHI	23.25 (4.79)	ab	bc		28.00 (4.08)	a	ab
90% CSI + 10% *B. velezensis* supernatant	33.50 (9.47)	a	ab		34.75 (6.65)	a	a
90% CSI + 10% *B. velezensis* cell suspension	11.50 (2.38)	b	d		12.25 (9.00)	b	de
75% CSI + 25% sterile BHI	33.25 (2.99)	a°	ab	*	23.00 (3.74)	a	bc
75% CSI + 25% *B. velezensis* supernatant	17.00 (5.35)	ab°	cd		16.25 (3.59)	b	cd
75% CSI + 25% *B. velezensis* cell suspension	14.75 (5.12)	b°	cd	*	6.00 (2.83)	c	e
50% CSI + 50% sterile BHI	18.00 (3.56)	a	cd		19.25 (3.20)	a	bcd
50% CSI + 50% *B. velezensis* supernatant	14.00 (2.16)	a	cd		12.50 (3.11)	b	de

° Non-parametric test according to Kruskal-Wallis

For both isolates, the highest percentage of spore germination after 24 hours was observed in 100 % CSI, while in general low percentages were observed in CSI to which *Bv* cell suspension was added.

Surprisingly, an increase of spore germination was observed in 90 % CSI with 10 % *Bv* supernatant for both isolates, but the difference with 90 % CSI with 10 % sterile BHI was not significant. When 75% of CSI was present in the culture medium, spore germination from both isolates was significantly lowered by 25 % *Bv* cell suspension compared to 25 % sterile BHI.

PR MUCL 46746 and PP CBS 112295 spore germination differed significantly after 24 hours of incubation in 75 % CSI complemented with 25 % sterile BHI or 25 % *Bv* cell suspension: in both media, conidiospore germination of the PP isolate was significantly lower than germination of the PR isolate.

Chitarra *et al.* (2003) demonstrated that *Bv* culture supernatant had a negative effect on *P. roqueforti s.s.* spore germination. The current experiment generally confirms this finding, but ascribes an even more potent inhibition of spore germination to *Bv* cell suspension. *Bv* cells can compete for nutrients with *P. roqueforti s.l.* in the culture medium, but the influence of this competition on spore germination is assumed to be low.

2.1.2.3. Fungal growth

Fungal growth was monitored spectrophotometrically by measuring the OD_{620} of all the microtiter plate's wells after 1, 2, 4, 8 and 24 hours and after five days. No straightforward increase in OD_{620} over time due to spore germination and mycelium growth was observed over the first 24 hours, probably due to little or no spore germination and subsequent mycelial growth: the OD data remained within the same narrow range. Therefore, only the OD_{620} data after five days of incubation (after subtraction of the initial OD_{620} value per well immediately after filling) are mentioned in Table 5.2.

Table 5.2. *In vitro experiment evaluating the antagonistic activity of Bacillus velezensis towards P. roqueforti s.l.: growth of P. roqueforti s.s. (PR) MUCL 46746 and P. paneum (PP) CBS 112295 after five days of incubation as registered spectrophotometrically by optical density at 620 nm (OD_{620})*. The mean OD_{620} per object is given, with its standard deviation between brackets. A significant interaction between infection and medium was found. Significant differences between the media are mentioned separately for both isolates by grey-colored lettercodes. Significant differences between the two isolates are indicated per culture medium by asterix (*) symbols. Additionally, black-colored lettercodes indicate significant differences between culture media with the same percentage of CSI.

Culture medium	PR MUCL 46746				PP CBS 112295			
	OD_{620}				OD_{620}			
100% corn silage infusion (CSI)	0.731 (0.047)	-	abc	*	0.819 (0.052)	-	ab
90% CSI + 10% sterile BHI	0.689 (0.055)	a	abcd		0.738 (0.049)	a	abc
90% CSI + 10% *B. velezensis* supernatant	0.553 (0.037)	b	cd		0.525 (0.021)	b	e
90% CSI + 10% *B. velezensis* cell suspension	0.516 (0.069)	b	d		0.534 (0.017)	b	de
75% CSI + 25% sterile BHI	0.878 (0.151)	a	a	*	0.726 (0.110)	a	abcd
75% CSI + 25% *B. velezensis* supernatant	0.600 (0.093)	b	bcd		0.488 (0.045)	b	e
75% CSI + 25% *B. velezensis* cell suspension	0.551 (0.074)	b	cd		0.466 (0.100)	b	ce
50% CSI + 50% sterile BHI	0.770 (0.067)	a	ab		0.845 (0.070)	a	a
50% CSI + 50% *B. velezensis* supernatant	0.592 (0.066)	b	bcd		0.500 (0.056)	b	e

Over the nine culture media, the two *P. roqueforti s.l.* isolates exhibited a different growth pattern. PR MUCL 46746 showed the highest growth on 75 % CSI with 25 % sterile BHI. The difference was not significant with 100 % CSI and with the 90 % and 50 % CSI complemented with sterile BHI, but was significant with all the media containing *Bv* supernatant or cell suspension. PP CSB 112295 growth was the highest on 50 % CSI with 50 % sterile BHI, but just like for PR MUCL 46746 the differences were only significant with media containing *Bv* supernatant or cell suspension. Therefore, it can be concluded that both *Bv* supernatant and cell suspension had an inhibiting effect on *P. roqueforti s.l.* growth registered as OD_{620}.

Per culture medium, only few differences in growth were detected between both *P. roqueforti s.l.* isolates. On 100 % CSI, PP CBS 112295 grew significantly stronger than PR MUCL 46746 during the 5-days incubation period, while on 75 % CSI with 25 % sterile BHI the opposite was found.

2.1.2.4. Quantitative screening of roquefortine C production

The antagonistic effect of *Bv* against *P. roqueforti s.l.* has been confirmed on multiple levels (*i.e.* conidiospore germination and survival, and fungal growth), rendering the tested *Bv* strain to an interesting candidate silage inoculant for *in vivo* inhibition of *P. roqueforti s.l.* growth in silages. However, it must be checked that growth inhibition of P*. roqueforti s.l.* by *Bv* does not trigger an increased mycotoxin production. Therefore, a quantitative screening of the production of the indicator mycotoxin ROC during the five-days incubation period has been performed (N = 1), as presented in Figure 5.3.

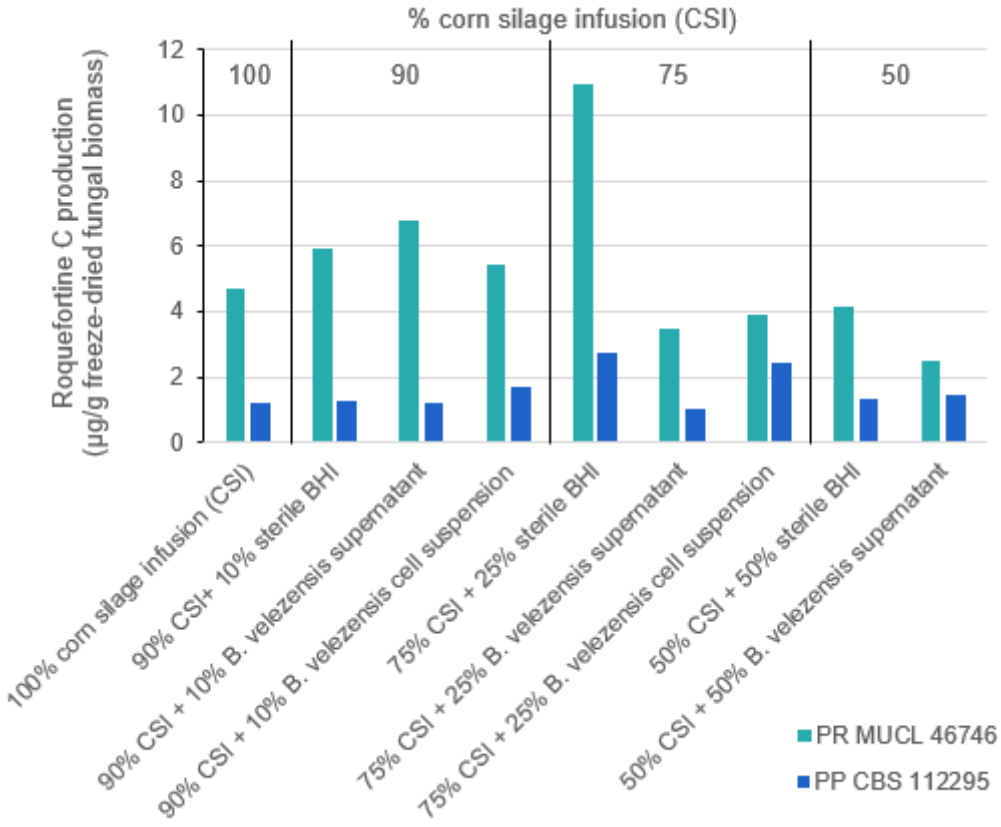


Figure 5.3. *In vitro experiment evaluating the antagonistic activity of Bacillus velezensis towards P. roqueforti s.s. (PR) MUCL 46746 and P. paneum (PP) CBS 112295: roquefortine C production (µg/g freeze-dried fungal biomass) after 5 days of aerobic incubation at 20 °C.*

PP CBS 112295 clearly produced less ROC than PR MUCL 46746. Addition of *Bv* supernatant or cell suspension generally did not result in higher ROC levels by any of the two *P. roqueforti s.l.* isolates compared to addition of sterile BHI to CSI.

For PR MUCL 46746, addition of 10 % sterile BHI, *Bv* supernatant or cell suspension to 90 % CSI increased ROC production compared to 100 % CSI. Addition of 25 % sterile BHI intensified ROC production even further, while addition of 50 % BHI resulted in lower ROC levels compared to 25 % BHI. The highest ROC production was detected on 75 % CSI with 25 % sterile BHI, which was also the culture medium exhibiting the strongest growth.

As for PP CBS 112295, the highest ROC levels were detected in 75 % CSI with 25 % sterile BHI or *Bv* cell suspension, but ROC did not differ much across the nine culture media compared to PR MUCL 46746.

Sterile BHI is a very nutritious culture medium. Due to *Bv* growth, the nutrient levels in the BHI in which the bacterium was cultured prior to the start of the experiment have dropped. Therefore, CSI supplemented with *Bv* supernatant or cell suspension contained less nutrients available for *P. roqueforti s.l.* growth and mycotoxin production. Since a positive correlation between growth of *P. roqueforti s.s.* and ROC production has been detected (Boichenko et al 2002a), this might explain the elevated ROC production by PR MUCL 46746 observed in the media containing 25 and 50 % sterile BHI, since growth was the highest on these media.

2.1.2.5. Growth of *Bacillus velezensis* in Corn Silage Infusion

The control media containing *Bv* cell suspension but no *P. roqueforti s.l.* conidiospores (indicated in white in Figure 5.1) have not been mentioned in Table 5.2, but the mean OD_{620} value at 10% *Bv* cell suspension addition to CSI (0.002 – st. dev. 0.002) was significantly lower compared to 25% *Bv* cell suspension addition (0.014 – st. dev. 0.004). However, in absolute values, both control media showed poor *Bv* growth in CSI, questioning its potential as a silage additive. It would be useful to include a survival assay for *Bv* cells in a future *in vitro* experiment.

In the current *in vitro* experiment, fungal growth as well as *Bv* growth were monitored spectrophotometrically at 620 nm. However, Kim *et al.* (1997) measured at 660 nm to quantify *Bv* growth. Nonetheless, no large difference between these two wavelengths regarding growth registration is expected. The optimum temperature for *Bv* growth is around 30 °C, whereas the current *in vitro* experiment was carried out at 20 °C. This relatively low incubation temperature can also help in explaining the low growth rate of *Bv* observed, despite the fact that *Bv* can growth in a temperature range of 15-45 °C (Kim *et al.* 1997; Ruiz-Garcia *et al.* 2005).

B. velezensis is an aerobic micro-organism (Liu *et al.* 2010; Ruiz-Garcia *et al.* 2005). Ruiz-Garcia *et al.* (2005) have found that *Bv* can grow in the pH range of 5-10. In the current *in vitro* experiment, CSI at pH 3.79 was used. This pH is well below pH 5, so the lack of *Bv* growth observed is very likely due to a too acidic growth medium. Velmurugan *et al.* (2009) have found that antifungal activity of *B. velezensis* remained stable in a pH range of 2-10 at 25 °C for 24 hours, but this was tested on culture supernatant and not on living bacteria.

2.1.3. Discussion and conclusion

- Contradictory results obtained with 50 % CSI and 50 % sterile BHI

When the results obtained with 50 % CSI and 50 % sterile BHI regarding *P. roqueforti s.l.* conidiospore survival, conidiospore germination and growth are put together, they appear to be somewhat contradictory.

P. roqueforti s.l. conidiospore germination after 24 hours in 50 % CSI with 50 % sterile BHI was at intermediate to low levels compared to the other culture media. For both PR MUCL 46746 and PP CBS 112295, fungal growth on the medium consisting of 50 % CSI and 50 % sterile BHI during a five-days incubation period was very good. So, conidiospore germination after 24 hours of incubation was reduced, whereas growth after five days of incubation was very good.
Conidiospore germination is an event situated in the lag phase of fungal growth, whereas after five days of incubation, the actual growth phase was observed. The lag phase encompasses time for germination plus the beginning of hyphal elongation (Gougouli and Koutsoumanis 2013). Studies investigating the effect of inoculum size, which is influenced by the conidiospore survival, on fungal growth parameters revealed that inoculum size affects the lag phase but not the growth rate in a given culture medium (Aldars-Garcia *et al.* 2017; Baert *et al.* 2007). Taking these observations into account, it is even more surprising that in the current *in vitro* experiment a reduced inoculum size (*cfr.* reduced conidial survival - no photographs taken of PR MUCL 46746 or PP CBS 112295 spore survival in 50% CSI and 50% sterile BHI) with an extended lag phase (*cfr.* relatively low conidiospore germination) exhibited a very strong growth over a five-days incubation period. This does suggest an elevated growth rate, most likely due to the specific composition of the culture medium consisting of 50 % CSI and 50 % sterile BHI (which is largely unknown).

- *B. velezensis* produces lipopeptides, acting as biosurfactants

In plant production, *Bacillus* species including *B. velezensis* have been proven by many authors to be promising antifungal biocontrol organisms (Nam *et al.* 2009b; Ongena and Jacques 2008; Romero *et al.* 2007; Velmurugan *et al.* 2009). They can produce thermostable cyclic **lipopeptides**, exerting antifungal effects both in alkaline and acidic conditions. Lipopeptides are synthesized by large non-ribosomal multi-enzyme complexes. The main families comprise iturins, surfactins and fengycins, all three exhibiting surfactant properties and antifungal activity (to a greater or lesser extent). As a member of the *B. subtilis* group, *B. velezensis* can produce all three families of lipopeptides (Ongena and Jacques 2008).

Surfactant molecules are characterized by causing a reduction of surface tension, modification of surface properties and perturbation of lipid bilayers. These properties are related to the amphiphilic character of lipopeptides, but also specific functions present in the peptidic moiety are important for their biological activity. The mechanisms of lipopeptide excretion are largely unknown, but is assumed to be driven by passive diffusion (Moyne *et al.* 2001; Ongena and Jacques 2008; Velmurugan *et al.* 2009).

Velmurugan *et al.* (2009) have found that antifungal activity of *B. velezensis* supernatant containing lipopeptides remained stable in a pH range of 2-10 at 25 °C for 24 hours, whereas Kim *et al.* (1997) found a lipopeptide from the surfactin family to be stable from pH 5.0 to 9.5 at 100 °C for one hour. At pH values around 4.0, the surface tension reducing activity of lipopeptides is reduced due to precipitation of the biosurfactants. In the current *in vitro* experiment, the pH of the CSI was 3.79, but the pH values of the other eight culture media have not been determined.

The carbon source used in the culture medium is very important in lipopeptide production *in vitro*. Carbohydrate substrates, e.g. sucrose and glucose, give a high lipopeptide yield: up to 40 gram of glucose per liter medium, lipopeptide yield increased linearly with increasing glucose concentration (Kim *et al.* 1997). In the same experiment, ammonium bicarbonate (at 13.5 g/l medium) proved to be an ideal nitrogen source since it buffers the pH of the culture medium. Phosphate ions added to this buffering effect.
For the current *in vitro* experiment, CSI was the major component of the culture media. Since whole-crop maize silage has been used to prepare the CSI and most water-soluble carbohydrates have been metabolized, the carbohydrate content of the media (which has not been determined) might have been rather low (with the exception of mannitol). However, taking the clear effect of *Bv* on conidiospore survival and germination into account, lipopeptides can be assumed to be actually present in the culture media – most likely introduced by the *Bv* supernatant or cell suspension at the start of the experiment, but possibly additionally produced by the *Bv* cell suspension in the CSI-based media during the five-days incubation period. Monitoring of lipopeptide production should definitely be included in future experiments.

- Bacteriostatic effect of ROC on Gram-positive bacteria

Like all *Bacillus* species, *B. velezensis* is a Gram-positive bacterium. ROC exhibits bacteriostatic activity towards Gram-positive bacteria (as mentioned in chapter 2 – section 3.5.3), so the ROC produced by *P. roqueforti s.l.* in the CSI-based media might have inhibited the growth of *Bv* cells. However, no detailed data were found in literature on the ROC concentrations inhibiting bacterial growth, especially of *Bacillus* species.

- Significant difference in ROC production between *P. roqueforti s.s.* and *P. paneum*

In both *in vitro* experiments discussed in chapter 3, *P. paneum* demonstrated a significantly stronger growth rate on the agar media PDA, CYA and MinM than *P. roqueforti s.s.*, whereas no significant differences in ROC production between the two species were encountered. In the current experiment, however, differences in growth were rather limited, but the PP CBS 112295 isolate produced significantly less ROC than the PR MUCL 46746 isolate.

In conclusion, it can be stated that based on the results of the current *in vitro* experiment *B. velezensis* appears to be a promising antagonist towards *P. roqueforti s.s.* as well as *P. paneum*: a clear effect on conidiospore survival and germination as well as on fungal growth has been detected in some of the tested culture media. Furthermore, nor *B. velezensis* supernatant nor cell suspension triggered an increased ROC production by *P. roqueforti s.l.*. *B. velezensis* growth in the Corn Silage Infusion was however very limited. This can be due to the fact that the strain was not adapted to acidic conditions prior to the experiment, but also to the intrinsic nature of the species. So, the question rises if *B. velezensis* will be able to grow and produce antifungal lipopeptides in an *in vivo* silage matrix. A first step towards answering this question has been taken in the context of an *in vivo* microsilo experiment, described further in this chapter. Moreover, if the answer to this question would appear to be positive, a whole lot of research and development activities will be required to successfully formulate a *B. velezensis* based silage inoculant.

2.2. Effect of HoLAB or HeLAB inoculant addition in a whole-crop maize based culture medium on *P. roqueforti s.l.* growth and roquefortine C production

Homo- and heterofermentative lactic acid bacteria are frequently used as silage inoculants, as already mentioned in chapter 1. HoLAB stimulate silage fermentation by very efficient production of lactic acid (LA). HeLAB are less efficient LA-producers, co-producing acetic acid (AA), ethanol, carbon dioxide and mannitol from water-soluble carbohydrates.

The fungal species *P. roqueforti s.s.* and *P. paneum* are well adapted to the conditions ruling in silages. In a preliminary *in vitro* experiment, the inhibitory effect of LA and AA on PR MUCL 46746 growth has been evaluated in a microtiter experiment (monitoring fungal growth spectrophotometrically at 620 nm over a three-days incubation period in double-concentrated Potato Dextrose Broth (PDB) supplemented with different amounts of LA and AA) in aerobic conditions at 20 °C. This experiment is not published in detail in this dissertation, but the main result was that AA unmistakably has stronger fungal inhibitory properties than LA: at 200 mM LA, fungal growth was still observed, whereas no growth was detected at 200 mM of AA despite the microscopical detection of spore germination was observed. To put this 200 mM concentration into perspective, the results of the on-farm sampling described in chapter 1 showed a mean LA content of 515 mM (varying between 135 and 835 mM) in the moisture of visibly non-mouldy whole-crop maize silage samples, and a mean AA content of 140 mM (varying between 40 and 265 mM). In non-mouldy grass silage samples, the mean LA level in the moisture was 550 mM (ranging from 15 to 1315 mM) and the mean AA content was 180 mM (varying between 0 and 435 mM). Regarding the interpretation of these concentrations, the same remarks should be made as in Chapter 4, section 3.2.2.3. Additionally, it must be mentioned that the antifungal properties of AA are not noticeably high compared to higher volatile fatty acids, like e.g. propionic acid (Moon 1983). Moreover, low AA concentrations stimulated *P. roqueforti s.s.* growth: in an *in vitro* experiment with PR MUCL 46746 aerobically incubated during 7 days at 20°C, fungal growth was stronger on Potato Dextrose Agar (PDA) supplemented with 17.5 mM of acetic acid compared to non-supplemented PDA (data not published). Taking all this into consideration, it is difficult to predict the effect of a given AA concentration on *P. roqueforti s.l.* in a silage matrix: it remains elusive whether the effect on growth is more inhibitory or more stimulatory.

Live LAB and *P. roqueforti s.l.* propagules can interact with one another. On the one hand, Lavermicocca *et al.* (2000) found that a ten-fold concentrated culture filtrate of *Lactobacillus plantarum* strain 21B possessed efficient antifungal activity against *P. roqueforti s.s..* On the other hand, *P. roqueforti s.l.* produces ROC, having bacteriostatic properties against Gram-positive bacteria. Since LAB are Gram-positive, their growth may be inhibited by ROC.

It would be very difficult to study the relationship between *P. roqueforti s.l.* and LAB as well as the effect of LA and AA produced by LAB on *P. roqueforti s.l. in vivo* in a real silage matrix. Therefore, an *in vitro* experiment mimicking silage conditions was carried out, as described below, to shed some light over these matters. On fresh crops prior to ensiling, the epiphytic lactic acid bacteria consist of a mixture of different species belonging to either HoLAB or HeLAB. However, in the current *in vitro* experiment the deliberate choice was made not to combine both types of LAB, in order to evaluate the effect of each of them separately.

2.2.1. Materials and methods

Since silage inoculants are added to fresh forage just before ensiling, fresh whole-crop maize was used in this experiment to make corn infusion agar (CIA), as described in Annex 1: freshly chopped whole-crop maize (dry matter content 350 g/kg FM) was dried at 60 °C and milled to 1-mm particles; subsequently corn infusion was made from this powder. After addition of agar and autoclaving, CIA was obtained.

Two commercially available silage inoculants were tested: the HoLAB inoculant was Pioneer Hi-Bred 1188, while Pioneer Hi-Bred 11A44 was used as a HeLAB inoculant. Pioneer HiBred 1188 contains *Lactobacillus plantarum* and *Enterococcus faecium* strains at $1.7*10^{10}$ cfu/g powder, while 11A44 contains *Lactobacillus buchneri* at $6.4*10^{9}$ cfu/g powder.
The inoculant powders were diluted in sterile physiological water to a concentration of $2.1*10^{7}$ cfu/ml. In standard 90-mm diameter Petri dishes, pour plates were made by distributing 1 ml of the appropriate inoculant solution and subsequent dispension of 20 ml of CIA at a temperature of 48-50 °C, resulting in a LAB concentration of $1.0*10^{6}$ per ml of agar medium. After solidification of the agar medium, an agar plug of 5 mm diameter was removed from the center of each plate.

Two *P. roqueforti s.l.* isolates were used in this experiment: *P. roqueforti s.s*. MUCL 46746 and *P. paneum* CBS 112295. Fungal infection of the pour plates containing LAB was accomplished by bringing 20 µl of spore solution (containing $0.5*10^{6}$ spores/ml, prepared as described in Annex 1) into the central opening in the agar medium, either before or after an anaerobic

incubation period of 7 days at 20 °C (in anaerobic jars containing Anaerocult® A) to facilitate LAB development in the fresh maize-based medium. Afterwards, all plates were incubated aerobically at 20 °C during 7 days to facilitate fungal growth. So, two incubation regimes were included in the experiment for *P. roqueforti s.l.*.

For both LAB inoculants, four plates were left uninfected, but were incubated anaerobically for 7 days at 20°C to be able to determine the production of lactic acid and acetic acid by the LAB inoculants during this incubation period. One blank CIA plate (made by distributing 1 ml of sterile physiological water before 20 ml of CIA was added) was included to determine the ROC content of the CIA. This plate was not infected with *P. roqueforti s.l.* and was not incubated.

Fungal growth was quantified by registration of the colony diameter at two transversal positions on the reverse side of the plates after 7 days of aerobic incubation (N = 4). The corresponding growth area was calculated.
ROC production was quantified (N = 4) by LC-MS/MS (as described in Annex 1), on freeze-dried agar plugs: three 9-mm diameter agar plugs were taken after 7 days of aerobic incubation: one plug near the center, one plug at the outer region of fungal growth and one plug at an intermediate position. The three agar plugs were transferred to a labeled 1.5 ml Eppendorf tube and stored at -20 °C prior to freeze-drying just before ROC analysis. From each tube, the empty weigth, the freshly filled weight and the weight after freeze-drying was noted. The ROC content detected in the three mycelium plugs (representing an area of 1.91 cm^2) was recalculated to the ROC content per cm^2 of growth area.
The remaining agar medium was used for chemical analysis (N = 4): the agar was cut into small pieces and was transferred into a labeled plastic cup. From each plastic cup, the empty and freshly filled weight was noted. Subsequently, distilled water was added to the ten-fold weight of the fresh agar weight and the cups were placed on a rotary shaker at 180 rpm for 1 hour at 20 °C. After 23 hours of storage at 4 °C, an aqueous extract was obtained by filtering the solution through a miracloth filter. The aqueous extracts were stored at -20 °C until analysis: the pH of the aqueous extract was measured, and the amount of lactic acid and acetic acid was determined by HPLC (as described in Annex 1).

The obtained data were statistically analysed as described in Annex 1.

The experimental setup of the *in vitro* experiment assessing the effect of a HoLAB and a HeLAB inoculant on *P. roqueforti s.l.* is summarized in Table 5.3.

Table 5.3. *Experimental setup of in vitro experiment assessing the effect of HoLAB or HeLAB inoculant addition on P. roqueforti s.l. growth and roquefortine C production in a whole-crop maize based medium.*

OBJECTS			
Incubation	**Additive**	**Infection**	**Monitoring**
Aerobic incubation for 7 days	HoLAB	*P. roqueforti s.s.* MUCL 46746	Fungal growth, chemical analysis and roquefortine C (N = 4)
	HeLAB		
Anaerobic incubation for 7 days, then aerobic incubation for 7 days	HoLAB		
	HeLAB		
Aerobic incubation for 7 days	HoLAB	*P. paneum* CBS 112295	Fungal growth, chemical analysis and roquefortine C (N = 4)
	HeLAB		
Anaerobic incubation for 7 days, then aerobic incubation for 7 days	HoLAB		
	HeLAB		
Anaerobic incubation for 7 days	HoLAB	no infection	Chemical analysis (N = 4)
	HeLAB		
No incubation	no additive	no infection	Roquefortine C (N = 1)

2.2.2. Results

2.2.2.1. Fungal growth and roquefortine C production

The results of the fungal growth monitoring and the ROC quantification are presented in Figure 5.4.

Growth of PP CBS 112295 was significantly higher compared to PR MUCL 46746, confirming the findings the *in vitro* experiment with four agar media described in chapter 3 and the nitrogen experiment described in chapter 4.

ROC production was significantly higher in case of HeLAB inoculation compared to HoLAB inoculation. A significant difference between the two *P. roqueforti s.l.* isolates was found: PR MUCL 46746 produced significantly more ROC than PP CBS 112295. In chapter 3, none of the two *in vitro* experiments detected a significant difference in ROC production between both species. However, the nitrogen experiment described in chapter 4 revealed a significantly higher ROC content of PR MUCL 46746 biomass on standard CYA after 6 days of incubation, whereas PP CBS 112295 biomass contained significantly more ROC at sampling after 9, 12 and 15 days of incubation. The *in vitro* experiment with *B. velezensis* discussed earlier in this chapter also pointed towards a higher ROC production by PR MUCL 46746 compared to PP CBS 112295 during a five-days incubation period. So, given the seven-days aerobic incubation period in the currently described *in vitro* experiment, the ROC production data are in line with the other *in vitro* experiments.

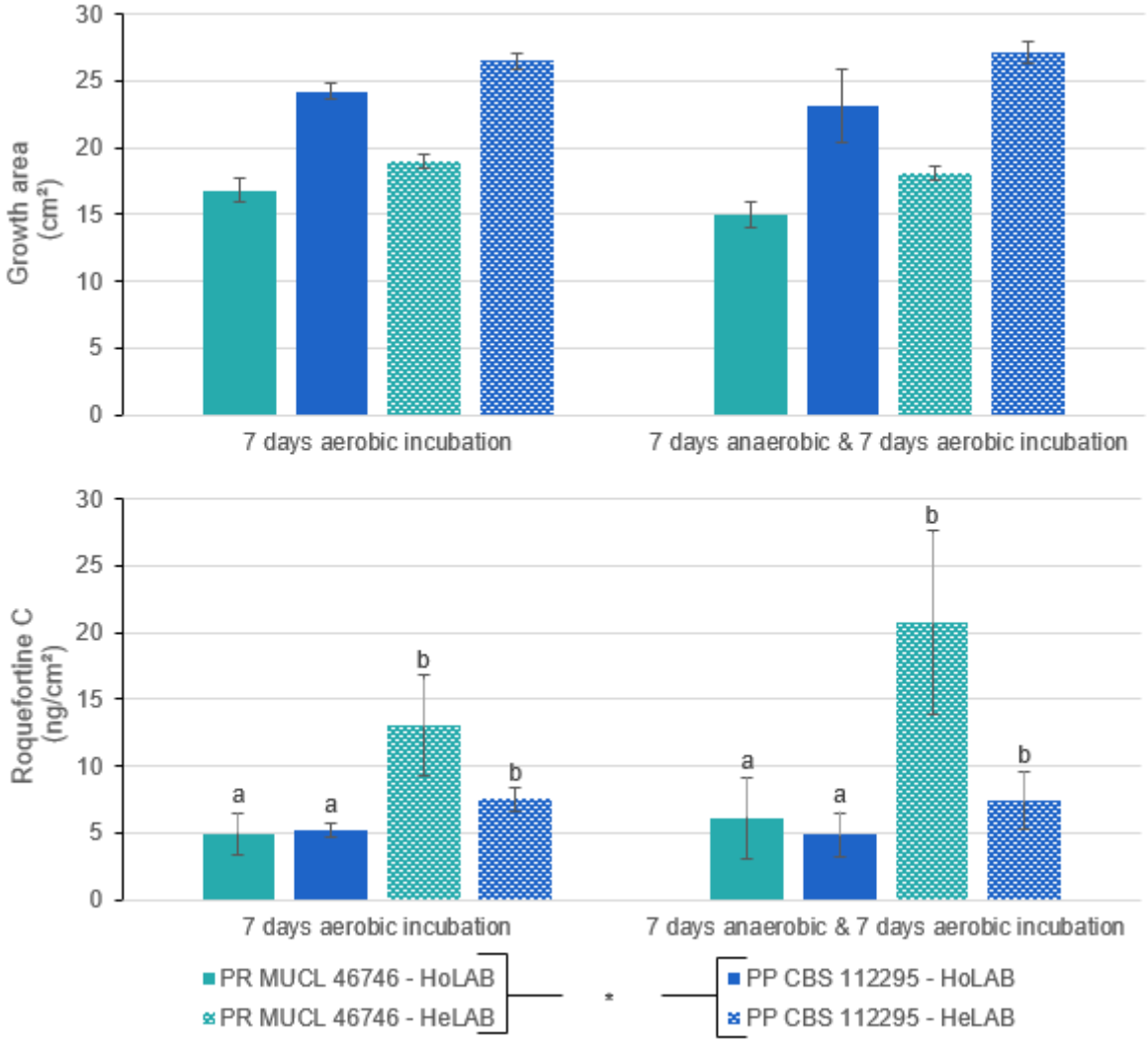


Figure 5.4. *In vitro experiment assessing the effect of HoLAB or HeLAB inoculant addition on P. roqueforti s.s. (PR) MUCL 46746 and P. paneum (PP) CBS 112295 in a whole-crop maize based medium: fungal growth (top) and roquefortine C production (bottom).* For both parameters, the mean values per object are presented as a bar chart, with error bars representing their resp. standard deviation. No significant interactions between infection, additive and incubation regime were found. Growth was not significantly influenced by additive or incubation regime. Roquefortine C production was not significantly influenced by the incubation regime, while the effect of additive is indicated by lettercode. Growth as well as roquefortine C production differed significantly between both *P. roqueforti s.l.* isolates, as indicated by an asterix (*) symbol in the legend.

Figure 5.4 confirms the limited effect of incubation regime on *P. roqueforti s.l.* growth. The effect on ROC production was greater, but not statistically significant. Although the mean ROC levels were relatively alike for the combinations PR-HoLAB, PP-HoLAB and PP-HeLAB, HeLAB inoculation of PR MUCL 46746 infected plates resulted in strongly elevated ROC concentrations in case of anaerobic and subsequent aerobic incubation compared to strictly aerobic fungal incubation. However, it should be noted that a high standard deviation was observed for the anaerobic-aerobic incubation.

2.2.2.2. Chemical analysis of agar medium

On aqueous extracts prepared from the agar media after registration of growth and sampling for ROC quantification, chemical analysis has been performed: the pH was determined as well as the lactic acid and the acetic acid content. These results are presented in Figure 5.5.

It was surprising to detect acetic acid in HoLAB inoculated media, since HoLAB usually don't produce acetic acid. Furthermore, the acetic acid cannot originate from the fresh whole-crop maize used to prepare CIA: if any acetic acid would have been present in the fresh whole-crop maize, it will have volatilized during the drying process (at 60 °C). However, *L. plantarum* can occasionally display heterofermentative metabolism, converting certain pentose sugars into lactic acid, acetic acid and ethanol (Oude Elferink *et al.* 2000).

Taking the uninfected plates inoculated with HoLAB or HeLAB into consideration, two remarks can be made:
As expected based on their difference in carbohydrate metabolism (described in Chapter 1), HoLAB inoculation resulted in a significantly lower pH of the culture medium, due to a significantly higher lactic acid and lower acetic acid level compared to HeLAB inoculation.

It is striking that the observed pH values of uninfected HoLAB containing medium were close to those observed in *P. roqueforti s.l.* infected media, whereas uninfected HeLAB medium had a lower pH than *P. roqueforti s.l.* infected media. For both additives, a significantly higher pH was detected in *P. roqueforti s.l.* infected media compared to uninfected medium, associated with a significant reduction of the lactic acid as well as acetic acid levels.

Upon growth of PR MUCL 46746, the pH of the culture medium was significantly lower compared to PP CBS 112295. This was reflected in a significantly higher lactic acid level observed in PR MUCL 46746 infected plates for both additives.
As could be expected (*cfr.* Chapter 1), HoLAB inoculation resulted in significantly lower pH values compared to HeLAB inoculation, which can be ascribed to significantly higher lactic acid production.

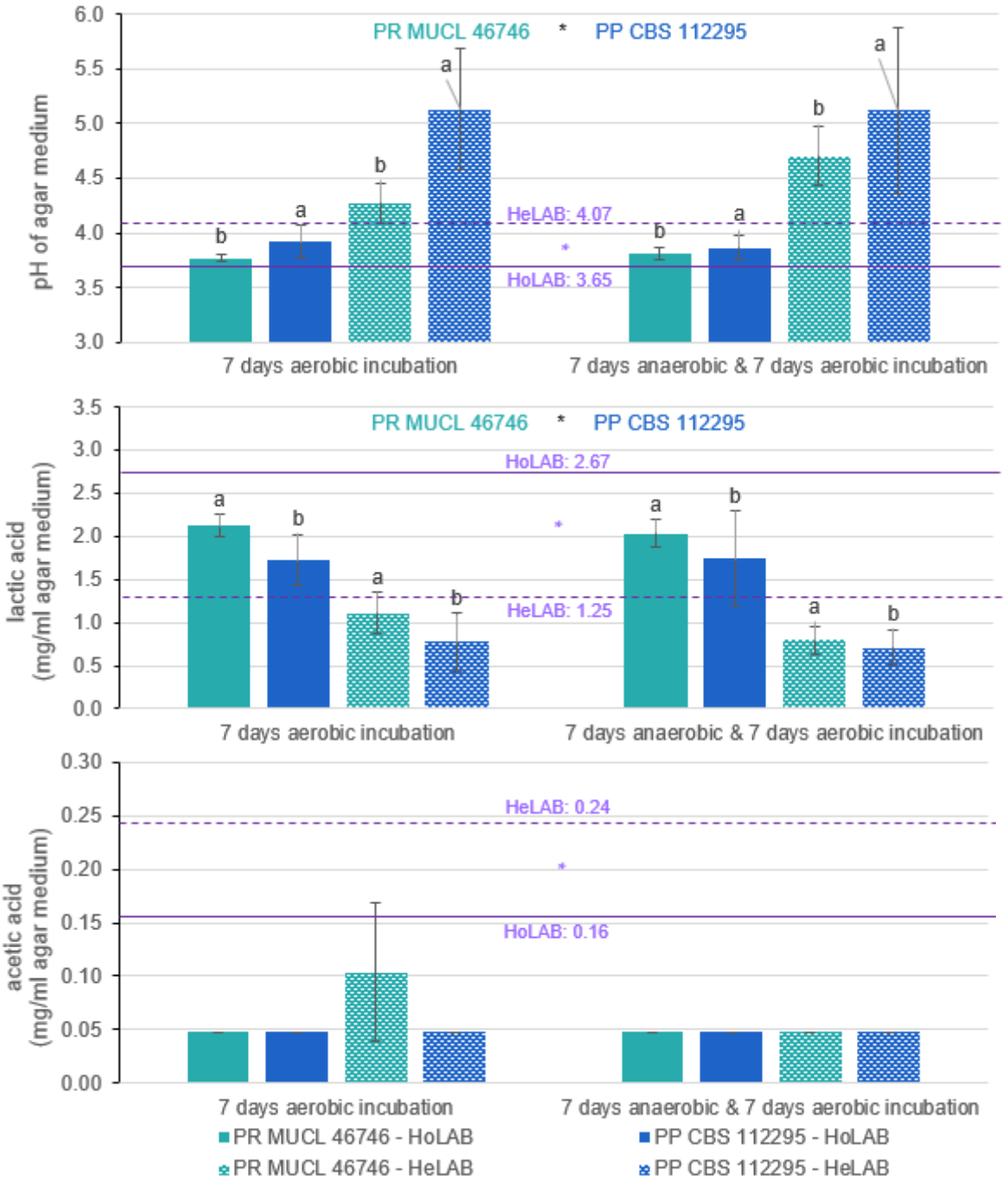


Figure 5.5. *In vitro experiment assessing the effect of HoLAB or HeLAB inoculant addition on P. roqueforti s.s. (PR) MUCL 46746 and P. paneum (PP) CBS 112295: pH (top), lactic acid content (middle) and acetic acid content (bottom) of agar medium.* For all parameters, the mean values of the *P. roqueforti s.l.* infected objects are presented in a bar chart, with error bars representing their resp. standard deviation. No significant interactions between infection, additive and incubation were detected. Incubation regime did not influence any parameter significantly, whereas a significant effect of both infection and additive was detected on pH and lactic acid content. The significant differences between both isolates are indicated by black-colored asterix (*) symbols, while the effect of additive is indicated by lettercode. Acetic acid levels were not significantly influenced by any of the three factors.
As a reference, uninfected plates containing HoLAB or HeLAB were also chemically analyzed after seven days of anaerobic incubation at 20 °C. These results are indicated in the graphs by horizontal lines. Significant differences between HoLAB and HeLAB inoculated plates are indicated by purple *-symbols.

2.2.3. Discussion and conclusion

In the current *in vitro* experiment, CIA was prepared from fresh whole-crop maize. By adding either HoLAB or HeLAB, a seven-days period of anaerobic incubation facilitated the production of lactic acid, acetic acid and other compounds in the medium, mimicking the fermentation phase of the ensiling process. After a subsequent seven-days aerobic incubation period (mimicking the feed-out phase of the ensiling process), *P. roqueforti s.l.* growth and ROC production were monitored and the culture medium was chemically analyzed. *P. roqueforti s.l.* growth did not differ significantly according to the applied LAB additive, whereas HeLAB inoculation significantly increased ROC production compared to HoLAB inoculation. Chemical analysis of the culture media revealed a reduction of lactic acid as well as acetic acid content upon fungal growth, so both acids were metabolized by *P. roqueforti s.l.* This confirms the findings of the *in vitro* experiment with different single carbon sources (chapter 3, section 4), that *P. roqueforti s.l.* can metabolize both lactic acid as acetic acid. The precise effect of these acids on ROC production by *P. roqueforti s.l.* cannot be unraveled from the current *in vitro* experiment, but acetic acid seems to trigger ROC production. As increased growth tends to result in increased ROC production (Scott *et al.* 1977 and the findings of the *in vitro* experiment with variable amounts of inorganic nitrogen, described in chapter 4 - section 2), this might help in explaining this observation. However, it cannot be ruled out that acetic acid itself has a direct or indirect effect on the expression of the ROC biosynthesis gene cluster.

In the context of the current *in vitro* experiment, HeLAB inoculation was associated with significantly higher ROC production by *P. roqueforti s.l..* However, it remains elusive whether HeLAB inoculants also might trigger ROC production by *P. roqueforti s.l. in vivo* in silages on-farm. Extensive sampling of both untreated and HeLAB treated on-farm silages might shed some light on this matter, but the critical remarks formulated in chapter 2 about the sampling of mycotoxin contaminated silages definitely need to be kept in mind.

3. *In vivo* effect of biotic factors on *P. roqueforti s.l.* growth: HoLAB, HeLAB, *B. velezensis* and propionic acid in grass silage

In chapter 4, the effect of HoLAB, HeLAB and propionic addition in whole-crop maize silage has already been evaluated in a microsilo trial focusing on the effect of prolonged anaerobic conditions on *P. roqueforti s.l.* numbers. In the microsilo trial that will be described hereafter, the effect of prolonged anaerobic conditions has not been tested, but an additional silage additive has been included: as *B. velezensis* proved efficient in reducing *P. roqueforti s.l.* conidiospore survival as well as conidial germination and subsequent fungal growth *in vitro* (as described in section 2.1 of this chapter), its potential as a silage additive is tested *in vivo*. *B. velezensis* growth in 100 % Corn Silage Infusion (CSI) during the five-days aerobic incubation period at 20 °C was very poor, so a microsilo assay would permit to determine whether or not *B. velezensis* can grow and produce antifungal lipopeptides *in vivo* in a silage matrix.

A microsilo experiment with grass was performed to assess the effect of HoLAB, HeLAB and *B. velezensis* inoculation as well as application of propionic acid.

3.1. Materials and methods

A mixture of perennial ryegrass and white clover (second cut) was mown and prewilted in the field. After chopping with a New Holland precision chopper to 10-12 cm particles, the starting material was homogenized well prior to ensiling of the different objects.

P. roqueforti s.l. spore solutions were freshly prepared as described in Annex 1 (but no glycerol was added and the conidiospore concentration was adjusted to $5*10^4$ spores/ml). The negative control was sprayed with 20 ml of sterile physiological water per kg FM. Per kg FM, 10 ml of PR MUCL 46746 or PP CBS 112295 conidiospore solution was applied to artificially infect the ryegrass - clover mixture, as well as 10 ml of the appropriate additive solution. Infection levels were low for both *P. roqueforti s.l.* isolates since usually low *P. roqueforti s.l.* numbers are encountered in fresh crops prior to ensiling.

For both Pioneer 1188 (HoLAB) and Pioneer 11A44 (HeLAB), a new commercial unit was used, containing resp. $1.25*10^{11}$ cfu/g and $1.0*10^{11}$ cfu/g of powder. The appropriate amount of inoculant powder was resuspended in sterile physiological water.

A *Bacillus velezensis* cell suspension (obtained after a three-days incubation period in 100 ml of Brain-Heart Infusion (BHI) at 30 °C on a rotary shaker at 130 rpm) was centrifuged in two 50-ml portions at 10 000 rpm during 5 min. The supernatant was discarded, whereas the bacterial pellet was resuspended in 50 ml physiological water containing 16 % glycerol and stored at -80 °C. Just before ensiling, both 50-ml portions of cell suspension were defrosted at 20 °C and combined with 100 ml of physiological water. The concentration of the obtained *B. velezensis* additive solution was determined by streak-plating 100 µl aliquots of a decimal dilution series on Plate Count Agar (PCA) (N=3). PCA plates were incubated aerobically at 30 °C for four days and *B. velezensis* was enumerated, taking the appropriate dilution factor into account: $5*10^5$ cfu of *B. velezensis* were present per milliliter of additive solution.

For propionic acid, a 45/55 mixture (vol/vol) with distilled water was prepared.

From the negative control object (*i.e.* no infection and no additive used), grass samples were taken prior to ensiling for determination of the dry matter (DM) content and the pH of the starting material, and for enumeration of the amount of *P. roqueforti s.l.* propagules. The DM content was 42 % and the pH was 6.09 (N=2), while no *P. roqueforti s.l.* propagules were found (N=3). On both objects without additive but with artificial infection with PR MUCL 46746 or PP CBS 112295, numbers of *P. roqueforti s.l.* were determined (N=3): on average 222 and 741 *P. roqueforti s.l.* spores were present on resp. *P. roqueforti s.s.* and *P. paneum* infected grass. Per object, four microsilos were filled with approximately 1.25 kg fresh matter. Mean silo density was 190 kg DM/m³.

The microsilos were stored at the attic of the Bottelare research center during 8 weeks, at an average temperature of 24 °C (min 20 °C - max 29 °C, registered with a temperature data logger) to facilitate fungal growth. The fermentation losses were monitored on a weekly basis. After an ensiled period of 42 days, aerobic stress was provided by opening the holes in the microsilos during 24 hours. All microsilos were desiled after 56 days.

From each individual microsilo, the dry matter content and the pH of the desiled material were determined and the desiled grass was subjected to determination of the aerobic stability. A sample from the center of each microsilo was subjected to *P. roqueforti s.l.* propagule enumeration.The obtained data were statistically analyzed as described in Annex 1.

The experimental setup of the microsilo trial is summarized in Table 5.4.

Table 5.4. *Experimental setup of microsilo trial evaluating the effect of silage additives on grass silage: negative control (no infection and no additive) versus additive applications after infection with P. roqueforti s.s. MUCL 46746 or P. paneum CBS 112295.*

OBJECTS			
Infection	**Additive**	Additive solution	**N**
negative control	no additive	sterile physiological water	4
P. roqueforti s.s.	no additive	sterile physiological water	4
MUCL 46746	HoLAB	Pioneer 1188: *L. plantarum* and *E. faecium* @ 1.10^6 cfu/g FM	4
@ 500 spores/g FM	HeLAB	Pioneer 11A44: *L. buchneri* @ 1.10^6 cfu/g FM	4
	B. velezensis	*B. velezensis* @ 5.10^3 cfu/g FM	4
	propionic acid	99% propionic acid @ 4.5 liter/ton FM	4
P. paneum	no additive	sterile physiological water	4
CBS 112295	HoLAB	Pioneer 1188: *L. plantarum* and *E. faecium* @ 1.10^6 cfu/g FM	4
@ 500 spores/g FM	HeLAB	Pioneer 11A44: *L. buchneri* @ 1.10^6 cfu/g FM	4
	B. velezensis	*B. velezensis* @ 5.10^3 cfu/g FM	4
	propionic acid	99% propionic acid @ 4.5 liter/ton FM	4
Desiling after 56 days			
Fermentation losses, *P. roqueforti s.l.* enumeration, dry matter, pH, aerobic stability and dry matter content after Honig			

3.2. Results

3.2.1. Fermentation losses

The microsilos were ensiled during 56 days, and aerobic stress was provided at 42-43 days after ensiling by removing of the Duct tape sealing the two holes in the microsilos during 24 hours. Since oxygen could ingress into the microsilos, the fresh weight losses during the ensiled period don't only reflect fermentation losses but also losses due to aerobic metabolism. Nonetheless, the term "fermentation losses" will be adopted.

The evolution of the fermentation losses during the 56-days ensiled period are presented in Figure 5.6.

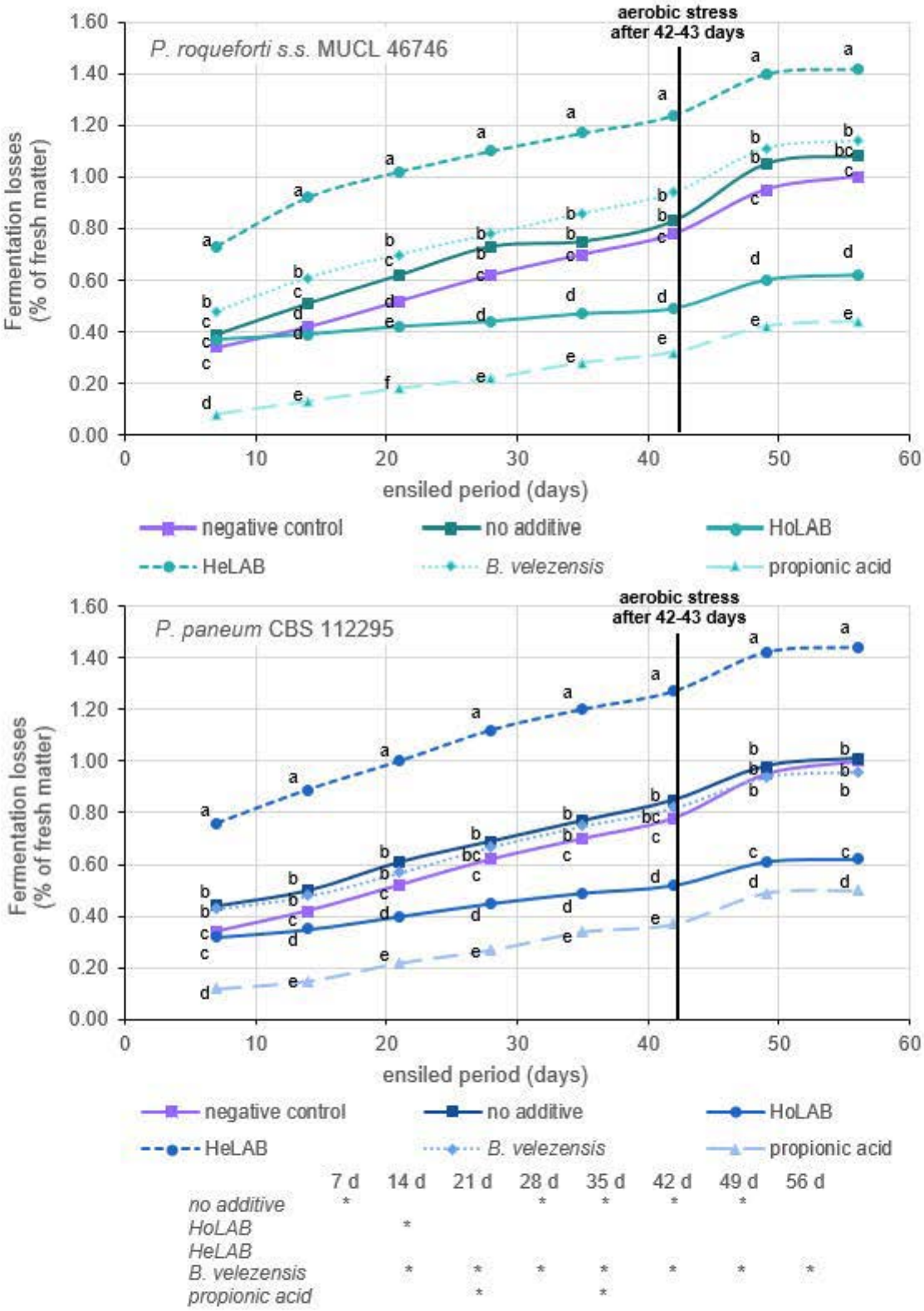


Figure 5.6. *Fermentation losses observed in a microsilo trial evaluating the effect of silage additives on grass silage: negative control (no infection and no additive) versus additive applications after infection with P. roqueforti s.s. MUCL 46746 (top) or P. paneum CBS 112295 (bottom).* The mean values per object are presented without error bars indicating their resp. standard deviation, but standard deviations were low for all objects (max 0.09 % on fresh matter). For both isolates, significant differences between the objects are indicated by lettercode per time-point. Significant differences between both isolates are indicated per object by asterix(*) symbols below the graphs.

In absolute value, the fermentation losses remained at a low level throughout the ensiled period for all objects (McDonald *et al.* 1991). However, there were relatively big differences according to the silage additive applied, as demonstrated by the multiple statistically significant differences indicated by lettercodes per time point.

The fermentation losses of the negative control were slightly lower compared to the fermentation losses in absence of a silage additive for grass artificially infected with *P. roqueforti s.l.*.

For both *P. roqueforti s.l.* isolates, application of propionic acid resulted in the lowest fermentation losses, being somewhat lower than the fermentation losses in case of HoLAB inoculation. The highest fermentation losses were for HeLAB inoculation. This is as can be expected by differences in carbohydrate metabolism between HoLAB and HeLAB, the latter producing carbon dioxide (described in chapter 1, section 1.2). In silages not treated with a silage additive, the epiphytic lactic acid bacteria on the grass consists of both HoLAB as well as HeLAB species (in an unknown ratio), resulting in intermediate levels of fermentation losses. Propionic acid is a fermentation inhibiting silage additive (chapter 1, section 1.5.6), reducing fermentation losses. Application of *B. velezensis* as a silage inoculant resulted in fermentation losses similar to those observed in silage not treated with a silage additive, suggesting that it has only a very limited effect on silage fermentation efficiency expressed as fermentation losses.

The fermentation losses of grass infected with PR MUCL 46746 differed significantly from those of PP CBS 112295 infected grass for nearly all objects at specific time points, as designated at the bottom of Figure 5.6. In case of *B. velezensis* inoculation, the fermentation losses of PR MUCL 46746 infected grass are significantly higher compared to PP CBS 112295 infected grass from 14 days after ensiling until desiling after 56 days. No possible explanation for this different behavior could be found in literature.

3.2.2. Analyses at desiling

At desiling no visible fungal growth was detected. *P. roqueforti s.l.* numbers were determined on desiled material, as well as the dry matter content and the pH. The aerobic stability was determined by Honig's protocol, and the dry matter content was determined again at the end of Honig's protocol. The results of these analyses are summarized in Table 5.5.

Table 5.5. *Analyses at desiling of microsilo trial evaluating the effect of silage additives on grass silage: P. roqueforti s.l. numbers, dry matter (DM) at desiling, aerobic stability and dry matter content after Honig's protocol.* For all parameters, the mean value per object is mentioned with its standard deviation between brackets. For the *P. roqueforti s.l.* numbers and the aerobic stability, no significant interaction was detected between infection and additive. The main effects of infection and additive are indicated by black-colored lettercodes. To address the effect of additive, lettercodes are equal for both *P. roqueforti s.l.* isolates. A significant interaction between infection and additive was found for the dry matter content and pH at desiling and the dry matter content at the end of the Honig protocol. For these parameters, the effect of one factor is evaluated per level of the other factor, as indicated by grey-colored lettercodes.

OBJECTS		*P. roqueforti s.l.*			DM at desiling				pH				aerobic stability		DM after Honig			
Infection	Additive	(log cfu/g FM)	*inf.*°	*add.*°	(g/kg FM)	*inf.*	*add.*			*inf.*	*add.*		(hours)	*inf.*°	(g/kg FM)	*inf.*	*add.*	
negative control	no additive	0.00 (0.00)	b	ab	431 (2)	-	a	a	4.83 (0.05)	-	a	a	144 (44)	b	443 (2)	-	a	a
P. roqueforti s.s.	no additive	2.08 (0.21)		ab	413 (0)	*	b		4.84 (0.04)	*	a		128 (58)		422 (4)	*	b	
MUCL 46746	HoLAB	0.22 (0.43)		b	403 (3)	*	b		3.96 (0.02)		c		> 175		413 (4)		b	
	HeLAB	1.43 (0.30)	ab	ab	389 (4)	*	c		4.62 (0.01)	*	b		> 175	a	400 (2)	*	cd	
	B. velezensis	1.79 (0.36)		a	377 (5)	*	c		4.53 (0.06)		b		> 175		393 (8)	*	d	
	propionic acid	1.75 (0.38)		ab	400 (8)		bc		4.68 (0.03)	*	b		> 175		409 (9)		bc	
P. paneum	no additive	2.40 (0.19)		ab	397 (3)	*		b	4.62 (0.07)	*		b	> 175		408 (7)	*		bc
CBS 112295	HoLAB	0.81 (0.94)		b	389 (8)	*		bc	3.98 (0.01)			c	> 175		401 (13)			bc
	HeLAB	1.95 (0.23)	a	ab	378 (3)	*		c	4.58 (0.02)	*		b	> 175	a	386 (3)	*		c
	B. velezensis	2.32 (0.14)		a	402 (5)	*		b	4.53 (0.08)			b	> 175		417 (3)	*		b
	propionic acid	1.49 (0.18)		ab	395 (9)			bc	4.48 (0.14)	*		ab	> 175		405 (8)			bc

° Non-parametric test according to Kruskal-Wallis

P. roqueforti s.l. numbers were significantly influenced by infection. On the uninfected grass, no *P. roqueforti s.l.* was detected, whereas artificial infection with PP CBS 112295 resulted in significantly higher *P. roqueforti s.l.* numbers. PR MUCL 46746 infected grass contained intermediate *P. roqueforti s.l.* propagule numbers at desiling, but the level of artificial infection with the PR isolate appeared to be somewhat lower than with the PP isolate.
Additive application had a significant effect on *P. roqueforti s.l.* counts: the significantly highest counts were registered after inoculation with *B. velezensis*, whereas HoLAB inoculation resulted in the significantly lowest *P. roqueforti s.l.* numbers. So, the applied *B. velezensis* cell suspension was apparently not capable to exert its *in vitro* observed inhibiting effect on *P. roqueforti s.l.* conidiospore survival *in vivo* in a grass silage matrix.

From a practical point of view, the differences in DM content were small, but both factors had a significant effect. A significant difference in DM content at desiling between PR MUCL 46746 and PP CBS 112295 was detected for all additive levels except for propionic acid. No additive, HoLAB and HeLAB application resulted in a significantly higher DM content in PR MUCL 46746 infected silage compared to PP CBS 112295 infected silage, whereas the opposite was observed for *B. velezensis* application.
The effect of the additives was evaluated per *P. roqueforti s.l.* isolate, taking the five additives upon artificial infection supplemented with the negative control into account. For both isolates, the significantly highest DM content at desiling was observed in the negative control. Grass artificially infected with PR MUCL 46746 had the lowest DM content at desiling after application of HeLAB, *B. velezensis* or propionic acid, indicating a less efficient silage fermentation. Upon artificial infection with PP CBS 112295, the lowest DM content was observed in case of HeLAB inoculation. HeLAB inoculation is known to cause a less efficient fermentation compared to HoLAB inoculation (as described in chapter 1). However, in absolute value, DM contents did not vary much between objects.

For PR MUCL 46746, the negative control and the infected grass without a silage additive had the significantly highest pH values, while HoLAB inoculation resulted in the significantly lowest pH. HoLAB inoculation also gave the lowest pH values for PP CBS 112295 infected grass, while the highest pH was observed in the negative control.
P. roqueforti s.l. infection also significantly influenced the pH of the silage samples: the pH differed significantly between grass infected with PR MUCL 46746 and with PP CBS 112295 for the additive levels no additive, HeLAB and propionic acid. For all three additives, the pH observed after artificial infection with PP CBS 112295 was significantly reduced compared to artificial infection with PR MUCL 46746. No reasonable explanation for this phenomenon could be found in literature. Furthermore, differences were quite small in absolute value.

The aerobic stability was not significantly influenced by additive application and was overall high. Therefore, it was decided not to determine supplementary fermentation characteristics on the silage samples obtained in the context of this *in vivo* microsilo experiment. Surprisingly, the uninfected silage had a significantly lower aerobic stability than artificially infected silage, but the standard deviation was substantial.

A significant difference in dry matter content after Honig between the *P. roqueforti s.l.* isolates used for artificial infection was found for silage not treated with an additive and for HeLAB or *B. velezensis* inoculated silage.
Additive application also had a significant influence on the dry matter content after Honig. The negative control had absolutely the highest DM recovery, whereas grass silage artificially infected with PR MUCL 46746 had the significantly lowest DM content after Honig upon treatment with HeLAB or *B. velezensis*. PP CBS 112295 infected grass treated with HeLAB had the lowest DM content after Honig. However, differences in dry matter content were very low in absolute value, being quasi irrelevant from a practical point of view.

3.3. Discussion and conclusion

Despite the significant differences observed in dry matter content and pH at desiling, aerobic stability and dry matter at the end of the Honig protocol according to *P. roqueforti s.l.* infection and additive application, these parameters did not show a large variation over the different objects. However, large differences in *P. roqueforti s.l.* propagules numbers on the desiled material have been detected, influenced by both infection and additive. Evidently, a more intense level of artificial infection with PP CBS 112295 compared to PR MUCL 46746 resulted in higher *P. roqueforti s.l.* numbers at desiling, whereas no *P. roqueforti s.l.* propagules were detected in uninfected grass silage without additive application (in which no *P. roqueforti s.l.* propagules were found at the time of ensiling either).

The *B. velezensis* cell suspension used in the current *in vivo* microsilo experiment could not live up to the great expectations which had arose in the *in vitro* experiment regarding its antagonistic activity towards *P. roqueforti s.l..* Most likely, this is due to a very short aerobic phase of the ensiling process in microsilos on the one hand and to a quick pH-drop. Another option is that the manufacturing process of the *B. velezensis* cell suspension was not optimal: HoLAB and HeLAB application solutions were prepared from commercially available products as a result of extensive R&D, contrary to the *B. velezensis* cell suspension.

Moreover, since *Bacillus* species can carry antibiotic resistance genes (Bernhard *et al.* 1978; Steinmetz and Richter 1994), it is of the outmost importance to select strains not bearing these genes as potential silage inoculant candidates.

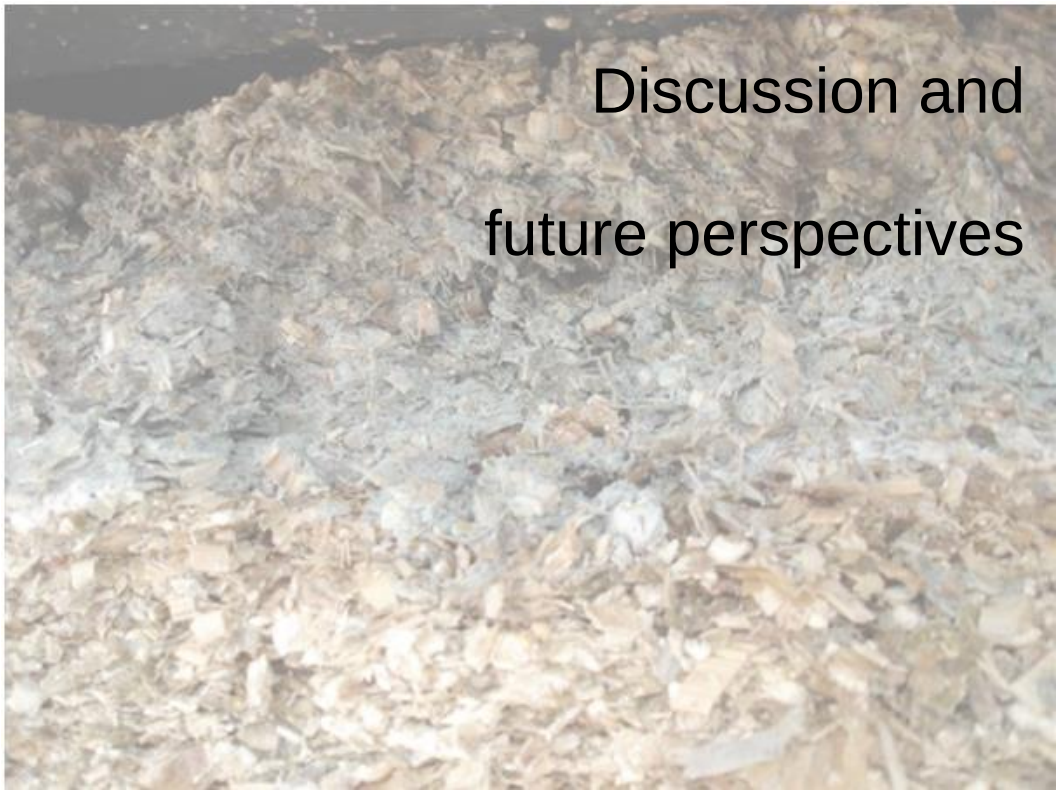
Finally, *B. velezensis* enumeration as well as quantification of lipopeptides at different timepoints during the ensiled period will definately provide more information about this bacterial species' capabilities of growth and lipopeptide production in a silage matrix.

4. Conclusions on the effect of biotic factors on *P. roqueforti s.l.* growth and roquefortine C production

This chapter on biotic factors kicked off with an *in vitro* experiment evaluating the antagonistic activity of a particular *B. velezensis* strain towards PR MUCL 46746 and PP CBS 112295 in a whole-crop based liquid medium: Corn Silage Infusion (CSI) was brought to pH 3.79 with a mixture of lactic acid and acetic acid according to the composition of the whole-crop maize silage used to prepare the CSI. Different volumes of sterile Brain-Heart Infusion (*i.e.* the medium used to culture *B. velezensis*), *B. velezensis* supernatant or *B. velezensis* cell suspension were added to CSI and these media were infected with 10 000 *P. roqueforti s.l.* conidia per ml. Fungal growth and ROC production were evaluated after static incubation at 20 °C during five days, whereas conidiospore germination and survival were monitored after 24 hours of incubation. Promising antagonistic effects were found regarding the reduction of *P. roqueforti s.l.* conidiospore germination and survival, as well as growth inhibition. Moreover, ROC production was not stimulated upon antagonistic activity of *B. velezensis*. However, *B. velezensis* growth in the CSI was very low over the five-days incubation period, raising serious doubts about its potential to exhibit its antagonistic activity towards *P. roqueforti s.l.* in an *in vivo* silage matrix. This matter has been addressed in an *in vivo* microsilo experiment with PR MUCL 46746 infected grass: the *B. velezensis* cell suspension was not able to live up to the great expectations created by the *in vitro* experiment since no significant reduction in *P. roqueforti s.l.* numbers in the desiled material were found.

The possible interaction between *P. roqueforti s.l.* on the one hand and lactic acid bacteria (LAB) on the other hand has been evaluated by an *in vitro* experiment with Corn Infusion Agar (CIA, prepared from fresh whole-crop maize) inoculated with LAB: HoLAB or HeLAB were incorporated in pour-plates of CIA and anaerobically incubated for seven days at 20 °C to mimic the silage fermentation phase. Afterwards, a seven-days aerobic incubation period facilitated growth of PR MUCL 46746 and PP CBS 112295. Fungal growth and ROC production were monitored, and the pH, lactic acid and acetic acid content of the culture medium was determined. HoLAB and HeLAB inoculation resulted in significantly different pH values and acid levels in the culture media. Fungal growth increased the pH of the medium by breakdown of lactic acid and acetic acid. Despite the fact that no significant effect of the two types of LAB on fungal growth was found, HeLAB inoculation was associated with significantly higher ROC production compared to HoLAB inoculation. If HeLAB inoculation *in vivo* in silages would also trigger ROC production, this would be an alarming finding. However, particular care should be taken in extrapolating the results of this *in vitro* experiment to *in vivo* silages.

Chapter 6



Discussion and

future perspectives

1. Discussion

Fungal growth and mycotoxin production are influenced by numerous abiotic and biotic factors. Abiotic factors comprise environmental factors (e.g. temperature, water activity, atmospheric gas composition, chemical composition, pH, carbon sources, nitrogen sources, the ratio of carbon over nitrogen, etc.). Biotic factors allude to the physiological state of the fungus, but also to interactions with silage microbiota (Hymery *et al.* 2014). To study the effect of abiotic and biotic factors on *P. roqueforti s.l.* growth and ROC production, both *in vitro* lab experiments as *in vivo* trials with microsilos have been executed. The main conclusions of these experiments are summarized at the end of Chapter 4 and Chapter 5.

The question may rise whether the microsilos used for the *in vivo* experiments do actually mimic on-farm silage conditions. O'Kiely and Wilson (1991) have made a comparison between small clamp silos and experimental plastic-pipe silos to evaluate the ensiling process of grass. They found a close agreement between both. Driehuis *et al.* (2001) have ensiled grass in clamp silos as well as in laboratory silos (*i.e.* 1-liter glass jars) and found comparable pH values at desiling after 90 days. Xiccato *et al.* (1994) have compared on-farm bunker silos with three types of experimental silos for ensiling of whole-crop maize. One type of experimental silo comprised a glass jar with a capacity of two liters, with airtight sealing and without effluent discharge valve. The results obtained with this particular silo type might be somewhat similar to those obtained with the microsilos used for the *in vivo* experiments in the context of this dissertation, since fermentation gasses could also escape while air could not ingress. In this study, experimental silos showed a more active fermentation compared to bunker silos. The researchers concluded that these experimental silos are useful to study silage conservation because they faithfully reflect the process normally occurring in on-farm bunker silos.
The standard microsilos used for the *in vivo* experiments described in this manuscript have proven to facilitate a good ensiling process, based on the pH-values obtained by ensiling whole-crop maize, grasses and other crops at variable dry matter contents (data not shown): due to their small scale and augmented air-tightness compared to on-farm silages, the LAB fermentation starts quicker and is more uniform throughout the silo. Therefore, it is definitely reasonable to assume that these microsilos mimic on-farm silages of excellent quality, under the conditions that the silage is well compacted, and no aerobic stress is provided during the ensiled period.

1.1. Answering of research questions

The first research question raised comprised the interaction of *P. roqueforti s.l.* with lactic acid bacteria and about the effect of some of their metabolites on fungal growth and ROC production. An *in vitro* experiment described in chapter 5, with HoLAB and HeLAB incorporated in pour-plates of Corn Infusion Agar (CIA), has shed some light on this matter, along with the findings of the *in vitro* experiment with eight different single carbon sources described in chapter 3. Conclusion of these experiments is that acetic acid can be used by *P. roqueforti s.l.* as a carbon source, facilitating good growth. Lactic acid as single carbon source was not very conducive for fungal growth. However, *in vivo* in a silage matrix, various carbon sources including the previously named ones are present.

As demonstrated by sampling of on-farm silages (chapter 1) fungal hot-spots are characterized by an elevated pH compared to visibly non-mouldy silage, attributable to a breakdown of acetic acid and lactic acid. In Chapter 1, a chicken-or-egg dilemma approach was suggested in order to assess this phenomenon: on the one hand, it can be assumed that silage fermentation was well in the mouldy hot-spots prior to fungal infestation. On the other hand, it might also be possible that in some specific locations within a silo (e.g. at air pockets) LAB could not perform a proper silage fermentation, locally resulting in silage with elevated pH-values due to low amounts of lactic acid and/or acetic acid. An *in vitro* experiment with CIA has clearly demonstrated that *P. roqueforti s.l.* can grow in the presence of both lactic acid and acetic acid at pH-values similar to those in silages. Aerobic incubation triggered a decrease of acid levels and an increased pH, corresponding to the findings of the on-farm sampling. Therefore, the first hypothesis seems to be the closest to the truth. Interestingly, ROC production by *P. roqueforti s.l.* was significantly higher on HeLAB-inoculated CIA than on HoLAB-inoculated CIA. Still, it remains elusive whether HeLAB inoculation of fresh feed commodities might also trigger *P. roqueforti s.l.* growth and ROC production *in vivo* during the feed-out period of on-farm silages.

The research question addressing the effect of oxygen on *P. roqueforti s.l.* growth and ROC production can be answered based on the results of multiple *in vitro* and *in vivo* experiments. At first, it was confirmed *in vitro* that growth was not possible in anaerobic conditions. Secondly, an *in vivo* microsilo experiment with whole-crop maize demonstrated that in artificially infected silage (containing 1500 conidia per gram fresh matter) without silage additive, *P. roqueforti s.l.* numbers at desiling after 100 and 150 days had dropped below the limit of detection, whereas at desiling after 50 days some *P. roqueforti s.l.* propagules (on average 66 per gram fresh matter) were still found. This confirms the outmost importance of keeping on-farm silages sealed long enough prior to starting the feed-out to the livestock.

P. roqueforti s.l. is not only well adapted to acidic conditions, but also to oxygen depletion and high carbon dioxide level. *In vitro*, *P. roqueforti s.s.* growth during normal aerobic incubation (*i.e*. 21 % oxygen and 0.04 % carbon dioxide) on Potato Dextrose Agar during seven days was higher compared to incubation in oxygen depleted and carbon dioxide enriched conditions (*i.e.* 6 % oxygen and 9 % carbon dioxide) at temperatures ranging from 5 °C to 30 °C. Optimum growth temperature was around 25 °C at normal aerobic incubation, whereas 20 °C was the temperature optimum in oxygen depleted and carbon dioxide enriched conditions. Muck and Huhnke (1995) found that the core temperature of silages in Wisconsin remained almost constant, around 20 °C. Wisconsin has a continental climate, with resp. minimum, mean and maximum annual temperatures of 4, 9 and 13 °C for Milwaukee from 1981 to 2010 (www.usclimatedata.com). In comparison, Belgium has a temperate climate and from 1901-2000 the minimum, mean and maximum annual temperatures were resp. 7, 10 and 14 °C (statbel.fgov.be). Since silages are characterized by anoxic or oxygen depleted conditions in a carbon dioxide enriched atmosphere, optimal growth of *P. roqueforti s.l.* can be expected at temperatures of 20-25 °C, which is precisely the expected silage core temperature range.
The effect of oxygen depletion and carbon dioxide enrichment of the atmosphere on mycotoxin production by *P. roqueforti s.l.* has not been investigated in the context of this PhD research. The *in vitro* experiment with HoLAB and HeLAB inoculant addition to Corn Infusion Agar did include an incubation regime where *P. roqueforti s.l*. conidia were incubated anaerobically during seven days followed by seven days of aerobic incubation. Compared to solely seven days of aerobic incubation, one week of anaerobic incubation did not significantly influence *P. roqueforti s.l.* growth, nor the ROC production.

In vivo microsilo experiments with microsilos have been conducted to evaluate the effect of imperfect silo sealing, and oxygen supply during 24 hours at 60 days after ensiling in combination with elevated temperature (*i.e.* 32 °C) at 61-64 days after ensiling: fungal counts at desiling after 76 days were significantly higher in case of oxygen supply and elevated

temperature upon infection with *P. roqueforti s.s.*, confirming the crucial role of oxygen in fungal infestation of silages.

Another research question was if any difference in sensitivity to *P. roqueforti s.l.* infection between whole-crop maize and grass silages could be explained by differences in carbon source composition. No scientific evidence exists that *P. roqueforti s.l.* infestation of silages is more severe in whole-crop maize than in grass silages. The nutritional composition (and accordingly the carbon and nitrogen source composition) of these two feed commodities is highly variable, before as well as after ensiling. During ensiling, the carbohydrates are largely converted into lactic acid, acetic acid, mannitol and ethanol in both silage types. The residual carbohydrate content is usually higher in whole-crop maize silages than in grass silages, so *P. roqueforti s.l.* generally has more sugars available for growth in whole-crop maize than in grass silages (Auerbach *et al.* 1998; McDonald *et al.* 1991; Nishino *et al.* 2003; Shao *et al.* 2005). It is impossible to extrapolate the results obtained *in vitro* with different single carbon sources (revealing a significantly higher ROC production on ethanol compared to lactic acid and acetic acid) towards *in vivo* silages, which are all composed of a diverse mix of carbon sources varying both in compounds and in levels over time and location within a silo.

The fourth research question aimed to determine the antagonistic potential of *Bacillus velezensis*. *B. velezensis* has displayed interesting antagonistic properties towards *P. roqueforti s.l.* in an *in vitro* experiment with Corn Silage Infusion (CSI), reducing conidiospore survival and germination as well as fungal growth. Moreover, its inhibitory properties were not associated with an increased ROC production by *P. roqueforti s.l..* However, *B. velezensis* growth in CSI proved to be very limited. This seriously questioned its practical applicability in an *in vivo* silage matrix, where upon the creation of anaerobic conditions a rapid acidification of the environment is established by the epiphytic microflora. A non-commercially available *B. velezensis* cell suspension has been applied as a silage additive on grass artificially infected with *P. roqueforti s.l.* in the context of an *in vivo* microsilo experiment. The *B. velezensis* cell suspension was unsuccessful in reducing the *P. roqueforti s.l.* numbers observed at desiling after 56 days compared to no additive application. Still, this failure does not imply that *B. velezensis* cannot be successfully applied as an antagonistic silage additive to help preventing P*. roqueforti s.l.* in silage: extended research on this matter is required.

1.2. Practice-orientated advice towards prevention

The goals of this PhD research towards the prevention of *P. roqueforti s.l.* growth and mycotoxin production in silages were ambitious. Despite the fact that no excessive breakthroughs or miracle solutions in solving the problems associated with mould infested silage were found, much more insight was gained into the well adapted nature of *P. roqueforti s.l.* to its natural silage habitat. It is exactly its great adaptation to conditions ruling in silages that make it difficult to prevent *P. roqueforti s.l.* infestation of silage.

It could be stated that it is easy to prevent the growth and mycotoxin production of *P. roqueforti s.l.* and other toxigenic fungi in silages, just by creating anaerobic conditions. However, creating anoxic conditions in silages is easier said than done since no silo coverage is 100 % airtight and finally all silos are opened for feed-out. Farmers can gain control on the rate and depth of air ingress between the silo particles through the ensiling and desiling practices applied (as described thoroughly in chapter 1). Therefore, it is of the outmost importance to apply good silage making practices “from field to feed” to combat the development of undesirable micro-organisms like *P. roqueforti s.l.*: the porosity of the silage mass must be kept as low as possible, e.g. by ensiling crops at an appropriate dry matter content, by quick and thorough silo compaction and sealing, by respecting a sufficiently long ensiled period, by unloading the silo properly and at a sufficiently high feed-out rate, *etc.* If preventive measures should fail and fungal hot-spots do arise in silages, it is certainly advisable to meticulously remove the visibly mouldy silage prior to feeding to the livestock.

2. Future perspectives

In this PhD dissertation, the effect of some abiotic and biotic factors on growth and mycotoxin production (with ROC as indicator) by *P. roqueforti s.l.* has been assessed by several *in vitro* lab experiments and *in vivo* trials with microsilos. *P. roqueforti s.l.* in silages are part of an ecological niche in which diverse microbial communities compete. Therefore, ROC production and mycotoxin production in general should be considered in an ecological context, which is difficult to elucidate *in vitro* (Hymery *et al.* 2014).

A whole multitude of both *in vitro* as *in vivo* experiments can assist in fully unraveling the mechanisms determining the growth and mycotoxin production by *P. roqueforti s.l.* in silage substrates. Evidently, this will take a lot of time, effort and budget. Furthermore, many factors need to be taken into account at the setup of different experiments.

At first, Aldars-Garcia *et al.* (2017) have studied the effect of single spore inoculum versus multiple-spore inoculum *in vitro* on growth an aflatoxin production by *Aspergillus flavus*. Studies on fungal growth are traditionally carried out with high inoculum levels of conidiospores, when in fact infection of silages by *P. roqueforti s.l.* generally occurs with a low number of spores and colonies most likely originate from single spores.

Secondly, the *in vitro* experiments described in chapter 3 have demonstrated that the selected four *P. roqueforti s.s.* isolates and two *P. paneum* isolates grown in standardized conditions significantly differ in growth and ROC production both within and between species. Therefore, the inclusion of several fungal isolates in future experiments would be advisable, in combination with the determination of not only ROC but also other mycotoxins produced by *P. roqueforti s.l..*

Several *in vitro* experiments performed in the context of this PhD study have demonstrated a stronger growth of *P. paneum* compared to *P. roqueforti s.s.* over a relatively short incubation period. Moreover, it appeared that ROC production by *P. paneum* increased more slowly over time than ROC production by *P. roqueforti s.s..* The factor time should be studied more in depth in future experiments, but it is difficult if not impossible to define a suitable time-frame related to the growth period of *P. roqueforti s.l.* in silages: fungal infestation of silage just below the silo coverage can stretch over a time period of weeks to months, whereas fungal infestation of the silage at the cutting face is in order of a matter of days.

Study of the behavior of *P. roqueforti s.l.* in a silage context is preferably executed in an *in vivo* silage matrix, especially when practice-orientated results are aimed for. Due to the heterogenic nature of on-farm silages (varying within silos and among silos on the same and on other farms), it will be far from evident to draw the right conclusions about the different influencing factors on *P. roqueforti s.l.* growth and mycotoxin production. Microsilos may be used to build bridges between *in vitro* laboratory experiments and *in vivo* on-farm silages. They have the advantage that they can be made in several replicates per object, and most importantly they do represent the ecological niche to which *P. roqueforti s.l.* is very well adapted: silage.

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Annex 1

General materials

and methods

1. Materials

anaerobic jars (Merck)

Anaerocult® A (Merck)

Anaerocult® C (Merck)

autoclave SA-260 MA (Sturdy)

Bürker chamber

cellophane: uncoated Natureflex NP 28µm (Innovia)

centrifugal filter units: ultrafree MC-GV Durapore PVDF 0,22 µm UFC30 GVNB (Millipore)

centrifugation tubes of 15 ml (SPL Life Sciences)

centrifugation tubes of 50 ml (SPL Life Sciences)

centrifuge: 5804 (Eppendorf)

cryopreservation cabinet: VX100 (Jouan)

Drigalsky spatula

Eppendorf tubes of 1.5 ml (Eppendorf)

Erlenmeyers of 250 ml

folded paper filters 597 ½ (diameter 125 mm and 185 mm) (Whatman)

glass tubes for slants (Novolab)

incubators (Memmert, Binder)

inoculation needles

laminar flow cabinet: EF/S (EuroFlow)

lyophilizer: Alpha 1-2 LD Plus (Christ)

microperforated bags (Sealed Air)

microtiter plates (Eppendorf)

mill (Retch)

miracloth (Millipore)

parafilm (Bemis)

Petri dishes 45-mm diameter (Gosselin)

Petri dishes 90-mm diameter (Gosselin)

pH electrode (Labconsult)

pincers

respiratory foil (AB Gene)

rocking shaker (Agiletec)

rotary shaker: KS-15 (Edmund Bühler GmbH)

stomacher: 400 circulator (Seward)

stomacher bags with lateral filter (Interscience)

syringe filters: cellulose acetate filter with 0.45 µm pore size and 25 mm diameter (GVS)

temperature data logger: EL USB-1 (Lascar Electronics)

2. Chemicals

acetic acid (min. 99 %, Sigma)

agar (Duchefa)

ammonium hydroxide: 25% NH_4OH in water (Sigma)

ammonium nitrate: NH_4NO_3 (Merck)

Brain-Heart Infusion broth: 12.5 g calf brains infusion, 5 g beef heart infusion, 10 g peptone, 2 g glucose, 5 g sodium chloride and 2.5 g disodium hydrogen phosphate per liter, with a pH of 7.4 ± 0.2 at 25 °C (Sigma)

boric acid: H_3BO_3 (min. 99.5 %, Fluka)

chloramphenicol (Duchefa)

citric acid (Sigma)

copper sulphate: $CuSO_4.5H_2O$ (Fluka)

dichloromethane (min. 99.5 %, Acros Organics)

4-dimethylamino benzaldehyde (DMAB) (min. 99 %, Fluka)

dipotassium phosphate: K_2HPO_4 (Duchefa)

ethanol: 70% in water (Sigma)

96% (Sigma)

ethanol gel for hand disinfection (Tork)

ethyl acetate (min. 99.5 %, Acros Organics)

fructose (Sigma)

glucose (Acros Organics)

glycerol (min. 99.5%, Scharlau)

hydrochloric acid 10N: HCl (Fluka)

iron sulphate: $FeSO_4.7H_2O$ (Sigma)

lactic acid (min. 99 %, Fluka)

magnesium sulphate: $MgSO_4.7H_2O$ (Sigma)

Malt Extract Agar: 30 g malt extract, 15 g agar and 5 g mycological peptone per liter, with a pH of 5.4 ± 0.2 at 25°C) (Sigma)

manganese sulphate: $MnSO_4.xH_2O$ (Sigma)

mannitol (Riedel-de Haën)

methanol (HPLC-grade, Sigma)

monopotassium phosphate: KH_2PO_4 (Duchefa)

potassium chloride: KCl (Duchefa)

Plate Count Agar (PCA): 9 g agar, 5 g tryptone, 2.5 g yeast extract, 1 g dextrose per liter, with a pH of 7.0± 0.2 at 25 °C (Sigma)

Potato Dextrose Agar (PDA): 20 g dextrose, 15 g agar and 4 g potato extract per liter, with a pH of 5.6 ± 0.2 at 25 °C (Sigma)

Potato Dextrose Broth (PDB): 20 g dextrose and 4 g potato extract per liter, with a pH of 5.6 ± 0.2 at 25°C (Sigma)

roquefortine C (min. 98 %, BioViotica)

sodium chloride: NaCl (Sigma)

sodium hydroxide: NaOH (Fluka)

sodium molybdate: $Na_2MoO_4.2H_2O$ (Sigma)

sodium nitrate: $NaNO_3$ (Sigma)

succinic acid (Sigma)

sucrose (Sigma)

Tween 80 (Duchefa)

yeast extract (autolysate, approx. 11 % total nitrogen and 3.5 % amino nitrogen, Sigma)

zearalanone (min. 98 %, Coring System Diagnostix)

zinc sulphate: $ZnSO_4.7H_2O$

3. Methods

3.1. Preparation of agar media

All agar media are prepared with distilled water and autoclaved at a temperature of 121 °C and a pressure of 1 bar during 15 min, except if mentioned otherwise.

Media are poured into appropriate recipients in a laminar flow cabinet. After solidification of the agar media, the filled recipients are stored in the dark until use.

3.1.1. Corn Infusion Agar (CIA)

Freshly chopped whole-crop maize is dried at 60 °C and milled to 1-mm particles. Corn infusion is prepared as described by Niderkorn (2007): milled fresh maize is infused in distilled water at 6 % (w/v) during 2 hours at 60 °C, followed by filtration through a miracloth filter and a folded Whatman 595 ½ filter. After centrifugation at 10 000 rpm during 15 min, the pellet is discarded and the supernatant fluid is collected. To this obtained corn infusion, 15 g/l agar is added prior to autoclaving.

3.1.2. Czapek-Dox Yeast extract Agar (CYA)

30 g sucrose - 15 g agar - 5 g yeast extract - 3 g $NaNO_3$ - 1 g K_2HPO_4 – 0.5 g $MgSO_4.7H_2O$ – 0.5 g KCl – 0.01 g $FeSO_4.7H_2O$ per liter

pH 7.00 at 20 °C

3.1.3. Malt Extract Agar (MEA)

50 g of Malt Extract Agar powder per liter

3.1.4. Mineral medium (MinM)

30 g sucrose - 15 g agar - 2 g NH_4NO_3 - 1 g KH_2PO_4 – 0.5 g $MgSO_4.7H_2O$ – 0.5 g KCl – 0.01 g $FeSO_4.7H_2O$ - 200 µl of trace element solution (containing 5 g citric acid, 5 g $ZnSO_4.7H_2O$, 0.25 g $CuSO_4.5H_2O$, 50 mg $MnSO_4.H_2O$, 50 mg H_3BO_3 and 50 mg $Na_2MoO_4.2H_2O$ per 100 ml) per liter

pH brought to 5.5 at 20 °C

3.1.5. Plate Count Agar (PCA)

17.5 g of Plate Count Agar powder per liter

3.1.6. Potato Dextrose Agar (PDA)

39 g of Potato Dextrose Agar powder per liter

3.1.7. PDA containing 5 ml acetic acid per liter (PDAA)

39 g of Potato Dextrose Agar powder is added to 995 ml of distilled water
after autoclaving and cooling to approx. 55 °C, 5 ml of filter-sterile acetic acid is added

pH 3.75 at 20 °C

3.1.8. Yeast Extract Sucrose agar (YES)

20 g sucrose - 15 g agar - 4 g yeast extract - 1 g KH_2PO_4 – 0.5 g $MgSO_4.7H_2O$ per liter

pH 5.60 at 20 °C

3.2. Preparation of liquid media and solutions

All liquid media are prepared with distilled water and autoclaved at a temperature of 121 °C and a pressure of 1 bar during 15 min, except if mentioned otherwise.

3.2.1. Brain-Heart Infusion broth (BHI)

37 g of Brain-Heart Infusion broth powder per liter

pH 7.4±0.2 at 25 °C

3.2.2. Corn Silage Infusion (CSI)

Whole crop maize silage is dried at 60 °C and milled to 1-mm particles. Corn infusion is prepared as described by Niderkorn (2007): milled maize silage is infused in distilled water at 6 % (w/v) during 2 hours at 60 °C, followed by filtration through a miracloth filter and a folded paper filter. After centrifugation at 10 000 rpm during 15 min, the pellet is discarded and the supernatant fluid is collected. The pH of the supernatant fluid is brought to the original pH of the silage used for the CSI preparation, using the same ratio of lactic acid to acetic acid as found in the silage.The supernatant is sterilized through a syringe filter and stored at 4 °C.

3.2.3. Ehrlich's reagent

0.2 g DMAB in 8.5 ml 96 % ethanol + 1.5 ml 10M HCl

no autoclaving or filter-sterilization

storage at 4 °C, protected from light

3.2.4. Physiological water

8.5 g NaCl per liter

3.2.5. Potato Dextrose Broth (PDB)

24 g of PDB powder per liter

pH 5.1±0.2 at 25 °C

3.3. Isolation of *P. roqueforti s.l.*

3.3.1. Direct plating

Silage samples are brought on PDA plates in a laminar flow cabinet using a sterile pincer, followed by aerobic incubation at 20-25 °C upright in the dark for mould isolation. To distinguish the *P. roqueforti s.l.* group from other *Penicillia*, selective PDA containing 5 ml of acetic acid per liter medium (PDAA) was used in combination with microscopic analysis (O'Brien et al., 2008). Using sterile inoculation needles, *P. roqueforti s.l.* isolates are subcultured on PDAA to confirm their purity, serving as inoculum for the preparation of a dilution series in physiological water, which is streak plated at 100 µl over the total surface of 90-mm diameter Petri dishes containing PDAA to obtain monospore colonies. On the one hand, these monospore *P. roqueforti s.l.* isolates are maintained on PDA slants at 4 °C and are subcultured periodically, while on the other hand spore solutions are prepared for long-term storage at -80 °C.

3.3.2. Dilution plating

A silage sample of 10-30 gram fresh matter is manually brought in a stomacher bag with lateral filter (wearing plastic gloves disinfected with ethanol gel) along with 90 ml of sterile physiological water supplemented with 0.01 % Tween 80. After placing the bag in a stomacher for homogenization during 1 min at 200 rpm, a dilution series is prepared in a laminar flow cabinet in 15-ml falcons filled with 9 ml of sterile physiological water. From this dilution series, 100 µl is streak-plated on standard 90-mm Petri dishes containing Potato Dextrose Agar supplemented with 5 ml of acetic acid per liter (PDAA) using a sterile Drigalsky spatula. After sealing of the plates with parafilm, they are aerobically incubated bottom-up, in the dark at 20 °C. During seven days, the plates are regularly checked for growth of *P. roqueforti s.l.*.

3.4. Preparation of *P. roqueforti s.l.* conidiospore solutions

For the preparation of spore solutions, monospore fungal inoculum is seeded in the centre of standard 90-mm diameter Petri dishes with PDA in a laminar flow cabinet. The plates are aerobically incubated upright in the dark at 25 °C during 14 days.
Spores are harvested in a laminar flow cabinet by washing with physiological water containing 0.01% Tween 80, with the aid of a sterile pipet tip. The suspension is transferred to a sterile 50-ml centrifugation tube. After centrifugation at 8 500 rpm during 15 min, the supernatant is discarded and the spore pellet is resuspended in sterile physiological water without Tween 80 added. The centrifugation and resuspension step are repeated, after which the spore suspension is filtered through a double layer of sterile miracloth to remove mycelial fragments and spore aggregates.

Unless mentioned explicitly, the concentration of spores is determined with a Bürker chamber and adjusted to $1*10^6$ spores/ml with sterile physiological water and further diluted to $5*10^5$ spores/ml with sterile glycerol. This spore solution is distributed in 1.5-ml Eppendorf tubes, 15-ml centrifugation tubes or 50-ml centrifugation tubes. After 1 hour of storage at 4 °C and 23 hours at -20 °C, the solutions are stored long-term at -80 °C.

3.5. Enumeration of fungal propagules in silage samples

3.5.1. Enumeration of total fungal propagules

To be able to enumerate the amount of fungal propagules in a fresh crop or silage sample, exactly 10, 20 or 30 grams of fresh matter is manually brought in a stomacher bag with lateral filter (wearing plastic gloves disinfected with ethanol gel) along with 90 ml of sterile physiological water supplemented with 0.01 % Tween 80. After placing the bag in a stomacher for homogenization during 1 min at 200 rpm, a dilution series is prepared in a laminar flow cabinet in 15-ml falcons filled with 9 ml of sterile physiological water. From this dilution series, 100 µl is streak-plated on standard 90-mm Petri dishes containing Potato Dextrose Agar (PDA) using a sterile Drigalsky spatula, in triplicate per dilution. After sealing of the plates with parafilm, they are aerobically incubated bottom-up, in the dark at 20 °C. During 7 days, the plates are regularly checked for growth of *P. roqueforti s.l.*.
Usually, after five days of incubation the number of fungal propagules can be counted in triplicate on PDA-plates from an appropriate dilution (*i.e.* propagule number below 50 per plate). The mean value of the three readings is determined and the number of fungal propagules in the original silage sample is calculated taking the particular dilution factor into account for each sample.

3.5.2. Enumeration of P. roqueforti s.l.

For enumeration of *P. roqueforti s.l.*, the same procedure as for total fungal counting is adopted, but the dilution series is streak-plated on standard 90-mm Petri dishes containing Potato Dextrose Agar supplemented with 5 ml of acetic acid per liter (PDAA).

3.6. Freeze-drying

Prior to lyophilisation, samples were sealed with micro-perforated parafilm and stored in racks at -80 °C. Samples were freeze-dried in an Alpha 1-2 LD Plus lyophilizer (Christ) according to the manufacturer's guidance.

3.7. Roquefortine C quantification by LC-MS/MS

Roquefortine C (ROC) was extracted from freeze-dried samples of *in vitro* experiments as described by Delmulle (2008) and quantified by LC-MS/MS based on the method described by Monbaliu *et al.* (2010).

ROC is expressed relatively to a known amount of internal standard, which is added to all samples: 0.2 ng of zearalanone (ZAN). To allow quantification of ROC, four reference samples (blank samples or ethyl acetate) were spiked with ROC (negative control and 3 known amounts). Based on the response factors (*i.e.* peak area for ROC / peak area for ZAN) for these reference samples, a linear regression is determined per run that is used for the quantification of ROC in the other samples.

Stock solutions of ROC (0.1 mg/ml) and ZAN (1 mg/ml) were prepared in methanol and stored at -20 °C. Working standard solutions of ROC (1 ng/ml) and ZAN (10 ng/ml) were freshly prepared in methanol prior to each run.

ROC was extracted with ethyl acetate and dichloromethane as solvents. Depending on the nature of the test samples, a different volume of ethyl acetate was added: 1 ml to agar plugs in 1.5-ml Eppendorf tubes, 2.5 ml to conidiospores in 15-ml centrifugation tubes and 3.5 ml to total fungal biomass in 50-ml centrifugation tubes. All samples were shaken on a rocking shaker for 15 min, protected from light. The ethyl acetate layer was removed and kept apart, followed by addition of an equal amount of dichloromethane to each sample. After 15 min of shaking on a rocking shaker (protected from light), the dichloromethane layer was combined with the ethyl acetate layer. This extract was filtered through a folded filter, wetted with 2 ml of

ethyl acetate. Subsequently, the filter was washed with 2 ml of ethyl acetate and 2 ml of dichloromethane.

The filtrate was evaporated to dryness in a water bath at 40°C under nitrogen, and redissolved in 200 µl of injection solvent (composition mentioned later) by vortexing during 1 min. The 200-µl extracts were filtered through centrifugal filter units (0.22 µm) by centrifugation at 14 000 rpm during 5 min and transferred to microvials. Microvials were stored at 4 °C prior to injection into the LC-MS/MS system.

LC-MS/MS analysis was performed with a Waters Acquity UPLC system coupled to a Micromass Quattro Premier XE triple-quadrupole mass spectrometer (Waters), equipped with Masslynx software for data processing. A 150 mm x 2.1 mm reverse-phase C_{18} column was used, with a 10 mm x 2.1 mm guard column of the same material (resp. 5 and 3.5 µm inner diameter, Waters). The column was kept at room temperature. The mobile phase consisted of variable mixtures of mobile phase A (water/methanol/acetic acid, 94/5/1 (v/v/v) and 5 mM ammonium acetate) and mobile phase B (methanol/water/acetic acid, 97/2/1 (v/v/v) and 5 mM ammonium acetate) at a flow rate of 0.3 ml/min with a gradient elution program, mentioned in Table A1.

Table A1. *Gradient elution program for roquefortine C determination.*

Time (min)	**% mobile phase A**	**% mobile phase B**
0-6	95	5
6-10	35	65
10-11	1	99
11-14	95	5
14-15	25	75
15-16	1	99
16-18	95	5
18-19	25	75
19-20	1	99
20-29	95	5

The injection solvent consisted of mobile phase A/mobile phase B (60/40, v/v) and 5 mM ammonium acetate. The injection volume of the samples on the analytical column was 20 µl.

The mass spectrometer was operated in the positive electrospray ionization (ESI+ mode). Capillary voltage was 3.2 kV. High-purity nitrogen was used as drying and ionization (ESI+) nebulizing gas, and argon was used as collision gas for collision-induced dissociation. Source and desolvation temperatures were set at 150 and 350 °C respectively. ROC was analyzed using selected reaction monitoring (SRM). The method was validated according to Commission Decision 2002/657/EC.

ROC eluted after approx. 7.8 min, while ZAN had a retention time of approx.9.2 min. In Masslynx, the peak areas for both mycotoxins was determined and the response factor was calculated for each sample. Based on the four spiked reference samples per run, a linear regression was fitted for quantification of the samples with unknown ROC content. The decision limit was 5 ng per ml, while quantification was possible from 10 ng per ml injection solution.

Quantification of ROC by LC-MS/MS comprised quantification of predominantly ROC, in combination with its stereo-isomer that can be formed under acidic, basic or photochemical conditions, and with roquefortine D (Richard et al 2007).

3.8. Chemical analyses

3.8.1. Dry matter content

The dry matter content of a sample is determined by air drying at 65 °C. The sample is placed in a perforated plastic bag along with an identification card, and the net fresh weight is noted. The samples are placed loosely on racks in a ventilated drier at 65 °C until constant weight. Immediately upon removal from the ventilated drier (to avoid re-uptake of moisture), the net dry weight from each sample is noted. If necessary, the samples are afterwards stored in cardboard boxes at ambient temperature until further analysis (*e.g.* mycotoxin analysis).

3.8.2. Determination of some fermentation characteristics

The pH of silage samples can be determined on fresh material or on defrosted material that was stored at -20 °C in sealed bags. An aqueous extract is prepared in an 250-ml Erlenmeyer: a net weight of approx. 20-25 grams of silage particles is brought into the Erlenmeyer and diluted with distilled water to the tenfold weight. All silage particles need to be submersed in the water. After sealing of the Erlenmeyer with parafilm, the Erlenmeyers are placed on a rotary shaker at 150 rpm during one hour at 20 °C, followed by 23 hours at 4 °C. After filtration of the suspension through a miracloth filter into a 250-ml cup, the pH of the aqueous extract is determined. The pH is determined at an air-conditioned room at 20 °C and is the device is calibrated with buffers at pH 7.00 and pH 4.00. Between measurements of different samples, the pH-electrode is rinsed thoroughly with distilled water and dried gently with a paper towel before each new measurement. After measuring the last sample and rinsing of the electrode, the electrode is submerged in potassium chloride solution (0.1 mol/liter) for storage.

The pH of culture media can be determined accordingly.

The following fermentation characteristics of silage samples are determined at CPAR (Centre Provincial de l'Agriculture et de la Ruralité – La Hulpe, Belgium) in an analysis pack:

- ammonia and ratio of ammonia nitrogen over total nitrogen: nitrogen and ammonia content are determined according to Kjeldahl (1983). Based on these results, the fraction of ammonia nitrogen over total nitrogen is calculated.
- pH: determination on an aqueous extract, as described earlier.
- fermentation acids: lactic acid, acetic acid and butyric acid are determined by HPLC according to Ohmomo *et al*. (1993).

3.9. Statistical analysis

Statistical analysis is performed with the SPSS Statistics 24 program. Significance is declared at 95%, with $p < 0.05$.

Per object, outliers are detected with box-plots and removed prior to further statistical analysis.

Per parameter, normality is checked by Shapiro-Wilk's test (applying Bonferroni correction) and homoscedasticity is checked with Levene's test. Depending on the number of fixed factors, a different strategy is applied:

- one factor:

If the normality as well as the equality of variances condition is met, a one-way Anova with Tukey as *post hoc* test is performed. Normally distributed parameters with unequal variances are subjected to Welch Anova with Dunnett T3 as *post hoc* test. Parameters that are not normally distributed are subjected to a non-parametric test according to Kruskal-Wallis with Dunn's test for pairwise comparisons (applying Bonferroni correction).

- two or more factors

A multiple Anova is performed to check if there are significant interactions between factors. If this is the case, an Anova analysis is performed for each level of one factor to assess the effect of the other factor(s). If variances are equal for normally distributed variables, Anova with Tukey as *post hoc* test is performed, otherwise a Welch Anova with Dunnett T3 as *post hoc* test is executed. Not normally distributed parameters are subjected to a non-parametric test according to Kruskal-Wallis with Dunn's test for pairwise comparisons (applying Bonferroni correction). When there are no significant interactions between factors and when variances are equal, the main effects of the factors are determined likewise over all levels of the other factor(s).

Annex 2

Curriculum vitae

Personal information

First name:	Eva
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E-mail:	eva.wambacq@ugent.be
Nationality:	Belgian
Place of birth:	Dendermonde
Date of birth:	August 30th, 1982

Educational record

2007: Three-day training program in morphological and molecular mould identification techniques, Mycology laboratory, Unit for Microbiology, Université Catholique de Louvain.

2005 – 2006: Postgraduate in Business Economy and Management, Hogeschool voor Wetenschap en Kunst, Brussels.

2000 – 2005: Master in Bioscience Engineering – cell and gene biotechnology, Universiteit Gent, Ghent.

Grade: Distinction

Master thesis: Antimicrobial compounds in mare's milk
promotor: prof. dr. ir. De Smet Stefaan

Professional record

2016 – present: Research associate at Hogeschool Gent / Universiteit Gent.

2009 – 2016: Research assistant at Hogeschool Gent / Universiteit Gent - PhD research in combination with teaching and contractual research.

2006 – 2009: Project engineer at Hogeschool Gent – PWO project "Identificatie en beheersing van schimmelontwikkeling in geconserveerde ruwvoeders".

Scientific output

Publications in peer-reviewed journals

Wambacq E., Audenaert K., Höfte M., De Saeger S. and Haesaert G. (2017) *Bacillus velezensis* as a biocontrol organism towards *Penicillium roqueforti s.l.* in silage: *in vitro* and *in vivo* evaluation. *Toxins* - special issue related to 1st Mycokey conference, in preparation.

Tangni E.K., Wambacq E., Bastiaanse H., Haesaert G., Pussemier L., De Poorter J., Foucart G. and Van Hove F. (2017) Survey of fungal diversity in silages supplied to cattle in Belgium over a two-year period. *Journal of Animal Science Advances* **7**: 1861-1873.

Wambacq E., Vanhoutte I., Audenaert K., De Gelder L. and Haesaert G. (2016) Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: a review. *Journal of the Science of Food and Agriculture* **96**: 2284-2302.

Wambacq E., Latré J. and Haesaert G. (2013) The effect of *Lactobacillus buchneri* inoculation on the aerobic stability and fermentation characteristics of alfalfa-ryegrass, red clover and maize silage. *Agricultural and Food Science* **22**: 127-136.

De Boever J., Dupon E., Wambacq E. and Latré J. (2013) The effect of a mixture of *Lactobacillus* strains on silage quality and nutritive value of grass harvested at four growth stages and ensiled for two periods. *Agricultural and Food Science* **22**: 115-126.

Participation to conferences and symposia

Wambacq E., Audenaert K., Höfte M., De Saeger S. and Haesaert G. (2017) *Bacillus subtilis* as a biocontrol organism towards *Penicillium roqueforti s.l.* in silage: *in vitro* and *in vivo* evaluation. **1st Mycokey Conference**, September 11-14, 2017, Ghent, Belgium. Oral presentation by E. Wambacq.

Sakarika M.*, Spanoghe J.*, Wambacq E.*, Gomes de Sousa G., Alloul A., Spiller M., Derycke V., Stragier L., Verstraete H., Fauconnier K., Grunert O., Haesaert G. and Vlaeminck S.E. (2017) Broadening the terrestrial application potential of MELiSSA phototrophs: feasibility as added-value organic fertilizer. **MELiSSA Mini-symposium** and masterclass 'Science and

Technology for Bioregenerative Life Support', 30 March 2017, Antwerp, Belgium. Poster presentation by J. Spanoghe, awarded with excellent poster prize (*equally contributed).

Croubels S. and De Saeger S. (2013) **35th Mycotoxin Workshop**, May 22-24, 2013, Ghent, Belgium. E. Wambacq as member of the organizing committee and attendant of conference.

Wambacq E., Latré J. and Haesaert G. (2012) Study of the effect of *Lactobacillus buchneri* inoculation on the aerobic stability and fermentation characteristics of alfalfa-ryegrass, red clover and maize silage. **16th International Silage Conference**, July 2-4, 2012, Hämeenlinna, Finland. Poster presentation by E. Wambacq.

Dupon E., Latré J., Wambacq E. and De Boever J. (2012) The effect of adding ferulate esterase producing *Lactobacillus* strains during ensiling on the quality of grass silage. **16th International Silage Conference**, July 2-4, 2012, Hämeenlinna, Finland. Poster presentation by E. Dupon.

Wambacq E., Latré J. and Haesaert G. (2011) Influence of elevated temperature and oxygen supply on the mycotoxin content of maize silage artificially infected with *Penicillium roqueforti s.s.* or *Penicillium paneum*. **4th International Symposium Mycotoxins**: Challenges and Perspectives, May 24, 2011, Ghent, Belgium. Poster presentation by E. Wambacq and member of the organizing committee.

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