CRANFIELD UNIVERSITY

MARIA GUTIÉRREZ POZO

IDENTIFICATION OF VOLATILE ORGANIC COMPOUNDS (VOCS) AS BIOMARKERS OF POTATO ROTS DURING COLD STORAGE AND ECOPHYSIOLOGICAL STUDY OF THE PATHOGENS INVOLVED

SCHOOL OF WATER, ENERGY and ENVIRONMENT
PhD/Full time

PhD in ENVIRONMENT and AGRIFOOD
Academic Year: 2017-2021

Supervisor: Ángel Medina Vaya
Associate Supervisor: Sofia Kourmpetli
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ABSTRACT

Potato tubers are stored for up to ten months. Therefore, controlling the conditions (temperature and relative humidity) in the storage environment is essential to reduce the risk of appearance of potato diseases. Fluctuations in the environmental conditions appear throughout the storage room, allowing the development of potato rots. This work aimed to identify Volatile Organic Compound (VOC) biomarkers of potato diseases to achieve an early identification of potato rots under commercial storage conditions. Furthermore, to achieve a deep understanding of the ecophysiology of the fungal and bacterial pathogens involved. Some of the objectives of this study were the isolation of fungal pathogens from potato tubers, the study of the mycotoxin production in the presence of dry rot, the development of a methodology for the VOC sampling and identification, and the study of the effect of storage time on the potato susceptibility to dry rot and soft rot.

A method for the untargeted detection and identification of VOCs produced by potatoes during storage was developed using Thermal Desorption-Gas Chromatography-Time of Flight- Mass Spectrometry. A range of different TD (Thermal desorption) tubes were evaluated, and the most suitable for the adsorption of VOCs under the study conditions were selected.

An *in vitro* study of the pathogens responsible for gangrene and dry rot was carried out using Natural Potato Dextrose Agar (PDA) and three different temperatures (5,10 and 15^{o}C) and water activities (a_w) (0.97, 0.98, 0.99). Higher temperature and a_w resulted in higher growth rate and lag time of both pathogens, *Fusarium* spp. and *Boeremia foveata.* Furthermore, mycotoxins, such as T-2, HT-2, diacetoxyscirpenol, beauvericin and neosolaniol, were detected in potato tubers infected with dry rot.

An *in vivo* study of dry rot and soft rot was carried out at 8.5^{o}C, using two cultivars with different susceptibility to these diseases (cvs. Record and Casablanca). The internal and external lesions resulting from the infection were assessed over time and the VOC fingerprint of each cultivar at each time point was determined. The

effect of storage time on the disease severity was also evaluated, demonstrating that at 8.5^{o}C, only dry rot's severity was affected by the longer storage time, while no effect was observed in soft rot.

Several VOCs were detected in higher abundance in presence of the pathogen in the *in vivo* and *in vitro* studies. They were selected as potential biomarkers of dry rot, soft rot and gangrene. Ethanol, acetone, ethyl acetate and acetic acid were detected *in vitro* as potential indicators of the presence of a fungal or bacterial disease. Furthermore, 2-methylpropan-1-ol was selected as a potential indicator of the presence of a fungal pathogen, such as *Fusarium* spp., responsible for dry rot in potato tubers. *In vivo*, 1-methoxy-3-methylbutane, 2-butanone, dimethyl disulfide and hydrogen cyanide were detected as potential biomarkers of dry rot or soft rot in potato tubers.

Lastly, based on the findings that have been achieved in this work, future research should be focused on the study of the growth and VOC production of *Pectobacterium carotovorum* spp. *in vitro* and the study of the disease severity and VOC production of gangrene *in vivo.* Furthermore, a validation of the VOC results achieved in this work under commercial storage conditions should be carried out. These studies will confirm and enhance the findings achieved in this work.

Keywords:

Gangrene, dry rot, soft rot, TD-GC-ToF-MS, *Fusarium sambucinum*, *Pectobacterium carotovorum atrosepticum, Fusarium oxysporum, Boeremia foveata*, potato storage, mycotoxins, disease severity

ACKNOWLEDGEMENTS

First of all, I would like to express gratitude to my supervisors, Dr Ángel Medina and Dr Sofia Kourmpetli, for the opportunity, guidance and support during my journey. I am thankful to my co-supervisor, Prof. Leon Terry for the expertise and advice provided.

To all Applied Mycology Group and Plant Science Laboratory Group, technicians and colleges from the lab, thank you all for the support and good time that we have spent in the lab. Thank you, Prof. Naresh Magan for all the support and expertise provided. I would like to thank the senior PhD students (already doctors) Alessandra, Alicia and Demi for their mentoring and support during the first year of my PhD. Thank you Senita, for all the support during this journey.

I am very grateful to Dr Carol Verheecke, not only for the support and expertise provided but also for her friendship. Thank you for allowing me to enjoy one of the most exciting experiences I have ever had, travelling to Algeria, get to know the culture and how they store potatoes. Thank you to everyone from ENS Kouba (Algiers, Algeria), who made me feel at home.

All of this would not have been possible without the little family we have created here at Cranfield. Thank you, Esther, Fernando and the little ladies, you made my journey easier. I cannot thank you more for all the support and love you have given me. Ángela (soon Dr. Ángela), you have been one of my main support during this journey, we started as office mates and we have finished as best friends. Thank you for supporting me in the good and bad moments, thank you for all the lab breaks we spent talking and talking.

I am grateful to Belén Patiño, Jéssica Gil, Covadonga Vázquez and Marta Garcia (Genetics, Physiology and Microbiology Department, Universidad Complutense, Madrid) for the opportunity you gave me to spend two weeks in your laboratory and learn about molecular identification techniques. It has been a pleasure to work with you and come back to the University where I did my master. Thank you, Marta, for letting me be your housemate for those two weeks and for all the support provided.

I would like to thank my partner, family and friends for unconditional love and encouragement during all of these years away from home.

Finally, it has been a pleasure to be part of the Interreg NWE “Real-time interactive storage quality control in fresh agro products (QCAP)” project. I have enjoyed every single meeting we had with the different partners.

TABLE OF CONTENTS

LIST OF FIGURES

differences between treatments at each specific day and stage (Record and Casablanca) (Wilcoxon test, p-values<0.03).

LIST OF TABLES

LIST OF APPENDICES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

μ_m	Growth rate
15-AS	15-Acetoxyscirpenol
ABE	Acetone-Butanol-Ethanol
Acetyl CoA	Acetyl Coenzyme A
AMDIS	Automated Mass Spectral Deconvolution and Identification System
ANOVA	Analysis of Variance
a_w	Water activity
CECT	Spanish Collection of Type Culture
cv.	Cultivar
cvs.	Cultivars
DAS	Diacetoxyscirpenol
dH_2O	Deionized water
DMDS	Dimethyl disulfide
DMS	Dimethyl sulfide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
e-nose	Electronic nose
FAIMS	Field Asymmetric Ion Mobility Spectrometry
FC	Fold Change
FID	Flame Ionization Detection
FISH	Fluorescence In Situ Hybridization
FT-IR	Fourier Transform Infrared microscopy
GC	Gas Chromatography
HCN	Hydrogen cyanide
HS	Headspace
ISO 1	Isolate 1
ISO 2	Isolate 2
ISO 3	Isolate 3
ISO 4	Isolate 4
ISO 5	Isolate 5
ISO 6	Isolate 6

LAMP	Loop-Mediated Isothermal amplification
LC	Liquid Chromatography
LFIA	Lateral Flow Immunoassay
LOM	Liquid Onion Medium
LPGA	*Levure* Peptone Glucose Agar
LTS	Low-Temperature Sweetening
MS	Mass Spectrometry
NB	Nutrient Broth
NIST	National Institute of Standard and Technology
NPDA	Natural Potato Dextrose Agar
OMA	Oat Meal Agar
PCA	Principal Component Analysis
PCA	*Pectobacterium carotovorum atrosepticum*
PCC	*Pectobacterium carotovorum carotovorum*
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PGPR	Plant Growth-Promoting Rhizobacteria
PR	Pathogenesis Related
PSA	Potato Sucrose Agar
PTR-MS	Proton-Transfer-Reaction Mass Spectrometry
QS	Quorum sensing
qTRAP	Quadrupolo Ion trap
RH	Relative Humidity
ROS	Reactive Oxygen Species
SPME	Solid-Phase Microextraction
TD	Thermal Desorption
TD-GC-ToF-MS	Thermal Desorption-Gas Chromatography-Time of Flight-Mass Spectrometry
ToF	Time of Flight
TTVP	Total Targeted Volatile Production
TSA	Trypticase Soy Agar
TZA	TZ- Agar
UHPLC	Ultrahigh Performance Liquid Chromatography
UK	United Kingdom

UV	Ultraviolet Light
VOCs	Volatile Organic Compounds

1 INTRODUCTION

Potato is one of the world's main food crops after maize, rice and wheat, which feeds a large part of the global population every year. Potato cultivars are grown in more than 100 countries, where Asia and Europe are considered the leading producers. In 2019 around 105 million tonnes of potatoes were produced in Europe. During the last ten years, potato production and its area harvested have fluctuated. Despite the reduction of the harvested area, the potato production was maintained. This was probably due, among others factors, to the improvement of potato diseases control management (Dehnen-Schmutz *et al.*, 2010; Fiers *et al.*, 2012, FAOSTAT, 2019).

The potato crop can be divided into three different categories depending on the destination of potato tubers; processed, fresh and seed potatoes. The potato crop is also classified based on their harvested time; first early, second early and maincrop. First early potatoes are planted in late March and harvested between June and July. Second early are planted in early to mid-April and harvested in July and August. While maincrop potatoes are planted from mid-to-late April and harvested from late August through October (Ivins and Bremner, 1965). There are around 80 different potato cultivars. In Great Britain, Maris Piper, Markies and Maris Peer were the main potato cultivars harvested in 2019. Maris Piper and Markies are maincrop, while Maris Peer is a second early potato cultivar.

Half of the potato crop is sold after harvest for immediate consumption, while the rest is stored at cold temperatures for up to 10 months. Potato tubers designated to the fresh and seed market are stored at temperatures below 4^{o}C, while for the processed market higher temperatures are needed (6-10^{o}C). A condition called Low-Temperature Sweetening (LTS) can occur when potato tubers are stored at temperatures below 4^{o}C. The LTS can result in undesirable darkened chips and fries for the processing market. However, lower temperatures reduce the chance of survival of potato pathogens, reduce the weight loss of tubers, and better control sprouting (Pinhero and Yada, 2016). A perfect skin finish is an essential requirement for the fresh market. A high relative humidity (RH) of 98% and controlled air ventilation is maintained during cold storage of potato tubers.

Storage is a dynamic environment due to potato tubers respiration and their interactions with the environment. A well-sealed and insulated potato storage must allow the crop to be kept at a constant temperature and unaffected by diurnal variation and weather conditions. The use of controlled ventilation is essential. Fans are positioned to create a flow of air through the crop or form a rolling mass of air, where the airflow should be as uniform as possible through the storage. The optimal environmental conditions management on storage is crucial to reduce the appearance of fungal and bacterial pathogens on the skin and flesh of potato tubers (Cargill, 1976; Cunnington, 2008; Cunnington and Pringle, 2012; AHDB Potatoes, 2019).

Potato storage diseases are a considerable threat to the potato market. Blemish control is a critical step, where potato tubers are required to present a skin finish without any kind of blemish or lesion. Any pathogen lesion present on the potato surface will result in rejection (Boyd, 1972; Cunnington, 2008). The loss estimated due to the presence of pathogens in potato tubers in the UK in 1999 was around 15%, corresponding to £95m annually (Bradshaw, Turner and Elcock, 2001). The development of plant protection policies played an important role in reducing the large generation of waste and their consequential economic losses (Dehnen-Schmutz *et al.*, 2010). In the UK, due to the long period of time that potato tubers spend in storage, one of the main causes of fresh potato waste in the retail supply chain is storage, around 3-5% of the potato crop is lost every year (Pritchard *et al.*, 2012). The early detection of potato storage diseases could reduce the large generation of waste and avoid the consequential economical losses

This review is focused on the different causal pathogens of diseases on potato tubers during storage, the environmental factors that affect their presence and the different methodologies developed to date for the early detection of potato storage diseases.

1. 1 Potato storage diseases

Potato storage diseases are generated by pathogens that can quickly adapt to different environmental conditions, such as low temperatures and relative humidity. The majority of pathogens that affect potatoes are from the fungi domain. These diseases are not considered as exclusively storage diseases, as they present two different stages, in the soil and the storage. The source of inoculum can be either soil or seed-borne. They also affect the potato plant and the potato progeny differently in the field. Many diseases presented in the soil stage can survive during the harvest process as a latent infection. Once potato tubers are stored at the optimal conditions, the development of the pathogen starts (Cunnington and Pringle, 2012).

The UK's main potato diseases are described in Table 1-1; six fungal diseases (silver scurf, black dot, skin spot, gangrene, dry rot and late blight) and three bacterial diseases (soft rot, brown rot and ring rot). Each disease is caused by a different pathogen that produces different symptoms on the surface and the interior of the potato tuber. Potato storage diseases can be classified based on how the pathogen is affecting the tuber. They are divided into: galls, blemishes, and rots; galls are based on outgrowth and tuber deformation; blemishes only affect the skin, and they appear as spots; and rots, where the tuber flesh is affected more deeply (Fiers *et al.*, 2012). Furthermore, they can also be classified based on their requirements to penetrate intact potato tubers or through their wounds, as it is shown in Table 1-1. Each pathogen will penetrate and infect tubers following different mechanisms. Some pathogens, such as *Helminthosporium solani* and *Colletotrichum coccodes*, can penetrate directly through the surface while the rest need to penetrate through natural openings (lenticels, stomata or eyes) or wounds produced by a lesion on the tuber during harvest, cooling or the storage process (Moore, 1924; Bain, Lennard and Wastie, 1982; Tweddell, Boulanger and Arul, 2003b; Al-Mughrabi, 2010).

Table 1-1 Fungal and bacterial rots affecting potato tubers during storage. The disease, pathogen involved, main symptoms in potato tubers, inoculum source, pathogen penetration and infection mechanism, temperature effect, relative humidity effect and other environmental factors effect are included per potato disease.

Fungal rots										
Disease	**Pathogen**	**Main symptoms in potato tubers**		**Disease cycle**			**Environmental factors**		**Other factors**	**References**
		External	**Internal**	**Inoculum source**	**Pathogen penetration**	**Pathogen infection**	**Temperature**	**Relative humidity**		
Silver scurf	*Helminthosporium solani*	Silvery and light brown lesions (1)	Weight loss due to the reduction of skin permeability (2)	Tuber-borne and soil-borne (1,3)	Lenticels or directly through periderm cells (4)	In soil a primary infection takes place, infected potato seed tubers spread the inoculum to the progeny (5,6,7) In storage, a secondary infection occurs, conidia produced during storage is transmitted through the air (1, 3)	Min T^{o}: 4^{o}C (5) Silver scurf severity increases with temperature (3, 8)	Optimal: 95-100% High RH (95-100%) increases silver scurf development with an increase in temperature. (5,8) High RH is needed for *H. solani* sporulation (5)	Condensation: Long period of condensation increases *H. solani* sporulation. Different positions of the storage room present different condensation levels (10) Planting density: A very high planting density can increase the spread of silver scurf (9) Harvest time: Silver scurf is more severe when it is harvested at late dates compared to early dates (9)	**1.** (Errampalli, Saunders and Holley, 2001) **2** (Read and Hide, 1984) **3** (Rodriguez *et al.*, 1996) **4** (Boyd, 1972) **5** (Lennard, 1980) **6**. (Firman and Allen, 1995b) **7** (Inglis and Gundersen, 2019) **8** (Hilton *et al.*, 2000) **9** (Firman and Allen, 1995a) **10** (Hardy, Burgess and Pringle, 1997)
Black dot	*Colletotrichum coccodes*	Brown-grey and silvery lesions with a poorly defined margin. Black microsclerotia are also produced (1, 2,3,4)	Alteration of the periderm due to shrivel and weight loss (4)	Tuber and soil-borne (5, 6)	Stolon or directly through periderm cells (2,7,8,9)	In soil, sclerotia from *C. coccodes* penetrate the host tissue for the infection and colonisation (8) While in storage, sclerotia presented on the surface germinates inward into the tuber (2,7) *C. coccodes* secretes a cell-wall degrading enzyme, pectate lyase (PL) (14)	*C. coccodes* germination: -Min T^{o}: 5-7^{o}C -Optimal T^{o}: 22^{o}C (9,10,11) Black dot development: Higher incidence and symptom extension at 15^{o}C compared to 5^{o}C. Once infected, if tubers are stored at temperatures below 1^{o}C deeper necrotic lesions appear (5,10, 12)	Soil water level: At higher soil water level an increase of the number of sclerotia produced by *C. coccodes* is detected (13)	Harvest time: Black dot severity increases through the season (5) Optimal pH: 6-7 (11)	**1**. (Jellis & Taylor, 1994) **2.** (A K Lees and Hilton, 2003) **3.** (Dillard, 1992) **4.** (Hunger and McIntyre, 1979) **5.** (Read and Hide, 1988) **6.** (Nitzan, Cummings and Johnson, 2008) **7.** (Tsror, Aharon and Erlich, 1999) **8.** (Wang and Higgins, 2005) **9.** (Dillard, 1988) **10.** (Glais-Varlet, Bouchek-Mechiche and Andrivon, 2004) **11.** (Nitzan and Tsror, 2003) **12.** (Mooi, 1959) **13.** (Cummings and Johnson, 2014)

Skin spot	*Polyscytalum pustulans*	Small black or purplish pustules, occurring singly or aggregated in groups. It mainly develops on the eyes (1,2,3,4)	In most of the cases there is no internal effect (3,4)	Soil-borne (4)	Lenticels, eyes and sprouts (4,5)	In soil, the pathogen infection occurs first through the eyes on tubers. Then in storage, the infection continues through the eyes and its development will depend on the environmental conditions. A potential spread of *P. pustulans* can occur in storage through the air (6)	*P. pustulans* optimal growth T°: 8-13°C (8) Higher lesions are produced at lower temperatures (3-4°C) compared with higher temperatures (10-15°C) (7,8)	Optimal RH: 95-100% (5,8) The infection of *P. pustulans* is favoured by humid conditions (95-100%) (5)	Tuber maturity: Tubers nearing maturity are most susceptible to skin spot (2,5)	**1.** (Owen, 1919) **2.** (Boyd, 1957) **3.** (Greeves and Muskett, 1939) **4.** (Graham and Hamilton, 1970) **5.** (Allen, 1957) **6.** (Boyd, 1972) **7**. (Lennard, 1967) **8.** (Hide and Adams, 1980)
Dry rot	*Fusarium* spp. (*F. solani coeruleum, F. oxysporum, F. sambucinum, F. avenaceum, F. sulphurous, F. oxysporum, F. equiseti, F. culmorum)*	Common symptoms: Wrinkling of the skin around the infection. Sometimes white patches and pustules appear in the wounds (1) *F. s. coeruleum* Dark brown without clearly defined edge lesions. Sporodochial pustules are frequently formed on the surface (2)	Brown staining, appearing in the vascular bundles and spreading into the flesh. The flesh begins to dry and shrink (1)	Soil- and seed-borne pathogen (3,4,5,6,7,8	Wounds or sections of the potato tuber where the skin is ruptured. How the pathogen penetrates through the wounds is slightly different between *Fusarium* spp. (2,9,10)	The main source of inoculum are seed tubers. After planting contaminated seed tubers, it results in soil infestation around the progeny tubers. Spores of *Fusarium* spp. keep attached to the surface of the potato tuber and it is not until a wound appears, after harvest, that allow the development of dry rot in storage when the environmental conditions are optimal (4, 8,7,10,11). Its propagation in storage has been discussed (12).	Its susceptibility increases with an increase of the storage T° (14). Low-temperature storage (4°C) before wounding reduces dry rot susceptibility (2, 13). *F.s. coeruleum* Optimal T° : 15-25°C (2, 10, 14)	Dry rot infection increases with an increase in wetness duration (15)	Pathogen effect: Each *Fusarium* spp. produces differences in their severity of the rots (6,8, 16, 17) Time between damage and infection (tuber maturity): Resistance to infection decrease as the storage season advance. The more time potato tubers spend between wounding and infection with *Fusarium* spp., the lowest would be their dry rot (18,19) Light effect: The exposure of potato tubers to light before the infection limits the fungal invasion of *F. sulphureum* and *F. s. coeruleum* (20)	**1**. (Longman, 1909) **2**. (Moore, 1924) **3**. (Jellis, 1975) **4.** (Secor and Gudmestad, 1999) **5.** (Nielsen and Johnson, 1972) **6**. (Adams and Griffith, 1983) **7**. (Leach, 1985) **8**. (Choiseul, Allen and Carnegie, 2001) **9**. (Pethybridge, 1917) **10.** (Mckee, 1954) **11.** (R. D. Peters *et al.*, 2008) **12.** (Manici and Cerato, 1994) **13.** (Boyd, 1952) **14**.(Theron and Holz, 1990) **15**. (Lui and Kushalappa, 2002) **16.** (Cullen *et al.*, 2005) **17.** (Heltoft *et al.*, 2015) **18.** (Heltoft *et al.*, 2016) **19**. (Mckee, 1954) **20.** (Percival, Karim and Dixon, 1998)

Gangrene	*Phoma exigua foveata* /*Boeremia foveata*	Black circular thumb mark type lesions and irregularly shaped depressions on the surface. At optimal conditions pycnidia can develop on the surface (1)	Invaded cavities often lined with dark brown or deep purplish mycelia. The infected tissue is black and well defined from the healthy area (1)	It is a seed-borne and soil-borne disease. (2,3,4, 5). Gangrene has also been considered as an air-borne disease (6,7)	Wounds (1,8,9)	As a soil-borne disease, potato tubers, damaged after harvest and handling, might present infested soil on their surface or the fungi is already in the periderm. Once in storage, the environmental conditions are optimal for *B. foveata* development in potato tubers (1) As tuber-borne disease, seed tubers gangrene-infected can develop disease on stems, when they grow they provide a source of pycnidiospores that can infect their progeny (8)	Optimal T^{o}: 5-7^{o}C This is the optimal T^{o} for the development of gangrene, because they are unfavourable to the wound-healing process (2) Low temperatures (5^{o}C) after wounding increase the incidence of gangrene and the size of the rots (2, 9, 10, 11, 12, 13).	Low humidity (60-70%) inhibits wound-healing, therefore increases gangrene incidence (14)	Potato stored in bags: The incidence of wounds infected with *B. foveata* increases inside a bag where one tuber was gangrene-infected (15, 16) Potato machinery: The incidence of gangrene increases if potato tubers are lifted using an elevator digger, instead of manually, in presence of one gangrene-infected tuber (15) Tuber maturity: The incidence and susceptibility to gangrene increase with the maturity of tubers (2)	**1.** (Malcomsom and Gray, 1968) **2.** (Malcolmson, 1958) **3.** (Boyd, 1972) **4.** (Adams, 1980) **5.** (Carnegie and Cameron, 1991) **6.** (Carnegie, 1980) **7.** (Carnegie, 1984) **8.** (Logan, 1974) **9.** (Adams and Griffith, 1978) **10.** (Hide, 1981) **11.** (Wigginton, 1974) **12.** (Adams and Griffith, 1983) **13.** (Gray and Paterson, 1971) **14.** (Croke and Logan, 1982) **15.** (Copeland, 1982) **16.** (Malcolmson and Gray, 1968b)
Late blight	*Phytophthora infestans*	Brown irregular lesions that tend to become sunken during storage (1)	Dry rusty-brown lesions that do not always penetrate beyond the vascular ring (1)	Soil-borne disease (2)	Eyes, lenticels and small wounds, but not through intact skin (1)	It presents two phases, in the haulm and on potato tubers. Tuber infection occurs when spores (sporangia) produced on the infected haulm are washed down by rainwater through the soil. The disease continues to develop in storage, but it does not spread from tuber to tuber (1,4,5,6)	Low temperatures are considered an important factor for the maintenance of latent infections of late blight in seed potatoes (7, 8,9, 10) Optimal T^{o} *in vitro:* 20^{o}C (11)	Sporangia of *P. infestans* survives to RH below 20% (12)	Tuber maturity: Resistance to late blight increases with maturity (3, 11, 13) Potato progeny location: Tuber infection significantly decreases with increasing soil depth (14). Time duration of optimal conditions: The period available for successful tuber infection is determined by the time where the soil is at the optimal temperature for the maintenance of viability of sporangia and precipitation (9)	**1.** (Boyd, 1972) **2.** (Andersson, Sandström and Strömberg, 1998) **3.** (Darsow, 2004) **4.** (Murphy, 1921) **5**. (Lacey, 1967a) **6.** (Lacey, 1967b) **7.** (Johnson and Cummings, 2009) **8.** (Crosier and Reddick, 1935) **9.** (Nyankanga *et al.*, 2007) **10.** (Kirk, Niemira and Stein, 2001) **11.** (Zan, 1962) **12.** (Sunseri, Johnson and Dasgupta, 2002) **13.** (Stewart, McCalmont and Wastie, 1983) **14.** (Porter, Dasgupta and Johnson, 2005)

Bacterial rots										
Soft rot	*Pectobacterium* spp. (*P. carotovorum carotovorum, P. carotovorum atrosepticum*)	Sunken, brownish-black, dry, necrotic lesions encircling lenticels (1)	The affected parenchyma below the epidermis is disorganised with the formation of small cavities. Tuber tissue is macerated to a creamy consistency which turns black in the presence of air (1,2)	Seed-borne disease (1,3)	Wounds and lenticels (4,5) Once the bacteria has penetrated, it is confined to the intercellular spaces (6,7)	Seed tubers already infected are transferred into stores. Once in storage, the initiation of the potato tuber infection only occurs if anaerobic conditions prevail. There is free water covering the potato surface and the temperature is above the minimum required for soft rot development (8,9). *Pectobacterium spp.* infection consists of its multiplication in the area and the production of numerous extracellular enzymes (10) The bacteria can survive in a quiescent form in tubers though all the storage period until planting time (3)	High T° (20-25°C) favour the multiplication of the bacteria (11,12,13, 14). There are differences between *Pectobacterium* spp. *P.c.a* is considered as a low-temperature strain (15) *P.c.a* optimal growth: 24°C *P.c.c* optimal growth: 28°C Optimal PEL (Pectate liase) activity: 12-15°C (10)	High relative humidity promotes bacterial multiplication in lenticels (1, 6, 11, 16)	Pre-packing procedures: There is an increase in the development of soft rots on potato tubers that have been stored in polyethene bags. Soft rot incidence is higher when potato tubers were just washed after harvested and bagged immediately (17) Manual or mechanical harvest: Tubers that are harvested by hand show a low decay due to soft rot compared to those mechanically harvested (20)	**1.** (Logan, 1964) **2.** (Czajkowski *et al.*, 2011) **3.** (Pérombelon, 2000) **4.** (Boyd, 1972) **5.** (Pérombelon and Lowe, 1975) **6.** (Fox, Manners and Myers, 1971) **7.** (Fox, Manners and Myers, 1972) **8.** (Pérombelon, 1972) **9.** (Perombelon and Kelman, 1980) **10.** (Smadja *et al.*, 2004) **11.** (Jones and Dowson, 1950) **12.** (De Boer and Kelman, 1978) **13.** (Moh *et al.*, 2011) **14.** (Lebecka, Flis and Murawska, 2018) **15.** (Graham and Dowson, 1960) **16.** (Cromarty and Easton, 1973) **17.** (Scholey, Marshall and Whitbread, 1968) **18.** (Lund and Nicholls, 1970; Lund and Wyatt, 1972) **19.** (Nielsen, 1968) **20.** (Lund and Kelman, 1977)

Brown rot	*Pseudomonas solanacearum /Ralstonia solanacearum* (Biotype 2)	Browning lesions around the vascular ring, vascular discolouration and excretion of bacteria slime. These symptoms occurs at late stages of the development of the disease (1,2,3)	No internal effects have been described	Soil-borne disease (3)	Mainly through wounds (1)	When contaminated water is used to irrigate the potato crop, it will result in a contamination of the soil and the crops growing on it. The bacterium enters through the roots (4,5). Once the bacteria reach the tubers, they can remain on the surface while they are stored. Potato tubers will remain symptomless and can even persist until the next generation (4,6)	The maximal survival of *R. solanacearum in vitro* is 12- 28ºC (7) Optimal Tº *in vitro* 25-37ºC (1)	Its development is favoured by high soil moisture levels (1)		**1.** (Rich, 1983) **2.** (Wullings *et al.*, 1998) **3.** (Tsror, Aharon and Erlich, 1999) **4.** (Stead, 1999) **5.** (Müller and Parusel, 1998) **6.** (Ciampi and Sequeira, 1980) **7.** (van Elsas *et al.*, 2001)
Ring rot	*Corynebacterium sepedonicum / Clavibacter michiganensis sepedonicus*	Ragged cracks on the surface. In those tubers where the stem was removed, the vascular ring may exhibit a yellowish discolouration (1,2)	Yellow or light brown creamy rot (1)	Seed-borne disease (1,3)	Stolon and wounds (1,2)	Infected seed tubers symptomless are planted in the soil. Those tubers will contaminate equipment involved in the handling of the potato crop (1, 3) Once potato tubers come in contact with the contaminated machinery they will become infected. In storage, ring rot bacteria can be spread by direct contact. The disease can keep more than a single generation of potato crops (4,5)	Soil temperature: The highest incidence has been detected between 14-18ºC (6) Optimal Tº for the development of ring rot: 18-22ºC (6,7)		Storage machinery: A good maintenance of the storage facility is a key control (4, 8) Survival of *Cms* in water: Cms can survive for 7 days in non-sterile surface water at low temperatures (10ºC) (9)	**1.** (Racicot, Savile and Conners, 1938) **2.** (van Elsas *et al.*, 2001) **3.** (Stead, 1999) **4.** (Nelson, 1978) **5.** (Kakau *et al.*, 2004) **6.** (Sherf, 1944) **7.** (Logsdon, 1967) **8.** (Nelson, 1980) **9**. (van der Wolf and van Beckhoven, 2004)

1.1. 1 Potato disease cycle

The potato disease cycle presents two different stages in most of the cases, one that takes place in the soil and the other during storage. Some of these pathogens, such as *Helminthosporium solani,* also infect the potato plant, infecting the progeny and finally transferring the disease to the storage. Depending on the pathogen, the infection of the potato plant in the soil, during potato handling or in storage will be different, as presented in Table 1-1. Besides, the development of a disease during storage is affected by different factors, such as the initial concentration of the pathogen, temperature and relative humidity of tubers. Once the combination of these factors, the natural resistance of the potato cultivar and the pathogen penetration requirements are suitable for pathogen growth, the infection starts (Cunnington and Pringle, 2012).

Each cultivar of potato presents a degree of susceptibility to different diseases. Potato tubers have a periderm and a narrow parenchyma cortex separating the periderm from the vascular ring. The periderm is considered a protective layer that prevents water loss of the tuber and provides the first barrier against pathogens (Peterson, Barker and Howarth, 1985). The thickness of this layer affects the susceptibility of the specific potato cultivar to the different diseases (Lulai, 2001).

1.1. 2 Pathogenicity of potato diseases

Enzymes are involved in pathogenicity, fungi and bacteria-causing rots produce a wide range of hydrolytic enzymes such as cellulases, pectinases, xylanases and proteases. They are responsible for tissue maceration and cell death, allowing the pathogen to access the nutritional resources of the plant tissues. *Pectobacterium* spp., responsible for soft rot in potato tubers, present an unconventional infection strategy based on *quorum sensing* (QS), using chemical signal molecules that allow the bacteria to synchronize through the production of virulence factors and make the pathogenic attack more efficient (Liu *et al.*, 2008).

Potato tubers present a robust immune system to defend themselves from the different pathogens. There are different general defence mechanisms, as the production of salicylic acid, jasmonic acid, Ca^{2+} and reactive oxygen species (ROS). There are unique responses in presence of a particular pathogen, in most of the cases related to the pathogenesis-related (PR) proteins. PR proteins are low molecular weight proteins with antimicrobial activities induced in presence of particular pathogens (Després *et al.*, 1995; Staskawicz *et al.*, 1995; Halim *et al.*, 2009; Tiwari *et al.*, 2020).

1.1. 3 Introduction of potato diseases in Great Britain

One of the first potato diseases reviews in the UK was in 1914 where 9 different diseases were reported, they were attributed to the potato tubers growing conditions (Horne, 1914). In the 40's losses due to the presence of skin spot, a blemish disease, was considered between 20 and 30% of the total production of seed potatoes (Greeves and Muskett, 1939). Later in the '80s another blemish disease, silver scurf, was considered a persistent disease detected in seed potato tubers (Errampalli, Saunders and Holley, 2001). From the '90s there was a large increase of blemish diseases (silver scurf, black spot, skin spot, black dot) due to the rise in demand of the fresh potato market, where potato tubers are required to have a high-quality appearance (Lees and Hilton, 2003). Considering those diseases that need a previous wound to infect the potato tuber, gangrene was considered with soft rot and dry rot as the most important potato storage disease in the UK from the '50s (McKee, 1952; Malcolmson and Gray, 1968; Boyd, 1972; Ebbels, 1983). The increase of soft rot was due to the introduction of bulk storage in 1972 (Pérombelon, 1972). Black dot, a blemish disease of potato tuber was first reported in the UK in the early '90s. However, until 2003 it was not considered as an economically significant disease in the UK (Lees and Hilton, 2003; Cunnington, 2008). The two final bacterial diseases presented in Table 1-1, brown rot and ring rot, were introduced in the UK later in 1992 and 2003. Both diseases are more common in tropical and subtropical regions due to their growth conditions than in colder regions(Müller and Parusel, 1998; Jones and Baker, 2007). Nowadays, ring rot must be eradicated if it appears under current

quarantine legislation (Jones and Baker, 2007). Therefore, at present, three main potato diseases are affecting potato storage in the UK; two fungal rots (dry rot, gangrene) and a bacterial rot (soft rot) (Dehnen-Schmutz *et al.*, 2010).

The three primary diseases that have just been mentioned, are wound diseases which responsible pathogens are able to infect the potato tuber only through wounds.

1.1.3. 1 Gangrene (*Boeremia foveata*)

Boeremia foveata, responsible for gangrene, is a fungal pathogen able to grow at low temperature, with an optimal temperature between 5 and 7ºC, similar temperature as in a potato storage facility. Potato tubers during storage present the optimal conditions for the growth of *Boeremia foveata.* However, the potato tuber needs to present a wound on the surface prior to the infection (Malcolmson, 1958). There is no recent data regarding *Boeremia foveata* in potato tubers, most of the literature dates from the 60s to the 90s.

1.1.3. 2 Dry rot (*Fusarium* spp.)

Different *Fusarium* spp. are responsible for dry rot in potato tubers. An extensive range of research has studied the susceptibility of different potato cultivars to dry rot. Different species of *Fusarium* are responsible for dry rot in potato*.* In the UK and Europe, the most common species are *Fusarium solani* var. *coeruleum*, *Fusarium sulphureum* and *Fusarium sambucinum.* In 2008, *Fusarium s. coeruleum* was considered the most prevalent pathogen responsible for dry rot in the UK (Choiseul, Allen and Carnegie, 2006; Peters *et al.*, 2008; Heltoft *et al.*, 2015). The optimal temperature for the development of dry rot is between 15 and 25ºC. The effect of temperature and relative humidity on its development has already been studied. An increase in the potato tuber susceptibility was detected with the increase of temperature and relative humidity (Moore, 1924; Mckee, 1954; Theron and Holz, 1990; Lui and Kushalappa, 2002). However, there was not enough information about the development of the fungus at low temperatures.

Some species of *Fusarium* can produce mycotoxins, a toxic secondary metabolite produced by fungi. These fungi can produce different types of trichothecenes (mycotoxins) with a high toxicity level for humans and animals (El-Banna, Scott and Lau, 1984). These mycotoxins have harmful consequences for the consumers, including immunosuppressive effects on humans and animals, due to their multiple inhibitory effects on eukaryotic cells (protein inhibition, DNA, and RNA synthesis, mitochondrial function, and interference in cell division and membrane function). Trichothecenes consumption can cause skin and gastrointestinal irritation, necrosis, haematological disorders, diarrhoea, vomiting and feed refusal, decrease of body weight, damage of the hematopoietic systems and even immunological alterations (Xue *et al.*, 2013). The production of trichothecenes in potato tubers infected with *Fusarium* spp. have already been studied (El-Banna, Scott and Lau, 1984; Ellner, 2002).

1.1.3. 3 Soft rot (*Pectobacterium carotovorum* spp.)

Soft rot is a potato disease produced by *Pectobacterium* spp. There are two main subspecies responsible for soft rot, *Pectobacterium carotovorum carotovorum* and *Pectobacterium carotovorum atrosepticum* (Boyd, 1972). Their optimal growth conditions varies between them, as it presented in Table 1-1, the optimal temperature for *P. carotovorum atrosepticum* is 25^{o}C while for *P.carotovorum carotovorum* is slightly higher, 28^{o}C (Graham and Dowson, 1960). The effect of high temperature and relative humidity has been studied, and it favours the multiplication of the bacteria (Jones and Dowson, 1950; De Boer, 2002; Moh *et al.*, 2011; Lebecka, Flis and Murawska, 2018).

There is a crucial requirement for the development of soft rot in potato tubers, the prevalence of anaerobic conditions (De Boer, 2002; Smadja *et al.*, 2004). Several studies have demonstrated the effect of low levels of O_2 and high levels of CO_2 on the growth of *Pectobacterium* spp. (Nielsen, 1968; Lund and Nicholls, 1970; Lund and Wyatt, 1972). Despite this, no data is available on the development of soft rot at low temperatures, those presented in storage conditions.

1.1. 4 Disease control

There has been an extensive range of studies where field applications of different fungicides, such as Thiabendazole, have been tested against these potato diseases to control the presence of the fungal or bacterial pathogens in the field and avoid their transfer to the storage (Carnegie *et al.*, 1988; Hilton *et al.*, 2000). However, it has been reported that many of the strains responsible for different diseases, such as dry rot or skin spot are already resistant to Thiabendazole (Hide and Ibrahim, 1994; Secor and Gudmestad, 1999; Choiseul, Allen and Carnegie, 2007; Carnegie, Cameron and Haddon, 2008). Different strategies for the control of potato diseases have been studied; most of them based on bacteria with antifungal activity against some of the fungal pathogens responsible for potato rots. As an example, many different *Pseudomonas* spp. have shown antifungal activity against *Helminthosporium solani* and *Fusarium* spp. (De la Cruz, Poplawsky and Wiese, 1992; Elson, Schisler and Bothast, 1997; Schisler *et al.*, 2000; Sadfi *et al.*, 2002; Slininger *et al.*, 2007; Al-Mughrabi *et al.*, 2013). Occasionally, those strategies are not enough; once a disease is already affecting potato tubers in storage, they are too late. In those cases, an early identification of those diseases is crucial to avoid generating a large amount of waste and consequential economic losses. In the next section, different mechanisms for the detection and identification of potato diseases are presented.

1. 2 VOCs (Volatile Organic Compounds) detection as a potential method for the early detection of potato rots

There are different methodologies for the detection and identification of plant diseases. They can be divided into two different categories: direct or indirect methods. Direct methods include molecular and serological methods where the pathogen is detected, allowing a rigorous identification of the pathogen responsible for the disease. Methods, such as Fluorescence *in situ* hybridization (FISH), the Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) are included in this group. Whereas indirect methods do not detect the pathogen itself, they detect changes in different parameters indicators of the presence of a disease. Different parameters such as

morphological, temperature, transpiration rate, changes or volatile compounds released by the infected potato are used in the indirect detection (Fang and Ramasamy, 2015).

The disease detection has been attempted at different levels of the potato production depending on the targeted potato disease, in the field or the storage. In the next subsections, we will focus on those methodologies that have been designed for the detection of diseases during storage, therefore on potato tubers instead of potato plants.

1.2. 1 Direct detection methods

Direct methods such as the Polymerase Chain Reaction (PCR) methodology has been extensively studied for the detection of fungal and bacterial potato pathogens, such as *Helminthosporium solani, Fusarium sambucinum, Colletotrichum coccodes, Phytophthora infestans, Clavibacter michiganensis* subsp. *sependonicus, Ralstonia solanacearum* and *Pectobacterium carotovorum* spp. (Tooley *et al.*, 1997; Pastrik, 2000; Weller *et al.*, 2000; Hyman *et al.*, 2000; Cullen *et al.*, 2001, 2002; Ozakman and Schaad, 2003; Atallah and Stevenson, 2006; El Hadrami *et al.*, 2007; Smith and De Boer, 2009; Qu, Wanner and Christ, 2011; Kettani-Halabi *et al.*, 2013; Li *et al.*, 2014; Massart, Nagy and Jijakli, 2014; Ranjan, Singh and Baranwal, 2016; Dinesh and Baranwal, 2016). In some cases, a multiplex PCR was proposed to detect several diseases by running a single reaction (Qu, Wanner and Christ, 2011; Ranjan, Singh and Baranwal, 2016). The PCR methodology allows not only the detection of the pathogen responsible of the disease with a very high sensitivity, but also its identification. The optimal selection of primers for the PCR provides a high specificity to this technique. However, it is a destructive method limited by lack of operational robustness; it depends on the efficacy of the DNA extraction, the selection or design of the optimal primers for the PCR, affected by the potential inhibitors presented in the sample assay and by the performance of the polymerase chain reaction (Fang and Ramasamy, 2015). Therefore, the need for specific equipment for the amplification at the different temperatures. Based on that, several authors studied the option of using Loop-Mediated Isothermal Amplification (LAMP) as a rapid

and sensitive technique that can be used without the need of specific equipment as the amplification is carried out at a single temperature, in some cases at room temperature. LAMP reaction consists of a hybridization of different primers followed by a strand extension at a single temperature (65^{o}C) in the presence of a strand displacement DNA polymerase forming a characteristic stem-loop DNA structure. The mains differences with PCR are the number of primers, the amplification at a single temperature and how the loop structures are formed during the amplification (Li *et al.* 2011). A specific LAMP assay was developed for the detection of *Pectobacterium carotovorum* spp. (Hu *et al.*, 2016; Ocenar *et al.*, 2019).

Other direct methods have been previously studied. A molecular detection technique based on Fluorescence *in situ* hybridization (FISH), a combination of microscopy and hybridization of DNA probes and target genes from plant samples, was studied for the detection of *Ralstonia solanacearum* in potato tubers (Wullings *et al.*, 1998). Similar disadvantages to the PCR methodology can be mentioned, such as the optimal design of the DNA probe and the appearance of false negatives due to the presence of autofluorescence materials. The Enzyme-Linked Immunosorbent Assay (ELISA), another molecular method based on antibodies, has also been studied for the identification of potato diseases such as *Clavibacter michiganensis* subs. *sependonicus* and *Ralstonia solanacearum* (Priou, Gutarra and Aley, 2006; Przewodowski and Przewodowska, 2017). The Fluorescence *in situ* hybridization (FISH) method presents a relatively low sensitivity for bacteria. Therefore its application is used mostly as a confirmation of the presence of a potato disease once a visual check has been carried out and not as an early detection method (Fang and Ramasamy, 2015). The different techniques mentioned are time-consuming, need to be carried out in specific facilities with the required equipment and the presence of a technician expert on this technology. In 2014, a Russian biotechnology research group developed a Lateral Flow Immunoassay (LFIA) for the detection of *Pectobacterium atrosepticum* spp. and *Clavibacter michiganensis* on potato tubers (Safenkova *et al.*, 2014; Safenkova, Zherdev and Dzantiev, 2015). This

technique, however, needed a previous step of sample preparation and lysis before its analysis through the LFIA test developed.

1.2. 2 Indirect detection methods

Indirect detection methods have also been developed for the detection of potato diseases. One of the advantages of some of those methods is that the detection of the disease could be achieved earlier as the processes measures are not directly related to the visual appearance of the potato tuber. The most relevant indirect method is that one based on VOCs. Volatile Organic compounds release by potato tubers when they are infected with different potato diseases. However, other indirect techniques have been developed for the detection of fungal diseases such as the Fourier Transform Infrared microscopy (FT-IR). It has been studied for the detection of *Colletotrichum coccodes* on the surface of potato tubers, with the possibility of a rapid and early identification of fungal pathogens *in vivo* (Erukhimovitch *et al.*, 2007, 2010). Hyperspectral techniques have been widely used for the detection of diseases at the field level in potato plants. However, they have not been used for the detection of potato storage diseases in tubers. The use of hyperspectral techniques in potato tuber have only been studied in the Zebra Chip (ZC) disease, a bacterial disease from Unites States, Mexico, Central America and New Zealand (Dacal-Nieto *et al.*, 2011; Garhwal *et al.*, 2020). It is a fast, non-destructive and non-contact method that appears to be a promising technique. However, until now high percentages of false positives have been detected in the studies mentioned.

1.2.2. 1 VOC detection methodology

The VOCs methodology, as previously mentioned, is one of the most studied indirect method for the early detection of potato diseases in storage. It is a non-optical method that involves the profiling of the volatile chemical signature of infected potatoes (Fang and Ramasamy, 2015). The infection of potato tubers by a pathogen could result in a release of VOCs by the potato tuber or by the pathogen, indicative of the disease. The analysis of these VOCs is normally carried out using Gas-Chromatography (GC) technology, in most cases coupled

with Mass Spectrometry (MS) to increase sensitivity and improve the identification of the compounds.

Different VOC collection methods have been widely used for the detection of potato rots. Based on the use of standard absorbent technologies, such as Tenax (Varns and Glynn, 1979; Ouellete *et al.*, 1990) or the use of Solid Phase Microextraction (SPME) (Kushalappa & Lui, 2002; Savelieva *et al.*, 2016: Blasioli *et al.*, 2013). The main difference between Tenax adsorbents and SPME is how VOCs are trapped into the adsorbent. In Tenax or any other TD tube the sample is passed through the tube and VOC are trapped, while for SPME the fiber is located inside the headspace and VOC will be retained on it (Fabre 2002, Marsili 1999). One of the limitations of SPME is the limited number of commercially available stationary phases.

Besides, different methods for the detection of VOCs have been used, such as Gas Chromatography with Flame Ionisation Detector (FID) coupled with MS (de Lacy Costello *et al.*, 2001) and other technologies, such as Field Asymmetric Ion Mobility Spectrometry (FAIMS) (Rutolo *et al.*, 2016: Sinha *et al.*, 2017) or electronic nose (Biondi *et al.*, 2014, Rutolo *et al.*, 2018). FAIMS characterizes the VOCs by fingerprinting the ion mobility of constituent ions of VOCs (Sinha *et al.,* 2017), while an electronic nose is an instrument that mimics the olfactory human system using a sensor array that detect mixtures of VOCs coming from the matrix due to the overlap of sensitivity of the different sensors present in the array (Biondi *et al.,* 2015). The use of FAIMS and e-nose will allow the detection of different pattern in presence and absence of a potato diseases, however, no identification of the VOCs is achieved in most of the cases. Indeed, they can be portable detector that will require less specialised equipment and it will be more cost-effective for its use in farms.

The production of VOCs from infected potato tubers have been widely studied. A summary of the different VOCs identified until now on different potato rots *in vitro* and *in vivo* are presented in Table 1-2. In the next two subsections different studies are presented based on the studied pathogens (bacterial or fungal pathogens).

Table 1-2 Volatile Organic Compounds (VOCs) selected as potential biomarkers of the presence of different potato pathogens *in vitro* (a) and *in vivo* (b). The disease, the pathogen involved, VOC methodology used, temperature, potato cultivar or media, incubation time, VOC name and the corresponded references are included. The synthetic and semisynthetic media presented were: PSA (Potato-Sucrose Agar), TZA (TZ-Agar), LPGA (*Levure* Peptone Glucose Agar) PDA (Potato Dextrose Agar) and NPDA (Natural Potato Dextrose Agar). For soft rot, PCA (*Pectobacterium carotovorum atrosepticum*) and *PCC (Pectobacterium c. carotovorum*) were included.

a) *In vitro*

Disease	Pathogen	Methodology	T°	Media	Time	VOCs	References
Dry rot	*Fusarium* spp. (*F. langsethiae, F. sporotrichioides*)	SPME -GC	23-24 °C	PSA	9-16 days	Ethanol	(Savelieva *et al.*, 2016)
						Ethyl acetate	
						Isobutanol	
						3-Methylbutan-1-ol	
						2-Methylbutan-1-ol	
						Ethyl-3-methylbutanoate	
						Terpenes	
						Sesquiterpenes	
						Trichodiene	
Brown rot	*Ralstonia solanacearum*	SPME-GC-MS	27 °C	TZA	5 days	Dimethyl disulfide (DMDS)	(Blasioli *et al.*, 2013)
				LPGA	2 days	2-Propanone	
						Dimethyl disulfide	
						Methyl 2- methylbutanoate	
				PDA	11 days	2-Butanone	
						3-Methylbutanoic acid	
						2-Furancarboxaldehyde	
						Propanoic acid	
						Methyl 2- methylbutanoate	

						Styrene	
Ring rot	*Clavibacter michiganensis sepedonicus*	SPME-GC-MS	27 °C	LPGA	2 days	Dimethyl trisulfide (DMTS)	(Blasioli *et al.*, 2013)
						3-Methylbutanal	
				PDA	11 days	2-Propanol	
						2-Methylpropanoic acid	
						3-Methylbutanoic acid	
						Benzaldehyde	
				NPDA	12 days	2-Propanol	
						2-Methylpropanoic acid	
						3-Methylbutanoic acid	
						2-Hydroxy-3-pentanone	

b) ***In vivo***

Disease	Pathogen	Methodology	To	Cultivar	Time	VOCs	References
Soft rot	PCA	GC-MS	20ºC	Russet Burbank	3- 6 days	Acetic acid ethenyl ester	(Lui, Vikram, Abu-Nada, *et al.*, 2005)
		TD-GC-FID-MS	15ºC	Chieftan	24-120 h	Acetone	(Varns and Glynn, 1979)
						Ethanol	
						2-Butanone	
		HS-GC	10 & 22ºC	Russet Burbank	1-5 days	3-Hydroxy-2-butanone	(Waterer and Pritchard, 1985)
		HS-GC-FID	4,10 & 22ºC	Russet Burbank	1-5 days	2-propanol	(Waterer and Pritchard, 1984a)
		TD-GC-MS	15ºC	Atlantic	4-16 days	Dimethyl sulfide (DMS)	(Ouellete *et al.*, 1990)
						Pentane	
		GC-FID-MS	20ºC	Maris Piper	4 weeks	Ammonia	(de Lacy Costello *et al.*, 1999)
						Trimethylamine	
						Dimethyl sulfide	
						Dimethyl disulfide	
						Dimethyl trisulfide	
						Acetone	

						Ethanol	
						Butan-1-ol	
	PCC	GC-MS	20°C	Russet Burbank	3 and 6 days	Diazene	(Lui, Vikram, Abu-Nada, *et al.*, 2005)
						Cyclohexene	
						Methoxy-(1,1-dimethyl-2-dihydroxy-ethyl)-amine	
		FAIMS	4 & 25°C	Burbank	21 days	Ethanol	(Sinha *et al.*, 2018)
						Acetone	
						2-Butanone	
						Ethyl acetate	
	PCA & PCC	GC-MS	20°C	Russet Burbank	3-6 days	2-Methyl-1-butanol	(Lui, Vikram, Abu-Nada, *et al.*, 2005)
						1-Pentanol	
						3-Hydroxy-2-butanone	
						2-Methylpropyl ester acetic acid	
						Methyl acetate	
						Acetone	
						Ethanol	
						Ethyl acetate	
						Methyl ester pentanoic acid	
						2-Methyl-butanoic acid	
						1-Butanol	
						1-Propanol	
		HS-GC-FID	22°C	Russet Burbank	1-5 days	Ethanol	(Waterer and Pritchard, 1984b)
						Methanol	
						1-Butanol	
		HS-GC	10 & 22°C	Russet Burbank	1-5 days	2-Propanol	(Waterer and Pritchard, 1985)
		SPME GC-MS & TD-GC-MS	20C	Pentand dell	14 days	Ammonia	(Jones, Ewen and Ratcliffe, 1998)
						Acetone	
						Ethanol	
						1-Butanol	
Dry rot	*Fusarium roseum*	TD-GC-MS	15°C	Atlantic	4-16 days	Dimethyl sulfide (DMS)	(Ouellete *et al.*, 1990)
						Pentane	

	Fusarium coeruleum	GC-MS & GC-FID	10ºC	Maris Piper	42 days	Butanal	(de Lacy Costello *et al.*, 2001)
						3-Methylbutanal	
						Undecane	
						Verbenone	
		GC-FID	10ºC	Maris Piper	42 days	Ethanol	
						2-Propanol	
	Fusarium sambucinum	GC-MS	20ºC	Russet Burbank	3-6 days	2,5-Norbornadiene	(Lui, Vikram, Abu-Nada, *et al.*, 2005)
						Styrene	
Late blight	*Phytophthora infestans*	GC-MS	10ºC	Maris Piper	42 days	2-Pentylfuran	(de Lacy Costello *et al.*, 2001)
						Copaene	
		GC-FID	10ºC	Maris Piper	42 days	Ethanol	
						2-Propanol	
		GC-MS	20ºC	Elite & Russet Burbank	3-6 days	Dimethyl disulfide	(Lui, Vikram, Hamzehzarghani, *et al.*, 2005)
						Chloroform	
Brown rot	*Ralstonia solanacearum*	SPME-GC-MS	4 ºC	Spunta & Kennebec	4 months	1-Hepten-3-ol	(Blasioli *et al.*, 2013)
						3,6-Dimethyl-3-octanone	
						3-Ethyl-3-methylpentane	
						Benzothiazole	
		HS-SPME-GC–MS	20ºC	Non specify	30 min	2-Propanone	(Stinson, Persaud and Bryning, 2006)
						2-Propanol	
						2-Butanone	
						2-Butanol	
						2-Pentanone	
						2-Pentanol	
Ring rot	*Clavibacter michiganensis sepedonicus*	SPME-GC-MS	4 ºC	Spunta & Kennebec	4 months	2-Propanol	(Blasioli *et al.*, 2013)
						3-Methyl-3-buten-2-one	
		HS-SPME-GC–MS	20ºC	Non specify	30 min	3-Methyl-2-pentanone	(Stinson, Persaud and Bryning, 2006)

Methodology: SPME: Solid Phase MicroExtraction, HS: Headspace. GC: Gas Chromatography, MS: Mass Spectrometry, FID: Flame Ionization Detection, TD: Thermal Desorption, FAIMS: Field Asymmetric Ion Mobility Spectrometry

1.2.2.1. 1 Bacterial diseases

The detection of VOC from potato rots have been mainly studied in presence of *Pectobacterium carotovorum* spp., responsible of soft rot in potato tubers. Varns and Glynn (1979) performed the first attempt of the detection of potato disease by VOC monitoring. They identified specific volatiles compounds released in soft rot infected potato tubers under controlled environmental conditions (15ºC) using Gas Chromatography and Mass Spectrometry (GC-MS). Samples were adsorbed in a Tenax GC adsorbent packed in an ID U-tube assembly. Then the samples were desorbed and analysed by GC-MS. Acetone, butanone and 2-butanone were produced as the tuber surface was becoming progressively soft. However, dry rot infections were also detected on the potato tubers analysed, indicating that the identified volatiles were not unique to *Pectobacterium* spp. Therefore they could not be considered as indicators exclusively of the presence of soft rot in potato tubers but instead as a general VOC indicator of the presence of an infection (Varns and Glynn, 1979). Waterer and Pritchard in 1984 added and extra pre-concentration process. They used a polymeric adsorbent (Chromosorb 105) for the concentration of the VOC samples (Waterer and Pritchard, 1984a). Different studies also used this adsorbent for the VOC sampling from potato tubers inoculated with *Pectobacterium carotovorum* spp. and stored at room temperature (15-22ºC). Once the VOC sample was collected using the Chromosorb 105 adsorbent, the sample was analysed by GC-MS (Waterer and Pritchard, 1984b, 1985; Ouellete *et al.*, 1990; Lyew *et al.*, 2001). The analysis of the VOCs carried out in these studies was mainly based on the Total Volatile Production and percentage of total peak area calculated by the integration of the chromatogram peaks. Most of the studies were not able to select VOCs as potential biomarker for the presence of soft rot on potato tubers, as they were also detected in presence of other pathogens or even in the healthy non-inoculated potato tubers. Compounds such as ethanol, acetone and 1-butanol were detected in common between those studies in presence of *Pectobacterium carotovorum* spp. However, there were some differences in their detection attributed to the difference in cultivar and the experimental design conditions.

Jones et al. (1998) compared two different adsorbents Tenax GR and Chromosorb 103 thermal desorption tubes for the detection of amine compounds on potato tubers infected with *Pectobacterium carotovorum* spp. and stored at 20^{o}C. They also studied a different extraction/ pre-concentration method known as Solid-Phase Microextraction (SPME), a passive adsorbent sampling technique that concentrates VOCs before direct transfer of the sample to the GC-MS (Jones, Ewen and Ratcliffe, 1998). The same extraction method was used in a different study for the discrimination of different diseases on potato tubers (*Pectobacterium carotovorum* spp., *Pythium ultimum, Phytophthora infestans* and *Fusarium sambucinum*). The VOC fingerprint was achieved using a SPME fibre to trap the VOC followed by GC with Flame Ionization Detection (FID) (Kushalappa *et al.*, 2002).

In a different study, Lui et al. (2005) studied the VOCs profile of potato tubers infected with soft rot and stored for 24h at 20^{o}C by GC-MS. Acetic acid ethenyl ester was identified for *P. c. atrosepticu*m, while for *P. c. carotovorum* three different compounds were found. When analysing *Pectobacterium* including both varieties, 13 different VOCs were found (Lui *et al.*, 2005). A similar study was carried out on the discrimination of three fungal diseases on potato tubers (Lui *et al.*, 2005b).

Storage at a lower temperature was studied by de Costello et al. (1999) where potato tubers were infected with *P.c. carotovorum* and stored at 10^{o}C. Twenty-two different VOCs were detected in potato tubers infected with the bacterium, but they were not considered as markers of soft rot because they were also detected in the control tubers (de Lacy Costello *et al.*, 1999).

Identifying pathogen-specific volatiles as markers for the presence of disease is challenging to achieve as many factors are affecting the VOC production (specific pathogen, aseptic conditions, temperature and relative humidity). Therefore, some of the studies have considered the increase in the total concentration of VOCs as a possible indicator of soft rot. Lyew et al. in 1999 developed a specific air sampling unit and studied changes in volatile production of tubers infected with *Pectobacterium c. carotovorum* stored at room temperature. They concluded

that differences between healthy and infected potatoes were observed after 24h and that the amount of VOCs depends on the initial amount of bacteria and the growth of the bacteria (Lyew *et al.*, 1999).

Detection of soft rot based on gas analysis can also be possible using Field Asymmetric Ion Mobility Spectrometry (FAIMS). It has been used in several studies for the detection of soft rot in potato tubers when comparing the results with non-inoculated tubers. However no identification of the biomarkers was carried out (Rutolo *et al.*, 2014, 2018; Sinha *et al.*, 2017). Recently, Sinha et al. identified some compounds in potato tubers infected with *P. c. carotovorum* stored at room temperature using FAIMS. Ethanol, acetone, 2-butanone and ethyl acetate were directly associated with the presence of this bacteria on potato tubers (Sinha *et al.*, 2018). One of the advantages of FAIMS is that it allows a differentiation between infected and non-infected potato tubers based on patterns. While for the identification of those VOC an extra step including VOC specific standards was needed to achieve the identification. Therefore, for the detection and quantification of that specific VOC, a previous selection of the targeted compounds will be needed. An untargeted VOC analysis is not possible using FAIMS.

Pectobacterium carotovorum spp., responsible for soft rot, is not the only bacterial pathogen whose VOC fingerprint has been studied. Two different bacterial pathogens, *Ralstonia solanacearum* and *Clavibacter michiganensis sepedonicus*, responsible for brown rot and ring rot, respectively, were also studied. The VOC production has been studied using two different techniques, SPME-GC-MS (Stinson, Persaud and Bryning, 2006) and SPME followed by Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) (Blasioli *et al.*, 2013). The previous study was carried out *in vivo* and *in vitro,* at room temperature and 27^{o}C*.* Three different VOCs, 1-hepten-3-ol, 3,6-dimethyl-3-octanone, 3-ethyl-3-methylpentane, 1-chloroctane and benzothiazole were considered as markers for brown rot, and 2-propanol and 3-methylbutanoic acid as marker for ring rot (Blasioli *et al.*, 2013).

1.2.2.1. 2 Fungal diseases

The number of studies based on the detection of VOC from potato tubers infected with fungal diseases is low. De Costello et al. (2001) studied for the first time the production of VOCs of potato tubers infected with fungal pathogens, *F. s. coeruleum* and *Phytophthora infestans,* at 10ºC and 95% of relative humidity (RH). The VOCs were measured 42 days after the inoculation. They measured VOCs using Gas Chromatography with FID to determine low-molecular-weight volatiles (C_1-C_3) that could not be identified by the Gas Chromatography-Mass Spectrometry (GC-MS). Two main VOCs were identified as specific for *F.s. coeruleum,* while four VOCs were found on potato tubers infected with *P. infestans* and those infected with *F.s. coeruleum* (de Lacy Costello *et al.*, 2001). In a recent study, the VOC production of *Fusarium* spp. cultured in different media (Potato sucrose Agar, PSA, and autoclaved kernels) was studied. The VOCs were sampled by SPME and followed by a GC-MS analysis. Ethanol and ethyl acetate were detected in higher abundance in presence of *Fusarium* spp. (Savelieva *et al.*, 2016).

1.2.2. 2 Electronic nose methodology

To date, the only VOC detection techniques available require expensive specialised equipment and trained personnel to carry them out. Different electronic sensing devices, known as electronic nose (e-nose) have been developed for the detection of potato diseases. One of them developed by de Lacy Costello et al. (2000) consisted of a ceramic sensor that could detect one soft rot diseased potato tuber from 100 kg potato tubers at room temperature (de Lacy Costello *et al.*, 2000). A recent study developed an array of metal-oxide based gas sensor for the early detection of soft rot on potato tubers. It allowed the discrimination between uninfected and soft rot infected tubers at 25ºC (Rutolo *et al.*, 2016). An optimized bionic electronic nose gas chamber and a sampling device were also designed for the detection of changes in VOC of soft rot infected potato tubers (Chang *et al.*, 2017). A different sensor focused on brown rot and ring rot was developed in 2006. It was an SPME automatic sampler combined with 8-metal oxide sensor array able to identify potato tubers infected with brown rot and ring rot (Stinson, Persaud and Bryning, 2006). A commercial electronic

nose equipped with a metal oxide sensor was also developed to detect those two diseases in potato tubers (Biondi *et al.*, 2014).

A commercial prototype sensor or e-nose requires high sensitivity and a pre-conditioning phase to reduce the effect of fluctuations in the ambient atmosphere. When a specific VOC sensor prototype is used under commercial conditions, it suffers problems associated with the fluctuation of temperature and relative humidity (RH), the high variability between replicates and the reduction of sensitivity at low temperatures. This reduction in sensitivity has been previously associated with the adaptation of the sensor to the cold temperatures, as after a period of time sampling at cold temperature, the sensitivity increases (de Lacy Costello *et al.*, 2000; Rutolo *et al.*, 2016). These sensors are a promising solution for commercial storage facilities; however further studies and development need to be carried out to achieve the optimal detection at low temperatures.

1.2.2. 3 VOC detection under commercial storage conditions

Most of the studies in VOC detection from potato rots have been carried out at room temperature, except for de Lacy Costello group study (de Lacy Costello *et al.*, 2001). The real problem is under potato storage conditions, where cold temperatures are maintained. Additional research needs to be carried out for the elucidation of VOC biomarkers of potato rots at cold temperatures. Until now, most of the technologies that have been used for the detection of potato diseases are based on GC, GC-MS, FAIMS or electronic nose. As it has already been mentioned, an extra step is introduced in some cases for the pre-concentration of the sample. Due to the low temperature of the storage facility, the abundance of VOC will be low. The incorporation of a pre-concentration method is needed for the VOC detection of potato tubers stored at cold temperatures. Thermal Desorption is an improved methodology for the pre-concentration of samples that will concentrate the VOC sample and increase the detection limit of the GC-MS analysis. It consists of an adsorptive enrichment of a specific solid adsorbent on thermal desorption tubes. The compounds of interest will be adsorbed on the sorbent surface (Cai *et al.*, 2015). In some of the studies, Thermal Desorption have already been used as a pre-concentrating technique, adsorbents such as

Tenax GC and Chromosorb 105 have been used. However, in most of these cases, they were part of the sampling unit, and no other sorbents were tried. Therefore, it could be that the most essential VOCs, were not able to be trapped in those specific sorbents.

The selection of the optimal adsorbent to be used as part of the pre-concentration method is needed. Most of the VOC detected at low temperatures will present a low molecular weight. In some cases, multi-bed sorbent tubes are designed to allow the broadest volatility range to be analysed within a single tube. A fundamental advantage of thermal desorption is that the sample preparation step is not required, and those problems associated with the use of solvents are eliminated (Lourenço *et al.*, 2020).

The use of Thermal Desorption- Gas Chromatography-Mass Spectrometry (TD-GC-MS) has been widely used in the detection of VOC from different matrixes and environments. Its use in the detection of VOC from plant material such as trees and flowers have been studied (Baraldi *et al.*, 1999; Howes, Kite and Simmonds, 2009; Li *et al.*, 2011; Dong, Lu and Cole, 2013; Durenne *et al.*, 2018; Zhou *et al.*, 2020). TD-GC-MS has also been widely used in food-related products such as honey, cut tobacco and even on the effect of heat treatments on the VOC released by different food matrixes (Gao *et al.*, 2012; Cacho *et al.*, 2015; Lee *et al.*, 2021). It has also been used on the VOC released by complex matrixes as those related to the olive oil production (Kanavouras, Kiritsakis and Hernandez, 2005; Hernández *et al.*, 2018). In some fruits as apples and pomegranate, it was used as an effective technique for producing a quick VOC “fingerprint” from freshly cut fruit without the need of further processing or solvent-based extraction (Jung, 2014; Roberts and Spadafora, 2020). It is also the technique used for the analysis of the aroma as esters of wines at different stages of the wine production (Gómez-Ariza, García-Barrera and Lorenzo, 2004; Callejón *et al.*, 2008; Tredoux *et al.*, 2008; Kelly *et al.*, 2012; Marquez *et al.*, 2014; Xiao *et al.*, 2015; González-Jiménez *et al.*, 2020). The only study where TD-GC-MS was used in the potato industry was to determine the hexanal content, as an indicator of lipid oxidation on potato crisps (Sanches-Silva *et al.*, 2004).

The identification of VOC from potato rots has mainly been studied at room temperature. Studies at lower temperatures need to be carried out considering the previously targeted VOC detected for soft rot and dry rot at room temperature. Furthermore, the identification of some potential VOC biomarker for gangrene at low temperature needs to be studied.

1. 3 Future developments and conclusion

Early identification of potato rots is a crucial step to avoid the generation of waste in the potato industry. Due to the appearance of new strains resistant to different fungicides and biocontrol agents, the development of new strategies is underway. An optimal early identification method is needed to detect as soon as possible when an infection occurs inside a storage facility. Those potato tubers from the storage room, where a disease has been detected, will be sold in the first place. This will avoid the spread of the disease and the complete loss of all the potato tubers from the storage room.

The use of VOCs as an indirect method for the detection of diseases on potatoes is a promising method. It would allow farmers to identify not only when an infection is present in the storage, but also, with the use of specific pathogen biomarkers, which disease is affecting their potato tubers. For the next batches of potatoes, prevention measurements could be taken as controlling temperatures and relative humidity or considering the control of this specific disease in the field for those pathogens whose primary origin is the soil.

The development of a compact and specific sensor to detect at real-time those specific biomarkers of potato rots, would be the suitable solution. They would be located inside the storage facility, in different locations throughout the whole storage room. As soon as one VOC biomarker is detected the sensor would give a specific signal, then the farmers will be aware of the presence of a disease.

1. 4 Research aim and objectives

The main aim of this project was the identification of Volatile Organic Compound (VOC) biomarkers of potato diseases (fungal and bacterial pathogens) under commercial storage conditions (5-10^{o}C) and gaining a deep understanding on the ecophysiology of important fungal and bacterial potato-pathogens under those conditions.

Specific objectives include:

- Optimisation of a methodology for the detection and identification of VOCs.
- Determination of the pathogen-specific VOCs *in vivo* and *in vitro.*
- Isolation of fungal and bacterial pathogens from potato tubers.
- Study of the ecophysiology of the microorganisms responsible for the targeted fungal or bacterial rots.
- Investigation of the mycotoxin production by specific potato tuber pathogens.
- Understanding the effect of storage time on potato susceptibility to the targeted pathogens.

2 MATERIALS, METHODS AND METHOD DEVELOPMENT

2. 1 Fungal and bacterial strains

A previous study on different fungal pathogens responsible for different diseases in potato tubers was carried out. Those pathogens, presented in Appendix A (Table A-1), were grown in different synthetic media (V8, Potato Dextrose Agar, Potato Sucrose Agar, Carrot Agar) and in slices of potato tubers autoclaved to check their development in a media similar to the potato tuber. Their sporulation was also stimulated by growing the fungal pathogen in V8 media and their exposure to UV A light. *Fusarium solani coeruleum,* responsible for dry rot, was studied in different synthetic media; however, it was not able to grow when it was inoculated in potato tubers. Finally, two different fungal pathogens and one single bacterial pathogen responsible for diseases in potato tubers during storage were used for this work. They are presented in Table 2-1.

Table 2-1 Pathogens responsible of different potato diseases and their supplier information

Strain	Disease	ID	Supplier
Boeremia foveata	Gangrene	CECT 20797	Spanish Collection of Type Culture (CECT), Spain
Fusarium sambucinum	Dry rot	Private collection-	Plant Breeding and Acclimatization Institute (IHAR) -National Research Institute, Mlochów, Poland
Pectobacterium carotovorum atrosepticum	Soft rot	CECT 314	Spanish Collection of Type Culture (CECT), Spain

Fungal strains: The fungal strains were subcultured in Potato Dextrose Agar (PDA, Sigma-Aldrich, Dorset, UK) for 7 days at 25^{o}C in the dark. A glycerol: water (70:30, v/v) stock solution of the previous culture was prepared separately and stored at -20^{o}C in the culture collection of the Applied Mycology Group, Cranfield University. This allowed its use for the different experiments, always using the same inoculum.

Bacterial strain: *Pectobacterium carotovorum atrosepticum* was cultured in Nutrient Broth (NB, Sigma-Aldrich, Dorset, UK) for three days in agitation at 30ºC. A glycerol: water (50:50, v/v) stock solution of the previous culture of *P. c. atrosepticum* was prepared and stored at -20ºC in the culture collection of the Applied Mycology Group at Cranfield University. This allowed its use for the different experiments, always using the same inoculum.

2. 2 Isolation and molecular identification of different fungal species from potato tubers

Six fungi were isolated from potato tubers that were stored for five days at 25ºC (Figure 2-1). Three isolates (ISO1, ISO2 and ISO3) were from the cv. Markies supplied by Glynn Harper (Sutton Bridge Crop Storage Research, UK) from the harvest season 2017. The others isolates (ISO4, ISO5 and ISO6) were from organic seed potato tubers cv. Record supplied by AGRICO UK (Scotland, UK) from the harvest season 2018.

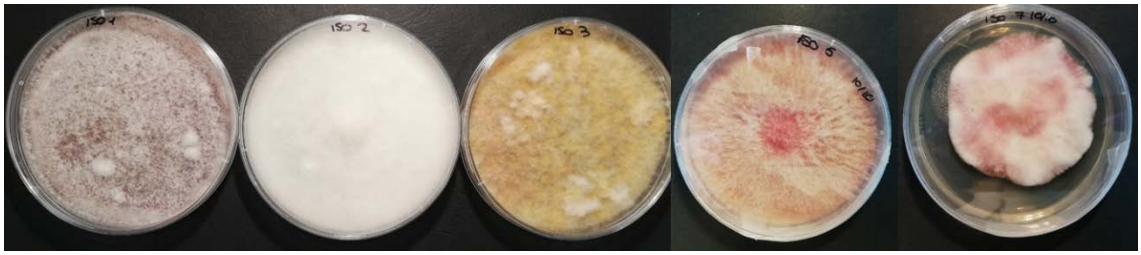


Figure 2-1 Images of five of the six fungal pathogens isolated from potato tubers. From left to right the different isolates are presented after 7 days of growth in PDA at 25ºC.

2.2. 1 Isolation methodology

Tubers were surface sterilised, following the same protocol as the one mentioned in Section 2.4. 2. Then, the tubers were incubated inside 12 L plastic boxes at a high relative humidity (98-99%) and 25ºC for five days. The high RH was achieved by including a beaker with sterile distilled water and a thin layer of water at the bottom of each box. After the incubation, pieces of tissue from potato tubers showing dry rot symptoms were placed on Potato Dextrose Agar (PDA) amended with chloramphenicol (0.1g/L, Sigma-Aldrich, Dorset, UK), and incubated for

seven days at 25ºC. The resulted culture was subcultured twice in PDA before obtaining the pure isolate.

After that, potato tubers cv. Maris Piper were surface sterilised, wounded in four different points and inoculated with a piece of agar from each of the isolates. They were incubated for three days at 25ºC and re-isolated from those potato tubers. A glycerol: water (70:30, v/v) stock solution with 5 mm of diameter plugs from similar areas of each of the isolate colony was prepared and stored at -20ºC until the molecular identification was carried out.

2.2. 2 Molecular identification methodology

Previous to the molecular identification, the isolates were exposed to UV A light to stimulate their sporulation and their potential identification by their observation under the microscope. The isolate 2 and isolate 4, presented in Figure 2-2, showed the typical macroconidia from *Fusarium* spp. Therefore, it can be expected that those two isolates will be *Fusarium* spp. Their specific specie was determined by molecular identification.

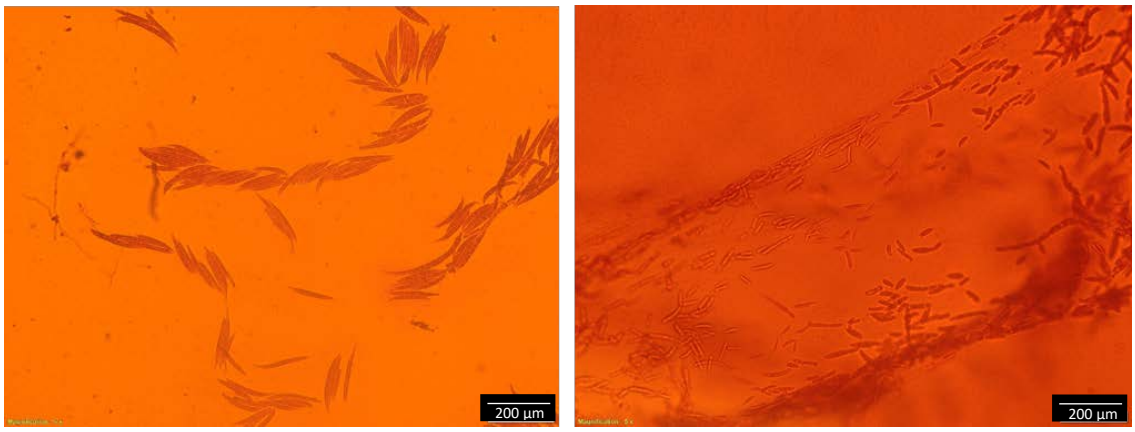


Figure 2-2 Microscope view from isolate 2 and 4 (left to right) after 7 days of incubation in PDA at room temperature under UV A light to stimulate their sporulation. The macroconidia, typical of *Fusarium* spp., can be observed.

The molecular identification was carried out in February 2019 in the laboratory of Microbiology in “Universidad Complutense” (Madrid, Spain). An aliquot of each of the isolates from the stock was inoculated in PDA for seven days at 25ºC. *Fusarium sambucinum,* supplied by the Plant Breeding and Acclimatization Institute (Mlochów, Poland) was also included with the isolates for their molecular

identification as a confirmation. The DNA of the isolates and *F. sambucinum* were extracted, a specific region of their genome amplified and sent for sequencing.

DNA extraction: Genomic DNA extraction was carried out following the protocol described by Querol et al. 1992 (Querol *et al.*, 1992). Before the DNA extraction, fungal mycelia from a seven-day-old cultures on PDA of each of the isolates were scraped off with a scalpel, frozen with liquid nitrogen and ground using a micropestle. DNA concentrations were determined using a NanoDrop ® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA). The resulted DNA concentrations are presented in Table 2-2.

Table 2-2 DNA concentration, 260/280 and 260/230 ration of six fungal isolates and *F. sambucinum.* DNA concentration was presented in ng of DNA per µl of sample.

Isolate/ strain	[DNA] (ng/µl)	260/280 ratio	260/230 ratio
Isolate 1	992.78	1.90	1.80
Isolate 2	104.88	1.86	0.54
Isolate 3	98.01	1.91	0.81
Isolate 4	511.41	1.90	1.56
Isolate 5	1091.17	1.84	2.24
Isolate 6	213.93	1.89	1.10
Fusarium sambucinum	121.48	1.89	0.67

PCR amplification for DNA integrity: The first PCR amplification was carried out to confirm the presence of fungal DNA and its integrity in all samples. It was carried out using universal primers encoding a partial fragment of the Internal Transcribed Spacer (ITS) that have been previously described by White et al. 1990 (ITS1/ITS4) (White *et al.*, 1990). After the PCR amplification, PCR products were detected and visualized under UV A light on 2% Agarose ethidium bromide gels in TAE 1X buffer (Tris Acetate 40mM and EDTA 1.0 mM). The NZYDNA Ladder V (Nzytech, Lisbon, Portugal) was used as the molecular size marker. The visualization under UV of the gel is presented in Appendix B (Figure B-1).

PCR amplification for sequencing: Genomic DNA was amplified using primers EF-1 and EF-2, described by O'Donnell 1998 (O'Donnell *et al.*, 1998). Those primers encoded for a partial fragment of the elongation factor gene, generally used for the molecular identification of *Fusarium* spp.

The amplification reactions were carried out in volumes of 25 µl, containing 100 ng of DNA per sample, 1 µl of each primer (20 uM: Metabion, Planegg, Germany) and 12.5 µl of NZYTaq II 2x Green Master Mix (Nzytech, Lisbon, Portugal).

PCR products were again visualised under UV light on 2% Agarose ethidium bromide gels and presented in Appendix B (Figure B-2). As it is shown, for the first isolate no band was observed; therefore the PCR products from the amplification of the region of the ITS were sent for sequencing.

DNA purification: The amplification products approximately 670 bp long for EF-1α encoding genes were purified using the NZYGelpure Kit (Nzytech, Lisbon, Portugal) and following the manufacturer's protocol.

Sequencing: The purified products were sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction in Macrogen facilities (Madrid, Spain). All amplification products were sequenced in both directions.

Sequence analysis: The resulted sequences were cleaned using Chromas DNA software (Technelysium, Australia) and assembled using the UGENE 1.29 Package (Russia). The sequences were then compared with those deposited on National Center of Biotechnology Information (USA) nucleotide database to obtain the closest match in nucleotide sequences (highest identity) to the same gene regions of the previously sequenced isolates. The sequence alignment for each of the isolates is presented in Appendix B (Figure B-3 to Figure B-9).

The results obtained after the sequences analysis are presented in Table 2-3. *Fusarium oxysporum*, *F. equiseti*, *F. culmorum, F. venenatum* and *F. avenaceum* have already been directly related to the presence of dry rot in potato tubers (Stefańczyk *et al.*, 2016). However, *Fusarium flocciferum* have not been previously isolated from potato tubers, as it has only been associated with fruit rot in papayas (Helal, Hosen and Shamsi, 2018). *Fusarium sambucinum* identification was confirmed after the sequencing analysis.

Table 2-3 Identification of the isolates after their sequencing

Isolate	Fusarium spp.
1	*Fusarium oxysporum*
2	*Fusarium equiseti*
3	*Fusarium venenatum*
4	*Fusarium flocciferum*
5	*Fusarium culmorum*
6	*Fusarium avenaceum*
F. sambucinum	*Fusarium sambucinum*

2. 3 *In vitro* studies

2.3. 1 Preparation of potato-based semi-synthetic media (Natural Potato Dextrose Agar)

A semi-synthetic growth media named Natural Potato Dextrose Agar (NPDA) was formulated to simulate the natural crop commodity frequently contaminated by the pathogens studied *Fusarium* spp. and *B. foveata* in storage. The semi-synthetic media was prepared with a 2% of glucose (Fisher Scientific, USA), 1.5 % of Agar (Sigma-Aldrich, Dorset, UK) and 30% of mashed potato in 1 L of potato infusion. Potato infusion was prepared with 200 g of peeled potatoes (cv. Maris Piper) boiled in 1 L of dH_2O for 20 minutes and filtered through a single layer of cheesecloth (Fisher Scientific, USA).

After filtration, the water activity (a_w) of the resulted solution was then modified with glycerol, based on a previously conducted water activity (a_w) regression line. The water activity of the NPDA was measured using the AQUALAB 4TE water activity meter (Decagon Instruments; Washington, USA). The correspondent grams of mashed potato, glucose and agar were finally added to the a_w adjusted media and autoclaved. After autoclaving the media, it was dispensed in Petri dishes (Ø 9 cm) (ThermoFisher Scientific, USA) and the a_w checked using the AQUALAB 4TE water activity meter.

2.3.1. 1 Optimisation of the Natural Potato Dextrose Agar (NPDA)

The growth of *B. foveata* and *Fusarium* spp. was studied in NPDA media containing different concentration of mashed potato.

2.3.1.1. 1 Percentage of mashed potato

An experiment was carried out to determine the optimal percentage of mashed potato to be used in the semi-synthetic potato-based media. Six different percentages of mashed potato (2, 3, 5, 10, 20 and 30%) were tested. *Boeremia foveata* and *Fusarium sambucinum* were grown on the different media and incubated at 25ºC. Results are shown in Appendix C (Figure C-1 and Figure C-2). No significant differences were observed in growth rate and lag time in any of the two pathogens studied (ANOVA, p-values>0.20). Therefore, the highest percentage of mashed potato (30%) was selected as it was the one that presented greatest similarity with the potato tuber.

2.3.1.1. 2 Modification of the a_w of the media

The a_w of the media was modified by the addition of different quantities of glycerol, based on a previously created correlation curve (Appendix D, Figure D-1). Different quantities of glycerol were added to the media, the media was prepared, and the a_w checked using the AQUALAB 4E a_w meter for the preparation of the curve. This curve allowed the determination of the quantity of glycerol needed to be added to the NPDA media.

2.3. 2 Inoculation of NPDA and incubation *in vitro*

The inoculation of the potato-based media was carried out differently for *Fusarium* spp. than for *Boeremia foveata.*

***Fusarium spp*. inoculation**: An aliquot of the glycerol stock (Section 2. 1) was inoculated on PDA and incubated for 7 days at 25ºC prior to each of the experiments. A spore suspension of 10^8 spores/ml was prepared by adding 1 mL of a 0.005% Tween 80 and sterile water solution and scraping with a spatula the *Fusarium* spp. colonies to achieve the release of the spores. The concentration was determined with a Helber Haemocytometer (Marienfeld, Germany). The inoculation of both *Fusarium* spp. on potato-based media was performed by

plating 1 µl of each of the spore suspensions in the middle of the potato-based media plate.

***Boeremia foveata* inoculation:** An aliquot of the glycerol stock (Section 2. 1) was inoculated in PDA and incubated for two days at 25ºC before each of the experiments. The inoculation of *B. foveata* on potato-based media was performed by placing a 5 mm diameter plug from a two days old culture in the centre of the agar plate.

Non-inoculated potato-based agar plates served as negative control. Inoculated and non-inoculated NPDA plates were distributed in 12L airtight containers (The Plastic Box Company Ltd., UK). Ten NPDA plates were included per container. The relative humidity of the boxes was modified by including a layer of glycerol:dH_2O with the targeted a_w at the bottom of each container. Every three to four days, freshly prepared glycerol:dH_2O solution was added under sterile conditions. The containers with NPDA plates were stored at the different temperatures (5,10 and 15ºC) for 30 days.

2.3. 3 Fungal growth assessment *in vitro*

Fungal growth was assessed daily after the inoculation from six different plates of each of the different conditions (temperature x a_w). Colonies diameter were measured in two perpendicular directions to each other. Two different growth parameters were calculated, growth rate (μ_m, mm of diameter per day) and lag time (λ, days) using Microsoft Excel (Microsoft Corporation, USA). Those parameters were computed by plotting the diameter against time and applying the primary linear model to those time points that represented the linear phase of the growth curve. The associated correlation coefficient was ≥0.98. The regression line slope was considered as the growth rate, while the lag time was estimated as the interception between the regression line and the *x*-axis (Garcia *et al.*, 2009).

2. 4 *In vivo* study

2.4. 1 Selection of potato cultivars

The resistance to different diseases of seven of the most planted cultivars between 2011 and 2016 (GB Plantings by Variety, AHDB, 2018) is indicated in Figure 2-3. The resistance data was obtained from the AHDB Potato Variety Database (AHDB, 2016). Markies presented higher resistance than Maris Piper in soft rot, dry rot and late blight.

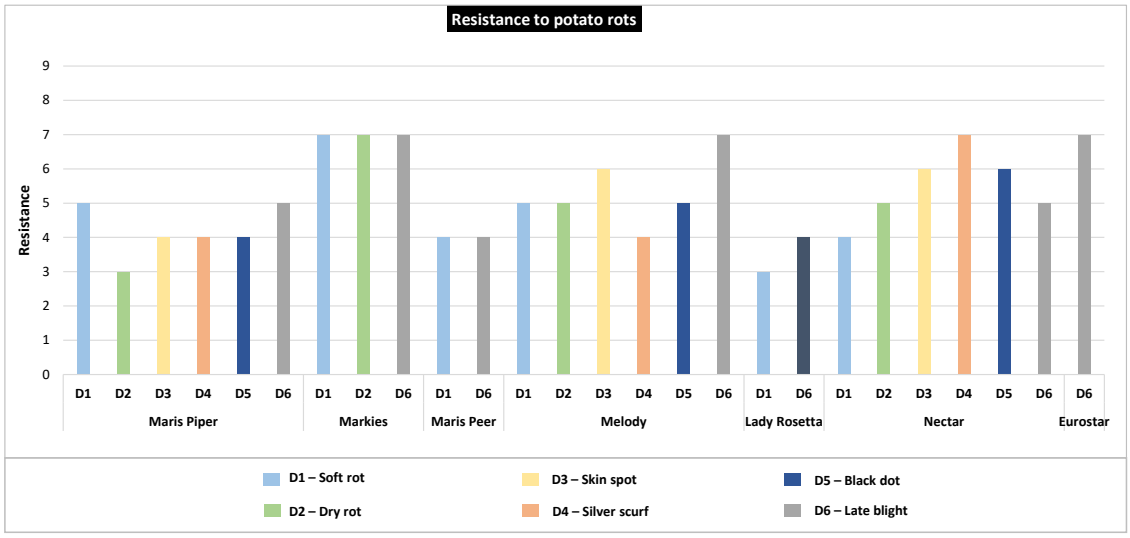


Figure 2-3 Resistance of Maris Piper, Markies, Maris Peer, Melody, Lady Rosetta, Nectar and Eurostar to six different potato diseases. Values from 1 to 9 represent the resistance of those cultivars, with being 9 the most resistant. This information was based on the UK National List testing and the AHDB Potatoes-funded Independent Variety Trials (IVT) programme (AHDB, 2017).

The first experiment that was carried out *in vivo* was with potato tubers from the harvest season 2017, cvs. Maris Piper, Nectar and Markies, provided by Glynn Harper (Sutton Bridge Crop Storage Research, UK). Potato tubers were inoculated with *Fusarium solani coeruleum, Pectobacterium c. atrosepticum* and *Boeremia foveata* separately and were incubated at 8.5ºC. However, no growth of any of the three pathogens was observed on potato tubers after 30 days. This was probably due to the application of different fungicides in the field that were directly affecting potato tubers.

After this problem, the decision was to use organic potato tubers. Therefore, a different study of the resistance of the different organic cultivars available was carried out based on the Potato Variety Database (AHDB, 2016) (Figure 2-4). There was a lack of data regarding the resistance of potato cultivars to gangrene.

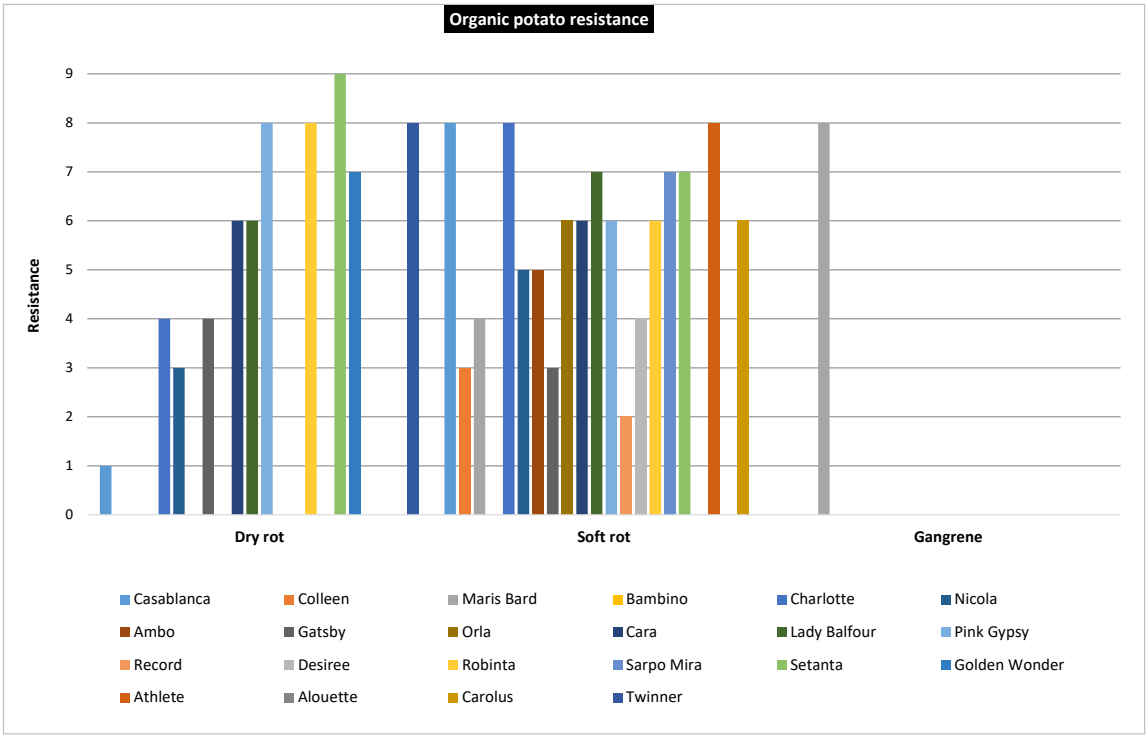


Figure 2-4 Resistance of 22 different organic potato cultivars to dry rot, soft rot and gangrene. Values from 1 to 9 represent the resistance of those cultivars, with being 9 the most resistant. This information was based on the UK National List testing and the AHDB Potatoes-funded Independent Variety Trials (IVT) programme (AHDB, 2018).

2.4.1. 1 Potato cultivars from the final *in vivo* experiment

In the final *in vivo* experiment, two different organic potato cultivars were used (cvs. Record and Casablanca). Organic potato tubers were selected due to the absence of any kind of fungicide during their growing and handling period. Both cultivars were seed potatoes and were supplied by Agrico UK Limited, Scotland. They were harvested on the 18th of September. Both cultivars presented different resistance to dry rot and soft rot based on the data provided by AHDB (AHDB 2017).

They arrived at Cranfield University on the 11th of October and were cooled down in Cranfield laboratory facilities by reducing one degree of temperature per day from 20.5ºC to 4.5ºC. Potato tubers were stored at 4.5ºC until the first experiment was started ("Early-stage"). The other half of potato tubers were kept at 4.5ºC and high relative humidity (98-99%) for 16 weeks until the start of the second experiment ("Mid-stage").

2.4. 2 Potato surface sterilisation

Different sterilisation treatments were tested. Two different reagents were used for the surface sterilisation: ethanol 70% (Ethanol 99%, Fisher Scientific, UK) or sodium hypochlorite (NaClO, 3%) at different concentrations (de Lacy Costello *et al.*, 2001; Kushalappa *et al.*, 2002; Tweddell, Boulanger and Arul, 2003a; Lui, Vikram, Abu-Nada, *et al.*, 2005).

The first trial consisted of a control with potato tubers non-treated and two different treatments where potato tubers were washed for 3 minutes with ethanol followed by their submersion in different concentrations of NaClO for ten minutes. The two different concentrations were 3 and 1.5% of NaClO. After their surface sterilisation, they were stored for seven days at 25ºC. The results, presented in Appendix E (Figure E-1), showed that those potato tubers treated with the highest concentration of NaClO were more rotten than the control after seven days of incubation. The concentration of NaClO and the time of exposure were damaging the skin of the potato.

The second trial consisted of the reduction of the concentration of NaClO and the increase in time, 15 minutes instead of 10. Results are shown in Appendix E (Figure E-2), where it can be observed that potato tubers sterilised with ethanol presented a more damaged skin that the ones treated with NaClO.

The last trial consisted of five different treatments: 1) a control, potato tubers not washed 2) a blank, potato tubers washed with sterile distilled water for 15 minutes 3) potato tubers treated with NaClO 1.5% for 15 minutes 4) treated with NaClO 0.5% for 15 minutes and 5) potato tubers washed for 3 minutes with ethanol

followed by 15 minutes in a solution of NaClO .0.5%. Pictures from the potato tubers are presented in Appendix E (Figure E-3).

In this case, the respiration rate of potato tubers was studied using a Sable Respirometer System (SABLE, Sable Systems, NV, USA) as described by Alamar et al. 2017 to ensure that the surface sterilisation of the potato tubers were not affecting their respiration rate during storage (Alamar 2017). Results, presented in Appendix E (Figure E-4), showed that those potato tubers treated with the highest concentration of NaClO and with ethanol presented a slightly high respiration rate, while the non-treated potato tubers and those tubers treated with NaClO 0.5% presented similar respiration rate.

Finally, potato tubers (cvs. Record and Casablanca) used in the *in vivo* experiment were surface sterilised as follow. Potato tubers were soaked in tap water for five minutes, and in distilled water for ten minutes. The rest of the soil presented on the surface of the tubers was removed with a scrubber. Once washed, they were submerged for 15 minutes in the sterilisation solution (0.5% NaClO). Afterwards, tubers were washed two times with sterile distilled water to eliminate all the residues from NaClO.

2.4. 3 Wounding, inoculation and incubation of potato tubers

Different inoculation methods were also tested prior to the *in vivo* study. Several methods previously used for the assessment of growth of different pathogens in potato tubers were tested (presented in Figure 2-5) (Jellis, 1975, 1982; Adams and Griffith, 1983; Bjor, 1987; de Lacy Costello *et al.*, 2001; Lui and Kushalappa, 2002; Choiseul, Allen and Carnegie, 2007). The inoculation method selected was the one with wounds of 5 mm in diameter. This specific size of lesion allowed the retention of the inoculum, while for the smaller lesion sizes, most of the inoculum suspension was all over the potato tuber. The grid methodology was discarded as it did not allow a uniform assessment of the internal and external lesion of the potato tuber.

Potato tubers were immersed in sterile distilled water for two to three hours before their inoculation. Subsequently, they were injured and inoculated under sterile

conditions. They were wounded with a 5 mm diameter cork borer. Four wounds were carried out on each potato tuber for the VOC monitoring and only one wound per tuber for the lesion assessment.

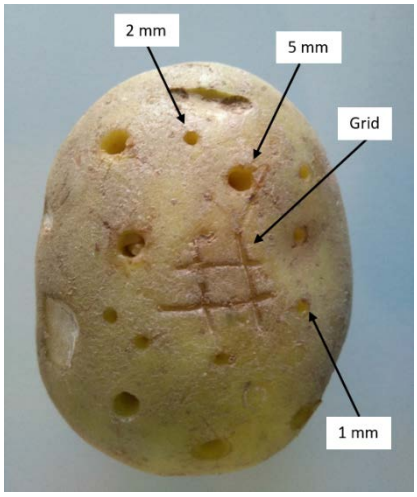


Figure 2-5 Potato tuber presenting the different inoculation methods tested. Wounds with different diameter sizes (1,2 and 5 mm) and the grid method.

***Fusarium sambucinum* inoculation** was carried out similarly as for the *in vitro* study (Section 2.3. 2). The inoculation of *Fusarium sambucinum*. on potato tubers was performed by adding 200 µl of the spore suspension on each of the 5 mm wounds previously carried out.

***Pectobacterium carotovorum atrosepticum* inoculation**: An aliquot of 500 µl of the glycerol stock was added in Nutrient Broth (NB) and incubated for one day at 30ºC. Then, they were subcultured in NB and incubated in a shaker at 30ºC and 120 rpm until a 10^6 cell/ml concentration was achieved. The inoculation of *Pectobacterium c. atrosepticum*. on potato tubers was performed by adding 200 µl of the culture on each of the 5 mm wounds previously carried out.

Twelve potato tubers were included in each replicate inside 12L air-tight boxes that were incubated at 8.5ºC under 98% of relative humidity. Each box had two different tubes, an inlet and an outlet. A 100 ml/min airflow was maintained through the inlet inside the 12L boxes to avoid the accumulation of CO_2. A relative humidity of 98% was achieved by including a layer of sterile distilled water at the bottom of each box. Every three to four days, new sterile water was added under sterile conditions. A 250 mL beaker with sterile distilled water was also included

on each box connected with the inlet of the box, creating bubbles in the water to increase the relative humidity inside the box.

2.4. 4 External and internal lesion assessment

The assessment of the lesions produced by *Fusarium sambucinum* and *Pectobacterium c. atrosepticum* was carried out differently for the external and the internal lesion.

External lesion assessment: Five potato tubers from the 12L boxes of each treatment were considered. Assuming the potato lesion to be round, the area of the rots (A) was calculated based on the area of a circle, where *r* was half of the average of the measured diameters of the rot. The value of the area of those potato tubers non inoculated (blank) was subtracted from the area of each rot at each specific day measured (Equation 1).

$$A = \pi r^2\ (Inoculated\ ) - \pi r^2\ (Blank)$$

Equation 1 Formula used to calculate the external infected area of the potato rots.

The infected area (mm^2) data was fitted to a linear model. This allowed the calculation of the infection rate (area mm^2/day) and lag time (days). Infection rate is the area of lesion that each of the pathogens was generating per day. While lag time, is represented in days, representing the time needed for those two specific pathogens to start generating any visible lesion on the potato tubers.

Internal lesion assessment: Three potato tubers from the 12L boxes of each treatment were cut in half. Pictures were recorded, and they were analysed using the ImageJ 1.52a software (National Institutes of Health, USA). The area of the rot of one half of the potato tuber was directly measured using the software. The first step consisted of setting up the scale (*Analyze>Set scale*), followed by the "Freehands selection" options presented in the menu. This tool allowed the selection of the part of the rot that will be considered for the calculation of the area. Once the area was drawn, the next step was measuring this area (*Analyze> Measure*). The mean of the three replicates (potato tubers) measured was considered for the internal lesion assessment.

2. 5 Volatile Organic Compound (VOC) methodology

The methodology used for the detection of the VOC produced in presence of the different pathogens was the Thermal Desorption coupled with Gas Chromatography, Time of Flight and Mass Spectrometry. Stainless-steel Thermal Desorption (TD) tubes were provided by Markes International (Markes International, UK).

2.5. 1 Thermal Desorption tubes selection

A selection of the optimal Thermal Desorption (TD) tube was carried out based on the structure of those VOCs expected in samples from potato tubers and at a low temperature (8.5^{o}C). Four different TD tubes were selected (Table 2-4) based on the adsorbent characteristics and the range of VOCs that were able to detect.

Table 2-4 TD tubes selected and their adsorbent characteristics (Markes International, UK)

Commercial name	**Adsorbent material**	**Sampling range (n^{o} of carbons)**
Tenax TA®	poly-(2,6-diphenyl)-p-phenylene oxide	C_7-C_{26}
Bio Monitoring®	poly-(2,6-diphenyl)-p-phenylene oxide + Carbon graphite	C_4-C_{30}
Air Toxics®	Carbon molecular sieve + carbon graphite	C_2-C_{14}
Universal®	poly-(2,6-diphenyl)-p-phenylene oxide + Carbon molecular sieve + carbon graphite	$C_{2/3}$-C_{30}
Tenax Sulficarb®	poly-(2,6-diphenyl)-p-phenylene oxide + sulphur + carbon molecular sieve	C_3-C_8

TD tubes were conditioned before sampling using a TC-20 tube conditioner (Markes International, UK) with Helium as a gas carrier. Their conditioning was carried out following the manufacturer's instructions. Each of the TD tube required a specific set of temperatures (Appendix F, Table F-1). After conditioning, tubes were capped with brass caps provided with PFTE ferrules (Markes International, UK) and stored at room temperature until they were required for sampling.

A first experiment was carried out using these five TD tubes. Cv. Maris Piper potato tubers (1200 g) were placed inside a nalophan bag (MediSense, Netherlands) and filled with 2 litres of compressed air. Bags were closed with flanges on one side and with a sampling tube made of an inert plastic material on the other side (Figure 2-6).

Figure 2-6 Potato tubers incubation in nalophan bags and air sampling using a TD tube connected to the Gil air plus Air Pump (Sensydine, USA).

Potato tubers were stored at room temperature for two hours, and after that VOC samples were sampled inside the TD with the help of a Gil Air Personal air pump (Sensydine, USA) at 100 ml/min. Three different volumes (1,2 and 3 L) were considered, the results are shown in Figure 2-7. It was observed that with Bio Monitoring and Tenax/Sulficarb a higher number of VOCs were detected. However, when those VOCs were classified depending on their number of carbons, differences were detected (Figure 2-8). In Air toxics TD tubes a high number of compounds with two and four carbons were detected, and in Bio Monitoring a high number of compounds of four and five carbons were detected.

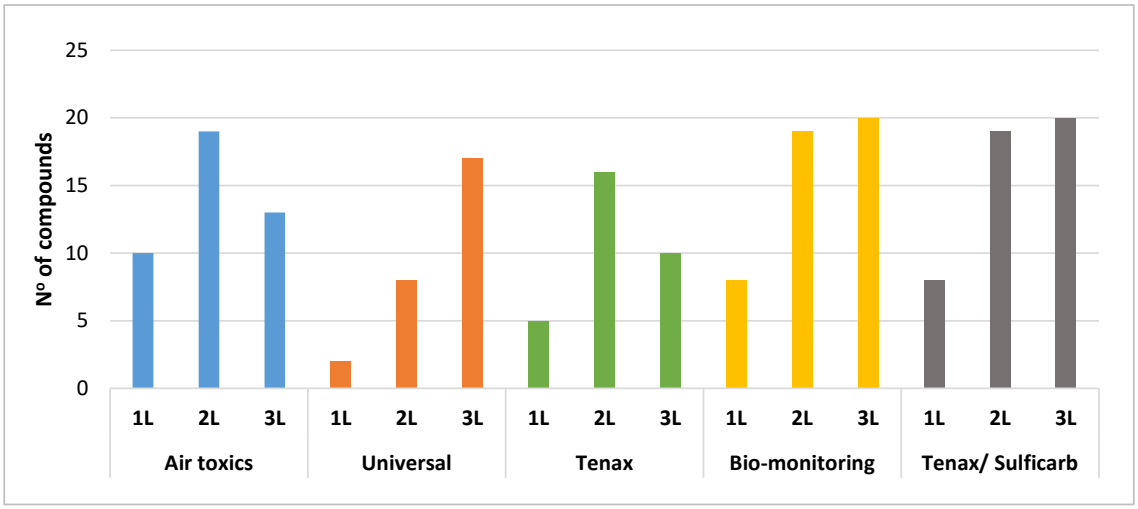


Figure 2-7 Number of Volatile Organic Compounds (VOCs) detected on samples from five different TD tubes (Air toxics, Universal, Tenax, Bio Monitoring and Tenax/Sulficarb) of potato tubers incubated at room temperature for two hours.

At low temperature during the storage of potato tubers, only small VOC will be volatile. Therefore, our interest was in those TD tubes able to absorb VOCs from C_2 to C_6. Bio Monitoring and Air toxics were selected as the optimal TD tubes for sampling VOC from potato tubers, as they detected the highest number of VOC with 2,4 and 5 carbons.

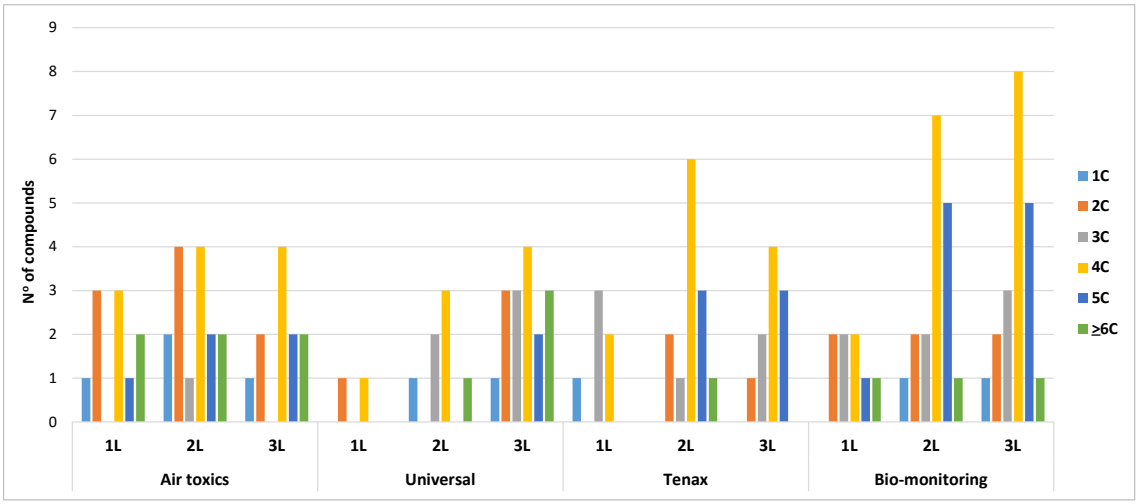


Figure 2-8 Number of Volatile Organic Compounds (VOCs), containing 1, 2, 3, 4, 5 or more than six carbons, detected on samples from five different TD tubes (Air toxics, Universal, Tenax and Bio Monitoring) of potato tubers incubated at room temperature for two hours.

2.5. 2 VOC sampling methodology

Volatile Organic Compound (VOC) samples were collected at different sampling points depending on the experiment from the 12 L containers. The containers were sealed, without any kind of ventilation through the inlet and outlet tubes, for two hours before the VOC sampling process to allow the VOCs to concentrate inside the box. Three replicates of VOC samples, one from each of the 12L boxes were considered.

Three litres of air were sampled from each container in a TD tube (Markes International, UK) using a Gil Air Personal air pump (Sensydine, USA) at 100mL/min.

Two different TD tubes were used in series in some of the experiments (Bio monitoring followed by Air toxics). VOCs that were not retained in the Bio Monitoring TD tube could be retained by Air toxics, as due to their different sorbent qualities (Table 2-4) they could adsorb compounds of different size. In addition, the use of two TD tubes also helped to account for the breakthrough volume, defined as the volume of sample that will purge an analyte through 1 gram of adsorbent resin in a desorption tube at a specific temperature. Therefore, if the breakthrough volume for any of the VOCs present in the samples was reached, we might be expecting them in the second in series TD tube.

The optimisation of the sampling procedure in series in order to determine the breakthrough volume would have been better using two TD tubes with the same adsorbent. However, as the VOCs that were expected in the samples were unknown it was very difficult to speculate which of them and at which concentration will be detected in our samples. As future work, a separate study on the breakthrough volume of the TD tubes for each of the selected VOCs would be needed.

Once the VOC samples were collected, tubes were capped with brass caps provided with PFTE ferrules (Markes International, UK) and stored at 4^{o}C until they were analysed (Figure 2-9).

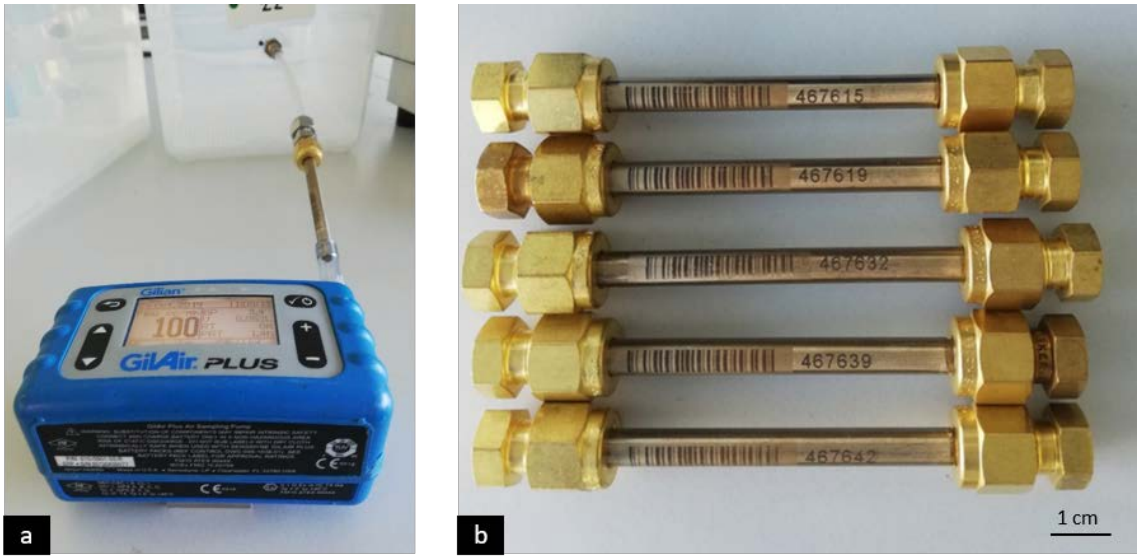


Figure 2-9 a) VOC sampling from 12L boxes using the Gil Air Personal air pump at 100 ml/min. b) Thermal desorption tubes with the brass caps (Markes International, UK)

2.5. 3 Thermal Desorption-Gas Chromatography-Time of Flight-Mass Spectrometry (TD-GC-ToF-MS)

Although no calibration curve based on standards was carried out for each of the VOCs, the identification of some of them was confirmed with standards (Appendix G, Table G-1).

To achieve the semi-quantification of the VOCs in the samples, an Internal Standard (IS), Toluene D-8 diluted in methanol (Sigma Aldrich, UK) was used. It was loaded on each of the tubes that contained air samples. TD tubes were attached to the calibration solution loading rig (Markes International, UK), loaded with 0.5 µl of Toluene D-8 (100 ng/µl) using a plunger-in-needle syringe (SGE Analytical Science, Australia) and supplied with a flow of 100 ml/min of Helium for three minutes. Once they were loaded with the IS, they were analysed by Thermal Desorption-Gas Chromatography-Time of Flight-Mass Spectrometry (TD-GC-ToF-MS).

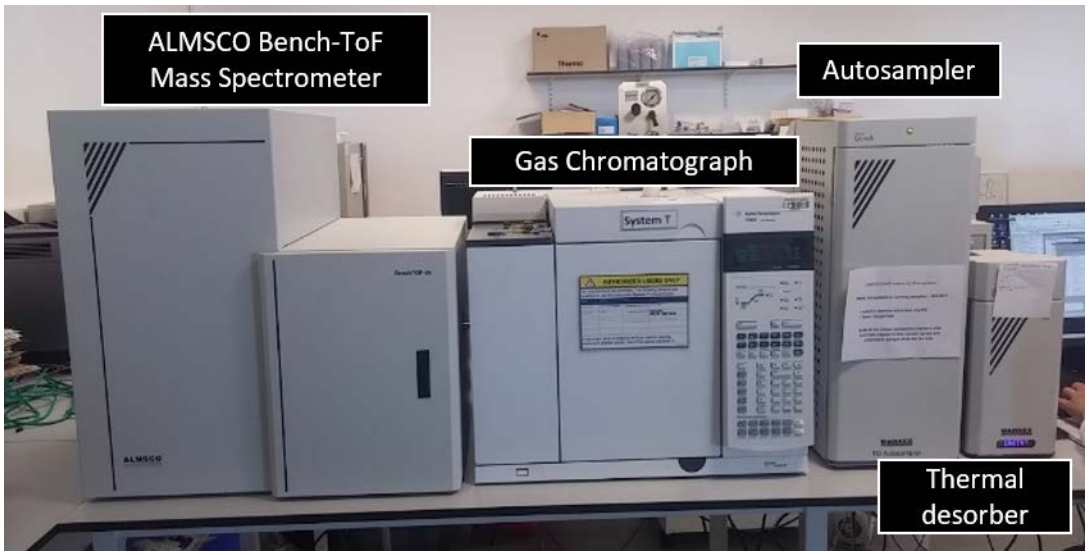


Figure 2-10 TD-GC-ToF-MS (Cranfield University)

Samples were placed on the UNITY-ULTRA-xr autosampler and tubes were desorbed using the thermal desorber UNITY-xr (Markes International, UK). Due to the different adsorbent loaded in each of the TD tubes used, two different cold traps were used for the desorption of Bio Monitoring and Air toxics, Material emissions and Sulficarb cold traps (Markes International, UK) were used respectively.

Once samples were desorbed they were analysed in an Agilent 7890 Gas Chromatograph using a DB6-624 semi-polar 60m x 0.4 mm x 0.25 mm column (Agilent Technologies LDA UK Ltd.), followed by the ALMSCO Bench-ToF Mass Spectrometer (ALMSCO International Ltd.) analysis.

The parameters set for each of the previous analysis is presented in Table 2-5. The split that was used for the desorption of the sample varied between 1:100 to 1:10 depending on the concentration of the samples. Prior to an analysis, five samples, expected to present the highest concentrations of VOC were run at the highest split and the non-analysed part of the sample was sent for recollection to a new conditioned TD tube. The resulted chromatograms were checked, and the next batch of samples were run at a specific split according to the previous results.

Table 2-5 TD-GC-ToF-MS parameters

Thermal Desorption (TD) parameters:
• 2 minutes of purge time
• Desorption at 280 °C for 10 minutes
Cold traps parameters:
• 2 minutes of purge time at 50 mL/min
• Trap low temperature: 25^{o}C
• Trap heat rate: 40^{o}C/s
• Trap high temperature: 300^{o}C
• Trap desorption time: 3 min
Gas Chromatography (GC) parameters:
• Effluent transfer time: 3 min/ 200°C/ 1.3 ml x min-1
• GC initial temperature: 50 °C
• 1.5 min 50 °C
• 2 °C / min increase until 75 °C
• 5 °C/ min increase until 140 °C
• 10 °C/ min increase until 200 °C
• GC oven temperature held at 200^{o}C for 2 minutes
Time of Flight (ToF)- Mass Spectrometry (MS)
• Eluted products transferred to the MS at 200 °C
• MS operated from 33- 480 amu / scan rate 2.2 Hz

2.5. 4 VOC data analysis

The VOC data analysis was conducted in two steps, untargeted and targeted analysis. The initial untargeted analysis consisted of the identification of VOCs that were detected in higher abundance in the presence of the fungal or bacterial pathogen compared to the control. The targeted analysis that followed consisted of the in depth study of each of the identified VOCs and the comparison of their abundance in presence and absence of the pathogen, between temperatures, between the a_w of the media and potato cultivars.

2.5.4. 1 Untargeted analysis

Different approaches have been used for the analysis of the VOCs during the period of this PhD. The first one consisted of the use of an open software, *Openchrom 1.3.0* (Openchrom®), that allowed the analysis of chromatograms and their correspondent mass spectrums. The chromatograms were filtered, a baseline was set, peaks were detected and then identified by their link with AMDIS (Automated Mass Spectral Deconvolution and Identification System, version 2.72) based on the National Institute of Standards and Technology (NIST)2004. The comparison between samples from infected potatoes and the control was carried out manually by the overlapping of the chromatograms.

The second approach consisted of the use of an online-based informatics platform, XCMS (https://xcmsonline.scripps.edu). A pairwise analysis was carried out, setting the parameters as presented in Table 2-6, based on previous studies (Smith *et al.*, 2006; Tautenhahn *et al.*, 2012; Forsberg *et al.*, 2018). No annotation neither identification was set as this software does not contain the VOC library needed for Gas Chromatography samples.

Table 2-6 Set of parameters for the XCMS pairwise analysis

XCMS section	Parameters
Feature detection ***Central Wave Detection Method***	Ppm: 10; Minimum peak width: 2; Maximum peak width: 7.5; Signal/Noise threshold: 6; mzdiff: 0.01; Integration method: 2; Prefilter peaks: 2; Prefilter intensity: 100, Noise filter: 0
Retention time Correction ***Obiwarp algorithm***	profStep: 1
Alignment	Bw: 5; minfrac: 0.5; mzwid: 0.025; minsamp: 1; max: 100
Statistics	Unpaired parametric t-test (Welch t-test) Perform post-hoc analysis: True; p-value threshold: 0.01; fold change threshold: 1.5
Visualization	EIC width: 200

The pairwise analysis was carried out for each of the different conditions, comparing the features detected on those samples from non-inoculated and inoculated NPDA or potato tubers. This analysis resulted in different retention times (RT), corresponded to a potential VOC, with a specific fold change (FC). The FC value represents the differences in peak abundance at this specific retention time between the absence and presence of the pathogen. Those peaks (RT) with a higher FC and a p-value lower than 0.05 were selected as potential compounds detected in presence of each of the specific pathogen studied compared to the non-inoculated plates or potato tubers. The elucidation of which compounds corresponded to each specific RT was achieved using AMDIS (AMDIS version 2.72), a deconvolution software that deconvoluted, integrated and identified each peak resulted from the TD-GC-ToF-MS analysis. The identification was carried out based on the 2014 NIST library (National Institute of Standard and Technology, US Government) linked with the AMDIS. However, after the targeted analysis of the selected VOCs, it was observed that in some cases their abundance was not higher in presence of the different pathogens as it was predicted by the untargeted analysis conducted using XCMS. Those inconsistences were attributed to the intended use of this online-based software as it was created for the analysis of Liquid Chromatography-Gas Chromatography data.

The final untargeted analysis approach, and the one adopted for the rest of the data of this thesis, was carried out using two different software packages; Mass Profiler Professional and AMDIS. Mass Profiler Professional software (*MPP 12.6.1*, Agilent Technologies) allows the exploration of relationships in GC/MS data, regardless of whether the peaks are identified or unknown. GC-MS data were processed into specific format files (ELU and FIN files) using AMDIS (Automated Mass Spectral Deconvolution and Identification System, version 2.72) based on the 2014 NIST library (National Institute of Standard and Technology, US Government). The MPP software has been previously used for the analysis of GC-MS samples in different studies (Gu *et al.*, 2011; Musharraf *et al.*, 2016; Wei, Gao and Ding, 2018). The information on extracted ions presented

in the ELU and FIN files was imported into the MPP software to process the resulted data.

The data was first grouped as blank and “inoculated” with the specific pathogen when comparing between treatments at each day, and grouped by sampling day when comparing between days. Different parameters were set as presented in Table 2-7. An important parameter is the ‘Minimum absolute abundance’ presented in counts; this allows the software to set a minimum peak intensity to be considered. Therefore, all of the VOCs that were detected at lower intensity will not be considered for the comparison analysis. The selection of the optimal minimum absolute abundance was carried out based on the counts detected for the IS (Toluene) on each group of samples and comparing it with the rest of the VOCs presented in the chromatogram. An analysis where the entities were filtered based on their FC calculated from statistical analysis (t-test, unpaired) was carried out. The results were observed in Fold Change (FC) values, providing information about how high the differences were between the selected groups. Those compounds detected significantly different (p-value<0.05) and with a FC higher than 1.5 were included in the results tables and selected as interesting compounds to study.

Table 2-7 Set of parameters for the MPP analysis

MPP section	Parameters
Filtering	Minimum absolute abundance: 800 counts; Retention time filtering: 4-27 min; Number of ions required: 3; Minimum Quality Score: 30
Normalization criteria	Normalization algorithm: Internal standard (Toluene D8)
Filtering flags	Present in at least 65% of the values in any 1 out of 2 conditions
Filter by Frequency	Retain entities that appear in at least 65% of samples in at least one condition
Significance analysis	T Test unpaired, Benjamini-Hochberg (Multiple Testing Correction)

An intermediate step in the VOC analysis consisted of the calculation of the Total Targeted Volatile Production (TTVP) of each of the treatments at each specific sampling day. The TTVP consisted of the sum of the relative peak area (area of each of the VOC normalised by the IS area) of a list of VOCs selected based on the previous untargeted analysis. Although it is named as total targeted volatile production is only including the production of the targeted VOCs. This TTVP could provide useful information regarding the effect of this group of VOCs in the presence and absence of the different pathogens studied.

2.5.4. 2 Targeted analysis

The second step was a targeted analysis, where a targeted library was created in AMDIS, including those compounds previously detected with higher abundance on the containers in presence of the “inoculated” plates or potato tubers. Those VOCs that presented higher FC than 1.5 in the previous untargeted analysis, where the absence and presence of the pathogens was compared, were selected. The chromatograms were filtered, a baseline was set, peaks were detected and then identified by its link with AMDIS (Automated Mass Spectral Deconvolution and Identification System, version 2.72) and the targeted library, previously created.

A data file with the potential compounds and their peak area was created in Microsoft Excel 2016 (Microsoft Office, US). The data was normalised against the IS (Internal Standard), resulting into the ‘Relative Peak Area’ or each VOC of interest. The abundance of each VOC was finally expressed as area per grams of potato tubers or NPDA and per litre of air sampled. A comparison between abundances of those targeted compounds was carried out between VOC samples from the containers in presence of a pathogen compared to its absence. It was considered as a semi-quantification.

2. 6 Mycotoxin extraction

Three different sampling points during the incubation time (7, 14 and 21 days) were selected for the detection of mycotoxins in presence and absence of *Fusarium sambucinum* and *Fusarium oxysporum.*

The extraction of the mycotoxins was carried out following the same protocol as Malachová 2014 (Malachová *et al.*, 2014). The preparation of the samples for the mycotoxin extraction consisted of three plugs of NPDA of 5 mm of diameter that were submerged in Liquid Nitrogen (N_2) and homogenized with 450-600 μm of glass beads using the Precellys 24 Tissue homogenizer (Bertin Instruments, France). They were agitated in the Tissue Homogenizer at 5500 rpm for 20 s followed by a 5 s interval and another 20 s of agitation. Once the samples were homogenized, a volume of extraction buffer (acetonitrile: water: formic acid, 79:20.9:0.1, v/v/v) was added to the samples and they were homogenized again in the Tissue Homogenizer to mix the sample with the extraction buffer. The volume of the extraction buffer was four times the weight of each of the samples. The samples were then left for incubation for 90 min at 25ºC at 300rpm on a rotary shaker in the dark. Afterwards, the extracts were centrifuged for 10 min at 22600 g. The supernatant (200μl) was then transferred to HPLC vials containing 250 μl microinserts (Fisher Scientific, USA) and kept at -20ºC until the analysis.

2. 7 Mycotoxin analysis by UHPLC-MS-MS

The analysis of the mycotoxins was performed in an Exion series Ultrahigh Performance Liquid Chromatography (UHPLC) system coupled with a 6500+ qTRAP-MS system coupled with IonDrive™ Turbo Spray (both Sciex Technologies, Warrington, UK) (UHPLC-MS-MS). Chromatographic separation was achieved on a reversed-phase ACE 3-C_{18} column (2.1x100mm, 3μm particle size; Hichrom) equipped with a C_{18} security guard cartridge (4x3mm, Gemini, Agilent) kept at 40ºC. A specific method was created based on Malachová 2014 method including the targeted mycotoxins (Malachová *et al.*, 2014), presented in Appendix H (Table H-2, Table H-2). The elution was carried out in a binary gradient mode, with two eluents, A: methanol/water/acetic acid 10:89:1 and B: methanol/water/acetic acid 97:2:1 (v/v/v), both containing 5mM of ammonium

acetate. A standard mix (5, 1, 0.5, 0.2, 0.1, 0.05 & 0.01 mg/L) including the 14 mycotoxins (Appendix H) that were studied, were analysed together with the samples to achieve the concentration of mycotoxins in the samples.

The analysis of the results obtained with the LC-MS were analysed using Multiquant 3.03 software (AB Sciex, Foster City, California, USA). The peaks were integrated and 1/x weighted linear calibration curves were carried out for each of the mycotoxins evaluated to evaluate the linearity of the method. The concentration of each of the mycotoxins was calculated and further data evaluation was carried out in Microsoft Excel 2016 (Microsoft office, US).

2. 8 Statistical analysis

Statistical analysis was performed using the JMP Pro 14 (SAS Institute, INC., Car, NC, USA) package. Data sets were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests, respectively. Those datasets that succeeded in the previous test were analysed using parametric test, ANOVA. When statistically significant differences were found (p-value<0.05), *post hoc* parametric comparisons for each pair using the Tukey-Kramer HSD method were performed.

Those datasets that did not succeed for the normality and homoscedasticity test were transformed. In those cases where after the transformation, the datasets failed both tests, a non-parametric test, Kruskal-Wallis, was performed. When significant differences were detected (p-value< 0.05), each pair was compared by a *post hoc* Wilcoxon test.

A Principal Component Analysis (PCA) was carried out for the VOC results as part of the VOC analysis using MPP software.

3 ECOPHYSIOLOGICAL STUDY AND VOLATILE ORGANIC COMPOUND PRODUCTION OF *BOEREMIA FOVEATA IN VITRO*

3. 1 Introduction

Gangrene is an important potato storage disease produced by *Boeremia foveata* (previously known as *Phoma exigua* var. *foveata*). It was one of the most serious fungal storage potato disease in the United Kingdom in the '70s. Potato tubers are harvested once a year and then stored at low temperatures (2-10ºC) and high relative humidity (90-100%) for up to 10 months. The temperature and relative humidity can vary between different positions of the storage rooms. Those potato tubers located at the top of the crates will be more exposed to the ventilation and refrigeration, therefore a lower temperature and relative humidity will be present. While the opposite occurs with those tubers located at the bottom of the crates, slightly higher temperature and relative humidity will prevail (Cunnington, 2018).

Maintaining the optimal environmental conditions in a storage room is essential to reduce the appearance of potato diseases. However, sometimes even under optimal storage conditions different potato pathogens, as *Boeremia foveata,* can appear on potato tubers. The main source of *B. foveata* inoculum during storage are affected tubers that by direct contact will infect the surrounding tubers (Schöber and Turkensteen, 1992). In 2007 a survey was carried out to decipher which fungi was responsible for most of the potato diseases in Scotland seed potatoes. *B. foveata* incidence on potato tuber was higher than *Fusarium avenaceum* (dry rot) and *Cylindrocarpon* spp. (pit rot) (Choiseul, Allen and Carnegie, 2007).

Boeremia foveata incidence on potato tubers has been considerably studied. The effect of different wounding methods, different *B. foveata* isolates, cultivars and different initial fungal inoculum concentration has been studied (Malcomsom and Gray, 1968; Entwistle, 1971; Rogers and Killick, 1975; Adams, 1980; Adams and Hide, 1980; Jellis, 1981, 1982; Bain, Lennard and Wastie, 1982; Bjor, 1987; Zhao and Shamoun, 2006). As it can be observed, no new research has been produced

in the last decade regarding the development of gangrene in potato tubers during storage.

The use of fungicides for the control of *B. foveata* has also been studied. Benomyl, thiabendazole and 2-aminobutane were the three main fungicides used for the control of gangrene in potato tubers in the '80s and '90s (Hide and Cayley, 1980, 1989; Carnegie *et al.*, 1988; Bång, 1992). Due to the ineffectiveness of some fungicides, the development of new methods for the detection of *B. foveata* in potato tubers has been studied. Many studies have been carried out based on a direct method, such as the Polymerase Chain Reaction (PCR) methodology. However, this is a destructive method where potato samples are required and consequently wasted (Cullen *et al.*, 2000, 2007; A'Hara, 2015).

A non-destructive method based on volatile organic compound (VOC) detection would be a potential solution for an early detection of gangrene in potato tubers during storage. Some of its advantages are the non-destructiveness of the sample and time-saving. The presence of a specific VOC, biomarker of gangrene in potatoes, can be detected as soon as it is produced without the need of a visual sign. Some VOC might be produced before the lesion can be visually detected. However, with the direct methods, it cannot be detected until farmers discover the presence of a rotten potato tuber.

Several studies have already reported the use of Gas Chromatography for the detection of VOCs from potato tubers infected with soft rot and dry rot (de Lacy Costello *et al.*, 1999, 2001; Sinha *et al.*, 2017; Rutolo *et al.*, 2018). One single study was carried out on *B. foveata* where the distinction between *B. foveata* infected and non-infected potato tubers was achieved based on the resulted Gas Chromatography-Mass Spectrometry fingerprint. However, an identification of the VOC detected was not included in this study (Weijman *et al.*, 1984).

The two aims of this research were 1) elucidate the effect of three different temperatures (5, 10 and 15^{o}C) and two different water activities (0.98, 0.99) on the ecophysiology of *B. foveata* in a semi-synthetic media (Potato Dextrose Agar) 2) study the VOC profile under those environmental conditions by Thermal

Desorption-Gas Chromatography-Time of Flight-Mass Spectrometry (TD-GC-ToF-MS).

3. 2 Materials and methods

3.2. 1 *Boeremia foveata* strain

Boeremia foveata, the principal pathogen responsible for gangrene on potato tubers, was previously prepared as mentioned in Section 2. 1. An aliquot of the glycerol stock was inoculated in PDA and incubated for two days at 25ºC prior to each of the experiments was used as inoculum.

3.2. 2 Inoculation and incubation of *Boeremia foveata*

The inoculation of *B. foveata* on potato-based media was performed as mentioned in Section 2.3. 2. Three different temperatures (5, 10 and 15ºC) and two different a_w (0.98, 0.99) were used as presented in Figure 3-1. The preparation of the NPDA and its a_w modification was carried out as mentioned in Section 2.3. 1 and 2.3.1.1. 2, respectively. The containers with 0.98 and 0.99 a_w NPDA plates were stored at 5, 10 and 15ºC for 15 days.

Three replicates (12L airtight boxes) were included per temperature and a_w with NPDA plates inoculated with *B. foveata*. One box was included per treatment with non-inoculated NPDA plates. All samples were included for the VOC sampling.

3.2. 3 Fungal growth assessment of *Boeremia foveata*

Fungal growth was assessed daily for 15 days after the inoculation as mentioned in Section 2.3. 3.

3.2. 4 Volatile Organic Compound (VOC) sampling and analysis

VOC sampling was carried out following the same procedure as the one indicated in Section 2.5. 2. Bio Monitoring TD tubes were used in this experiment for the sampling of the VOC from potato-based media inoculated with *B. foveata* and non-inoculated. VOC samples were collected after 0, 1, 2, 3 and 15 days of incubation at 5, 10 and 15ºC.

The VOC samples were analysed by Thermal-Desorption-Gas Chromatography-Time of Flight-Mass Spectrometry (TD-GC-ToF-MS) as mentioned in Section 2.5. 3.

3.2. 5 Volatile Organic Compounds (VOCs) data analysis

The data analysis followed was similar to the one mentioned in Section 2.5. 4. First an untargeted analysis, followed by a targeted analysis where those compounds that were detected in higher abundance in presence of *Boeremia foveata*, compared to the blank were selected as the most interesting VOC to be studied.

3.2. 6 Statistical analysis

The statistical analysis performed was the same as mentioned in Section 2. 8. The comparisons were carried out to check if significant differences were detected between treatments (*Boeremia foveata*. inoculated and non-inoculated NPDA), between the three temperatures (5, 10, 15ºC), the two a_w (0.98, 0.99) and the different sampling points (0, 1, 2, 3 and 15 days).

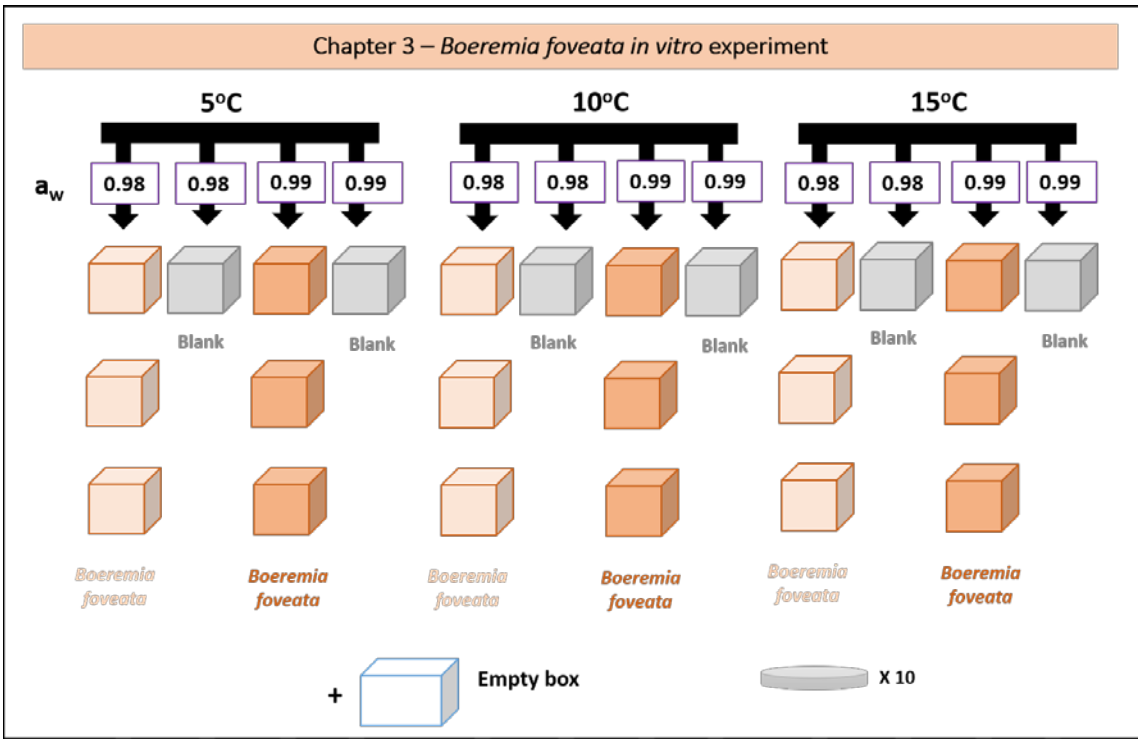


Figure 3-1 Experimental design of *Boeremia foveata in vitro* experiment. Three temperatures (5,10,15ºC) and two a_w (0.98, 0.99) were used. Ten NPDA plates inoculated with *B. foveata* or non-inoculated (blank) were included on each 12L box. VOCs were sampled after 0,1,2,3 and 15 days of incubation.

3. 3 Results

3.3. 1 Effect of temperature and a_w on *Boeremia foveata* growth parameters on potato-based media

Boeremia foveata growth on potato-based media was highly influenced by temperature and water activity. Figure 3-2 shows the effect of three different temperatures and two water activities on the growth rate (mm of diameter/day) and lag time (days) of *B. foveata* in NPDA. The maximum growth rate was achieved at the highest temperature with a value of 10 mm of diameter per day. In contrast, the lag time was less than two days and presented the lowest value at 15ºC where *B. foveata* started to grow in less than a day.

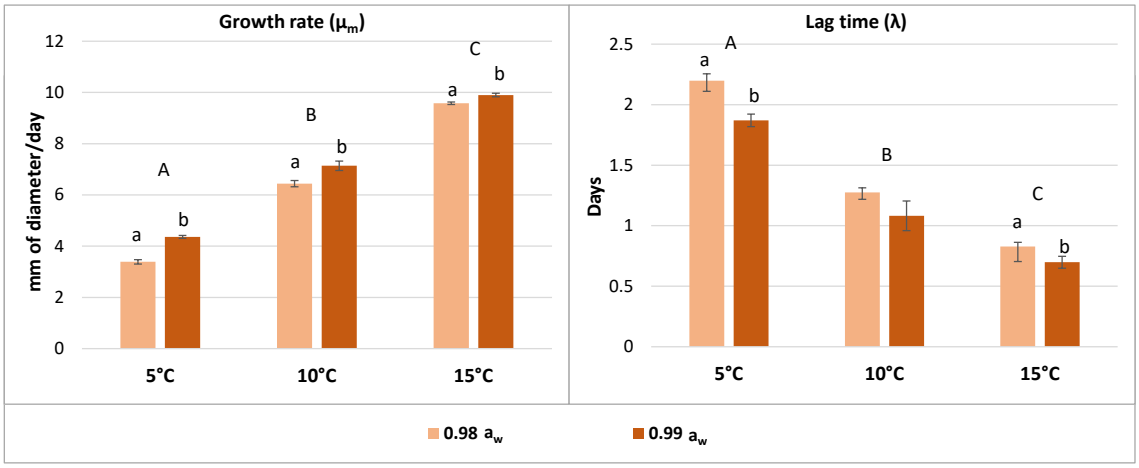


Figure 3-2 Effect of temperature (5, 10, 15ºC) and a_w (0.98, 0.99) on *Boeremia foveata* growth rate (mm of diameter/day) and lag time (days) on potato-based media. Data shows means of six replicates (colonies) with bars indicating standard deviation (SD). A,B,C: Significant differences detected between temperatures at each a_w (t-test, Wilcoxon test, p-values<0.05). a,b: Significant differences between a_w at each specific temperature (ANOVA, Kruskal-Wallis test, p-value<0.05).

Overall, the growth rate of *B. foveata* was significantly higher (p-values<0.01) at higher temperatures and a_w. While the reverse tendency was observed on lag time when it was incubated at higher temperatures and a_w (p-values<0.05).

3.3. 2 Volatile Organic Compound (VOC) detection in presence of *Boeremia foveata* under different environmental conditions

3.3.2. 1 Untargeted analysis

Differential VOCs were detected in presence and absence of *Boeremia foveata* in potato-based media at different stages of the incubation time (0, 3 and 15 days). Three and fifteen days of incubation showed different VOCs and with different Fold Change (FC) regarding the abundance in those samples from plates with *B. foveata* compared to the non-inoculated plates. The FC, was an indicator of how were the differences between the two different samples that were compared.

Results from the first analysis carried out with MPP software, comparing VOCs detected at the start of the experiment (day 0) and after 15 days of incubation from *B. foveata* inoculated plates are shown in Table 3-1.In this comparison, the abundance of each of the VOC detected in the samples were compared. The FC values of those compounds, how high was the difference after 15 days of storage compared to the start of the incubation, were between 10^4 to 10^6. Two of those compounds, ethanol and methyl acetate, were detected in common at the three different temperatures with different FC values. A direct positive relationship was observed between the FC detected and the environmental conditions of the incubation (temperature, a_w). Acetic acid and 3-methyl-1-butanol were only detected with significantly higher abundance after 15 days of incubation only at the highest temperature (15ºC), as presented in Table 3-1.

When comparing VOCs detected after 15 days of incubation from non-inoculated plates and *B. foveata* inoculated plates, results are shown in Table 3-2. At 5ºC compounds as ethanol, acetone, methyl acetate, 1-propanol and 2-methylpropan-1-ol were detected with a Fold Change higher than 10^5 times compared to the one found in the non-inoculated plates. At 10ºC, few VOCs were detected with a higher abundance in presence of *B. foveata*. While at the highest temperature (15ºC), there was an increase in the number of VOCs detected with higher abundance in presence of *B. foveata.*

Table 3-1 Fold change (FC) values of VOCs detected in significantly higher abundance in presence of *Boeremia foveata* incubated in NPDA at 5, 10 and 15ºC and two different a_w (0.98, 0.99) after 15 days of incubation compared to the start of the incubation period (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected between the two stages of the incubation period (p-value<0.05).

RT (min)	Volatile Organic Compound (VOC)	5ºC		10ºC		15ºC	
		0.98 a_w	0.99 a_w	0.98 a_w	0.99 a_w	0.98 a_w	0.99 a_w
8.86	Ethanol	6.66E+04	2.14E+05	1.03E+05	4.97E+06	8.45E+06	4.68E+06
8.92	Acetone	NS	1.13E+07	1.63E+07	NS	NS	NS
10.68	Methyl acetate	2.23E+05	2.90E+05	1.08E+06	4.27E+06	4.42E+06	2.84E+06
13.21	1-Propanol	5.96E+04	NS	NS	NS	NS	NS
14.81	Ethyl Acetate	4.06E+04	NS	NS	NS	NS	2.45E+06
17.44	Propan-2-yl acetate	3.42E+05	NS	NS	NS	6.62E+05	NS
18.49	Acetic acid	NS	NS	NS	NS	9.75E+05	NS
23.08	3-Methyl-1-butanol	NS	NS	NS	NS	3.12E+05	NS

Table 3-2 Fold change (FC) values of VOCs detected in significantly higher abundance in presence of *Boeremia foveata* compared to the non-inoculated plates after 15 days of incubation at 5, 10 and 15ºC and two different a_w (0.98, 0.99) (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected between the absence and presence of *B. foveata* (p-value<0.05). *: VOCs detected in common with the previous analysis (Table 3-2)

RT (min)	Volatile Organic Compound (VOC)	5ºC		10ºC		15ºC	
		0.98 a_w	**0.99 a_w**	**0.98 a_w**	**0.99 a_w**	**0.98 a_w**	**0.99 a_w**
8.86	Ethanol *	NS	6.56E+06	1.56E+06	1.86E+07	2.75E+07	1.56E+07
8.92	Acetone *	NS	NS	NS	NS	9.11E+02	NS
10.68	Methyl acetate *	NS	8.90E+06	NS	NS	1.44E+07	9.47E+06
12.04	2-Methyl-1-pentene	NS	NS	NS	NS	1.75E+06	NS
13.21	1-Propanol *	NS	8.17E+05	NS	NS	NS	NS
14.81	Ethyl Acetate *	NS	NS	NS	NS	NS	8.16E+06
17.11	2-Methylpropan-1-ol	NS	3.64E+06	NS	2.84E+07	4.26E+07	2.72E+07
17.44	Propan-2-yl acetate*	NS	NS	NS	NS	2.15E+06	NS
18.49	Acetic acid *	NS	NS	NS	NS	3.17E+06	NS
23.08	3-Methyl-1-butanol *	NS	NS	NS	NS	1.01E+06	NS

When comparing the results based on the a_w of the media (Table 3-2), significant differences on the abundance of each of the VOC between the presence and absence of *B. foveata* were mainly detected at the highest a_w (0.99) at 5 and 10ºC, while at 15ºC higher FC was observed at the lowest a_w (0.98).

Some of the VOCs detected with higher abundance when comparing day 0 with day 15, were also detected when the comparison was carried out between those VOCs detected in presence of *B. foveata* compared to the non-inoculated plates (Table 3-2).

A comparison between day 0 and day 3 was carried out at the different temperatures and a_w (Table 3-3). The number of VOCs detected with significant differences in abundance after 3 days of incubation were lower compared to those detected after 15 days of incubation. No VOCs were detected at 5ºC. VOC as methyl acetate and propan-2-yl acetate were detected in common with the results after 15 days of incubation. Different VOCs at the ones that were detected after 15 days of incubation were found as acetaldehyde, methyl formate, 2-methylpentane and 3-methylpentane.

The detection of the VOCs in presence of *B. foveata* compared to the non-inoculated plates was also studied after 3 days of incubation (Table 3-4). Similar results were achieved as after 15 days of incubation, although in this case most of the compounds were detected at the highest temperature (15ºC). The FC values were also lower than the ones after 15 days. Besides, after 3 days VOCs detected in significantly higher abundance in presence of *B. foveata* were only detected at the highest a_w at 10ºC, while at 15ºC a higher number of VOCs were detected at the lowest a_w.

In Table 3-5, a summary of the main VOCs of interest related with *B. foveata* detected after three and fifteen days of incubation (early and late stage) are presented. Compounds as 2-methylpropane and butane were found with the highest FC value after three days of incubation. While dimethyl carbonate and acetic acid were detected after 15 days of incubation at different temperatures. Compounds as methyl acetate, hexane and 2-methylpropan-1-ol were found in common between both days.

Table 3-3 Fold change (FC) values of VOCs detected in significantly higher abundance in presence of *Boeremia foveata* incubated in NPDA at 5, 10 and 15ºC and two different a_w (0.98, 0.99) after 3 days of incubation compared to the start of the incubation period (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected between the two stages of the incubation period (p-value<0.05).

RT (min)	Volatile Organic Compound (VOC)	5ºC		10ºC		15ºC	
		0.98 a_w	0.99 a_w	0.98 a_w	0.99 a_w	0.98 a_w	0.99 a_w
6.89	Methyl formate	NS	NS	NS	9.41E+04	NS	NS
10.68	Methyl acetate	NS	NS	NS	6.49E+05	1.17E+06	NS
10.86	2-Methylpentane	NS	NS	NS	NS	6.74E+05	NS
11.55	3-Methylpentane	NS	NS	NS	NS	1.04E+04	NS
12.77	Propan-2-yl formate	NS	NS	1.12E+07	NS	NS	NS
17.44	Propan-2-yl acetate	NS	NS	NS	4.23E+05	3.57E+05	NS

Table 3-4 Fold change (FC) values of VOCs detected in significantly higher abundance in presence of *Boeremia foveata* compared to the non-inoculated plates after 3 days of incubation at 5, 10 and 15ºC and two different a_w (0.98, 0.99) (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected between the absence and presence of *B. foveata* (p-value<0.05). *: VOCs detected in common with the previous analysis (Table 3-3)

RT (min)	Volatile Organic Compound (VOC)	5ºC		10ºC		15ºC	
		0.98 a_w	0.99 a_w	0.98 a_w	0.99 a_w	0.98 a_w	0.99 a_w
6.89	Methyl formate*	NS	NS	NS	3.01E+05	NS	NS
7.28	2-Methylbutane	NS	NS	NS	2.34E+00	2.17E+06	NS
10.68	Methyl acetate*	NS	NS	NS	NS	2.85E+06	NS
10.86	2-Methylpentane*	NS	NS	NS	NS	1.64E+06	1.42E+06
11.55	3-Methylpentane*	NS	NS	NS	NS	2.52E+04	6.68E+04
12.04	2-Methyl-1-pentene	NS	NS	NS	NS	7.26E+05	NS
12.08	Isopropyl Alcohol	NS	NS	NS	NS	1.61E+06	NS
17.44	Propan-2-yl acetate*	NS	NS	NS	1.35E+06	8.66E+05	1.96E+07
18.49	Acetic acid	NS	NS	NS	NS	NS	5.67E+06

Table 3-5 VOC fingerprinting of *Boeremia foveata* culture in a potato-based media after 3 and 15 days of incubation at three different temperatures (5,10 and 15ºC) and two water activities (0.98,0.99). The retention time (RT) of each of the VOC is included in minutes. * VOC detected at both stages.

Early-stage		Late-stage	
RT	**Compound**	**RT**	**Compound**
		8.86	Ethanol
		8.92	Acetone
10.68	Methyl acetate *	10.68	Methyl acetate*
10.86	2-Methylpentane	14.81	Ethyl Acetate
11.55	3-Methylpentane	17.11	2-Methylpropan-1-ol
17.44	Propan-2-yl acetate *	17.44	Propan-2-yl acetate *
18.49	Acetic acid*	18.49	Acetic acid*

3.3.2. 2 Targeted analysis results using AMDIS

A targeted analysis was then carried out with those compounds included in Table 3-5. In Figure 3-3, those VOCs detected in presence and absence of *Boeremia foveata* after 15 days of incubation at the different conditions are shown. As a tendency, the VOC abundance was higher at the highest temperature. In all cases, the relative abundance of the VOC was higher in presence of *Boeremia foveata* compared to its absence. Significant differences (p-value < 0.05) in VOC abundance between B. *foveata* inoculated and non-inoculated plates were detected mainly at 10 and 15ºC only for ethanol and ethyl acetate. At 15ºC and 0.98 a_w, ethanol abundance was significantly higher (p-value < 0.02) compared to the rest of the temperatures. Temperature only presented a significant positive effect (p-value<0.05) on the production of acetone, while no significant differences were detected in the rest of the VOCs represented (p-values>0.10). No significant differences were detected between both water activities (0.98, 0.99) (p-values>0.05).

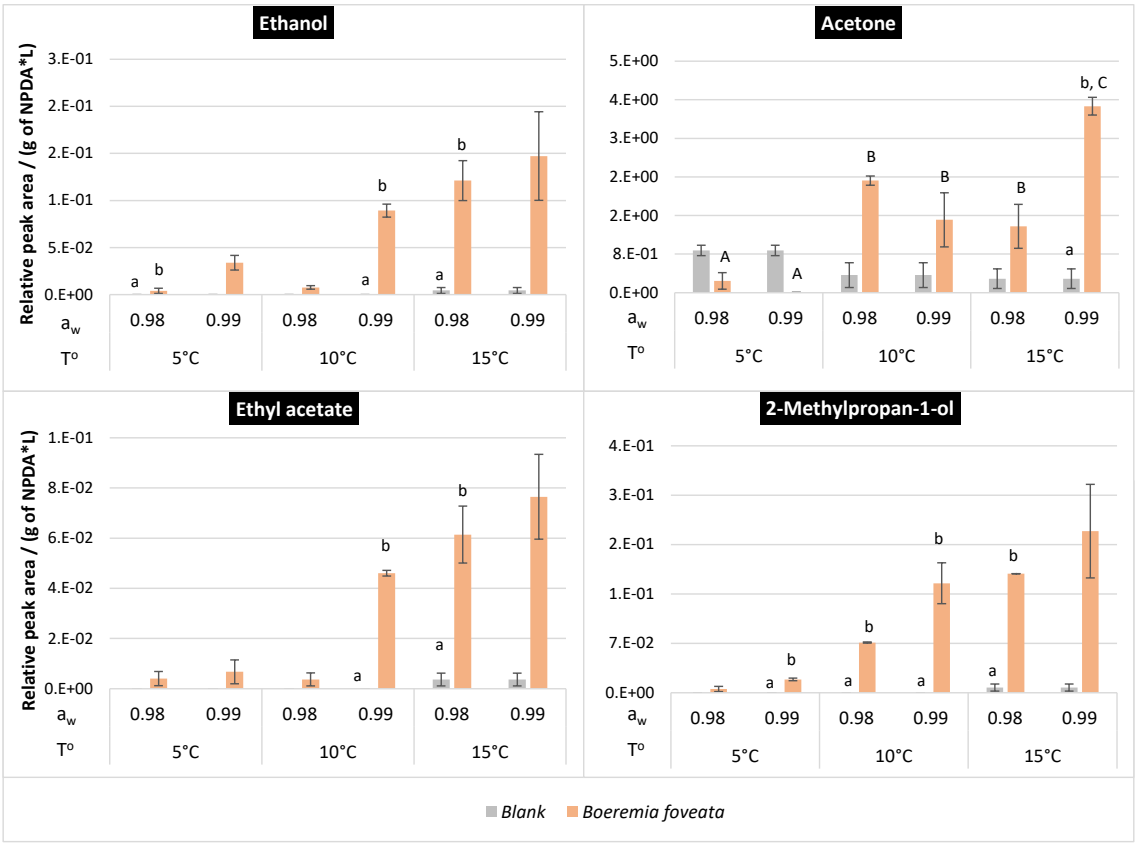


Figure 3-3 VOC abundance of ethanol, acetone, ethyl acetate and 2-methylpropan-1-ol after 15 days of incubation at three different temperatures (5, 10 & 15ºC) and two a_w (0.98 & 0.99). VOC abundance was represented in relative peak area per grams of NPDA and per litre of air sampled. VOC samples from potato-based media non-inoculated (Blank) and inoculated with *Boeremia foveata* were compared. a,b: Significant differences between blank and *B. foveata* inoculated (Kruskal Wallis, p-value<0.05). A,B: Significant differences between temperatures in *B. foveata* inoculated plates at each a_w (Wilcoxon, p-value<0.05).

In Figure 3-4, 2-methylpentane, 3-methylpentane, propan-2-yl acetate and propan-2-yl formate abundances were represented in presence and absence of *Boeremia foveata* in potato-based media after three days of incubation. 2-Methylpentane and 3-methylpentane were detected in higher abundance at the highest temperature (15ºC), however no significant differences (p-values>0.10) were detected between non-inoculated and *B. foveata* inoculated plates. Significant differences between a_w were detected at propan-2-yl formate at 10ºC, observing higher abundance at the highest a_w (0.99).

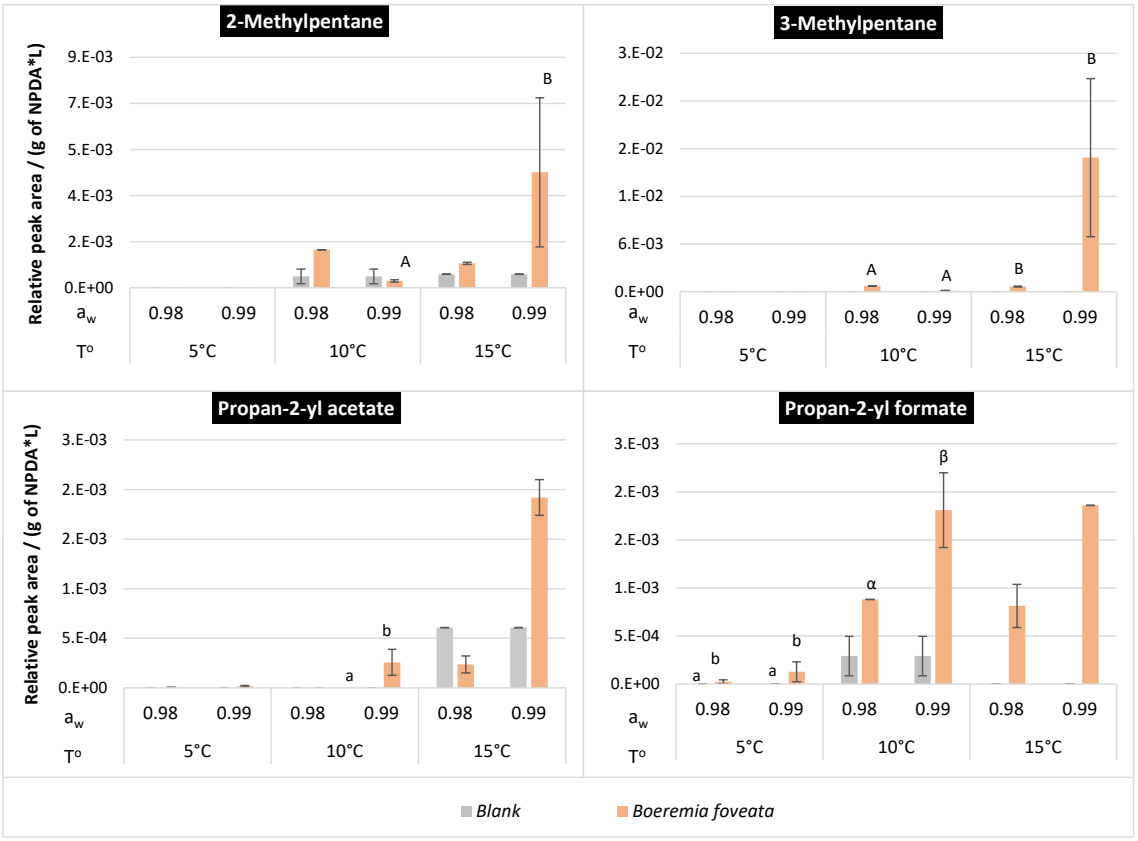


Figure 3-4. VOC abundance of 2-methylpentane, 3-methylpentane, propan-2-yl acetate and propan-2-yl formate after 3 days of incubation at three different temperatures (5, 10 & 15ºC) and two a_w (0.98 & 0.99). VOC abundance was represented in relative peak area per grams of NPDA and per litre of air sampled. VOC samples from potato-based media non-inoculated (Blank) and inoculated with *Boeremia foveata* were compared. a,b: Significant differences between blank and *B. foveata* inoculated (Kruskal-Wallis test, p-value<0.05). A,B: Significant differences between temperatures at each specific a_w (Wilcoxon test, p-value<0.05). α,β: Significant differences between a_w (0.98,0.99) at each specific temperature in presence of *B. foveata* (Kruskal-Wallis test, p-value<0.05).

Once results after 15 and 3 days of incubations were obtained, a temporal evolution of three VOC, 2-methylpropan-1-ol, methyl acetate and acetic acid was represented in Figure 3-5. 2-Methylpropan-1-ol was mainly detected after 15 days of incubation, however at 15ºC it was also detected after three days of incubation. Significant differences (p-value<0.05) were found after 15 days of incubation between non-inoculated plates and *B. foveata* inoculated at the three different temperatures. The only exception was at 15ºC and 0.99 a_w.

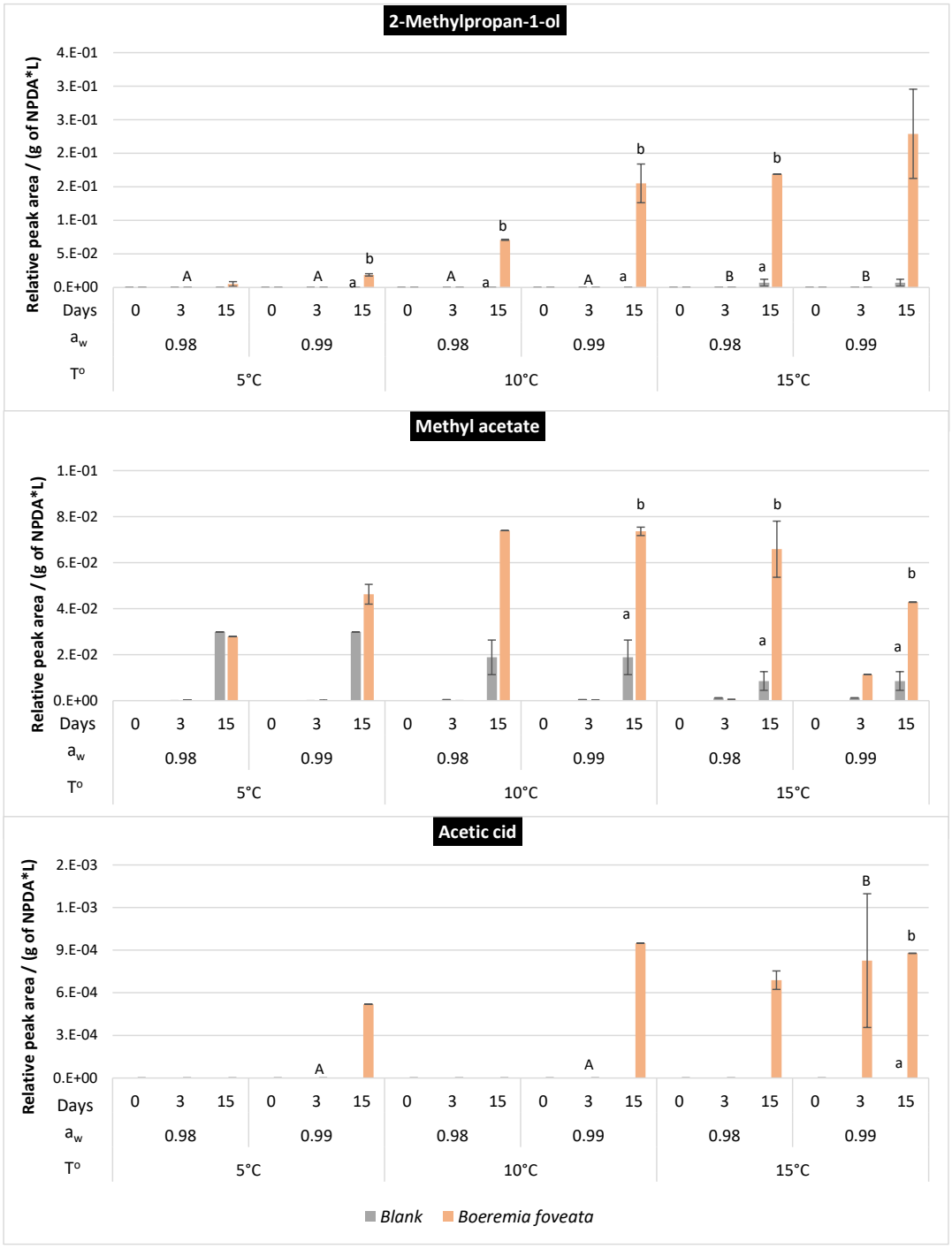


Figure 3-5 Temporal evolution of 2-methylpropan-1-ol, methyl acetate and acetic acid abundance. VOC abundance was presented in relative peak area per grams of NPDA and per litre of air sampled. VOC samples from NPDA inoculated with *B. foveata* and non-inoculated plates (Blank) were compared a,b: Significant differences between treatments at each specific T^o and a_w (Kruskal-Wallis test, p-value<0.05). A,B: Significant differences between temperatures at each specific a_w (Wilcoxon test, p-value<0.05).

The production of 2-methylpropan-1-ol by *B. foveata* was affected (p-values<0.05) by temperature at both water activities. As it is shown significant differences (p-values<0.05) were detected between non-inoculated plates and *B. foveata* inoculated plates at 10ºC and 15ºC at 0.99a_w after 15 days of incubation (p-value< 0.01). The temporal evolution presented an increasing tendency. After 15 days of incubation the detection of methyl acetate was higher than after three days at the three different temperatures.

The abundance of acetic acid, as presented in Figure 3-5, was only detected in presence of *B. foveata* at the highest a_w (0.99) of each temperature after 15 days of incubation, except from 15ºC and 0.99a_w where it was also detected after 3 days of incubation. Significant differences (p-values<0.05) were detected between temperatures at 0.99a_w, higher production of acetic acid was detected at the highest temperature compared to the others.

No significant differences (p-values>0.3) were detected in the production of 2-methylpropan-1-ol, methyl acetate and acetic acid between both water activities.

3. 4 Discussion

3.4. 1 Effect of temperature and a_w on *Boeremia foveata* growth parameters on potato-based media

There is not much research about the ecophysiology of *B. foveata in vitro*. The vast majority of the studies have been carried out *in vivo.* Understanding how the fluctuations of the environmental conditions can affect the growth of *B.foveata* without the effect of any other factors (potato cultivar, potato immune response, weather conditions) could be very useful.

Boeremia foveata growth was directly affected by temperature and a_w of the media (Figure 3-2). It was observed that at higher temperatures and a_w, higher was the growth rate of *B. foveata* and fewer days were needed to start growing in potato-based media. The main impact of those results is directly related to the presence of gangrene in commercial storages. *B. foveata* infection will expand faster and start in fewer days in those potato tubers located at the bottom of the crates, with higher temperature and relative humidity. Furthermore, those potato tubers are the ones less accessible to the farmer, therefore, until the whole crate is affected the farmer will not be able to detect the presence of the disease in the storage.

Two previous studies have been carried out on the growth of *B. foveata in vitro* where the effect of temperature was studied (Logan and Khan, 1969; Otazú *et al.*, 1979). The colony diameter of *B. foveata* was studied at the same three different temperatures (5,10,15ºC) as in our experiment. Their results were in accordance with ours, temperature was positively affecting the growth of *B. foveata.*

Another work on *B. foveata* growth *in vitro* also studied the growth rate at 15ºC in a freeze-dried potato agar media. While we observed a growth rate of 10 mm/day in NPDA, they observed 1mm/day in their media. This difference will be due to the different media preparation, the water is more available in NPDA than in a media with potato tubers that have been previously freeze-dried (Percival, Karim and Dixon, 1999).

The a_w of the media affected the growth of *B. foveata* in our study, at the highest a_w (0.99) higher was the growth of *B. foveata* and lower the time needed to start growing. A previous old study *in vitro* observed results that were in accordance with ours results, at higher relative humidity higher was the development of gangrene in potato tubers (Henriksen, 1975).

Those three studies were the only one carried out *in vitro,* while most of the studies based on *B. foveata* growth have been done *in vivo.* Malcolmson et al. (1958) reported that lower temperatures were more favourable for the development of gangrene in potato tubers, with the minimum temperature tested being 5^{o}C. This is contradictory to the results obtained in this study; however, the development of gangrene in potato tubers is not only affected by temperature and relative humidity. A fluctuation in storage temperature was shown to affect gangrene development, while varietal/genetic differences and the weather conditions before and during harvest can also affect *B. foveata* growth on potato tubers (Malcolmson, 1958). Several authors found that storing potato tubers at a higher temperature before their inoculation with *B. foveata* produced larger rots (Gray and Paterson, 1971; Adams and Griffith, 1983).

Most of the studies that have been carried out on *B. foveata* are from more than 20 years ago. It needs to be considered that nowadays there are different potato cultivars, with different susceptibilities to *B. foveata.* Therefore, our results provide up to date information regarding the effect of temperature and relative humidity on the development of gangrene in potato tubers. It presents the consequences of an increase of temperature and RH in a storage facility, a higher development of gangrene. Further studies will need to be carried out to confirm our results, including an *in vivo* study where the development of gangrene could be studied at different temperatures and water activities.

3.4. 2 Volatile Organic Compound (VOC) data analysis

The fact that gangrene will not be visually detected until later stages of the development of the disease is a relevant problem. However, if specific VOCs can be detected in presence of *Boeremia foveata* in potato tubers before the visible lesion, this would be and indicator of the presence of gangrene somewhere in the storage room.

The results presented in Table 3-1, Table 3-2, Table 3-3 and Table 3-4 showed that a higher number of VOCs were detected after 15 days of incubation compared to 3 days. This is reasonable when considering that the growth of *B. foveata* was higher after 15 days of incubation compared to 3 days. These two sampling points provide an early and a late stage of the development of *B. foveata* volatile production in NPDA.

To date, only one study has been carried out focused on the VOC production of *B. foveata* in potato tubers. However, no identification of the volatiles detected was achieved (Weijman *et al.*, 1984).

At a late stage of the development of *B. foveata* in media, compounds as ethanol, acetone, ethyl acetate and 2-methylpropan-1-ol were detected with higher abundance in presence of *B. foveata* compared to the non-inoculated plates. Their higher abundance was detected mainly at the two highest temperatures (10 and 15^{o}C), this is an important factor to be considered. As it has been mentioned, the fluctuation of temperatures during storage can create the optimal environment for the quicker development of gangrene, higher temperatures and relative humidity. Besides, the production of ethanol, acetone, ethyl acetate and 2-methylpropan-1-ol will also be higher at those temperatures.

Ethanol is a simple alcohol that can be naturally produced during microbial fermentation of sugars. It has been previously detected in presence of bacterial pathogens responsible of soft rot (Lui, Vikram, Abu-Nada, *et al.*, 2005; Blasioli *et al.*, 2013) and ring rot (Blasioli *et al.*, 2013) in potato tubers. It has also been previously detected in synthetic media (oatmeal agar, cellulose-based media and PDA) in presence of different fungal pathogens as *Muscodor albus* (Strobel *et al.*, 2001), *Gliocladium roseum* (Strobel, 2011) and *Myrothecium inundatum*

(Banerjee *et al.*, 2010) at room temperature. Its production is mainly due to the fermentation of the sugar, any fungal or bacterial pathogen able to ferment will produce ethanol. Therefore, the detection of ethanol in a potato storage could be a potential indicator of a fungal or bacterial disease.

Acetone was the VOC detected with the highest abundance, although it was also detected in less abundance in the non-inoculated plates. It is a product from the Acetone-Butanol-Ethanol (ABE) fermentation process that some strains of the anaerobic bacterium *Clostridium acetobutylicum* carry out. In the 90's it was studied the ability of this bacterium to use as a carbohydrate substrate potato and convert it into a mixture of acetone, butanol and ethanol (Grobben *et al.*, 1993; Gutierrez *et al.*, 1998). Until now, *B. foveata* has not been associated with the ABE fermentation. However, acetone has been detected and associated with the presence of soft rot in potato tubers (Varns and Glynn, 1979; Lui, Vikram, Abu-Nada, *et al.*, 2005; Sinha *et al.*, 2018). It has been previously detected in many *in vitro* studies, in presence of different bacteria, as rhizobacteria, *Bacillus* spp. and *Ralstonia solanacearum* (Bruce *et al.*, 2004; Spraker *et al.*, 2014; Chaves-López *et al.*, 2015; Giorgio *et al.*, 2015). There are several studies where it was detected in presence of different microorganisms as *Phomopsis* spp., *Trichoderma* spp., actinomycetes, *Truffle* spp. and *Nodulisporum* spp.(Wheatley *et al.*, 1997; Schöller *et al.*, 2002; March, Richards and Ryan, 2006; Singh *et al.*, 2011; Hung, Lee and Bennett, 2013; Ul-Hassan *et al.*, 2013). Therefore, it can also be used as an indicator of spoilage in potato tubers due to the presence of fungal or bacterial pathogens.

Ethyl acetate was detected in our study at the three different temperatures and a_w (0.98, 0.99), with the highest abundance at the highest temperature. It is an ethyl ester that resulted from the reaction of ethanol and formic acid. It is a byproduct of the fermentation of must in the wine-making process (Baiano *et al.*, 2016; Gabur *et al.*, 2020). The different pathways leading to the spoilage aroma of wines have been broadly studied by several authors (Sponholz, 1993;Francis and Newton, 2005; Bartowsky and Henschke, 2008; Bartowsky, 2009; Bartowsky and Pretorius, 2009). It was elucidated that the presence of ethanol from the alcoholic fermentation and acetic acid from the Acetyl CoA, reacted and produced

ethyl acetate during the wine-making (Bartowsky and Pretorius, 2009). It has been previously reported in synthetic media inoculated with *Fusarium* spp. (Wang *et al.*, 2018), *Muscodor albus* (Strobel, 2011)*, Lichtheimia ramose*(Chung *et al.*, 2017) and *Bacillus* genus (Chaves-López *et al.*, 2015) after room temperature incubation. It has also been reported *in vitro* in potato tubers and grapes inoculated with *Pectobacterium carotovorum* spp. and different fungal and bacterial pathogens, respectively (Varns and Glynn, 1979; Lui, Vikram, Abu-Nada, *et al.*, 2005; Blasioli *et al.*, 2013; Tasin *et al.*, 2018). Similar behaviour as ethanol and acetone, it was detected in presence of fungal and bacterial pathogens. Therefore, it can be considered with ethanol and acetone as an indicator of spoilage in the potato storage.

At an early-stage of the development of *B. foveata* compounds as 2-methylpentane, 3-methylpentane were detected with higher abundance in presence of *B. foveata*. Their abundances were lower than the ones detected in the four previous VOC after 15 days of incubation. This is probably due to the differential growth achieved after 3 and 15 days of incubation at the different temperatures.

2-Methylpentane and 3-methylpentane were detected at 10 and 15^{o}C, with a very low abundance. They are organic compounds known as branched alkanes; they are both isomers of hexane. Those two compounds have been mainly related to the petroleum refinery (Zhang *et al.*, 2017). The resulted compound of the methylation of 3-methylpentane, 3-ethyl-3-methylpentane was considered in a study from 2013 as markers of potato brown rot. However, there was no information regarding the origin of this compound (Blasioli *et al.*, 2013). These two compounds would therefore potentially be good biomarkers for gangrene, although further studies will be required to consider their suitability and investigate their biological origin.

The temporal evolution of methyl acetate, 2-methylpropan-1-ol and acetic acid was presented in Figure 3-5, as those three VOC were detected in presence of *B. foveata* at the two different stages. Its detection was mainly detected after 15 days of incubation, although at the highest temperature and highest a_w (0.99),

they were also detected after three days of incubation. This is due to the size of the colony of *B. foveata* at the two different sampling points, after three days of incubation, the higher colony was at 15ºC, in consequence, the detection of some of the three VOC after three days. The main information provided by those results are the highest production of methyl acetate, 2-metylpropan-1-ol and acetic acid after 15 days of incubation. In a potato storage facility, only in those areas of the store where a higher temperature is present, an early the detection of those VOCs would be possible.

There are several studies where methyl acetate has been detected in presence of a fungal or bacterial pathogen. It was detected in a few studies where potato tubers were inoculated with *Pectobacterium carotovorum* spp. However, no quantification was carried out (Varns and Glynn, 1979; Lui, Vikram, Abu-Nada, *et al.*, 2005). Methyl acetate was also detected in synthetic media inoculated with *Muscodor albus* (Strobel, 2011) and *Fusarium poae* (Savelieva *et al.*, 2016). In our experiment, methyl acetate was detected at the end of the development of *B. foveata*. It was also detected with a relatively high abundance in the non-inoculated plates; therefore, it cannot be considered as a general biomarker of the presence of a pathogen in potato tubers.

2-Methylpropan-1-ol was mainly detected at a late stage of the growth of *B. foveata*. It is considered as one of the microbial volatile organic compounds (MVOC) as it is produced in the metabolism of some fungi and bacteria (Korpi, Järnberg and Pasanen, 2009). It has already been detected in presence of different fungal pathogens *in vitro*, most of them in presence of entophytic fungi (Mitchell *et al.*, 2010; Ting, Mah and Tee, 2010; Singh *et al.*, 2011; Strobel, 2011). In a recent study, it was detected at an early-stage of the fungal development of two *Fusarium* spp. (*F. oxysporum* and *F. proliferatum)*, after two days of incubation at 20ºC in Liquid Onion Medium (LOM) (Wang *et al.*, 2018). In our study, it was detected at a late stage in presence of *B. foveata*, and, different from the previous VOCs mentioned, it was not detected in the non-inoculated plates. Therefore, it can be considered as an excellent biomarker of fungal pathogens in different matrixes, as potato tubers.

The last VOC studied was acetic acid; its detection was mainly at a late stage of the growth of *B. foveata*, although at the highest temperature it was detected at an early-stage. It has been previously detected in synthetic media inoculated with endophytic fungi *Gliocladium roseum* and *Muscodor albus* (Ezra, Hess and Strobel, 2004; Strobel, 2011), with different isolates of *Rhizobacteria* from the rhizosphere of *Solanum tuberosum* (Velivelli *et al.*, 2015) and with *Myrothecium inundatum* (Banerjee *et al.*, 2010). Acetic acid was also found *in vitro* in soft rot onions (Wang *et al.*, 2016). Acetic acid has been detected in presence of different fungal and bacterial pathogens, mainly *in vitro.* Therefore, it can be considered a biomarker of the presence of fungal or bacterial pathogens in potato tubers.

None of the VOCs detected could be considered as potential biomarkers specific for gangrene. However, the VOC fingerprint detected in presence of *B. foveata in vitro* could be used as an indicator of the presence of gangrene in storage facilities. The identification of specific biomarker for each of the potato diseases is challenging, as most of the VOCs produced during the infection of a potato tuber will be common to many fungi and bacteria, as they resulted from the metabolism of the same media, the potato tuber. However, identifying potential VOCs that can be considered biomarkers of the presence of a disease in storage is already an achievement. Its identification in higher abundances during storage can be an indicator of the presence of a disease.

3. 5 Conclusions

The main conclusions achieved in this research were:

- Gangrene development is directly affected by temperature and a_w of the media *in vitro.* Higher temperatures and a_w results in quicker development of gangrene and less time needed to start developing.
- Different VOCs were detected at different stages of the development of gangrene (3 and 15 days) at the different environmental conditions.
- Ethanol, acetone, acetic acid and ethyl acetate could be considered as an indicator of spoilage during storage. However, they could not be considered specific biomarkers of gangrene.
- 2-Methylpentane and 3-methylpentane could be considered as potential biomarkers of gangrene *in vitro* at an early stage of the disease development. However, further studies will be required on the biological origin of those two VOCs.
- 2-Methylpropan-1-ol could be considered as a potential biomarker of the presence of a fungal disease in potato tubers at a late stage of the development of the disease. Further studies will need to be carried out to confirm this conclusion.

4 ECOPHYSIOLOGICAL STUDY, VOC AND MYCOTOXIN PRODUCTION BY *FUSARIUM OXYSPORUM* AND *FUSARIUM SAMBUCINUM IN VITRO* AT DIFFERENT ENVIRONMENTAL CONDITIONS

4. 1 Introduction

Dry rot is one of the main diseases of potato tubers in Europe. It is produced by different *Fusarium* spp., such as *Fusarium avenaceum, Fusarium solani* var. *coeruleum, Fusarium sambucinum* and *Fusarium oxysporum.* In Great Britain, two of the primary pathogens responsible for dry rot in potato tubers are *F. avenaceum* and *F. solani* var. *coeruleum* (Choiseul, Allen and Carnegie, 2007; J. C. Peters *et al.*, 2008). Although in a previous study, *F. sambucinum* was considered the most common *Fusarium* spp. (Secor and Gudmestad, 1999).

In different surveys carried out in 2008 *F. sambucinum* was observed as one of the most aggressive pathogens responsible for dry rot (Ocamb, Hamm and Johnson, 2007; J. C. Peters *et al.*, 2008; R. D. Peters *et al.*, 2008). Furthermore, as it was mentioned in Section 2. 2, *F. oxysporum* was one of the pathogens isolated in Cranfield laboratory facilities from the harvest season 2018 of potato tubers. This pathogen was considered as a potential threat during the storage of potato tubers in Italy (Manici and Cerato, 1994), South Africa (Theron and Holz, 1989; Venter *et al.*, 1992) and USA (Ocamb, Hamm and Johnson, 2007). In a recent study, it was detected in Poland as the most abundant *Fusarium* spp. that was affecting potato tubers (Stefańczyk *et al.*, 2016).

Potato tubers are stored for long period of time, therefore maintaining the optimal environmental conditions (temperature and relative humidity) is essential to reduce the incidence of fungal or bacterial rots. Understanding the ecophysiology *in vitro* of the pathogens responsible for those diseases, in this case, *F. sambucinum* and *F. oxysporum,* responsible for dry rot, could provide valuable information that can be extrapolated to the potato tuber. This will allow the understanding of how different environmental conditions will affect the development of dry rot in potato tubers during storage. Once a potato storage

room is already affected by dry rot, its early detection is essential to avoid the complete loss of those batches of potato tubers. The elucidation of the VOC fingerprint detected in presence of *F. sambucinum* or *F. oxysporum in vitro*, could provide information about the kind of VOC that will be expected *in vivo,* in a potato storeroom.

Some of the *Fusarium* spp. responsible for dry rot in potato tubers are mycotoxigenic fungi, able to produce secondary metabolites called mycotoxins. As mentioned in Chapter 1 (Section 1.1.3. 2), there is limited research on the accumulation of mycotoxins in potato tubers at different environmental conditions (temperature and relative humidity). Therefore, elucidating not only the VOCs release in presence of these fungi, but also how the environmental conditions can affect the accumulation of mycotoxins will provide information about the food safety of potato tubers.

The aims of this study were 1) to elucidate the effect of three different temperatures (5, 10 and 15^{o}C) and two different water activities (0.97, 0.99) on the ecophysiology of *F. sambucinum* and *F. oxysporum* in a potato-based semi-synthetic media; 2) study the VOC production profile in presence of both *Fusarium* spp. at 10^{o}C and two different water activities (0.97, 0.99) by Thermal Desorption- Gas Chromatography- Time of Flight Mass of Spectrometry; and 3) elucidate the effect of temperature and water activity on the mycotoxin accumulation of both species under different environmental conditions (temperature x a_w).

4. 2 Material and methods

4.2. 1 Fungal pathogens (*Fusarium sambucinum* and *Fusarium oxysporum)*

4.2.1. 1 *Fusarium sambucinum*

Fusarium sambucinum, responsible for dry rot on potato tubers, was supplied from a private collection in the Plant Breeding and Acclimatization Institute (Poland). The inoculum preparation was carried out as mentioned in Section 2. 1.

4.2.1. 2 *Fusarium oxysporum*

Fusarium oxysporum, responsible for dry rot in potato tubers, was isolated from rotten potato tubers cv. Markies in the Applied Mycology Group facilities at Cranfield University (UK), as presented in Section 2. 2.

4.2. 2 Inoculation and incubation of *F. sambucinum* and *F. oxysporum* on potato-based media

The inoculation of both *Fusarium* spp. on potato-based media was performed as mentioned in Section 2.3. 2. Potato-based media consisted of Natural Potato Dextrose Agar (NPDA) that was prepared following the procedure mentioned in Section 2.3. 1. Three different temperatures of incubation were used (5,10 and 15^{o}C) and two different a_w conditions (0.97, 0.99) as presented in Figure 4-1. The modification of the a_w of the media was achieved following the same procedure as mentioned in Section 2.3.1.1. 2. The containers with 0.97 and 0.99 a_w NPDA plates were stored at 5, 10 and 15^{o}C for 30 days.

Three replicates (12 L airtight boxes) were included per temperature and a_w with NPDA plates inoculated with *F. sambucinum, F. oxysporum* and non-inoculated (Figure 4-1).

4.2. 3 Diametric growth rates of *F. sambucinum* and *F. oxysporum* on potato-based media

Fungal growth was assessed daily for 30 days after the inoculation following the methodology mentioned in Section 2.3. 3.

4.2. 4 Volatile Organic Compounds (VOCs) sampling and analysis

VOC sampling was carried out following the same procedure as the one indicated in Section 2.5. 2. Two different TD tubes were used, Bio Monitoring and Air-toxics, in series one after the other and connected with a tube. Bio Monitoring TD tube was the first in the line, followed by the Air-toxics. VOC samples were collected after 0, 5, 10 and 15 days of incubation at 10^{o}C.

VOC samples were analysed by Thermal-Desorption-Gas Chromatography-Time of Flight-Mass Spectrometry (TD-GC-ToF-MS) following the same procedure as the one mentioned in Section 2.5. 3.

4.2. 5 Volatile Organic Compounds (VOCs) data analysis

The data analysis followed was similar to the one mentioned in Section 2.5. 4. A first untargeted analysis was carried out, followed by the calculation of the Total Targeted Volatile Production (TTVP) of each of the treatments and finally a targeted analysis where those compounds that were detected in higher abundance in presence of *Fusarium* spp. compared to the blank, were selected as the most interesting VOCs to be studied.

4.2. 6 Mycotoxin analysis

At three different sampling points (7, 14 and 21 days) the mycotoxin content was measured from NPDA inoculated with both *Fusarium* spp. The extraction of the mycotoxins was carried out as mentioned in Section 2. 6. Its analysis was performed in an Exion series Ultrahigh Performance Liquid Chromatography (UHPLC) system coupled with a 6500+ qTRAP-MS system coupled with IonDrive^{TM} Turbo Spray (both Sciex Technologies, Warrington, UK) (UHPLC-MS-MS), as mentioned in Section 2. 7. The analysis of those results was also mentioned in the same Section.

4.2. 7 Statistical analysis

The statistical analysis performed was the same as the one presented in Section 2. 8. The comparisons were carried out to check if significant differences were detected between treatments (*Fusarium* spp. inoculated and non-inoculated NPDA), between the three temperatures (5, 10, 15ºC), the two a_w (0.97, 0.99) and the different sampling points (0, 5, 10 and 15 days).

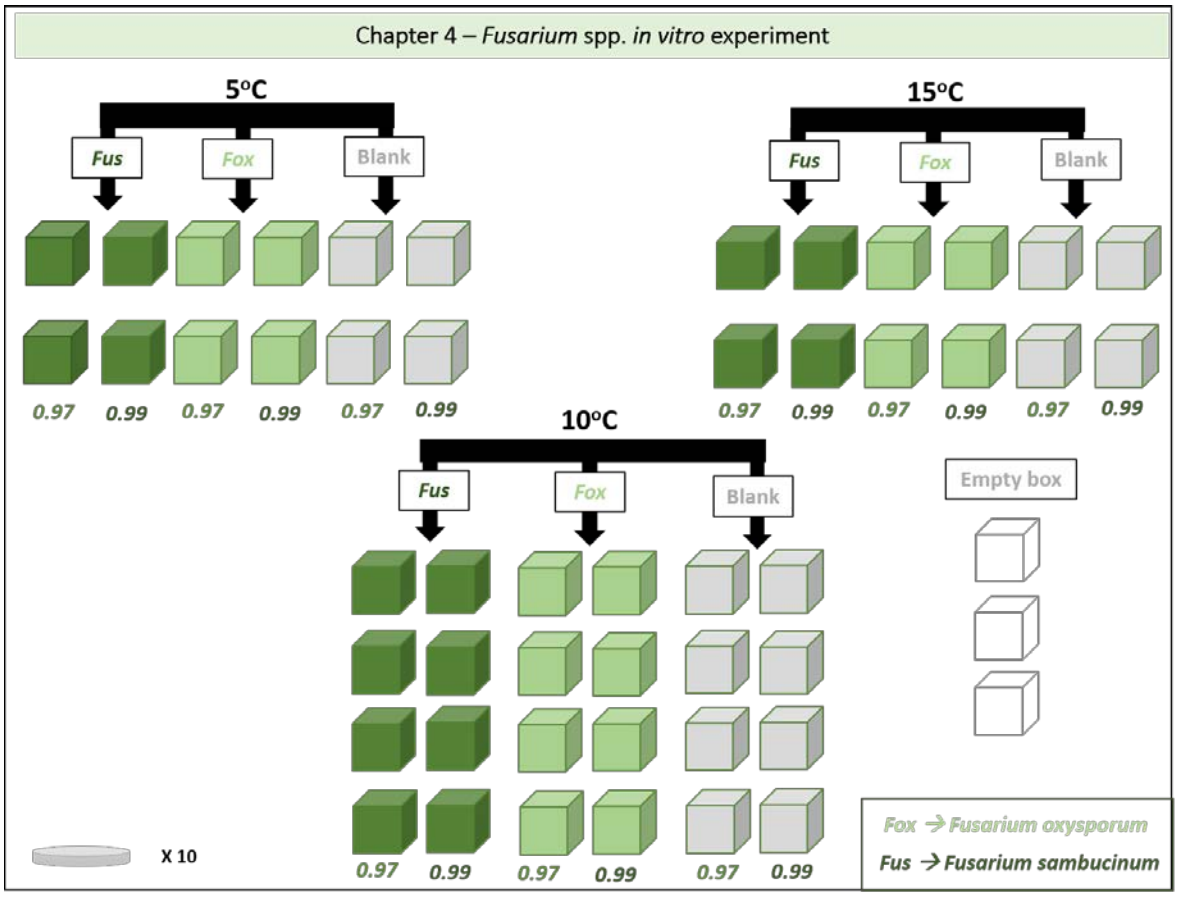


Figure 4-1 Experimental design of *Fusarium sambucinum (Fus)* and *Fusarium oxysporum (Fox) in vitro* experiment. Three different temperatures (5, 10 ,15ºC) and two different a_w (0.97,0.99) were used. Ten NPDA inoculated or non-inoculated plates were included in each 12L box. Two box replicates were used at 5 and 15ºC, while four boxes were used at 10ºC where the VOC fingerprint was studied. Samples taken at 0,5,10 and 15 days of incubation.

4. 3 Results

4.3. 1 Effect of temperature and a_w on two *Fusarium* spp. growth parameters on potato-based media

The effect of temperature and a_w on growth rate (μ_m) of *F. oxysporum* and *F. sambucinum* is presented in Figure 4-2. The higher growth rate was achieved by *F. sambucinum* at 15ºC and 0.99 a_w, with 9 mm of diameter per day. Overall, there was an increase in growth rate with temperature and water activity. Significant differences (all p-values<0.004) were detected for each of the *Fusarium* spp. between water activities (0.97 and 0.99) at the three different temperatures (5, 10,15ºC) with higher growth rate at the highest water activity (0.99). Significant differences (p-values<0.0001) in the growth rate of both *Fusarium* spp. were detected between the three different temperatures, with a higher growth rate at the highest temperature (15ºC). When the growth rate was compared between both *Fusarium* spp., significant differences (p-values<0.003) were only detected at the highest a_w (0.99) at 5 and 10ºC, while at the highest temperature,15ºC, significant differences were detected at both a_w (0.97, 0.99). At 5ºC x 0.99 a_w, *F. oxysporum* presented higher growth rate when compared with *F. sambucinum*, conversely at 10ºC x 0.99 a_w and 15ºC at both a_w, *F. sambucinum* grew faster.

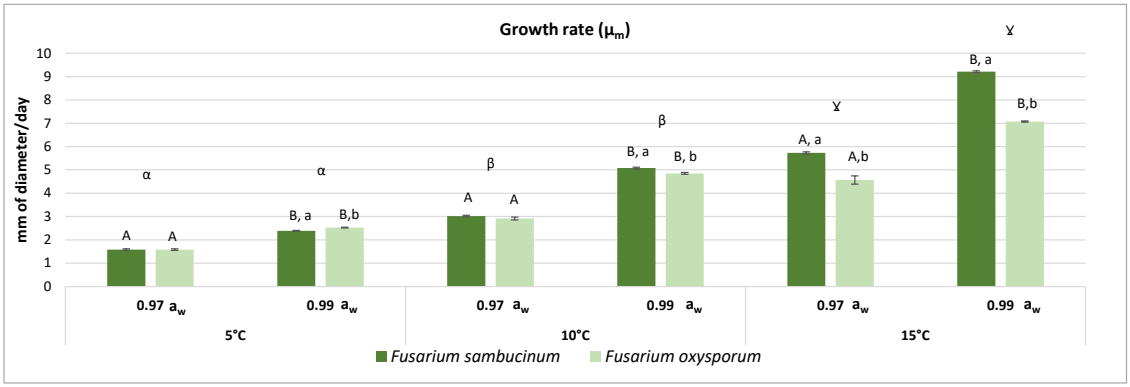


Figure 4-2 Effect of temperature (5, 10, 15ºC) and a_w (0.97, 0.99) on *F. sambucinum* and *F. oxysporum* growth rate (μ_m) on potato-based media. Data shows means of six replicates (colonies) with bars indicating SD. a,b: Significant differences between both *Fusarium* spp. at each specific a_w and temperature, A, B: Significant differences between a_w at each temperature. α, β, Ɣ: Significant differences between temperatures at each specific *Fusarium* spp. and a_w (t-test, p-value<0.05)

The inverse tendency of the growth rate was observed for the lag time (λ) (Figure 4-3); the higher the temperature was, the lower was the time needed for both *Fusarium* spp. to start growing in NPDA. At the lowest temperature, 5ºC, different water activities did not affect the lag time of both *Fusarium* spp. At 10ºC, water activity only significantly affected *Fusarium sambucinum* (p-value<0.007), which was shorter at the highest water activity (0.99). Same results were obtained at the highest temperature where significantly shorter λ were found for both *Fusarium* spp. and a_w (p-values<0.004).

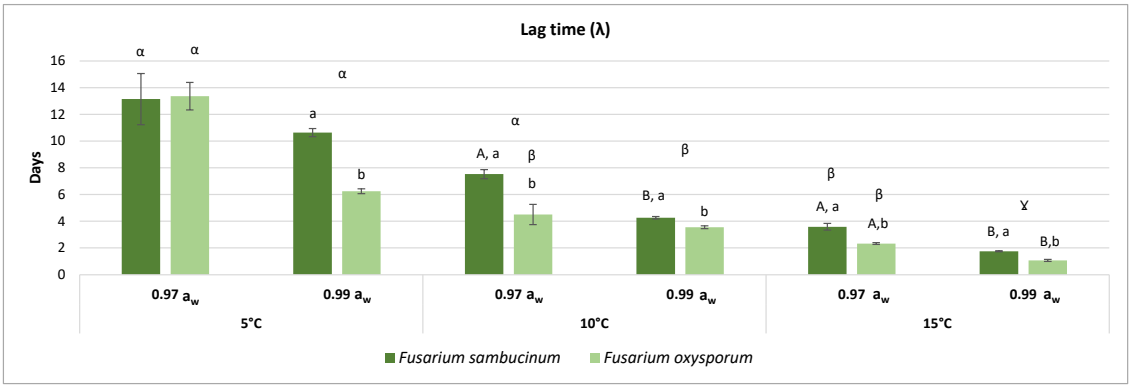


Figure 4-3 Effect of temperature (5, 10, 15ºC) and a_w (0.97, 0.99) on *F. sambucinum* and *F. oxysporum* lag time (λ) on potato-based media. Data shows means of six replicates with bars indicating SD. a,b: Significant differences between both *Fusarium* spp. at each specific a_w and temperature, A, B: Significant differences between a_w at each temperature. α, β, Ɣ: Significant differences between temperatures at each specific *Fusarium* spp. and a_w (t-test, p-value<0.05).

The effect of temperature on λ of both *Fusarium* spp. differed between a_w; at 0.97 a_w, significant differences were only detected between 5ºC and 15ºC and 10ºC and 15ºC (p-values<0.008). While at 0.99 a_w, significant differences (p-values<0.006) were detected between the three temperatures, with shorter λ.

Differences in the lag time were observed between both *Fusarium* spp.; significant differences were detected at the highest a_w (0.99) at 5ºC, and at both a_w at 10 and 15ºC (p-values<0.007). *F. sambucinum* presented higher lag time than *F. oxysporum.*

In Figure 4-4, the colony diameter of both *Fusarium* spp. after 5, 10 and 15 days of incubation at 10ºC and at the two different a_w, is presented. The three sampling points (5, 10 and 15 days) presented in Figure 4-4 corresponded to the same time when VOCs were sampled. Significant differences (p-value<0.05) were detected between the colony size of both *Fusarium spp.,* except for the highest temperature and a_w, where no significant differences were detected (p-value>0.80). A higher colony size was observed for *F. oxysporum* compared to *F. sambucinum* at three different sampling points (5, 10 and 15ºC). Images of the colony size after 0, 5, 10 and 15 days of incubation are presented in Appendix I (Figure I-1Figure I-2).

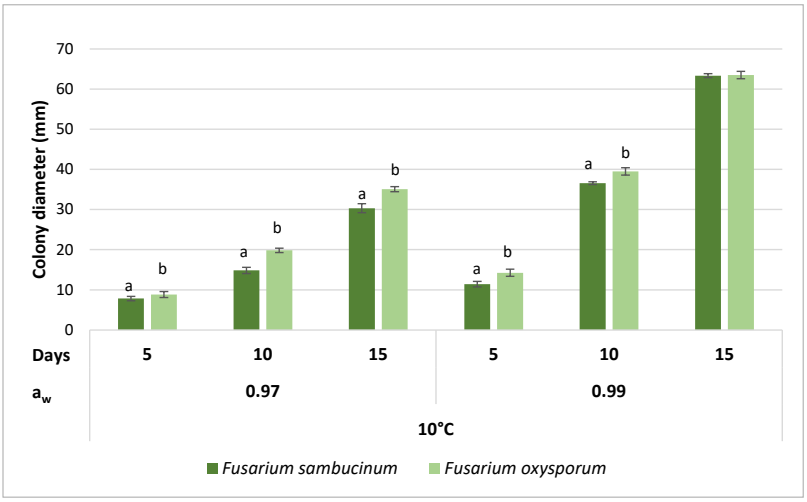


Figure 4-4 Effect of temperature (5, 10, 15ºC) and a_w (0.97, 0.99) on *F. sambucinum* and *F. oxysporum* colony diameter after 5, 10 and 15 days of incubation at 10ºC. Data shows means of six replicates with bars indicating standard deviation (SD). a,b: Significant differences between both *Fusarium* spp. at each specific a_w (Kruskal-Wallis, p-value<0.05).

4.3. 2 Volatile Organic Compound (VOC) detection in presence of the two *Fusarium* spp. under different environmental conditions

The detection of VOCs from NPDA plates inoculated with *F. sambucinum, F. oxysporum* and non-inoculated was studied at 10ºC and each of the a_w selected (0.97, 0.99 a_w) after 0, 5, 10 and 15 days of incubation.

4.3.2. 1 Untargeted analysis

Firstly, an untargeted analysis was carried out, where different pairwise comparisons were undertaken between samples. The initial group of comparisons were performed between those VOC samples in presence of *F. sambucinum* collected at the start of the experiment (day 0) and at each sampling point (5,10 and 15 days). The second group of comparisons consisted of VOC samples from those non-inoculated plates compared with those in presence of *F. sambucinum* at each sampling point (0,5,10 and 15 days). The same groups of comparisons were carried out for *F. oxysporum.*

The first comparison, presented in Table 4-1 was carried out between VOC samples from *Fusarium* inoculated NPDA at the start of the experiment (day 0) and after 15 days of incubation at 10ºC and at two different water activities (0.97, 0.99). The results are shown in Fold Change (FC) values of those VOC that were detected with higher abundance in presence of *Fusarium* spp. after 15 days of incubation. When comparing the number of VOC detected in higher abundance after 15 days between both *Fusarium* spp., overall, *F. oxysporum* presented a higher number of significant (p-values<0.05) VOC at 0.97 and 0.99 a_w. Some VOCs were detected in presence of both *Fusarium* spp. such as carbon dioxide, acetaldehyde, acetone, ethanol, isopropyl alcohol, 2-methylpentane, 4-methyl-1-hexene, ethyl acetate, 2-methylpropan-1-ol, 3-methylhexane and ethenyl acetate.

The a_w did not affect the VOC detected with higher abundance in presence of *Fusarium* spp as it was shown in Table 4-1. It can be considered that in most of the cases, the Fold Change was higher at 0.99 a_w compared to 0.97 a_w. Therefore, the differences that are detected between the abundance of each of the VOCs were higher at 0.99a_w than at 0.97a_w.

Table 4-1 Fold change (FC) values of VOCs detected in significantly higher abundance in presence of *Fusarium* spp. incubated in NPDA at 10ºC and two different a_w (0.97, 0.99) after 15 days of incubation compared to the start of the incubation period (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected between the two stages of the incubation period (p-value<0.05).

RT	Volatile Organic Compound (VOC)	*F. sambucinum*		*F. oxysporum*	
		0.97 a_w	0.99 a_w	0.97 a_w	0.99 a_w
5.17	Carbon dioxide	3.67E+01	5.93E+02	NS	4.30E+05
5.88	Isobutane	NS	NS	8.08E+00	NS
6.48	Acetaldehyde	5.48E+03	2.45E+08	NS	1.69E+07
6.96	Methyl formate	1.73E+02	NS	NS	NS
7.86	Ethanol	7.16E+06	3.98E+03	1.29E+01	2.84E+02
8.77	Acetone	8.61E+00	6.69E+01	2.98E+01	3.40E+00
8.92	Isopropyl alcohol	3.32E+04	3.68E+06	1.60E+01	NS
9.11	Ethyl formate	NS	NS	7.45E+01	3.93E+08
9.47	Methyl acetate	NS	NS	3.34E+02	4.08E+01
10.08	2-Methylpentane	2.45E+05	1.14E+05	3.65E+10	NS
10.66	1-Hexene-4-methyl	7.64E+03	6.80E+06	1.10E+10	NS
11.47	1-Propanol	NS	NS	NS	4.26E+06
13.16	Ethyl Acetate	2.20E+06	NS	NS	1.27E+08
15.17	2-Methylpropan-1-ol	NS	3.06E+08	7.02E+00	2.58E+08
15.43	3-Methylhexane	1.87E+05	NS	2.06E+02	9.27E+05
16.18	3-Methylbutanal	NS	NS	6.44E+07	6.97E+06
18.22	Ethenyl acetate	2.18E+05	NS	1.29E+09	NS
18.83	n-Propyl acetate	NS	NS	1.17E+07	3.42E+06
21.4	2-Methylbutan-1-ol	NS	NS	1.01E+10	NS
22.12	Isobutyl acetate	NS	NS	1.03E+08	7.14E+07

The results of the second comparison are presented in Table 4-2. The second analysis consisted of a comparison between non-inoculated plates and the *Fusarium* inoculated plates VOCs detected after 15 days of incubation at both a_w. This comparison provided information about which VOCs from those ones detected with higher abundance after 15 days (Table 4-1), were directly related

to the presence of *Fusarium* spp. Compounds, such as carbon dioxide, acetaldehyde, ethanol, methyl acetate were detected as significantly different between the non-inoculated plates and the presence of *Fusarium* spp. New compounds also appeared, such as sulfur dioxide and trichloromethane. 2-Methylpropan-1-ol and ethyl formate were the two VOC considered as the ones with the highest FC value. Therefore, their abundance in presence of *F. sambucinum* and *F. oxysporum* were 10^8 times higher than their abundance in the non-inoculated plates.

Table 4-2 Fold change (FC) values of VOCs detected in significantly higher abundance in presence of *Fusarium* spp. compared to the non-inoculated plates after 15 days of incubation at 10ºC and two different a_w (0.97, 0.99) (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected (p-value<0.05).

RT	Volatile Organic Compound (VOC)	*F. sambucinum*		*F. oxysporum*	
		0.97a_w	**0.99 a_w**	**0.97 a_w**	**0.99 a_w**
5.17	Carbon dioxide	NS	NS	NS	1.68E+05
5.76	Sulfur dioxide	NS	4.42E+01	NS	NS
5.88	Isobutane	NS	1.38E+01	NS	NS
6.48	Acetaldehyde	NS	NS	NS	2.04E+04
7.86	Ethanol	3.00E+04	1.24E+03	2.27E+02	5.35E+01
8.92	Isopropyl alcohol	4.63E+06	5.33E+01	NS	NS
9.11	Ethyl formate	NS	NS	1.47E+09	1.53E+08
9.47	Methyl acetate	NS	NS	4.00E+02	4.94E+01
10.08	2-Methylpentane	NS	2.94E+01	NS	NS
10.66	1-Hexene-4-methyl	NS	2.64E+06	NS	NS
11.47	1-Propanol	NS	NS	NS	1.66E+06
13.16	Ethyl Acetate	2.60E+05	NS	5.31E+09	4.96E+07
14.10	Trichloromethane	NS	2.02E+02	NS	NS
14.81	2-Methylhexane	NS	NS	4.15E+07	2.15E+00
15.17	2-Methylpropan-1-ol	NS	1.19E+08	1.60E+09	1.00E+08
15.43	3-Methylhexane	NS	3.93E+01	2.26E+06	3.61E+05
15.79	Isopropyl acetate	NS	NS	NS	3.68E+05
16.18	3-Methylbutanal	NS	NS	1.49E+07	NS

The same comparisons were carried out after 10 days of incubation presented in Table 4-3 and Table 4-4. The number of VOC that were shown in Table 4-3, where those VOC detected in presence of *Fusarium* spp. with significantly higher abundance after 10 days of incubation compared to the start of the experiment. They were lower than after 15 days of incubation (Table 4-1). However, the only new VOC detected was carbon disulfide. A similar tendency to the one observed after 15 days of incubation was detected; a higher number of VOC were detected with significantly higher abundance in presence of *F. oxysporum* and at 0.99 a_w. Very few VOCs were detected with significantly higher abundance in presence of *F. sambucinum* after 10 days of incubation compared to the start of the experiment.

Table 4-3 Fold change (FC) values of VOCs detected in significantly higher abundance in presence of *Fusarium* spp. incubated in NPDA at 10ºC and two different a_w (0.97, 0.99) after 10 days of incubation compared to the start of the incubation period (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected between the two stages of the incubation period (p-value<0.05).

RT	Volatile Organic Compound (VOC)	*F. sambucinum*		*F. oxysporum*	
		0.97 a_w	0.99 a_w	0.97 a_w	0.99 a_w
5.17	Carbon dioxide	NS	NS	NS	1.57E+06
5.79	Sulfur dioxide	NS	NS	1.41E+02	8.11E+01
6.48	Acetaldehyde	NS	1.02E+07	7.65E+01	1.30E+08
7.86	Ethanol	NS	NS	NS	8.62E+01
8.77	Acetone	NS	2.34E+00	NS	2.82E+01
9.11	Ethyl formate	NS	NS	NS	4.86E+08
9.47	Carbon disulfide	4.33E+06	3.51E+06	2.96E+07	2.78E+08
9.50	Methyl acetate	NS	NS	NS	2.82E+02
10.66	1-Hexene-4-methyl	NS	NS	NS	8.49E+03
13.16	Ethyl Acetate	NS	NS	1.44E+08	1.67E+10
15.17	2-Methylpropan-1-ol	NS	NS	5.38E+04	3.32E+08
16.18	3-Methylbutanal	NS	NS	NS	9.23E+04
18.83	n-Propyl acetate	NS	NS	NS	2.78E+07

Table 4-4 shows the VOCs detected after 10 days of incubation, where the non-inoculated plates were compared with the *Fusarium* spp. inoculated plates. Ethyl acetate was the VOC detected with the highest FC, with a value of 10^8, and it was detected in presence of *F. oxysporum.*

Table 4-4 Fold change (FC) values of VOCs detected in significantly higher abundance in presence of *Fusarium* spp. compared to the non-inoculated plates after 10 days of incubation at 10ºC and two different a_w (0.97, 0.99) (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected between the absence and presence of *Fusarium* spp. (p-value<0.05).

RT	Volatile Organic Compound (VOC)	*F. sambucinum*		*F. oxysporum*	
		0.97 a_w	**0.99 a_w**	**0.97 a_w**	**0.99 a_w**
5.17	Carbon dioxide	NS	NS	NS	7.61E+03
5.76	Sulfur dioxide	2.09E+00	NS	2.76E+01	7.70E+01
5.88	Isobutane	NS	NS	1.71E+01	8.29E+01
7.86	Ethanol	NS	1.14E+01	NS	1.67E+02
8.77	Acetone	NS	NS	NS	7.91E+00
9.45	Carbon disulfide	NS	NS	9.53E+00	7.17E+01
9.50	Methyl acetate	NS	5.20E+00	9.04E+00	5.09E+02
10.08	2-Methylpentane	NS	NS	NS	2.11E+04
10.66	1-Hexene-4-methyl	NS	NS	NS	3.00E+04
13.16	Ethyl Acetate	NS	NS	1.92E+07	1.79E+09
14.10	Trichloromethane	1.89E+00	NS	4.92E+00	NS
15.17	2-Methylpropan-1-ol	NS	NS	NS	3.56E+07
18.83	n-Propyl acetate	NS	NS	NS	2.98E+06

The last VOC analysis was carried out after 5 days of incubation. Only four VOC were detected with significantly higher abundance after 5 days of storage compared to the start of the experiment (Figure 4-5). Ethyl acetate was only detected with significantly higher abundance in presence of *F. oxysporum* at 0.99 a_w.

Table 4-5 Fold change (FC) values detected on those VOCs detected in significantly higher abundance in presence of *Fusarium* spp. incubated in NPDA at 10ºC and two different a_w (0.97, 0.99) after 5 days of incubation compared to the start of the incubation period (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected between both stages of the incubation period (p-value<0.05).

RT	Volatile Organic Compound (VOC)	*F. sambucinum*		*F. oxysporum*	
		0.97 a_w	**0.99 a_w**	**0.97 a_w**	**0.99 a_w**
5.17	Carbon dioxide	8.98E+05	NS	NS	NS
6.45	Acetaldehyde	5.97E+04	1.86E+05	2.61E+04	NS
8.77	Acetone	NS	NS	NS	3.26E+07
13.16	Ethyl acetate	NS	NS	NS	1.12E+07

When the comparison was carried out between those VOCs detected in the non-inoculated plates compared to the inoculated with *Fusarium* spp. only acetone, isopropyl alcohol and ethyl acetate were detected (Table 4-6). Isopropyl alcohol appeared in significantly higher abundance in presence of *F. sambucinum* and *F. oxysporum* at the highest water activity (0.99).

Table 4-6 Fold change (FC) values of VOCs detected in significantly higher abundance in presence of *Fusarium* spp. compared to the non-inoculated plates after 5 days of incubation at 10ºC and two different a_w (0.97, 0.99) (t-test, p-value<0.05). The Retention Time (RT) of each VOC is presented in minutes. NS: No significant differences were detected between the absence and presence of *Fusarium* spp. (p-value<0.05).

RT	Volatile Organic Compound (VOC)	*Fusarium sambucinum*		*Fusarium oxysporum*	
		0.97 a_w	**0.99 a_w**	**0.97 a_w**	**0.99 a_w**
8.77	Acetone	NS	NS	NS	1.47E+07
8.99	Isopropyl alcohol	NS	7.44E+00	NS	1.05E+01
13.16	Ethyl acetate	NS	NS	NS	5.06E+06

Those VOCs that were that were detected in our previous comparisons in presence of *F. sambucinum* and/or *F. oxysporum,* were selected for the next step of the VOC analysis (Appendix J, Table J-1). Considering those 20 VOCs

included in the table, the Total Targeted Volatile Production (TTVP) calculated as the sum of all the relative peak areas, was represented at the different sampling points, after 0, 5, 10 and 15 days of incubation at 10ºC (Figure 4-5).

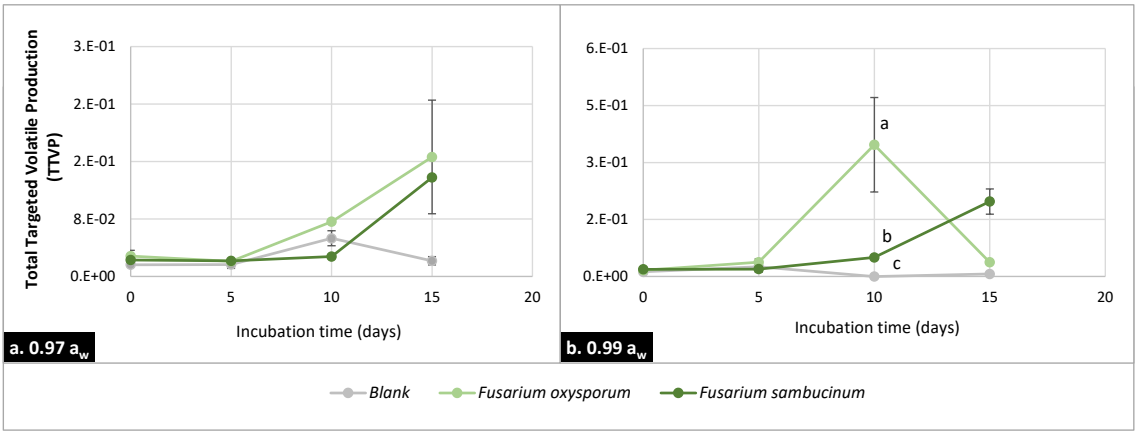


Figure 4-5 Total Targeted Volatile Production (TTVP) of targeted VOCs detected in absence (blank) and presence of *F. sambucinum* and *F. oxysporum* incubated at 10ºC in potato-based media at 0.97 a_w (a) and 0.99 a_w (b) at different points of the incubation period. a,b: Significant differences between treatments (t-test, p-values<0.05).

The results showed an increase in the TTVP at 0.97 a_w in presence of *F. oxysporum* and *F. sambucinum.* At 0.99 a_w, there was also an increase in the TTVP for *F. sambucinum.* However, the main increase in the TTVP was achieved for *F. oxysporum* after 10 days of incubation where significant differences (p-values<0.05) were detected between the presence and absence of *Fusarium* spp.

4.3.2. 2 Targeted analysis

The VOC abundance of ethanol, 2-propanol and acetone after 5 days of incubation are presented in Figure 4-6. The abundance was presented in Relative Peak Area per grams of NPDA and per litre of air sampled. No significant differences between the presence and the absence of *Fusarium* spp. was observed for ethanol and 2-propanol. While acetone abundance was significantly

higher in presence of both *Fusarium* spp. compared to their absence (blank). Higher abundance of acetone was detected at the highest a_w.

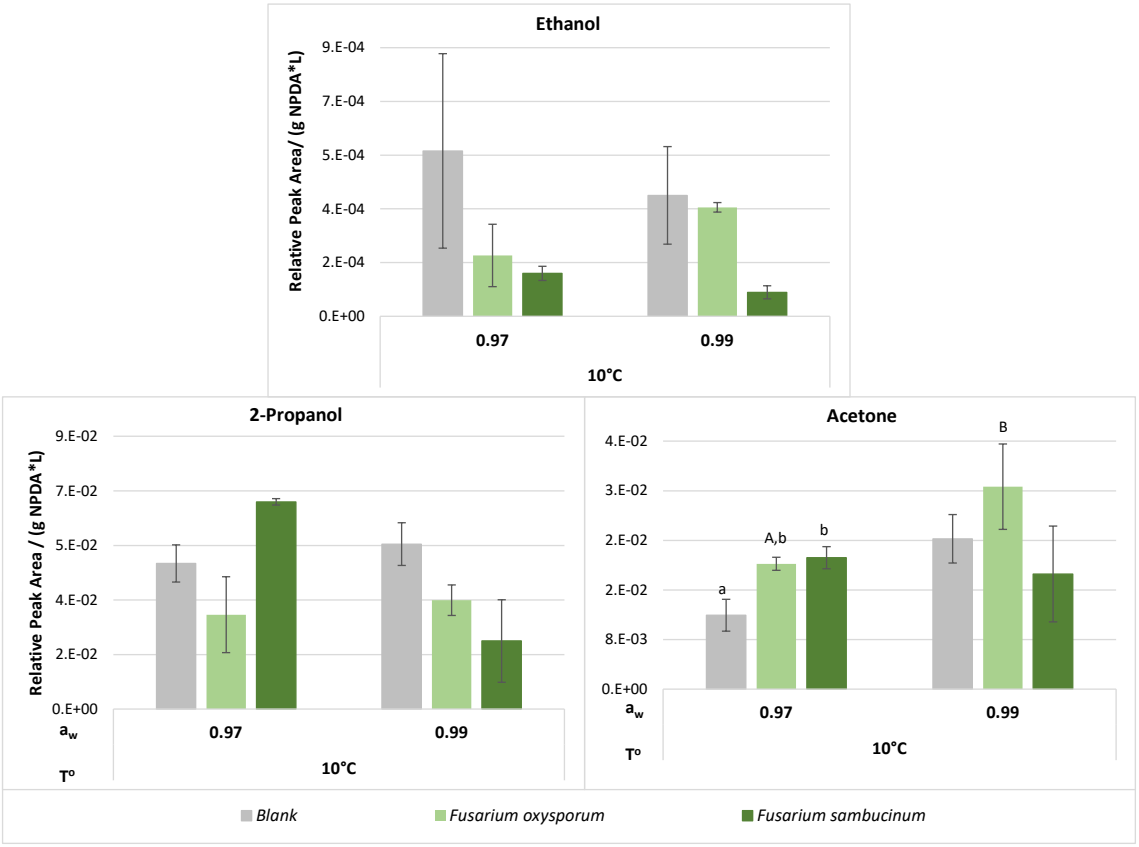


Figure 4-6 VOC abundance of ethanol, 2-propanol and acetone after 5 days of incubation at 10°C at two different a_w (0.97 & 0.99). VOC abundance was represented in relative peak area per grams of Natural Potato Dextrose Agar (NPDA) and per litre of air sampled. VOC from NPDA non inoculated (Blank) and inoculated with *F. sambucinum* and *F. oxysporum* were compared. a,b,c: Significant differences between treatments (Wilcoxon, p-value<0.05). A, B, C: Significant differences between the two a_w (Kruskal-Wallis, p-value<0.05).

After 10 days of incubation at 10°C, the abundance of sulfur dioxide, methyl acetate, ethyl acetate and 2-methylpropan-1-ol was presented at the two different a_w (0.97, 0.99) (Figure 4-7). Although higher overall abundances of those four VOCs were detected mainly in presence of *F. oxysporum,* significant differences (p-value<0.05) were only detected for ethyl acetate and 2-methylpropan-1-ol, with a higher abundance of both in presence of *F. oxysporum* at both a_w (0.97, 0.99).

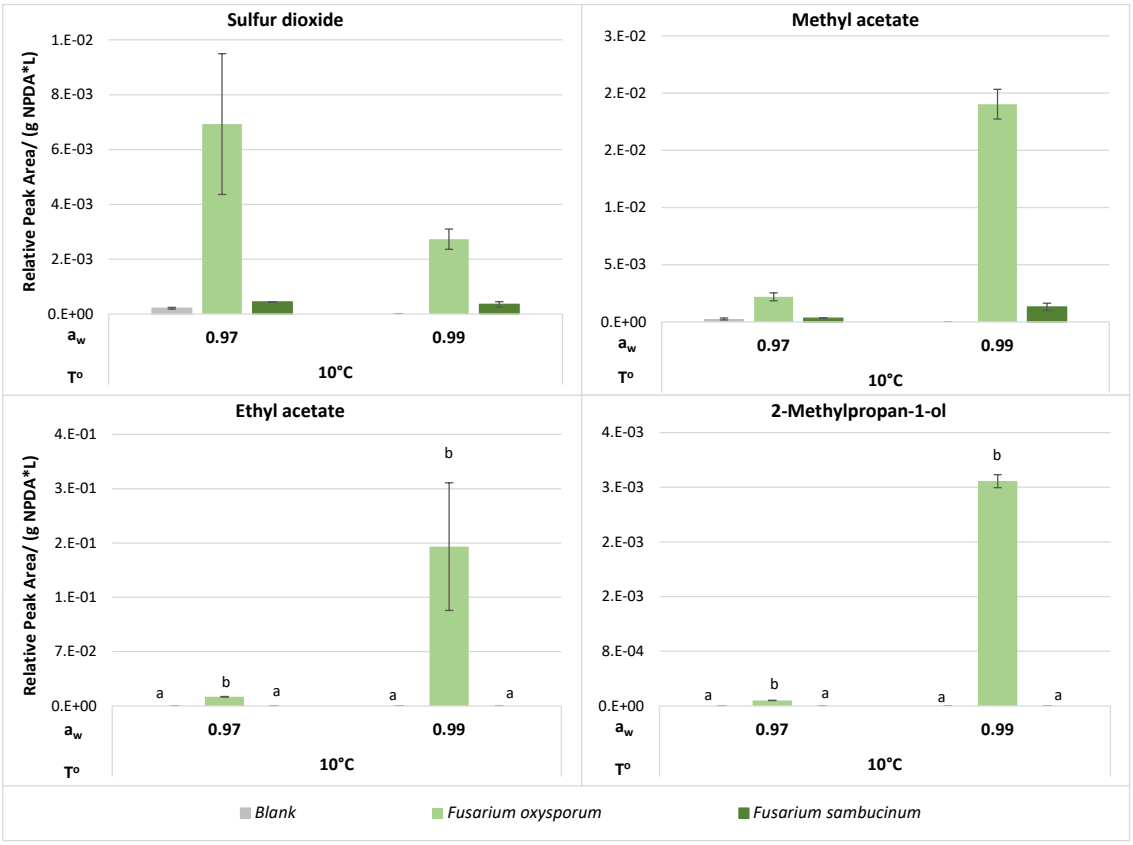


Figure 4-7 VOC abundance of sulfur dioxide, methyl acetate, ethyl acetate and 2-methylpropan-1-ol after 10 days of incubation at 10°C at two different a_w (0.97&0.99). VOC abundance was represented in relative peak area per grams of Natural Potato Dextrose Agar (NPDA) and per litre of air sampled. VOC from NPDA non inoculated (Blank) and inoculated with *F. sambucinum* and *F. oxysporum* were compared. No significant differences were detected between a_w. a,b,c: Significant differences between treatments (Wilcoxon, p-value<0.05).

After 15 days of incubation at 10°C, five VOCs with the highest FC values were selected from Table 4-2. Methyl acetate, ethanol, methyl acetate, 2-methylpropan-1-ol and ethyl formate abundances at 10°C were higher in presence of *Fusarium* spp. compared to their absence (Figure 4-8). The production of ethanol and methyl acetate by *F. sambucinum* in NPDA was affected by the a_w of the media, a higher abundance of both VOCs was detected at the highest water activity (0.99 a_w). 2-Methylpropan-1-ol, ethyl acetate and ethyl formate were only detected in presence of *F. oxysporum* at 0.97 a_w and in presence of *F. sambucinum* at 0.99 a_w.

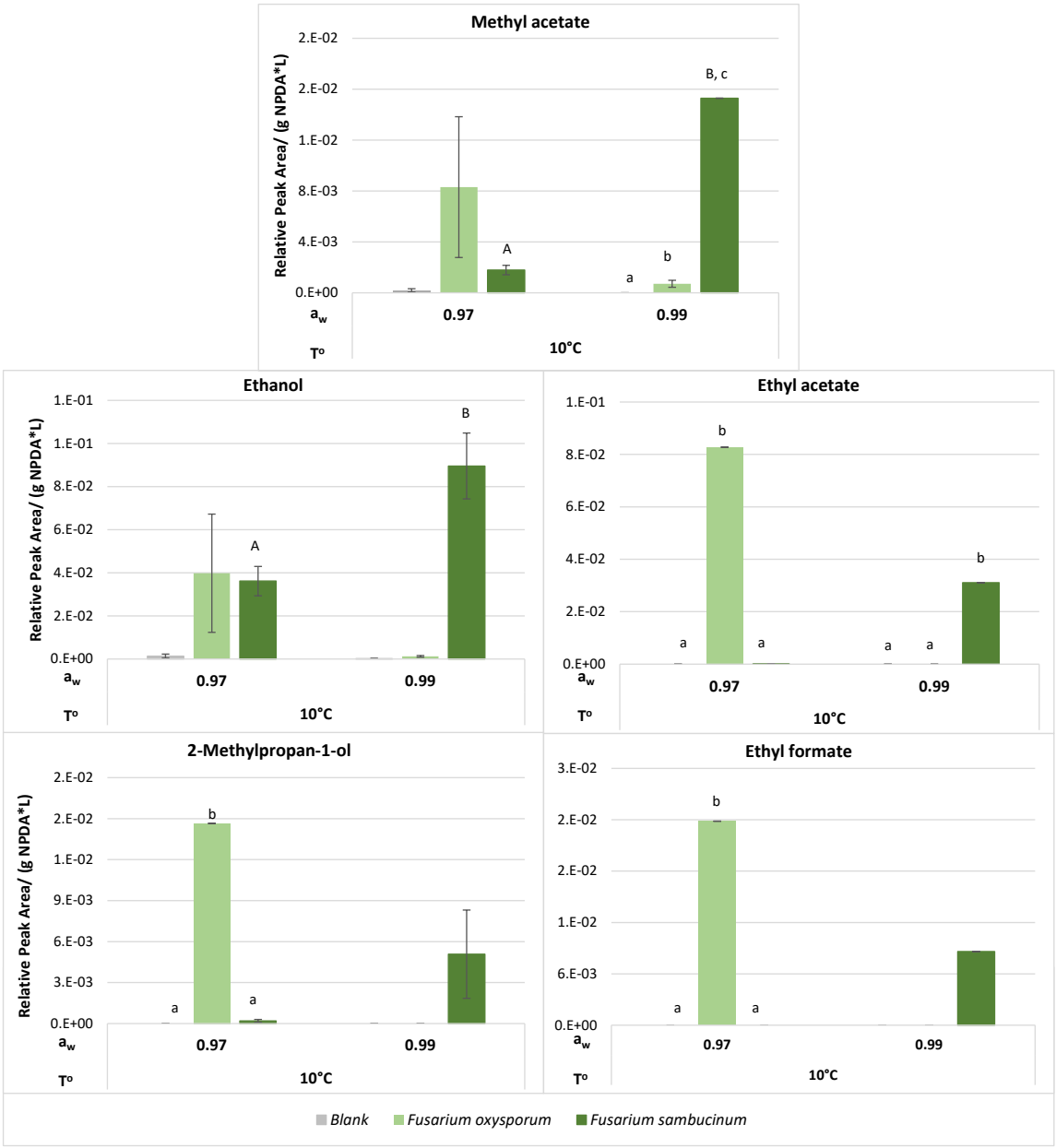


Figure 4-8 VOC abundance of methyl acetate, ethanol, ethyl acetate, 2-methylpropan-1-ol and ethyl formate after 15 days of incubation at 10°C at two different a_w (0.97 & 0.99). VOC abundance was represented in relative peak area per grams of Natural Potato Dextrose Agar (NPDA) and per litre of air sampled. VOC from NPDA non inoculated (Blank) and inoculated with *F. sambucinum* and *F. oxysporum* were compared. a,b,c: Significant differences between treatments (Wilcoxon, p-value<0.05). A, B, C: Significant differences between the two a_w (Kruskal-Wallis, p-value<0.05).

In Figure 4-9, a temporal evolution of ethanol, ethyl acetate and 2-methylpropan-1-ol abundance is presented. Those three VOCs were detected throughout all the sampling points (5, 10 and 15 days) at 10ºC in higher abundance in presence of *Fusarium* spp. Their detection was higher at the two latest sampling points, after 10 and 15 days of incubation. Ethanol was detected in presence of both *Fusarium* spp, while ethyl acetate and 2-methylpropan-1-ol were detected mainly in presence of *F. oxysporum.*

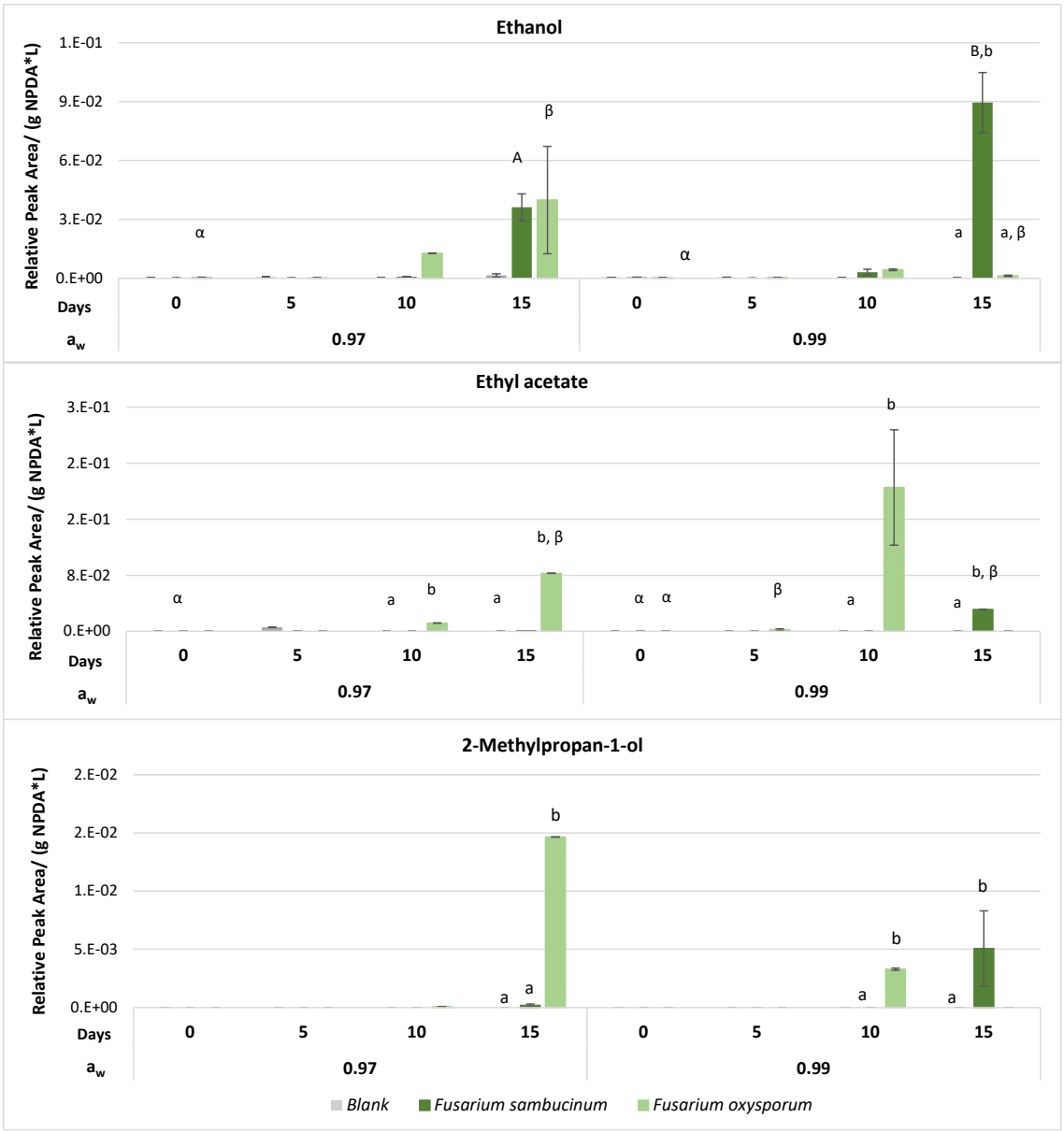


Figure 4-9 Temporal evolution of ethanol, methyl acetate and ethyl acetate abundance when they were stored at 10ºC. VOC samples from potato-based media inoculated with *F. sambucinum, F. oxysporum* and non-inoculated plates (Blank) were compared. a,b: Significant differences between treatments at each specific day and a_w (Wilcoxon, p-value<0.05) A, B: Significant differences between aw at each specific day and treatment. α, β: Significant differences between days at each specific a_w (Kruskal-Wallis, p-value<0.05).

4.3. 3 Effect of temperature and a_w on mycotoxin (T-2, HT-2, Diacetoxycirpenol, Neosolaniol, Beuvericin and Alternariol methyl ether) accumulation of *Fusarium sambucinum* and *Fusarium oxysporum*

The mycotoxin accumulation on the growth media was studied at three different sampling points, after 7, 14 and 21 days. Seven mycotoxins, known to be produced by *Fusarium* spp., were detected in presence of *F. sambucinum* and *F. oxysporum* at the different sampling points (Table 4-7). HT-2, T-2, diacetoxyscirpenol, neosolaniol, beauvericin and 15-acetoxyscirpenol were detected in presence of *F. sambucinum* after 7, 14 and 21 days of incubation. While only T-2 and alternariol methyl ether were detected in presence of *F. oxysporum.*

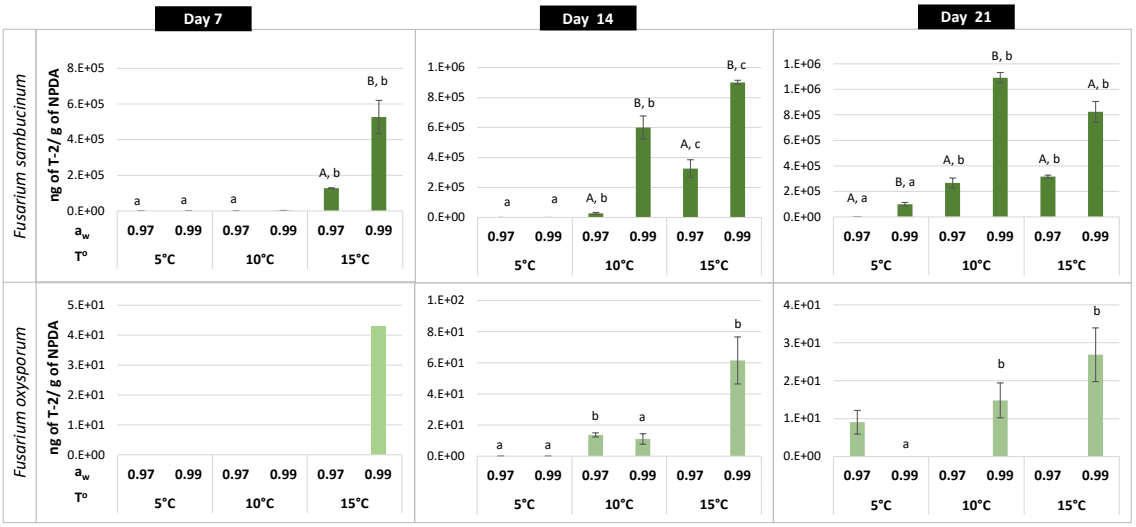
Table 4-7 Mycotoxins detected in presence of *F. sambucinum* and *F. oxysporum* after incubation in NPDA at different temperatures (5,10,15ºC) for 7, 14 and 21 days.

Day 7	Day 14	Day 21
	Fusarium sambucinum	
T-2	T-2	T-2
HT-2	HT-2	HT-2
Diacetoxyscirpenol	Diacetoxyscirpenol	Diacetoxyscirpenol
Neosolaniol	Neosolaniol	Neosolaniol
Beuvericin	Beuvericin	Beauvericin
15-Acetoxyscirpenol	15-Acetoxyscirpenol	15-Acetoxyscirpenol
	Fusarium oxysporum	
	T-2	T-2
		Alternariol methyl ether

Subsequently, the accumulation of each of these mycotoxins was studied at the three different temperatures (5, 10, 15ºC) and two a_w (0.97, 0.99). T-2, was detected in presence of both *Fusarium* spp. and its accumulation at the different conditions is presented in Figure 4-10. T-2 was mainly detected in presence of *F. sambucinum* at the highest temperatures.

After 7 days it was only detected at 15ºC (p-values<0.03), after 14 days it was also detected at 10ºC (p-values<0.04) and after 21 days it was detected in all the conditions (temperature x a_w). After 21 days, significant differences were only detected between 5 and 15ºC and 5 and 10ºC at both a_w (p-values<0.05). At the highest a_w (0.99) the concentration of T-2 detected was significantly higher (p-values<0.05) than at 0.97 a_w at the three different sampling points.

Figure 4-10 T-2 accumulation in presence of *F. sambucinum* and *F. oxysporum* after 7, 14 and 21 days of incubation at different environmental conditions (temperature and water activities). a,b: Significant differences between temperatures at each specific a_w. A, B: Significant differences between a_w at each specific temperature (t-test, p-values<0.05).



The accumulation of T-2 in presence of *F. oxysporum* differed from *F. sambucinum.* After 7 days of incubation, it was only detected at the highest a_w and temperature (15ºC x 0.99 a_w). After 14 days of incubation, it was detected at both a_w at 10ºC and only at 0.99 a_w at 15ºC. Those samples from 0.97 a_w, in presence of *F. oxysporum* were discarded after 14 days of incubation. After 21 days of incubation T-2 detection was observed at the three different temperatures. Significant differences (p-values<0.04) were only detected between temperatures after 14 and 21 days of incubation.

The accumulation of HT-2, diacetoxyscirpenol (DAS) and 15-acetoxyscirpenol (15-AS) in *F. sambucinum* inoculated NPDA is shown in Figure 4-11. Their accumulation was similar to the one presented for HT-2. After 7 days of incubation its presence was detected at 15ºC, except for 15-acetoxyscirpenol that was also detected at 10ºC x 0.99 a_w. After 14 days its detection was also predominant at 10 and 15ºC and after 21 days, its presence was detected at almost all the conditions, with the lowest concentration detected at 5ºC x 0.97 a_w.

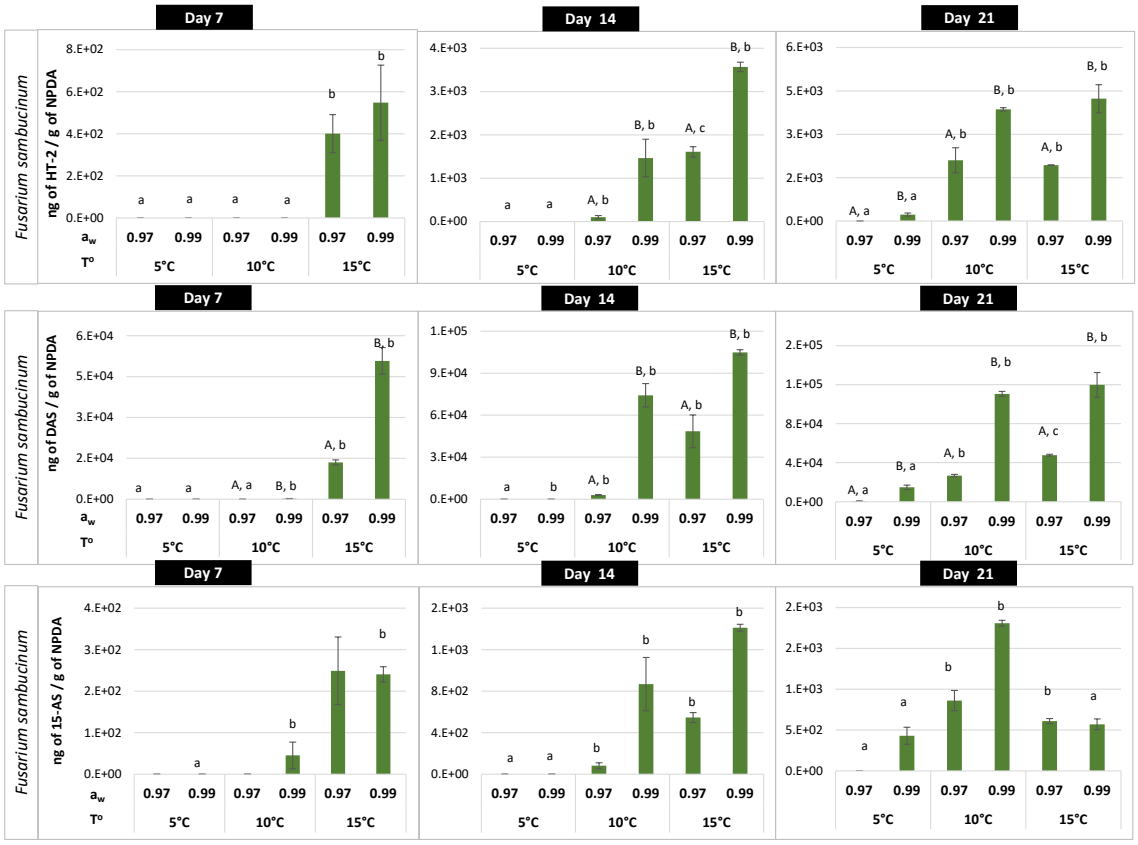


Figure 4-11 HT-2, diacetoxyscirpenol (DAS) and 15-acetoxyscirpenol (15-AS) accumulation in presence of *F. sambucinum* after 7, 14 and 21 days of incubation at different environmental conditions (temperature and water activities) a,b: Significant differences between temperatures at each specific a_w. A, B: Significant differences between a_w at each specific temperature (t-test, p-value<0.05).

Significant differences (p-values<0.05) were detected between temperatures (5, 10 & 15ºC) for these three mycotoxins (HT-2, DAS and 15-AS). The a_w of the media affected significantly (p-values<0.05) the concentration of HT-2 and diacetoxyscirpenol after 14 and 21 days of incubation, with higher accumulation of the mycotoxins at the highest water activity (0.99) compared to 0.97 a_w.

The accumulation of neosolaniol and beauvericin is presented in Figure 4-12. Neosolaniol accumulation presented similar behaviour as T-2, HT-2, diacetoxyscirpenol and 15-acetoxyscirpenol. At the latest sampling points (14 and 21 days) the detection of them was observed at the three temperatures (5,10,15ºC). However, its detection at 5ºC after 21 days was significantly lower (p-values<0.03) compared to 10 and 15ºC. Significant differences (p-values<0.01) were also detected between water activities, in general, the concentration of neosolaniol was higher as higher was the water activity.

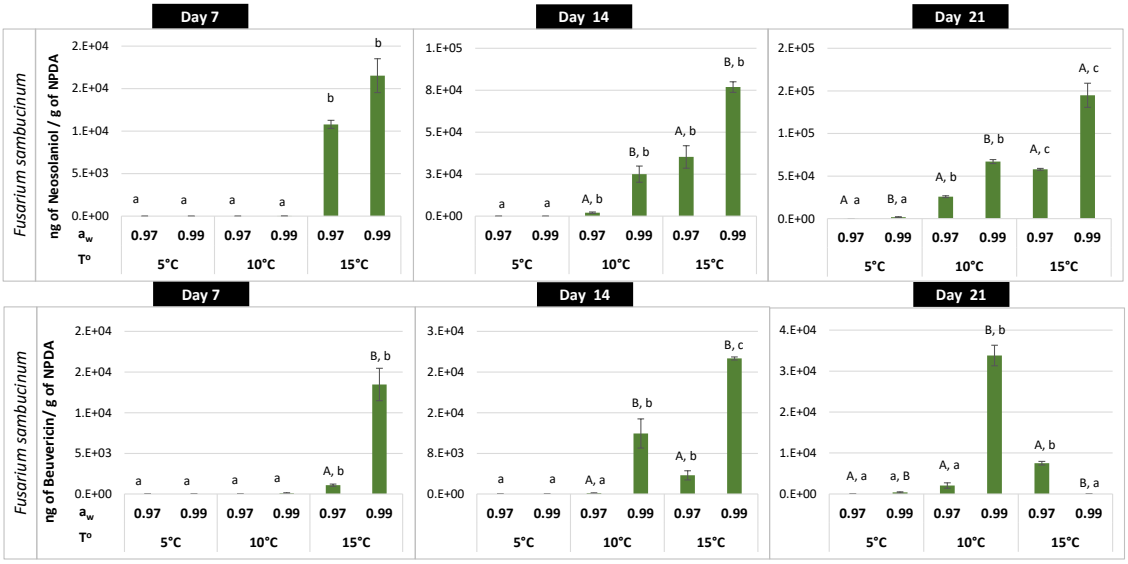


Figure 4-12 Neosolaniol and beauvericin production in presence of *Fusarium sambucinum* after 7, 14 and 21 days of incubation at different environmental conditions (temperature and water activities). a,b: Significant differences between temperatures at each specific a_w. A, B: Significant differences between a_w at each specific temperature.

The accumulation of beauvericin was significantly affected by the a_w of the media. After 7 days of incubation, it was mainly detected at 15ºC x 0.99 a_w, after 14 days of incubation it was mainly detected at 10 and 15ºC at the highest a_w (0.99). After 21 days its detection was significantly higher (p-values<0.04) at 10º C x 0.99 a_w.

Alternariol methyl ether was only detected in presence of *Fusarium oxysporum* after 21 days of incubation at 15ºC x 0.99 a_w (Figure 4-13).

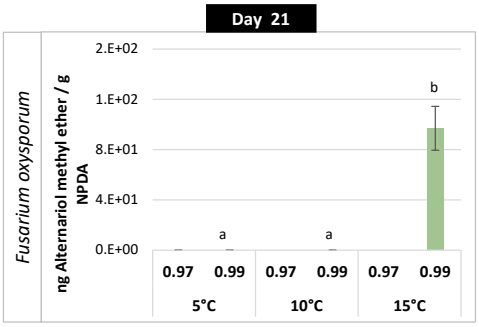


Figure 4-13 Alternariol methyl ether accumulation in presence of *Fusarium sambucinum* after 21 days of incubation at different environmental conditions (temperature and water activities). a,b: Significant differences between temperatures at each specific a_w

The temporal evolution of mycotoxins produced by *Fusarium sambucinum* is presented in Figure 4-14. At the lowest temperature (5ºC), there was no mycotoxin accumulation after 7 and 14 days, with little accumulation detected after 21 days. At 10ºC an increasing tendency from 7 days to 21 days of incubation was observed at both a_w. However, at 10ºC x 0.99 a_w the increase was more noticeable, with concentrations of T-2, HT-2 and diacetoxyscirpenol similar to those achieved at the highest temperature and water activity (15ºC x 0.99 a_w). At 15ºC, the tendency was different between the two different water activities. At 0.97 a_w the toxin accumulation increased until day 14, and the concentrations maintained at similar levels after 21 days. Interestingly at 15ºC and 0.99 a_w, there was a noticeable increasing tendency in diacetoxyscirpenol and neosolaniol concentration until day 21.

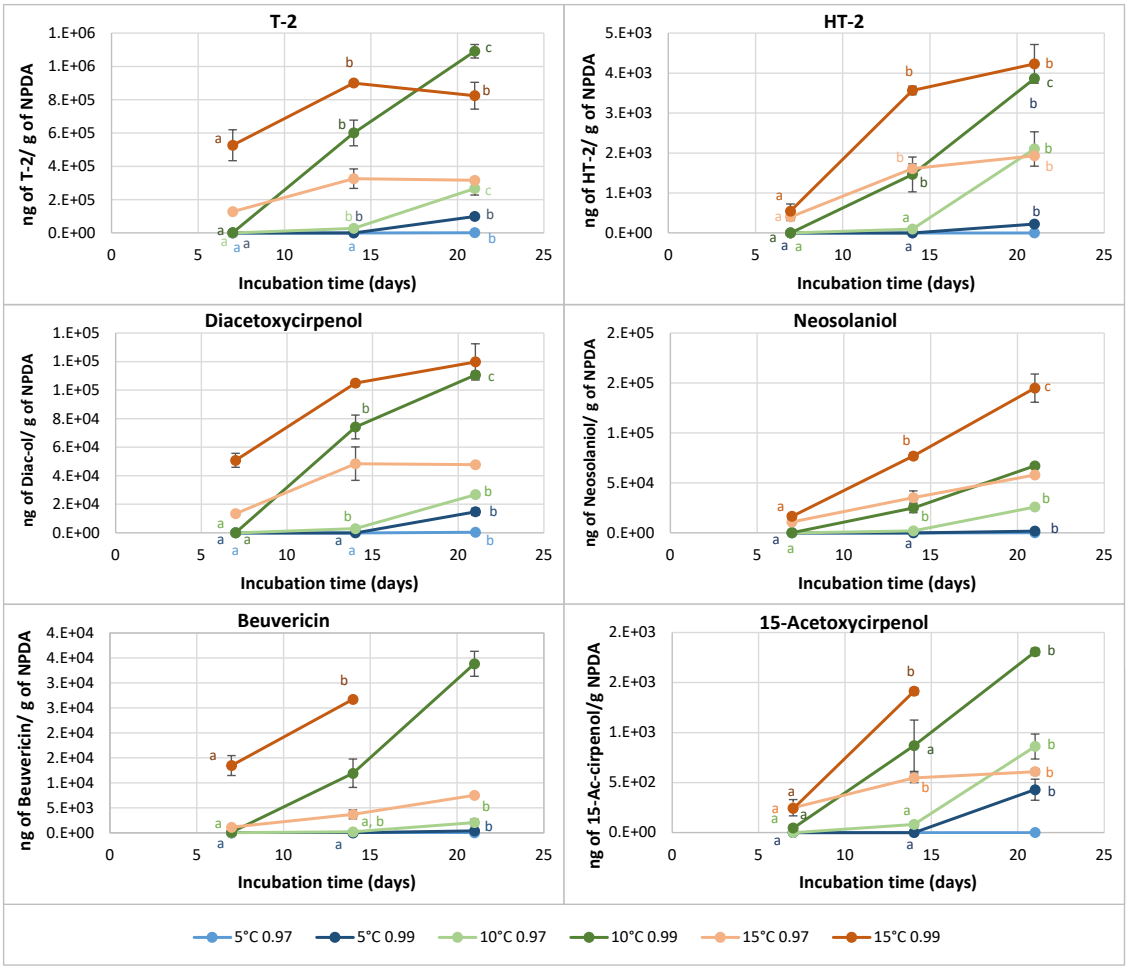


Figure 4-14 Temporal evolution of the accumulation of six different mycotoxins (T-2, HT-2, Diacetoxyscirpenol, Neosolaniol, Beuvericin and 15-Acetoxyscirpenol) in presence of *F. sambucinum* incubated in NPDA at three different temperatures (5, 10, 15ºC) and two water activities (0.97, 0.99) represented with different colours. a,b,c: Significant differences between days at each specific temperature and water activities.

4. 4 Discussion

In this chapter, the effect of temperature and water activity on two different *Fusarium* spp. was studied *in vitro*. *Fusarium sambucinum* and *Fusarium oxysporum* are both pathogens responsible for dry rot in potato tubers. Potato tubers for the processed market are stored at temperatures between 6-10ºC and high relative humidity conditions (98-99%). Understanding the ecophysiology of those pathogens responsible for dry rot in these conditions is important. Maintaining the optimal environmental conditions during storage is essential. Although, there might be fluctuations in temperature and relative humidity throughout the whole storeroom. Fluctuations that can directly affect the development of potato diseases, as dry rot produced by *Fusarium* spp. As it was previously mentioned, the environmental conditions of all the potato tubers, either in bulk or in boxes, are not uniform through all the different levels of the storage (Cargill, 1976; Cunnington and Pringle, 2012). Therefore, elucidating how the fluctuations in the environmental conditions can affect the development, VOC and mycotoxin production of both *Fusarium* spp., responsible of dry rot, could be beneficial for its management.

Furthermore, a natural potato-based media with a percentage of mashed potato was used to understand the ecophysiology of both fungi in a media with similar nutrients content than potato tubers during storage. In a potato store, there are a large range of factors that will affect the growth of *Fusarium* spp. The growth of *Fusarium* spp. also depends on the potato cultivar, the temperature of curing and handling, the soil where it was grown, the weather conditions during this year and how it was harvested, among others (Boyd, 1967; Morris, Forbes-Smith and Scriven, 1989; Percival, Karim and Dixon, 1998; Choiseul, Allen and Carnegie, 2001; Heltoft *et al.*, 2015). Studying the behaviour of *Fusarium* spp. *in vitro,* in absence of any other parameters, can be considered as a first step, before studying the development of *Fusarium* spp. *in vivo* in potato tubers.

4.4. 1 Effect of temperature and a_w on *Fusarium* spp. growth

F. sambucinum growth *in vitro* was directly affected by temperature and a_w of the media, as higher was the temperature and a_w higher was the growth of the fungi. Therefore, an increase in the temperature and relative humidity of a potato store will result in the higher development of dry rot produced by *F. sambucinum.* Therefore, those potato tubers located at the bottom of the crates will potentially present a higher risk of development of dry rot.

Most of *in vitro* studies of *Fusarium* spp. have assessed the diameter of the colonies achieved after a certain number of days. However, there are no studies on *F. oxysporum* or *F. sambucinum* lag time. Elucidating the growth rate of both fungi under different environmental conditions (temperature, a_w) provides useful information; however, knowing the time needed for the fungi to start growing is another important factor that needs to be considered.

The study of the *F. sambucinum* lag time in our work showed that at higher temperature less days were needed for the fungus to start growing *in vitro.* Similar tendency with a_w at the two highest temperatures, 10 and 15ºC, at 0.99 a_w less number of days were needed. Therefore, an increase in temperature and a_w during the storage of potato tubers benefit the development of *F. sambucinum* by a reduction in the time needed to start developing the disease.

Similar results were achieved for *F. oxysporum.* However, when both *Fusarium* spp. were compared, the growth rate of *F. sambucinum* was higher than *F. oxysporum.* However, *F. sambucinum* requires a longer time to start its development. Therefore, *F. sambucinum* grows quicker than *F. oxysporum,* although it will take longer to start growing. Depending on the pathogen causing dry rot in a storage room, its development will be quicker or lower and it will need more or less time to start the infection.

Most of the studies based on the development of *Fusarium* spp. responsible for dry rot in potato tubers, have been carried out *in vivo* (Boyd, 1952; Ayers and Robinson, 1954). Some of them suggested that temperature was directly affecting the susceptibility of potato tubers to *Fusarium* spp. responsible for dry rot as *F. coeruleum, F. sambucinum* and *F. avenaceum* from 5ºC to 20-25ºC

(Boyd, 1952; Mckee, 1954; Venter *et al.*, 1992; Sangalang, Backhouse and Burgess, 1995; Lui and Kushalappa, 2002; Gachango *et al.*, 2012). The effect of the relative humidity or duration of wetness has also been studied, with an increase in the severity of dry rot produced by different *Fusarium* spp., such as *Fusarium sambucinum* (Lui and Kushalappa, 2002). Although these results were observed *in vivo,* they were in accordance with the results found in our study.

The effect of temperature on the growth of *Fusarium* spp. *in vitro* has been studied several times on different media as PDA or Rice Extract Agar (REA), among others. Daami-Remadi et al. (2006) studied the effect of temperature on both pathogens, but different isolates, *in vivo* and *in vitro.* In their *in vitro* experiment, both *Fusarium* spp. were inoculated in non-modified PDA (0.995 a_w), and the diameter of colonies observed six days after incubation at the same temperatures as our experiment (5,10,15ºC) is presented in Table 4-8 (Daami-Remadi *et al.*, 2006). The main differences between these results and the one achieved in this chapter were for *F. oxysporum*. They did not observe any growth at 5ºC and the colony diameter was very low at 10 and 15ºC compared to our results. Also, they observed larger colonies for *F. sambucinum* compared to *F. oxysporum,* the opposite to what was observed in our study. Those differences might be due to the inoculum concentration, as in their study a fragment of mycelia (6 mm) was directly inoculated on PDA. Moreover, the differences in the media content, as the media used in our study presented a mashed potato, and the fact that they were not the same isolates could also be affecting the differential growth between both experiments. The increase tendency in colony size with temperature was also achieved. Therefore, the findings achieved in this chapter suggest that higher temperatures in storage will directly increase the growth of *F. sambucinum* and *F. oxysporum.*

Table 4-8 Comparison of colony diameter (mm) of *F. sambucinum* and *F. oxysporum* after their incubation at three different temperatures (5,10 and 15ºC). Results from this chapter experiment and Daami-Remadi et al. 2006 are included in the table (Daami-Remadi *et al.*, 2006)

Tº	Pathogen	Our experiment	Daami-Remadi 2006
5ºC	*F. sambucinum*	0.97a_w: 0 mm 0.99a_w: 8.4±0.4 mm	5 mm
	F. oxysporum	0.97a_w: 8.9±0.3 mm 0.99a_w: 10±0.3 mm	0 mm
10ºC	*F. sambucinum*	0.97a_w: 9.5±0.6 mm 0.99a_w: 18.3±0.5 mm	20 mm
	F. oxysporum	0.97a_w: 10.4±0.7 mm 0.99a_w: 22±0.6 mm	10 mm
15ºC	*F. sambucinum*	0.97a_w: 26.2±1.6 mm 0.99a_w: 52.3±0.6 mm	50 mm
	F. oxysporum	0.97a_w: 27.8±0.8 mm 0.99a_w: 48.6±0.6 mm	15 mm

The effect of temperature and a_w on *F. sambucinum* in rice extract agar (REA) was studied by Ferre et al. (2007). They calculated the growth rate of *F. sambucinum* at 15ºC at 0.95, 0.98 and 0.995 a_w. They observed an increasing tendency, as higher was the a_w of the media higher was the growth rate of *F. sambucinum.* This data was in accordance with what we observed in our study. They observed values between 1 and 3 mm per day at 0.95 and 0.995, respectively. Values that were almost 1/6 of our results, achieving a growth rate at 15ºC x 0.99 a_w of 9 mm per day. This difference might be due to the media and the specific strain of *F. sambucinum,* as it was isolated from maize grains from Valencia (Ferre, Caselles and Siurana, 2007). These results are in accordance with our findings regarding the effect of the relative humidity on the growth of *F. sambucinum.* The increase of the relative humidity of the potato storage rooms will benefit the growth of *F. sambucinum.*

There have been several studies where different *Fusarium* spp. responsible for dry rot in potato tubers were studied on synthetic media. Moore et al. (1924) observed similar results; an increase in temperature was increasing the growth

of *F. coeruleum* in vegetable media (Moore, 1924). In a different study, *F. coeruleum* and *F. sulphureum* growth were studied at 20ºC in freeze-dried potato powder media (Percival, Karim and Dixon, 1999), observing a lower growth rate than the one we observed. Those differences could be due to the differences in media, temperature and *Fusarium* spp.

4.4. 2 VOC detection in presence of *Fusarium spp.*

The study of the VOCs produced by *Fusarium* spp. *in vitro* provide different information as the provided from an *in vivo,* experiment. An *in vitro* study allows the detection of those VOCs produced from the metabolic activity of the fungi in a semi-synthetic media, such as the Natural Potato Dextrose Agar used in our work. Most of the factors involved in the growth and VOC production of both fungi *in vitro* can be controlled. However, for the *in vivo study,* there are different factors that cannot be controlled. Factors such as the presence of other microorganisms in the internal part of the potato tuber, the response of the potato tuber against the presence of the pathogen and the specific physiological conditions of the potato tuber among others. All of those factors are directly affecting the growth of the fungi and therefore the production of VOCs. Those VOCs detected in presence of infected potato tubers (*in vivo*) are a result of the presence of both, the fungi and the potato. While in an *in vitro* study those VOC detected will be exclusively produced by the *Fusarium spp., as F. sambucinum* and *F. oxysporum,* used in our work. The elucidation of those VOC *in vitro,* could be considered as a first step for the identification of VOC biomarkers of the presence of dry rot in potato tubers.

Most of the VOCs detected at 10ºC from *F. sambucinum* and *F. oxysporum* were the same, they only differed in their abundance. This might be expected as both pathogens were metabolizing the same substrate, potato-based media. Furthermore, both pathogens belong to the same family (*Nectriaceae*), therefore they are genetically similar to each other and probably both sharing many biochemical processes. Their differences in abundance was probably due to their different colony size observed at the different sampling points as presented in Figure 4-4. *F. sambucinum* colony size was higher than *F. oxysporum,* therefore,

at the same sampling point at which VOC were collected, the colony size of both *Fusarium* spp. was different.

The variability between VOC samples is an important factor when the VOC are considered. There is a high variability between replicates and that might explain the differences between different sampling days. Only three replicates were considered, for further studies at least five will be needed to reduce the variability. The sampling of VOC was carried out at a low temperature, 10^{o}C, that might also explain the reason of the detection of few compounds. The limit of detection of the TD-GC-ToF-MS needs to be considered, as well as the overloading of the Thermal Desorption tubes during the sampling procedure.

The most abundant VOCs produced by both *Fusarium* spp. were 2-methylpropan-1-ol and ethyl acetate. Most of the VOCs were detected in common after 15 and 10 days of incubation. However, carbon disulfide was only detected after 10 days of incubation. It was only produced by *F. oxysporum* and at both a_w but in very low abundance. Carbon disulfide has been previously detected in onions infected with *F. oxysporum* (Wang *et al.*, 2019). However, its absence after 15 days of incubation is difficult to explain. Further studies will need to be carried out to check the production of carbon disulfide in presence of different *Fusarium* spp.

The Total Targeted Volatile Production (TTVP) presented in Figure 4-5, showed different tendencies at the two different a_w (0.97, 0.99). At 0.97 a_w, there was an increase in the TTVP after 15 days of incubation, while at 0.99 a_w, the increase in the TTVP was already observed after 10 days of incubation. The data of *F. oxysporum* TTVP at 0.99a_w and after 15 days of incubation is not truly representative, as only 6 of the 20 VOC that were included for the calculation of the TVP were detected in those samples from these conditions. The TTVP has been previously used for the distinction between the presence and the absence of pathogens in different food matrixes (Varns and Glynn, 1979; Waterer and Pritchard, 1984b, 1984a, 1985; Lyew *et al.*, 2001; Kushalappa *et al.*, 2002; Wang *et al.*, 2019). However, the TTVP results obtained in this chapter did not elucidate the distinction between the presence and the absence of both *Fusarium* spp.

Elucidating which VOCs were produced at each of the stages of the incubation period could provide different VOC biomarkers for the presence of dry rot in potato tubers at the start of their growth or once they are already at a late stage of their development. Although some of the VOCs can be shared at the three different stages, maybe their abundance is higher compared to the non-inoculated plates at a specific stage of the incubation.

At the earliest stage of the incubation period, after 5 days of storage, no potential biomarkers were detected in presence of *Fusarium* spp. (Figure 4-6). However, acetone and ethanol have been previously detected in presence of potato diseases pathogens as *F. coeruleum* and *Pectobacterium* spp. (de Lacy Costello *et al.*, 2001; Blasioli *et al.*, 2013; Savelieva *et al.*, 2016).

At a middle stage of the incubation period, after 10 days of incubation, once the development of both *Fusarium* spp. was higher, sulfur dioxide, methyl acetate, ethyl acetate and 2-methylpropan-1-ol abundances were observed (Figure 4-7).

It was found that the highest production of those four VOCs was achieved by *F. oxysporum,* while the production of *F. sambucinum* was very low. This was again, due to the differences in colony growth at each of the sampling points, being higher for *F. oxysporum* compared to *F. sambucinum.*

At the latest stage of the incubation period, after 15 days of incubation, methyl acetate, ethanol, ethyl acetate, 2-methylpropan-1-ol and ethyl formate abundance was higher in presence of both *Fusarium* spp. compared to the non-inoculated plates (Figure 4-8). Similar results were observed in abundance of those five VOCs in presence of both of the *Fusarium* spp. studied. This is probably related to the fact that after 15 days of incubation, *F. sambucinum* that grows quicker than *F. oxysporum* will have already achieved the same level of growth than *F. oxysporum.*

VOCs as ethanol, ethyl acetate and 2-methylpropan-1ol were detected at two of the three different stages of the incubation period. Therefore, elucidating the abundance evolution of these three VOCs could provide useful information. However, due to the great variability between replicates and the different

conditions of the TD-GC-ToF-MS at the different sampling point, a great variability was observed. A slight increase in the VOC produced by both *Fusarium* spp. was observed with time. However, an extra sampling point at 20 days of incubation could have helped to elucidate if those VOCs were produced in higher concentration with time. Therefore, in a potato storage facility, those potato tubers that present higher development of dry rot might be producing higher concentrations of VOCs.

Those three VOCs, ethanol, ethyl acetate and 2-methylpropan-1-ol were produced in higher abundance in presence of *Fusarium* spp., therefore they are probably directly related to the presence of the pathogen in the media. Ethanol has been previously detected in several studies in presence of *F. oxysporum,* inoculated in onion bulbs (Vikram, Prithiviraj and Kushalappa, 2004; Vikram *et al.*, 2006; Wang *et al.*, 2019), in Liquid Onion Extract Medium (LOM) (Wang *et al.*, 2018) and presence of soft rot on potato tubers (Lui, Vikram, Abu-Nada, *et al.*, 2005). In a recent study, it was detected in Malt Agar Medium inoculated with *F. oxysporum* strain 21, non-pathogenic, at 25^{o}C (Terra *et al.*, 2017) and it was detected by e-nose in pure cultures of *F. culmorum* (Loulier *et al.*, 2020). Therefore, it can be considered as a potential VOC biomarker of the presence of a disease in potato tubers, not only caused by fungi but also bacterial pathogens.

Ethyl acetate has also been previously detected in presence of *F. oxysporum* and *F. culmorum in vitro* (Terra *et al.*, 2017; Loulier *et al.*, 2020). It is the acetate ester formed in presence of acetic acid and ethanol and one of the most common esters detected in wines during the fermentation (Marquez *et al.*, 2014). Therefore, it could also be considered as a potential VOC biomarker of potato diseases, as it has also been associated with the presence of bacteria.

The last VOC, 2-methylpropan-1-ol, one of the most interesting compounds detected in our study, was considered in 2009 as one of the most often reported microbial volatile organic compounds. Its origin was previously related to the metabolism of valine (Korpi, Järnberg and Pasanen, 2009). It was already detected in presence of *Penicillium* and *Aspergillus* species growing in Oat Meal Agar (OMA) in the ’90s (Börjesson, Stöllman and Schnürer, 1993). It was also

detected in presence of an endophytic fungi, *Muscodor albus* (Corcuff *et al.*, 2011). In a recent study, 2-methylpropan-1-ol was related to the presence of *F. oxysporum* in onion media, it was detected in very high concentrations around 50 nmol/L (Wang *et al.*, 2018). Therefore, in our study its presence was consistent after 10 and 15 days of incubation and its production was higher in presence of *Fusarium* spp. compared to its absence. It could be considered as a potential VOC biomarker of dry rot in potato tubers, although further studies will be needed to confirm these results. Its detection in presence of bacterial pathogens as *Pectobacterium carotovorum* spp., responsible for soft rot in potato tubers, will need to be studied to discard the possibility of its detection due to the presence of other bacteria in potato tubers, as it was detected in one study by de Lacy Costello et al.(de Lacy Costello *et al.*, 1999).

Although those three VOCs can be considered as potential biomarkers of the presence of a disease in a commercial storage facility. It needs to be considered that its production is directly related to the presence of the fungi, therefore an *in vivo* study will be required.

4.4. 3 Effect of temperature and a_w on both *Fusarium spp.* mycotoxin accumulation

Different mycotoxins were detected in presence of both *Fusarium* spp., responsible for dry rot in potato tubers. Toxins produced by *Fusarium* spp. are normally classified as trichothecene and non-trichothecene mycotoxins (Mills, 1990; Logrieco *et al.*, 1998; Bojanowski *et al.*, 2013). The trichothecenes mycotoxins detected in this study were diacetoxyscirpenol (DAS), HT-2, T-2, neosolaniol and 15-acetoxyscirpenol, while the only non-trichothecene mycotoxin detected was beauvericin. Elucidating the accumulation of those mycotoxins by *F. sambucinum* and *F. oxysporum,* both responsible for dry rot in potato tubers, at the different environmental conditions studied can provide interesting information regarding the food safety of potato tubers infected with dry rot.

In presence of *F. sambucinum* a larger number of mycotoxins were detected, T-2, HT-2, DAS, neosolaniol, beauvericin and 15-acetoxyscirpenol. However, in presence of *F. oxysporum*, only three mycotoxins were detected, T-2, DAS and

alternariol methyl ether. This is in accordance with what has already been reported regarding the mycotoxins produced by both *Fusarium* spp. In presence of *F. oxysporum* T-2 and beauvericin were detected on different sources, while in presence of *F. sambucinum,* T-2, HT-2, neosolaniol, diacetoxyscirpenol, 15-acetoxyscirpenol and beauvericin were detected (Bojanowski *et al.*, 2013).

In this study, the accumulation of most of the mycotoxins was directly related to the growth of both *Fusarium* spp.*,* as higher was the growth of the fungi higher was the production of mycotoxins. Therefore its production was directly affected by the temperature and a_w. The detection of mycotoxins in potato tubers has been previously studied (El-Banna, Scott and Lau, 1984; Ellner, 2002; Delgado *et al.*, 2010; Xue *et al.*, 2013, 2014). Besides, Xue et al. 2014 studied the effect of temperature on the production of trichothecenes by *Fusarium* spp. in potato tubers. Their results were in accordance with the results observed in this chapter, a higher concentration of trichothecenes was detected at room temperature compared to low temperatures (Xue *et al.*, 2014).

The temporal evolution of the different mycotoxins produced by *Fusarium sambucinum*, growing on NPDA media at the different temperatures (Figure 4-14) presented an increasing tendency. At the highest temperatures, 10ºC and 15ºC mainly, it can be observed the increasing tendency in the concentration. While for diacetoxyscirpenol, after 21 days of incubation, it has already reached the maximum concentration of toxin. That might be related to the quantity of nutrient left for the fungi to grow in the NPDA, and therefore to produce diacetoxyscirpenol.

The effect of temperature is interesting as mycotoxins were already detected at low temperatures (5, 10ºC). Twenty-one days were needed for both *Fusarium* spp., mainly *F. sambucinum* to start producing mycotoxins at the lowest temperature studied (5ºC). Therefore, considering that potato tubers can be stored for up to 10 months, if the environmental conditions are optimal for the development of *Fusarium* spp. the accumulation of mycotoxins can take place in the potato tuber. Moreover, once those potato tubers are sold, they will spend a period of time at a temperature close to 15ºC until they are consumed, where the

growth and accumulation of mycotoxins, as it has been presented, is even higher than at lowest temperatures.

Not all of those mycotoxins present a regulation for human consumption. There are EU recommendations on maximum contamination limits for the sum of T-2 and HT-2 in different commodities as cereals. As an example, in maize, the maximum level for direct human consumption is 100 µg/kg (European Commission 2013/165/EU, 2013).

Considering that a relative concentration of 10^6 and 10^3 of T-2 and HT-2 ng/g NPDA were observed (Figure 4-10, Figure 4-11), if the accumulation of mycotoxins increases with time, a high concentration of mycotoxins could be present once potato tubers are sold. This is also directly affecting the production of mycotoxins at higher temperatures and higher a_w, the production of those mycotoxins will be even higher. Considering those values and comparing them with the EU recommendation in maize they will be exceeding the limit. However, it needs to be considered that those values were achieved without considering the matrix effect during its detection in UHPLC-MS-MS, it might be that the real values are lower than the observed.

4. 5 Conclusions

The main conclusions from this chapter were:

- ✓ *Fusarium sambucinum* and *Fusarium oxysporum* growth on NPDA was directly affected by temperature and water activity. A higher temperature and water activity increased their growth rate and reduced their lag time. Therefore, higher temperature and relative humidity in potato storage facilities will result in higher development of dry rot.
- ✓ The incubation time was directly affecting the VOC fingerprint detected in presence of *Fusarium* spp. at different water activities (0.97,0.99).
- ✓ Ethanol and ethyl acetate were detected in presence of *F. sambucinum* and *F. oxysporum.* They could be considered as potential biomarkers of the presence of a disease (fungal or bacterial) in potato tubers. Although, an *in vivo* study will be needed to confirm this conclusion.
- ✓ 2-Methylpropan-1-ol was detected in presence of both *Fusarium* spp., with higher abundance in presence of *F. oxysporum.* It could be considered as a potential VOC biomarker of the presence of dry rot in potato tubers. Further analysis will need to be carried out to confirm these conclusions.
- ✓ Temperature, water activity of the media and time were directly affecting the accumulation of mycotoxins in presence of both *Fusarium* spp. A higher temperature and a_w increased the mycotoxins accumulation *in vitro*. Therefore, potato tubers stored at higher temperatures, as home storage, will present a higher risk of accumulation of mycotoxins in presence of mycotoxigenic *Fusarium spp*.

5 SEVERITY OF POTATO SOFT AND DRY ROTs & VOC FINGERPRINT DURING COLD STORAGE

5. 1 Introduction

Potato tubers are stored for a long period of time at low temperatures (3-10^{o}C) and at high relative humidity (90-100%) where controlled air ventilation is maintained. An optimal management of the environmental conditions in storage is essential to reduce the appearance of fungal and bacterial pathogens on the skin and flesh of potato tubers (Cargill, 1976; Cunnington and Pringle, 2012; Cunnington, 2018).

As previously mentioned, there are around 80 different potato cultivars currently used in Great Britain. Each of them will present a specific susceptibility to potato diseases as dry rot, soft rot or gangrene, among others. Elucidating the severity of the lesion of those potato pathogens in the current potato cultivars will allow the farmers to decide which cultivars to plant. Depending on the main diseases that are affecting their area at that time or in the previous years, they will select the optimal cultivar.

The development of a potato disease, as dry rot or soft rot, during the storage of potato tubers might not be visible until the end of the storage period, once a large number of potatoes are already affected. An early detection of potato storage diseases could reduce the large generation of waste and avoid the consequential economic losses.

Since 1972, many different studies have been undertaken on potato tubers diseases, mainly on dry rot (*Fusarium* spp.), gangrene (*Boeremia* spp.) and soft rot (*Pectobacterium* spp.) (Boyd, 1972; Secor and Gudmestad, 1999; Tsror, Aharon and Erlich, 1999; Schisler *et al.*, 2000; Kushalappa *et al.*, 2002; Choiseul, Allen and Carnegie, 2006; Cunnington, 2008; J. C. Peters *et al.*, 2008; Cunnington and Pringle, 2012; Fiers *et al.*, 2012; Bojanowski *et al.*, 2013; Safenkova *et al.*, 2015). Recently potato diseases incidence have been studied in seed potato tubers in Colorado (Zeng, 2018). However, only a few studies have focused on the effect of storage time on the severity of those two specific

diseases, soft rot and dry rot. Recently, a study was carried out regarding the effect of long term storage on soft rot, however, the temperature of incubation for the assessment of the severity of soft rot was carried out at 23ºC (Chung *et al.*, 2013), while the real conditions during cold storage are temperatures between 3 and 10ºC.

There are different methods for the early detection of potato diseases, however, the identification of Volatile Organic Compound (VOC) biomarkers of potato diseases in storage has been considered as a potential method (Fang and Ramasamy, 2015). Several studies have been carried out on VOCs identification from potato tubers infected with different pathogens. Most of the studies have been undertaken on *Pectobacterium* spp. (Waterer and Pritchard, 1984b; Ouellete *et al.*, 1990; de Lacy Costello *et al.*, 1999, 2000; Lyew *et al.*, 2001; Lui, Vikram, Abu-Nada, *et al.*, 2005; Rutolo *et al.*, 2014, 2016; Sinha *et al.*, 2017). However, the release of VOCs have mainly been studied at a range of temperature between 15 to 25ºC, while the released VOCs from potato tubers infected and incubated under cold storage conditions (4-10ºC) have been barely studied (Varns and Glynn, 1979; Weijman *et al.*, 1984). As it has been presented in previous chapters, the growth of three different fungi, *Boeremia foveata, Fusarium sambucinum* and *Fusarium oxysporum* was directly affected by the different environmental conditions (temperature and water activity). Therefore, the study of the release VOCs at the actual temperature where potato tubers are stored in the storage facilities (3-10ºC) will provide more accurate information and will allow the identification of biomarkers of the presence of different potato diseases.

Studying the development of soft rot and dry rot and their VOC production at an intermediate temperature as 8.5ºC could clarify the effect of an increase in the temperature in storage, as the worst-case scenario. Considering that at those conditions potato tubers will probably present a higher risk of development of diseases.

Thus, the overall aim was to elucidate the development of dry rot and soft rot on potato tubers under cold storage. The objectives of this study were:

a) study the severity of soft rot and dry rot on two potato cultivars (Record and Casablanca) **b)** elucidate the effect of storage time on the severity of soft rot and dry rot on potato tubers **c)** determine the VOC fingerprint of potato tubers infected with soft rot and dry rot, separately, and **d)** study the effect of storage time on the VOC fingerprint of infected soft rot and dry rot potato tubers.

5. 2 Material and methods

The same experiment was carried out twice at different stages of the storage of potato tubers. Once the potato tubers from the harvest season of 2018 were received at Cranfield University, they were cooled down and stored at 4ºC until the start of the experiment (10 weeks). Half of those potato tubers were used for the first experiment, the one considered as “Early-stage” of the storage. While the other half was stored at 4.5ºC for 16 extra weeks until the second experiment was carried out (“Mid-stage”).

5.2. 1 Potato cultivars

Two different organic potato cultivars were used (Record and Casablanca). Its origin and handling once they arrived at Cranfield University was mentioned in Section 2.4.1. 1.

5.2. 2 Potato surface sterilisation

Potato tubers (cvs. Record and Casablanca) were surface sterilised before their inoculation with the pathogen as mentioned in Section 2.4. 2.

5.2. 3 Fungal and bacterial pathogens

Two different pathogens were used: *Pectobacterium carotovorum atrosepticum* (soft rot) and *Fusarium sambucinum* (dry rot).

5.2.3. 1 *Pectobacterium carotovorum atrosepticum* strain

P. carotovorum subsp. *atrosepticum*, responsible for soft rot in potato tubers, was previously cultured as mentioned in Section 2. 1.

5.2.3. 2 *Fusarium sambucinum* strain

Fusarium sambucinum, responsible for dry rot in potato tubers, was previously cultured as mentioned in Section 2. 1.

5.2. 4 Potato tubers inoculation and incubation

Potato tubers were wounded, inoculated and incubated at 8.5^{o}C and a high relative humidity as mentioned in Section 2.4. 3.

Four different treatments were included per potato cultivar as presented in Figure 5-1**;** non-wounded potato tubers (control), wounded and inoculated with sterile distilled water (blank) and tubers wounded and inoculated with *Pectobacterium c. atrosepticum* or *Fusarium sambucinum*. Another control was included without potato tubers (empty box). Three replicates were included per treatment, for the monitoring of the VOCs and an extra box was included per treatment for the external and internal assessment of the observed rots.

5.2. 5 External and internal lesion assessment

External and internal lesion assessment was carried out following the procedure mentioned in Section 2.4. 4. The external lesion was assessed after 4, 8, 10, 11, 12, 13, 14, 15, 16 and 32 days, while the internal lesion was assessed after 10, 20 and 30 days.

5.2. 6 Volatile Organic Compound (VOC) sampling methodology

VOC samples were collected after 0, 8, 16 and 32 days of incubation following the same methodology as the one mentioned in Section 2.5. 2. VOC samples were taken in two different Thermal Desorption (TD) tubes (Bio Monitoring and Air toxics) (Markes International, UK). Both TD tubes were placed in series one after the other and connected with a tube, Bio Monitoring TD tube was the first in the line followed by the Air-toxics. Two different TD tubes were included to allow the recovery of different kinds of compounds, depending on their affinity they were retained by Bio Monitoring or Air toxics as mentioned in Section 2.5. 1.

The storage of the volatile samples, the addition of the internal standard and the analysis of the samples by Thermal Desorption-Gas Chromatography-Time of Flight-Mass Spectrometry (TD-GC-ToF-MS) was carried out as described in Section 2.5. 3.

5.2. 7 VOC data analysis

The first experiment, at an Early-stage of the storage period, included VOCs that were sampled using two different TD tubes, Bio Monitoring and Air toxics. For the second experiment, at a Mid-stage of the storage period, VOCs were only analysed on Bio Monitoring tubes, due to a contamination issue with the Air toxics samples. A contamination of the equipment (TD-GC-ToF-MS) with 2-Pentanone resulted in the contamination of some of the Air toxics samples that were run at that time. Therefore, those samples were not able to be analysed due to the presence of a high peak of 2-Pentanone.

The data analysis of the results from the TD-GC-ToF-MS was carried out as described in Section 2.5. 4. A first untargeted analysis was carried out followed by the calculation of the Total Targeted Volatile Production (TTVP) of each of the treatments. However, in this case the data was grouped by stage of the storage period (Early-stage, Mid-stage), by cultivar (Record, Casablanca) and by treatment (Control, Blank, *Fusarium, Pectobacterium*) when comparing between treatments at each specific day (8, 16, 32). The comparisons were carried out for each stage of the storage and cultivar between treatments at each specific sampling day.

Based on the initial comparisons, a selection of VOC was carried out. Those VOC with the highest values of FC were selected and included in a specific library in AMDIS. New comparisons were carried out between the peak areas of these compounds. The comparisons were performed between VOC samples from the containers with non-wounded potato tubers (control), wounded non-inoculated potato tubers (blank), *Fusarium* inoculated and *Pectobacterium* inoculated potato tubers at the four different sampling points (Day 0, 8, 16 and 32). At the same time, the comparison between those specific VOC abundances was carried out between the two different stages of the storage (Early and Mid-stage).

5.2. 8 Statistical analysis

Statistical analysis was performed similarly as it was described in Section 2. 8.

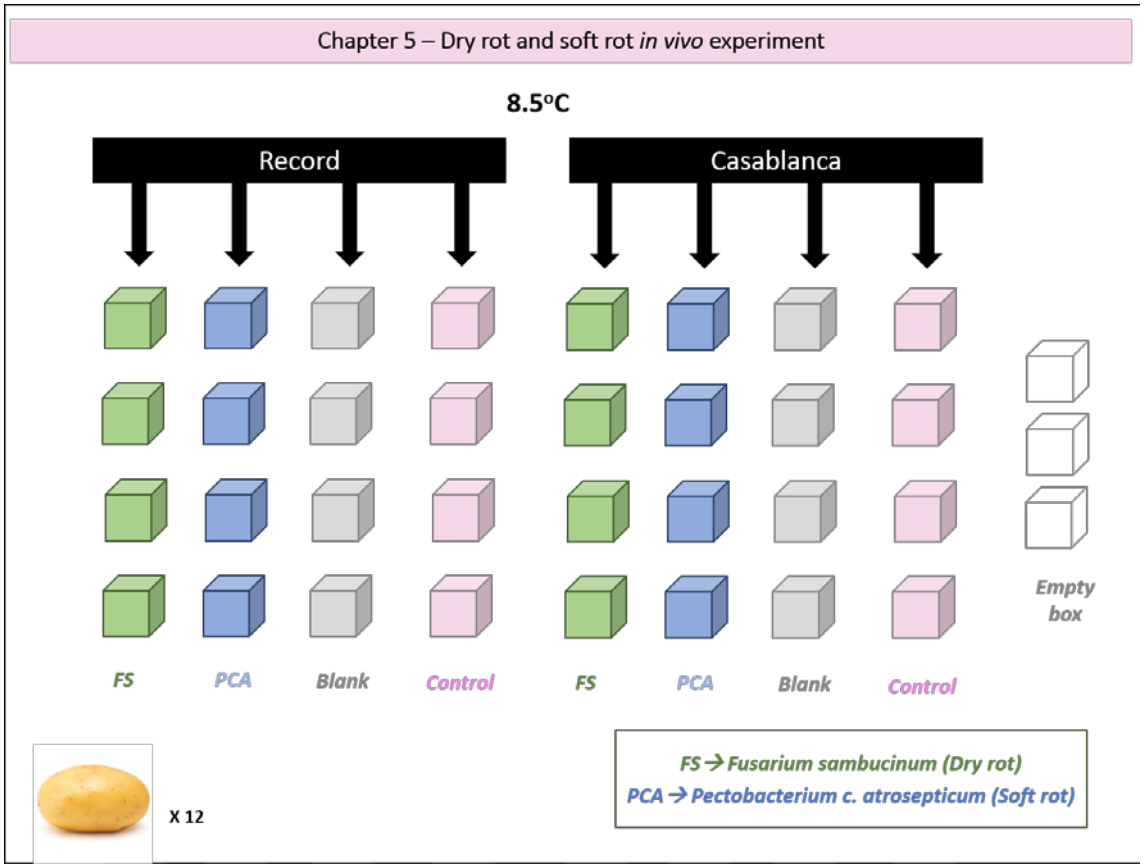


Figure 5-1 Experimental design of dry rot and soft rot *in vivo* experiment associated with Chapter 5. Four different treatments were included: non-wounded potato tubers (Control), wounded and non-inoculated potato tubers (Blank), *Fusarium sambucinum* (FS) and *Pectobacterium c. atrosepticum* inoculated potato tubers (PCA). Twelve potato tubers were included on each 12L boxes and incubated at 8.5°C. VOCs were sampled after 8, 16 and 32 days.

5. 3 Results

5.3. 1 Disease severity of dry rot and soft rot

The effect of cultivar and stage of the storage period on the disease severity of dry rot and soft rot was studied based on the size of the internal and external lesion produced by *Fusarium sambucinum* and *Pectobacterium c. atrosepticum,* respectively.

5.3.1. 1 Effect of cultivar and stage of the storage period on the dry rot and soft rot external lesion

The external lesion of dry rot and soft rot started to be visible after four days of incubation at 8.5ºC. In Figure 5-2 and Figure 5-3, potato tubers from the two cultivars (Record and Casablanca) were captured after 5, 10, 15 and 30 days of incubation. As it can be observed in the images, cv. Casablanca presented more extended lesions on both diseases, dry rot and soft rot compared to cv. Record Furthermore, those potatoes from the Mid-stage of the storage (Figure 5-3) presented larger lesions compared to those potato tubers from the Early-stage of the storage (Figure 5-2).

Results on the external infected area of potato tubers in presence of *F.sambucinum* and *Pectobacterium c. atrosepticum* at an Early-stage of the storage are presented in Figure 5-4. No significant differences (p-value>0.24) in the severity of dry rot lesions were observed between cultivars except for day 12 and 13 where cv. Record was significantly (p-values<0.05) more infected than cv. Casablanca. However, cv. Casablanca presented significantly (p-values<0.05) higher soft rot lesion compared to Record. *Pectobacterium c. atrosepticum* after 4,10, 15 and 32 days of incubation.

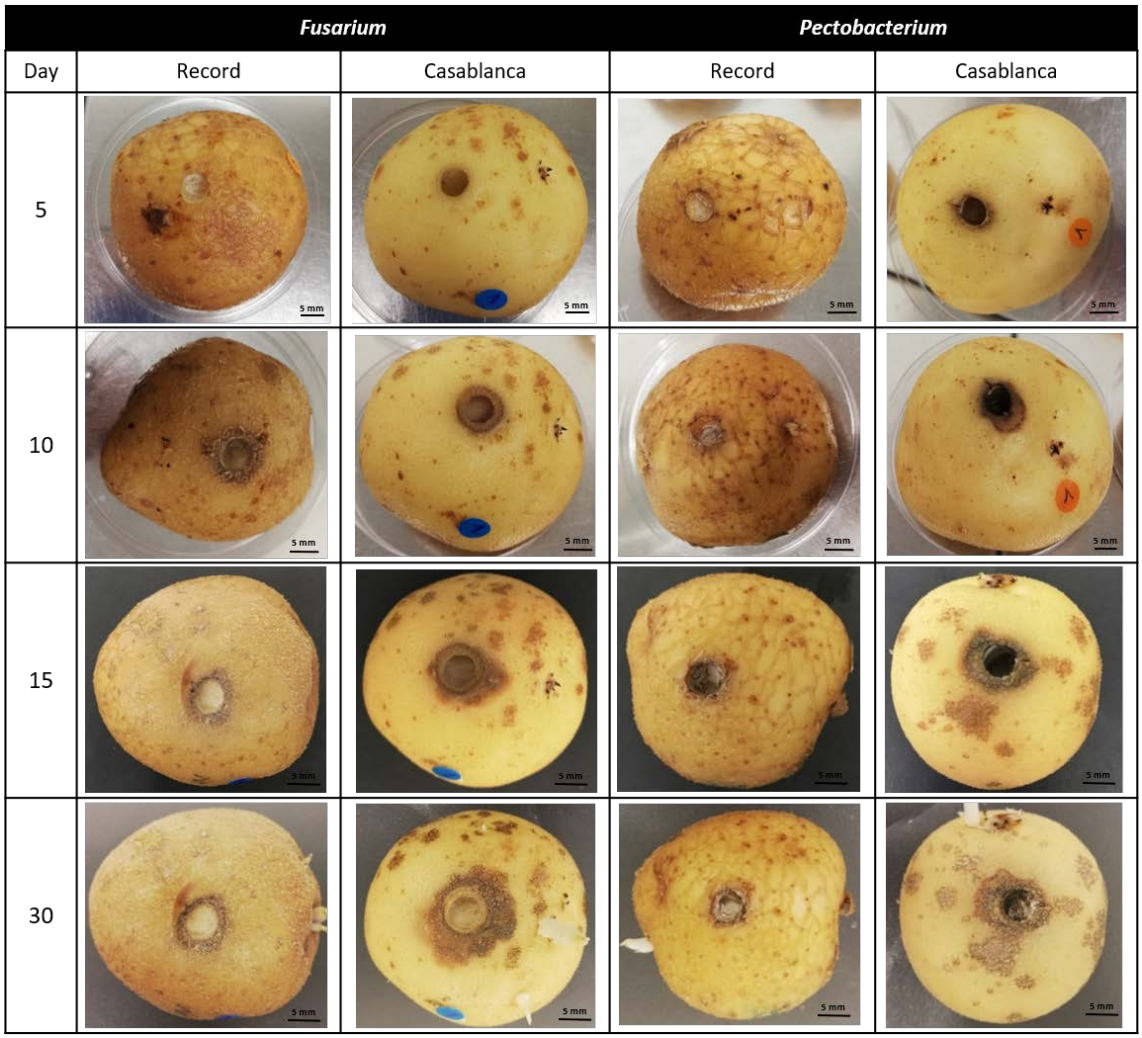


Figure 5-2 Early-stage of the storage period. Images of potato tubers cvs. Record and Casablanca inoculated with *Fusarium sambucinum* and *Pectobacterium c. atrosepticum* after 5, 10, 15 and 30 days of incubation at 8.5ºC.

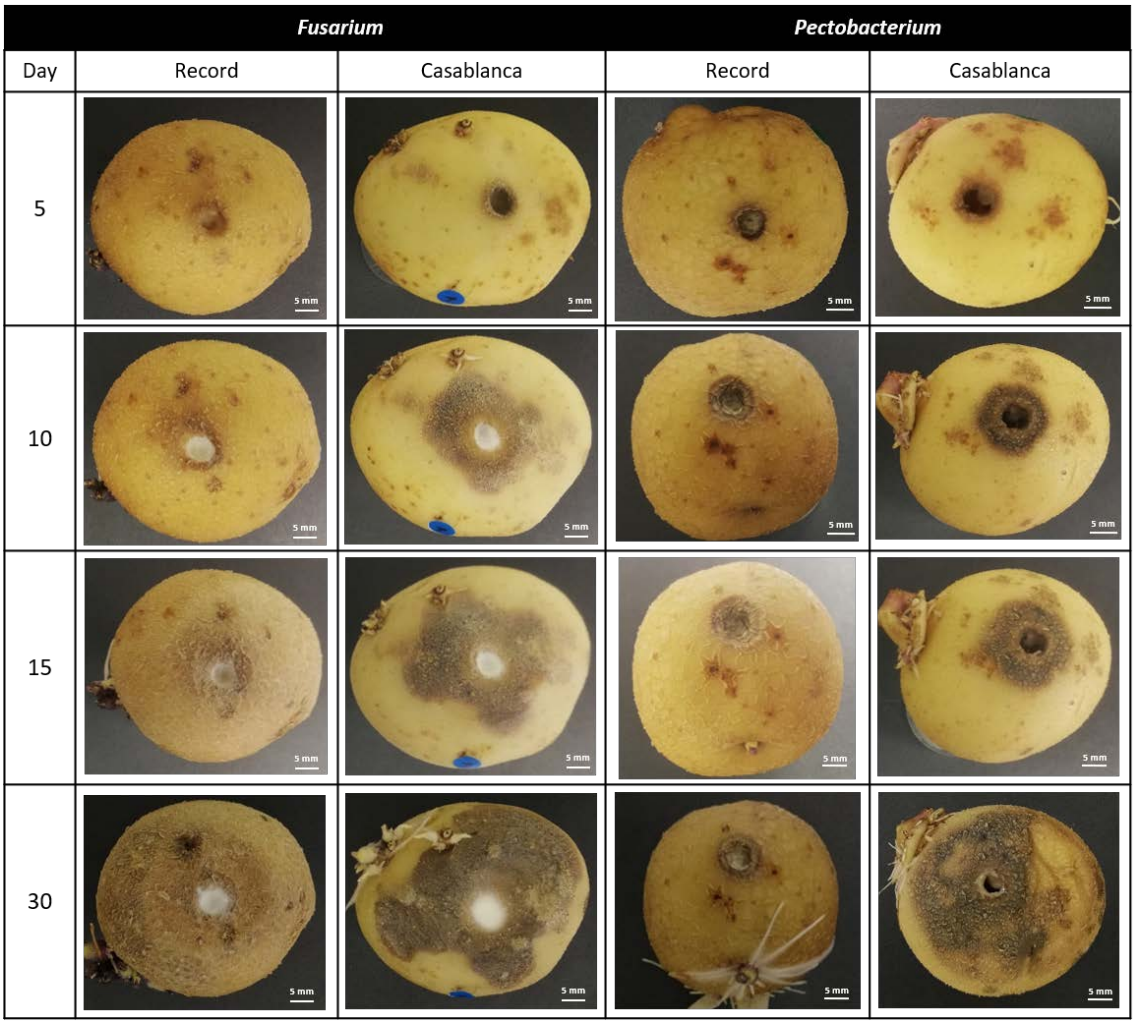


Figure 5-3 Mid-stage of the storage period. Images of potato tubers cvs. Record and Casablanca inoculated with *Fusarium sambucinum* and *Pectobacterium c. atrosepticum* after 5, 10, 15 and 30 days of incubation at 8.5ºC.

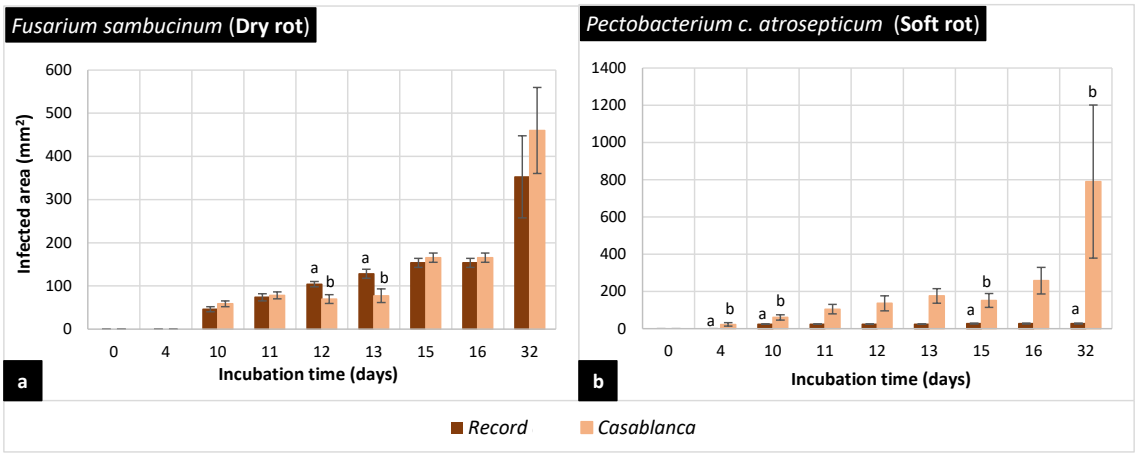


Figure 5-4 Early-stage of the storage. Effect of *Fusarium sambucinum* (a) and *Pectobacterium c. atrosepticum* (b) on two different cultivars of potato tubers (Record and Casablanca). Infected area was presented at the different assessment days. Cultivars with different letters are significantly different at each specific assessment day (Kruskal-Wallis, p-value<0.05).

When the same experiment was carried out at a Mid-stage of storage, results on the severity of dry rot and soft rot differed between cultivars. As shown in Figure 5-5, the severity was significantly higher (p-values<0.05) in cv. Casablanca compared to cv. Record. As in the Early-stage of the storage, the development of soft rot on cv Record was very low.

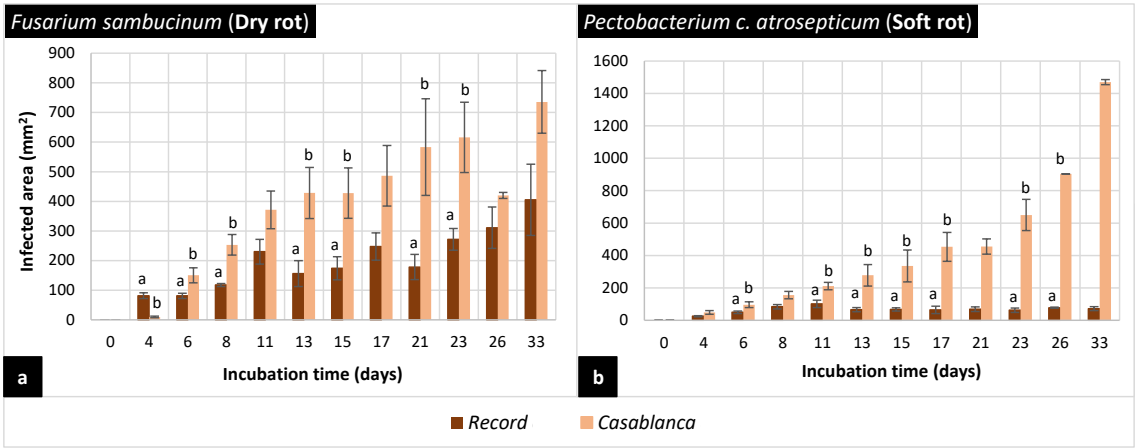


Figure 5-5 Mid-stage of the storage. Effect of *F. sambucinum* (a) and *P. c. atrosepticum* (b) on two different cultivars of potato tubers (Record and Casablanca). Infected area were presented at the different assessment days. Cultivars with different letters are significantly different at each specific assessment day (Kruskal-Wallis, p-value<0.05).

In Figure 5-6, a comparison of the severity of soft rot and dry rot on the two different cultivars (Record and Casablanca) at the two different stages of the storage is presented. For dry rot (Figure 5-6 a), significant differences (p-values<0.009) in disease severity between the two stages of storage were mainly detected in cv. Casablanca. Those cv. Casablanca potato tubers from the Mid-stage of storage presented higher infected area compared to those from an Early-stage of the storage. However, cv. Record presented higher infected area during the early-stage of the development of dry rot, after four and eight days of incubation.

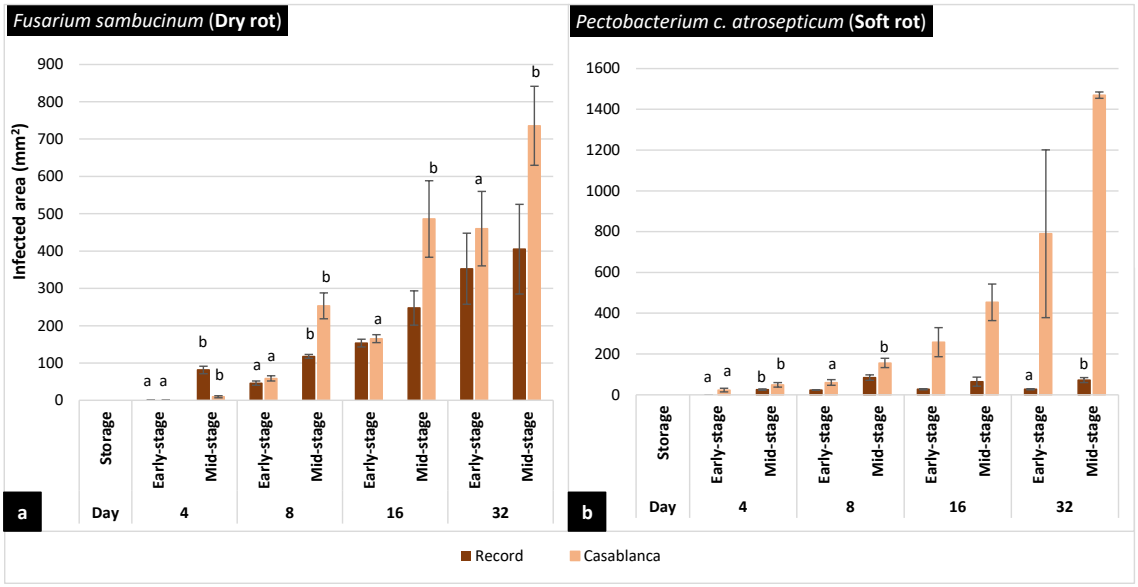


Figure 5-6 Infected area produced by *Fusarium sambucinum* (a) and *Pectobacterium c. atrosepticum* (b) after four, eight, sixteen and thirty-two days of storage at cold temperatures at two different stages of the storage. a,b: Significant differences between both stages of the storage (Early and Mid-stage) at each specific cultivar and assessment day (Kruskal-Wallis, p-value<0.05).

Considering the lesion produced by soft rot (Figure 5-6 b) significant differences (p-value<0.05) between the two stages of the storage period were only detected at an early-stage of the incubation period, after four and eight days of incubation for cv. Casablanca Therefore, storage was affecting the development and severity of soft rot on cv. Casablanca only at the early-stage of the incubation. Cv. Record could not be considered as its development was very low. Although significant differences were detected, higher severity of the extent of the lesion

produced by soft rot, on those potato tubers that were stored for four months before their inoculation only at a late stage of the development of the disease, after 32 days in cv. Record.

Two different parameters about the external lesion (infection rate and lag time) were calculated for each of the potato diseases in both cultivars (Record, Casablanca) (Figure 5-7). The fitted linear models used for the calculation of those two parameters are included in Appendix K. Results of dry rot showed no significant differences (p-values>0.60) in infection rate between cultivars. When the comparison was carried out between storage stages, significant differences (p-values<0.05) were detected in infection rate, being higher on those potato tubers from the Mid-stage storage. While for the lag time opposite results were achieved (p-value<0.005), more days were needed for dry rot to produce visible lesion at an Early-stage of the storage compared to the Mid-stage.

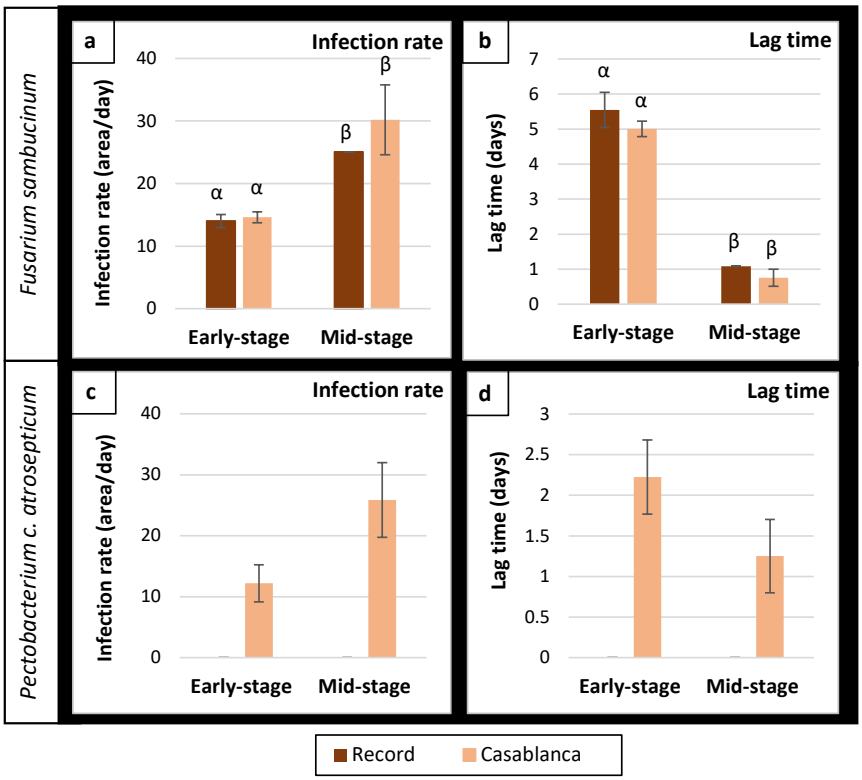


Figure 5-7 Infection rate and lag time of *Fusarium sambucinum* (a, b) and *Pectobacterium c. atrosepticum* (c, d) on potato tubers of two different cultivars (Record and Casablanca cv) at the two different stages selected of the storage (Early and Mid-stage). α, β: Significant differences between stages of the storage for each cultivar (T-test, p-value<0.05).

The results of soft rot infection rate and lag time (Figure 5-7 b,c) were only considered for cv. Casablanca. The data resulted from soft rot in cv. Record did not fit the linear model due to the very slow or almost no development of the disease in this cultivar. No significant differences (p-values>0.10) were detected between both stages of the storage; the infection rate and lag time on both cases were very similar.

5.3.1. 2 Effect of cultivar and stage of the storage period on the dry rot and soft rot internal lesion

Internal lesion assessment in presence of dry rot and soft rot was carried out after 10 and 30 days of incubation at low temperature (8.5ºC). In Figure 5-8 and Figure 5-9, the internal lesion of potato tubers (cv Record and Casablanca) at the two different stages of the storage are presented. At an Early-stage of the storage (Figure 5-8), a higher internal lesion was visually observed in presence of soft rot and dry rot in cv. Casablanca compared to cv. Record. However, at a Mid-stage of the storage (Figure 5-9), images were only taken after 10 days of storage. In the case for dry rot, both cultivars seemed to have a similar extend of internal lesion, while for soft rot the internal lesion was higher for cv. Record.

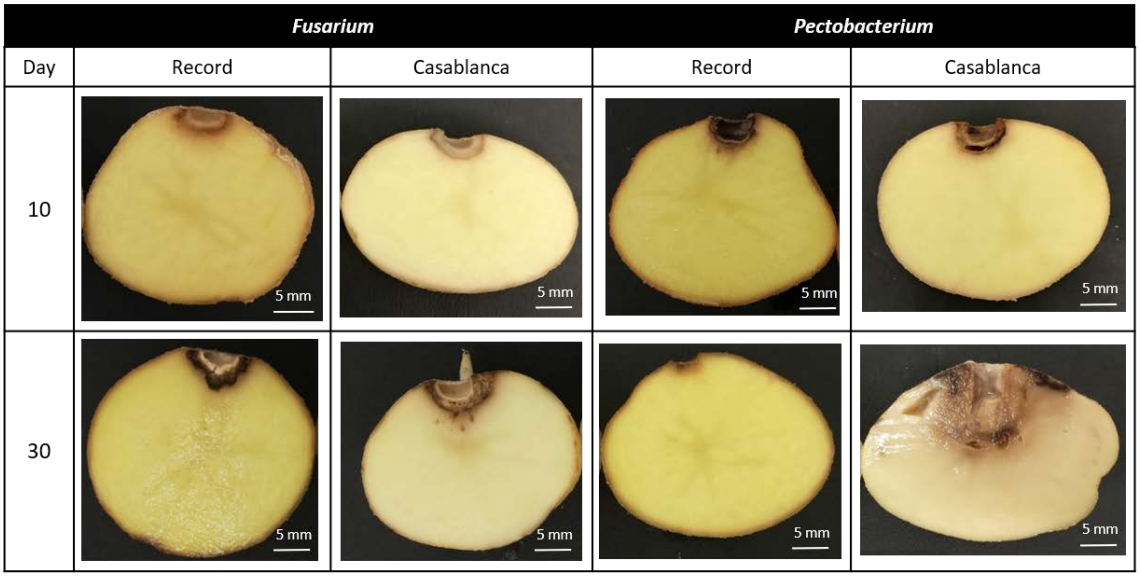


Figure 5-8 Early-stage of storage. Internal lesion of potato tubers inoculated with *Fusarium sambucinum* and *Pectobacterium c. atrosepticum* after ten and thirty days of incubation at 8.5ºC.

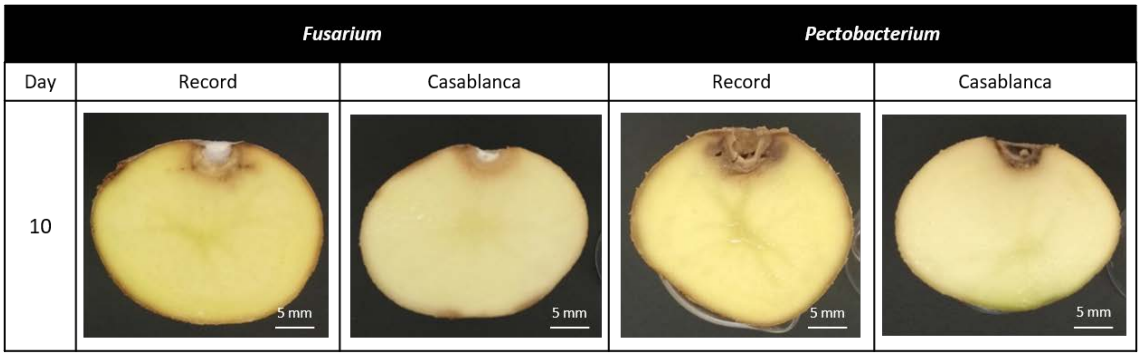


Figure 5-9 Mid-stage of storage. Internal lesion of potato tubers inoculated with *Fusarium sambucinum* and *Pectobacterium c. atrosepticum* after ten days of incubation at 8.5ºC.

The internal lesion, based on infected area, was represented for both experiments, the Early and Mid-stage of storage (Figure 5-10). The internal lesion produced by dry rot and soft rot were represented at the start of the experiment, after 10 and 30 days of incubation at 8.5ºC. No significant differences (p-values>0.15) were detected in the internal lesion produced by dry rot (Figure 5-10 a) between cultivars (Record and Casablanca.). No data was available from day 10 at Mid-stage of the storage; therefore, no comparison was carried out in this case.

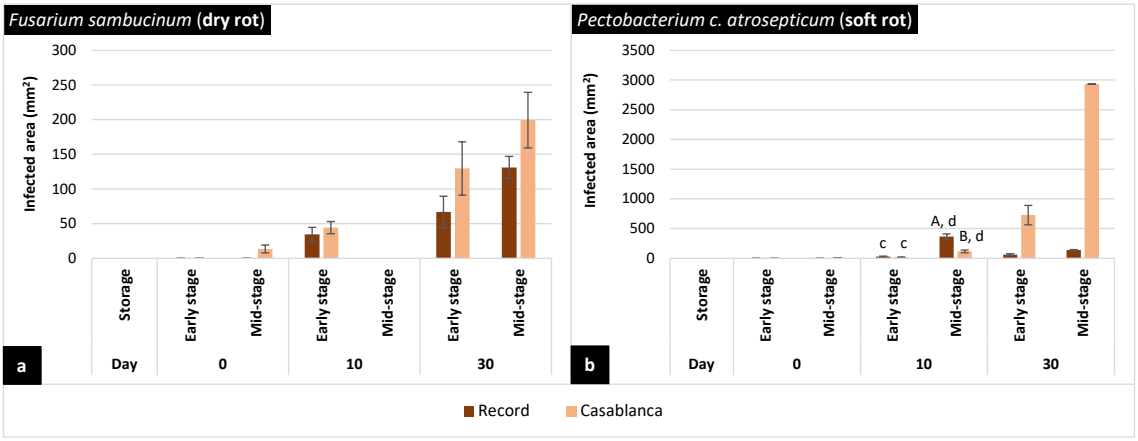


Figure 5-10 Internal lesion of potato tubers inoculated with *Fusarium sambucinum* (a) and *Pectobacterium c. atrosepticum* (b) at the start of the experiment, after ten and thirty days of incubation at 8.5ºC. c,d: Significant differences between stages of the storage for each cultivar. A, B: Significant differences between cultivars at each stage of the storage (Kruskal-Wallis, p-value<0.05).

Regarding the internal lesion produced by soft rot (Figure 5-10 b), significant differences were detected between Early and Mid-stage of the storage only after 10 days of incubation, where higher (p-value<0.05) internal lesion was detected in those potato tubers (cvs. Record and Casablanca) from the Mid-stage of storage. At the same time, significant differences in internal lesion (p-values<0.04) were detected between cultivars, at day 10 cv. Record presented higher internal lesion than cv. Casablanca at the Mid-stage of the storage.

The depth of the internal lesion was studied in those potato tubers from an Early-stage of the storage (

Figure 5-11). Significant differences (p-values<0.002) in rot depth were detected between treatments, with a higher rot depth detected in cv. Record in presence of *Pectobacterium c. atrosepticum* at both sampling days. While in cv. Casablanca presented a higher rot depth in presence of both pathogens compared to the non-inoculated potato tubers after 30 days of incubation. No significant differences (p-value>0.08) were detected between cultivars.

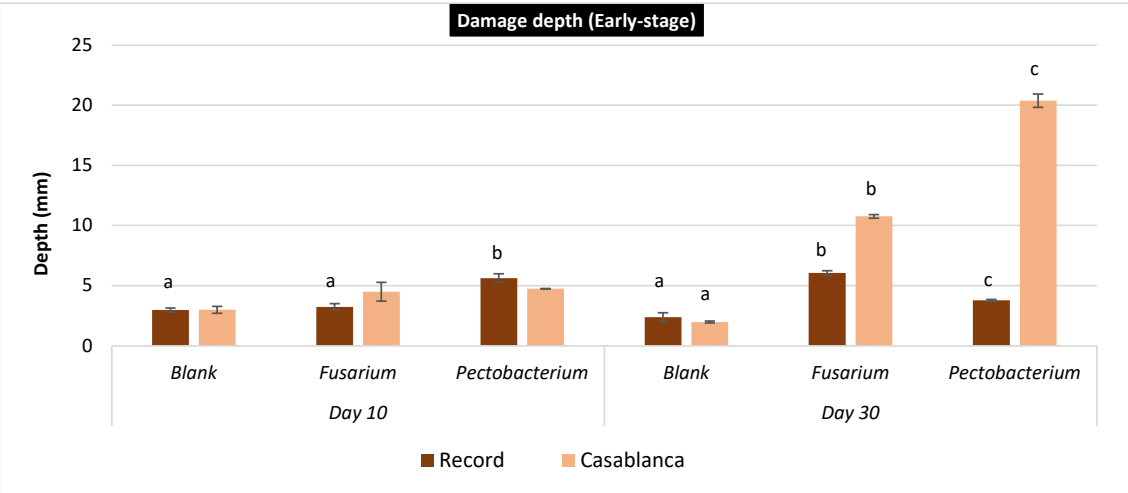


Figure 5-11 Depth of internal lesion of potato tubers non-inoculated (blank) and inoculated with *F. sambucinum* and *Pectobacterium c. atrosepticum* after 10 and 30 days of incubation at 8.5ºC. a,b,c: Significant differences detected between treatments at each specific day (t-test, p-value<0.05).

5.3. 2 Volatile Organic Compound (VOC) results

Different VOCs were detected in the presence and absence of *Fusarium sambucinum* and *Pectobacterium c. atrosepticum* at different stages of the potato diseases development. Different VOCs were also detected at Early-stage and Mid-stage of storage.

The two different steps carried out for the VOC analysis are presented in different sections. The first one included the untargeted analysis with the results from the comparisons between those VOC samples from non-inoculated potato tubers (blank) and inoculated with the two different potato pathogens. Moreover, the second one, the targeted analysis, where those VOCs selected from the previous analysis were represented on each specific treatment, at each specific day and storage stage.

5.3.2. 1 Untargeted analysis

The untargeted analysis consisted of a comparison between potato tubers that were wounded but non-inoculated (blank) and those that were inoculated with *F. sambucinum* and *Pectobacterium c. atrosepticum,* separately*.* This analysis was carried out for each experiment, at an Early and Mid-stage of the storage period.

In both experiments, a Principal Component Analysis (PCA) was carried out for each cultivar considering all the different treatments, potato tubers non-wounded and non-inoculated (control), wounded but non-inoculated (blank), inoculated with *F. sambucinum* and inoculated with *Pectobacterium c. atrosepticum* and at the three different sampling points (Appendix L*).* The distribution of the treatments (control, blank, *Fusarium* and *Pectobacterium*) could be observed for each cultivar and at each of the stages of the storage period (Early and Mid-stage). However, VOC samples from the same treatment were not clearly clustered, probably due to the high number of VOCs detected and the variability between replicates.

At an Early-stage of the storage, those VOCs that were detected in significantly higher abundance (p-values< 0.05) in presence of each of the pathogens (*Fusarium* and *Pectobacterium*), compared to its absence, at the three different

stages of the incubation period (8, 16 and 32 days) in both cultivars (Record and Casablanca) are presented in Table 5-1 (Bio Monitoring TD) and Table 5-2 (Air toxics TD). Each VOC is presented with a fold change (FC) value, representing how much the VOC abundance is changing between the presence and absence of each of the pathogens. As higher is the FC value higher differences in abundance were detected for this specific VOC.

The results from the Bio Monitoring TD tubes are presented in Table 5-1. Different VOCs were detected in the two cultivars and with different FC values. Compounds as methyl formate, acetone, trichloromethane, benzene and 1-methoxy-3-methyl-butane were detected in both cultivars. However, their pattern was not the same regarding the pathogen and the day of its detection. Methyl formate was detected in both cultivars in presence of *Fusarium sambucinum* but at different days.

In cv Record. (Table 5-1) isopropyl alcohol was the VOC detected with a higher FC in presence of dry rot with a FC value of 10^8. It was followed by 1-methoxy-3-methyl-butane with an FC of 10^6 after 16 days of incubation at cold temperature. While in cv. Casablanca, 1-methoxy-3-methyl-butane was also detected with the highest FC, followed by sulfur dioxide and 2-butanone.

In Table 5-2, the untargeted analysis from the Air toxics TD tubes samples is presented. As Air toxics was the second TD tube in line, the number of VOCs detected was lower than those detected using the Bio Monitoring TD tubes. In this case, the only compound that resulted significantly higher in presence of the pathogens compared to their absence in both cultivars was butanoic acid methyl ester. Comparing between both pathogens, more VOCs were detected with significantly higher abundance in the Air toxics TD in presence of dry rot compared to the presence of soft rot.

Table 5-3 shows the VOCs identified through the untargeted analysis of the Mid-stage samples. Compounds, such as hydrogen cyanide, 2-butanone, formic acid, 1-methyl propyl-ester and 1-methoxy-3-methyl-butane were detected in both cultivars in presence of the two different pathogens. A similar pattern as the one presented in the Early-stage of the storage experiment was found, where different

VOCs with different FC were detected at different stages of the incubation period and between the two cultivars.

In cv Record, 1-methoxy-3-methyl-butane was also detected with the highest FC, followed by hydrogen cyanide and acetone. While in cv. Casablanca hydrogen cyanide was detected with the highest FC, but it was only detected in presence of soft rot at the start of the incubation period, after 8 days. It was followed by different VOCs, such as acetaldehyde, 3-penten-2-ol and methyl acetate, among others.

As it has been shown in the tables (Table 5-1, Table 5-2, Table 5-3), resulted from the untargeted analysis, results were very variable not only between cultivars, but also between incubation days. This is due to the variability between the VOC replicates. Only three replicates were considered and, in some cases, each of the specific VOC was not detected in all replicates.

Considering these results, those VOCs that were detected as significantly higher in presence of *F. sambucinum* or *Pectobacterium c. atrosepticum* compared to the non-inoculated tubers with higher FC (FC>1.5) were selected. A list of 15 and 20 VOC were selected for the Early and Mid-stage of storage experiments, respectively (Appendix J, Table J-1).

Table 5-1 Untargeted analysis results from Bio Monitoring TD at an Early-stage of storage from both cultivars (Record, Casablanca). Fold change (FC) values obtained on those VOCs detected in higher abundance in presence of *Fusarium* (Fus) and *Pectobacterium* (Pect) at each specific sampling point (after 8,16 and 32 days of incubation at 8.5ºC). The Retention Time (RT) of each VOC is presented in minutes (t-test, p-value<0.05). NS: No significant differences were detected between the absence and presence of pathogens in potato tubers. (p-value<0.05).

RT (min)	Volatile Organic Compound (VOC)	Fold Change (FC)					
		Day 8		Day 16		Day 32	
		Fus	Pect	Fus	Pect	Fus	Pect
cv. Record							
7.09	Methyl formate	NS	NS	1.03E+05	NS	NS	NS
9.01	Acetone	NS	NS	NS	NS	NS	7.77E+02
9.22	Isopropyl alcohol	4.05E+08	NS	1.66E+08	NS	NS	NS
9.55	1,4-Pentadiene	NS	NS	NS	NS	3.24E+07	NS
9.66	Methyl acetate	2.32E+01	NS	4.04E+00	NS	NS	NS
10.88	3-Methylpentane	NS	NS	NS	2.67E+06	NS	NS
14.5	Trichloromethane	NS	NS	2.02E+01	NS	NS	NS
16.20	Benzene	NS	NS	2.46E+07	NS	NS	7.91E+02
17.14	1-methoxy-3-methyl-butane	NS	NS	1.02E+06	5.57E+05	NS	NS
24.95	2,4-Dimethyl-1-heptene	NS	NS	NS	NS	NS	1.37E+03

cv. Casablanca

5.91	Sulfur dioxide	1.45E+05	1.75E+06	1.70E+06	NS	NS	NS
6.29	1-Propene, 2-methyl-	1.45E+05	NS	NS	NS	NS	NS
6.42	Hydrogen cyanide	NS	9.67E+04	2.26E+07	NS	NS	NS
7.11	Methyl formate	1.42E+05	NS	NS	NS	NS	NS
8.08	Ethanol	NS	1.46E+05	NS	NS	NS	NS
9.09	Acetone	NS	NS	NS	NS	2.61E+06	NS
9.66	Methyl acetate	2.36E+00	NS	NS	NS	NS	NS
12.93	Ethenyl acetate	NS	2.01E+03	NS	NS	1.00E+06	NS
13.46	2-Butanone	NS	1.36E+06	NS	NS	NS	NS
13.79	2-Butanol	NS	NS	NS	NS	2.85E+04	NS
15.37	Acetic acid	NS	3.44E+05	NS	NS	NS	NS
15.43	Trichloromethane	1.62E+04	3.50E+03	NS	NS	NS	NS
16.23	Benzene	NS	9.28E+04	NS	NS	NS	NS
17.14	1-methoxy-3-methyl-butane	NS	NS	8.02E+07	NS	3.00E+02	NS
17.59	1-Butanol	NS	NS	NS	NS	NS	1.11E+05
21.41	Disulfide dimethyl	NS	2.47E+04	NS	NS	1.04E+01	NS
21.78	1-Butanol, 2-methyl-	NS	NS	NS	NS	NS	6.96E+04

Table 5-2 Untargeted analysis results from Air toxics TD at an Early-stage of storage from both cultivars (Record, Casablanca). Fold change (FC) values obtained on those VOCs detected in higher abundance in presence of *Fusarium* (Fus) and *Pectobacterium* (Pect) at each specific sampling point (after 8,16 and 32 days of incubation at 8.5ºC). The Retention Time (RT) of each VOC is presented in minutes (t-test, p-value<0.05). NS: No significant differences were detected between the absence and presence of pathogens in potato tubers. (p-value<0.05).

RT (min)	Volatile Organic Compound (VOC)	Fold Change (FC)					
		Day 8		Day 16		Day 32	
		Fus	Pect	Fus	Pect	Fus	Pect
cv. Record							
6.3	1-Propene, 2-methyl	NS	NS	NS	NS	NS	1.23E+05
6.96	Hydrogen sulfide	NS	NS	2.04E+07	NS	NS	NS
9.05	Acetone	NS	NS	4.31E+05	NS	NS	NS
9.31	Dimethyl sulfide	3.71E+06	NS	NS	NS	NS	NS
12.81	Ethenyl acetate	NS	NS	9.74E+07	NS	NS	NS
12.97	Butanal	NS	NS	1.78E+06	NS	NS	NS
17.04	Heptane	NS	NS	NS	6.00E+04	NS	NS
19.7	Butanoic acid, methyl ester	NS	NS	2.28E+05	NS	NS	NS
cv. Casablanca							
5.94	Dimethyl ether	NS	NS	NS	NS	1.31E+07	NS
6.55	Acetaldehyde	NS	4.49E+03	NS	NS	NS	NS

9.52	Carbon disulfide	NS	NS	NS	NS	NS	8.25E+03
9.67	Methyl acetate	NS	5.95E+05	NS	NS	NS	NS
14.37	Trichloromethane	NS	NS	NS	4.09E+05	NS	NS
16.33	Formic acid	NS	NS	NS	1.86E+04	NS	NS
19.11	Acetic acid	NS	NS	NS	NS	1.04E+05	NS
19.7	Butanoic acid, methyl ester	NS	NS	NS	NS	5.60E+04	NS
21.34	Dimethyl disulfide	NS	NS	NS	NS	1.39E+05	NS
21.56	4-Methylpentan-2-one	NS	NS	NS	NS	2.62E+05	NS
24.95	2,4-Dimethyl-1-heptene	NS	NS	NS	NS	3.43E+04	NS

Table 5-3 Untargeted analysis results from Bio Monitoring TD at a Mid-stage of storage from both cultivars (Record, Casablanca). Fold change (FC) values obtained on those VOCs detected in higher abundance in presence of *Fusarium* (Fus) and *Pectobacterium* (Pect) at each specific sampling point (after 8,16 and 32 days of incubation at 8.5ºC). The Retention Time (RT) of each VOC is presented in minutes (t-test, p-value<0.05). NS: No significant differences were detected between the absence and presence of pathogens in potato tubers. (p-value<0.05).

RT (min)	Volatile Organic Compound (VOC)	Fold Change (FC)					
		Day 8		Day 16		Day 32	
		Fus	Pect	Fus	Pect	Fus	Pect
cv. Record		NS	NS	NS	NS	NS	NS
5.51	Formaldehyde	1.67E+04	NS	NS	NS	NS	NS
6.27	Hydrogen cyanide	NS	NS	NS	NS	4.97E+05	NS
8.83	Propanal	NS	NS	1.09E+05	NS	NS	NS
8.99	Acetone	NS	3.29E+05	6.18E+05	NS	9.06E+04	NS
9.23	Dimethyl sulfide	NS	2.51E+05	NS	NS	NS	NS
13.31	2-Butanone	NS	NS	NS	NS	5.40E+04	NS
13.66	1-Methylpropyl ester formic acid	NS	NS	1.50E+05	NS	9.92E+04	NS
15.25	Acetic acid	NS	NS	NS	NS	1.18E+05	NS
17.04	1-methoxy-3-methyl-butane	NS	NS	9.12E+06	1.95E+06	3.04E+06	NS

cv. Casablanca			NS	NS	NS	NS	NS
6.37	Hydrogen cyanide	NS	9.09E+06	NS	NS	NS	NS
6.5	Acetaldehyde	NS	NS	NS	NS	4.75E+05	1.59E+06
8.81	Propanal	NS	NS	NS	1.10E+05	NS	NS
9.12	4-Penten-2-ol	NS	NS	NS	5.72E+06	NS	NS
9.64	Methyl acetate	NS	NS	NS	8.38E+06	NS	NS
11.68	1-Propanol	NS	NS	NS	NS	NS	3.58E+06
12.82	Ethenyl acetate	NS	NS	NS	8.51E+05	NS	NS
13.2	2-Butanone	NS	7.82E+04	NS	NS	NS	NS
13.66	1-Methylpropyl ester formic acid	NS	NS	NS	NS	NS	1.53E+02
15.42	1-Propanol, 2-methyl-	4.55E+05	3.28E+06	NS	NS	NS	NS
17.04	1-methoxy-3-methyl-butane	NS	NS	3.52E+05	NS	NS	NS
21.2	Dimethyl disulfide	NS	NS	NS	NS	NS	6.09E+04
21.47	1-Pentanol	NS	NS	2.53E+05	NS	NS	NS

Those VOCs that were detected in significantly higher abundance in presence of *Fusarium sambucinum* or *Pectobacterium c. atrosepticum,* included in the previously mentioned list (Appendix M, Table M-1) were selected for the calculation of the Total Targeted Volatile Production (TTVP). The TTVP evolution of each of the cultivars (Record and Casablanca) at an Early-stage of the storage period is presented Figure 5-12. The TTVP of cv. Record after 32 days of incubation increased in presence of dry rot and soft rot, while in cv. Casablanca the TTVP decreased with time. No significant differences (p-values>0.16) were detected between treatments in cv. Casablanca at each specific incubation day. While for cv. Record significant differences (p-value<0.02) were detected between the treatments, with a higher TTVP in presence of *Pectobacterium c. atrosepticum.*

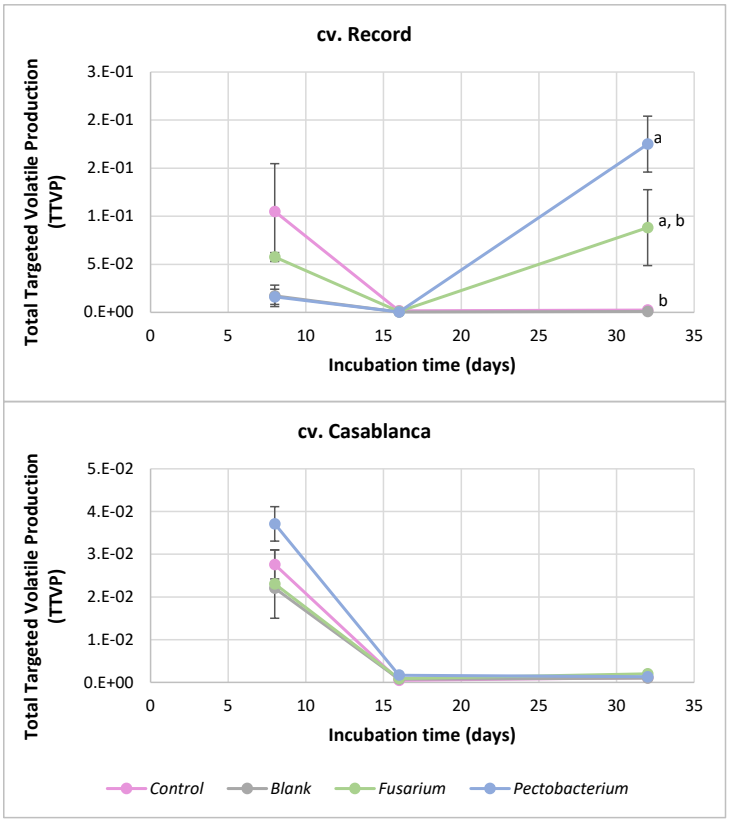


Figure 5-12 Total Targeted Volatile Production (TTVP) of selected VOCs in potato tubers non-wounded and non-inoculated (control), wounded and non-inoculated (blank) and in presence of *Fusarium* and *Pectobacterium* at an Early-stage of the storage period after their incubation at 8.5ºC. a,b: Significant differences detected between treatments at each specific day (t-test, p-value<0.05)

The results from the comparison of TTVP between cultivars in presence of soft rot and dry rot are shown in Figure 5-13. No significant differences (p-values>0.05) in the TTVP between cultivars were detected at an Early-stage of the storage.

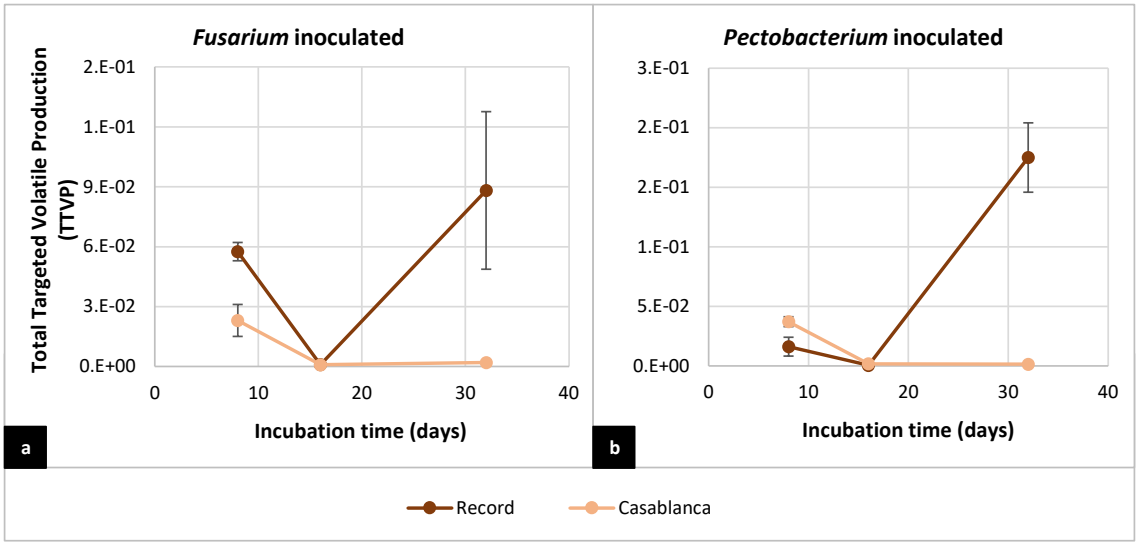


Figure 5-13 TTVP of selected VOCs in presence of *Fusarium* (a) and *Pectobacterium* (b) at an Early-stage of the storage period after their incubation at 8.5ºC (Kruskal-Wallis, p-value<0.05)

The TTVP results at a Mid-stage of the storage period differed from those ones that have just been presented at an Early-stage. Those results, presented in Figure 5-14, show a decrease with time in the TTVP in cv. Record and an increase in cv. Casablanca. No significant differences (p-values>0.12) were detected between treatments.

Figure 5-15 shows the comparison of the TTVP between cultivars on each specific disease. In those potato tubers inoculated with *F. sambucinum* (a), significant differences (p-value<0.02) in the TTVP were only detected after 32 days of storage at cold temperature. While in presence of *Pectobacterium c. atrosepticum* (b) significant differences (p-value<0.05) were detected since the start of the pathogen development, after 8,16 and 32 days. In both cases, cv. Casablanca presented a higher TTVP compared to cv. Record.

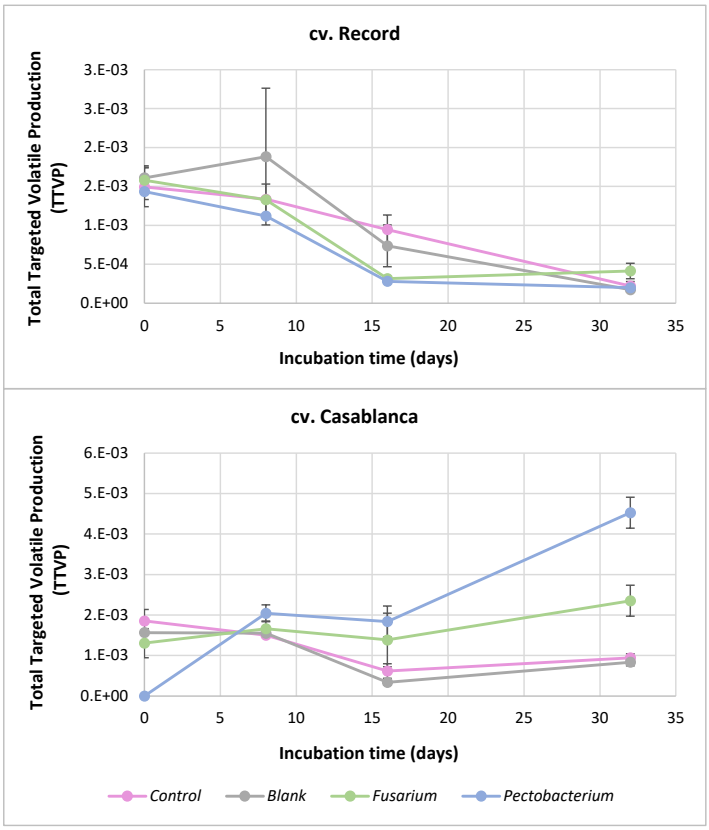


Figure 5-14 Total Targeted Volatile Production (TTVP) of selected VOCs detected in potato tubers non-wounded and non-inoculated (control), wounded and non-inoculated (blank) and in presence of *Fusarium* and *Pectobacterium* at a Mid-stage of the storage period after their incubation at 8.5ºC (Wilcoxon, p-values<0.05).

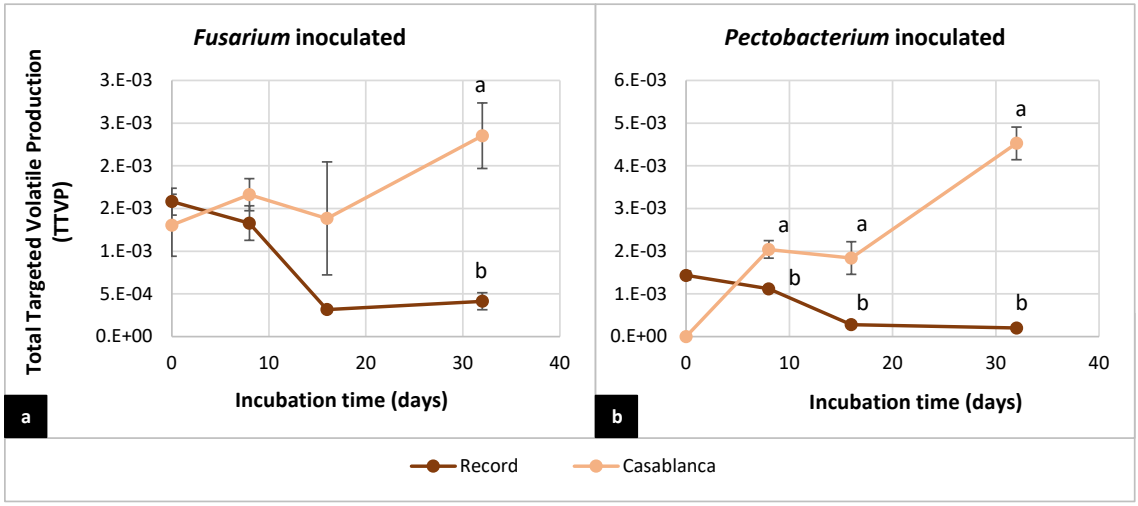


Figure 5-15 TTVP of specific selected VOCs in presence of *Fusarium* (a) and *Pectobacterium* (b) at a mid-stage of the storage period. The TTVP is presented after 8, 16 and 32 days of storage at 8.5ºC. a,b: Significant differences between cultivars (ANOVA, Wilcoxon, p-values<0.05).

5.3.2. 1 Targeted analysis

Based on the untargeted analysis, a VOC library was built to target the analysis on the individual detection of each of the identified VOCs.

The relative abundance of three VOCs, acetic acid, ethanol and sulfur dioxide at an Early-stage of the storage is presented in Figure 5-16. They were selected as their abundance was higher in presence of the diseases compared to their absence. As it is shown acetic acid and sulfur dioxide were mainly detected after 32 days in presence of dry rot and soft rot. However, significant differences (p-value<0.05) were only detected in sulfur dioxide, its abundance was higher in the presence of soft rot compared to the rest of the treatments. Ethanol was detected with a very high abundance after 8 days of incubation in cv. Casablanca, although no significant differences (p-value>0.05) were detected compared to the absence of both pathogens (blank). Regarding the differences between cultivars, cv. Record presented a significantly higher (p-value<0.004) abundance of acetic acid after 32 days of incubation in presence of soft rot and ethanol after 16 and 32 days in presence of dry rot, respectively.

In Figure 5-17, results from the Mid-stage of the storage period are shown. The four most abundant VOCs were dimethyl sulfide, butan-2-yl formate, 2-methylpropan-1-ol and 2-methyl-1-butanol, its abundance was detected in higher intensity in presence of dry rot and soft rot. Dimethyl sulfide was not only detected in presence of both pathogens, it was also detected at a late stage of the incubation period in the non-wounded and non-inoculated potato tubers. It might be due to the contamination of some of those potato tubers after almost a month stored at 8.5^{o}C. Apart from this case, butan-2-yl formate, 2-methylpropan-1-ol and 2-methyl-1-butanol were only detected with higher intensity in presence of each pathogen. As it is shown, cv. Casablanca presented higher abundance values on those four VOCs compared to cv. Record, this is directly related to the development of the disease in both cultivars, as it was mentioned in the growth assessment section.

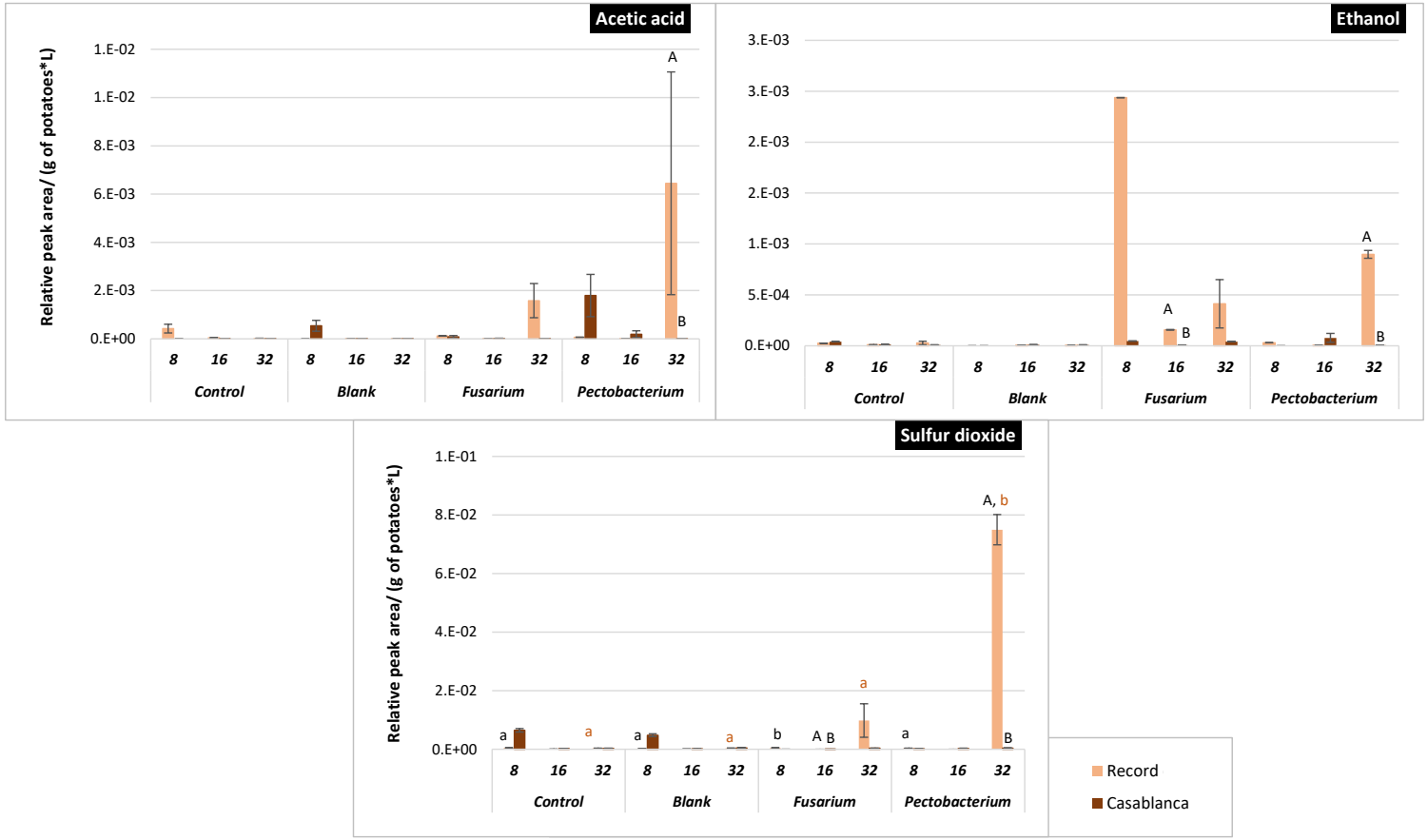


Figure 5-16 Acetic acid, ethanol and sulfur dioxide relative abundance after 8, 16 and 32 days of incubation at 8.5ºC of two potato cultivars (Record and Casablanca) at an Early-stage of the storage. Control: non-wounded and non inoculated; blank: wounded and non inoculated; *Fusarium* inoculated and *Pectobacterium* inoculated. a,b: Significant differences between treatments at each specific day and cultivar (Record and Casablanca) (Wilcoxon, p-values< 0.051). A, B: Significant differences between cultivar at each specific treatment and day (t-test, p-value< 0.05).

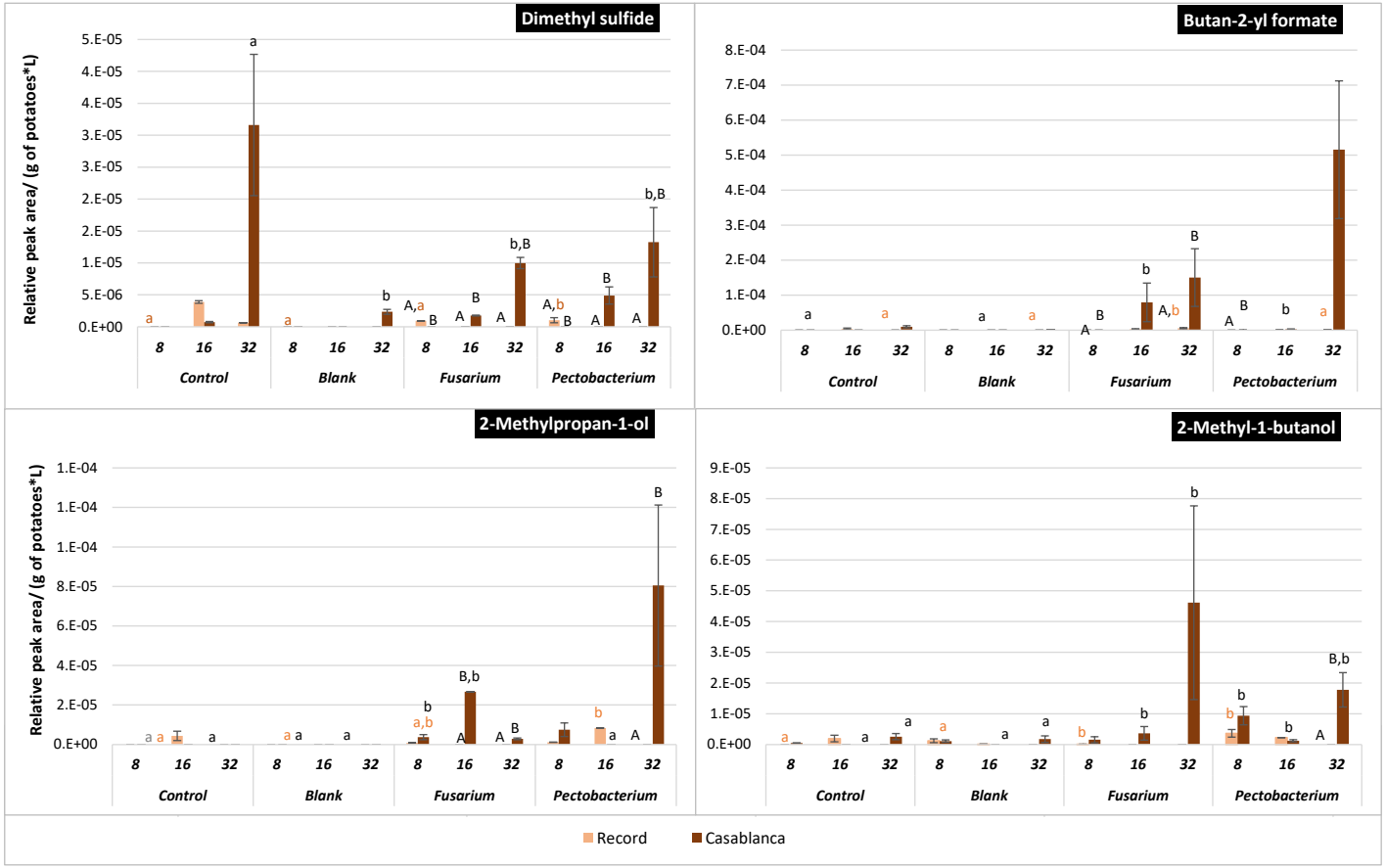


Figure 5-17 Dimethyl sulfide, butan-2-yl formate, 2-methylpropan-1-ol and 2-methyl-1-butanol relative abundance after 8, 16 and 32 days of incubation at 8.5ºC of two different potato cultivars (Record and Casablanca) at a Mid-stage of the storage. Control: non-wounded and non inoculated; blank: wounded and non inoculated; *Fusarium* inoculated and *Pectobacterium* inoculated. a,b: Significant differences between treatments at each specific day and cultivar (Record and Casablanca) (Wilcoxon test, p-values< 0.05). A, B: Significant differences between cultivar at each specific treatment and day (Kruskal-Wallis, p-values<0.05).

The next comparison was carried out between the two different stages of the storage period, five different VOCs that were detected at both stages of the storage were represented individually (1-methoxy-3-methylbutane, methyl acetate, dimethyl disulfide, 2-butanone and hydrogen cyanide).

1-Methoxy-3-methylbutane abundance is presented in Figure 5-18, except for its detection on the control, its higher detection was in presence of *F. sambucinum* and *Pectobacterium c. atrosepticum.* After 32 days of incubation at cold temperature it was detected at Mid-stage in cv. Record and at both stages in cv. Casablanca Significant differences (p-values<0.05) were detected after 32 days in cv. Record between Early and Mid-stage of storage in presence of dry rot and soft rot. A higher abundance of 1-methoxy-3-methylbutane was detected at a Mid-stage of the storage. When the comparison was carried out between treatments, after 16 and 32 days, its detection was higher in presence of *Fusarium* in cv. Casablanca, at a Mid-stage of the storage, and only after 32 days in presence of *Pectobacterium.*

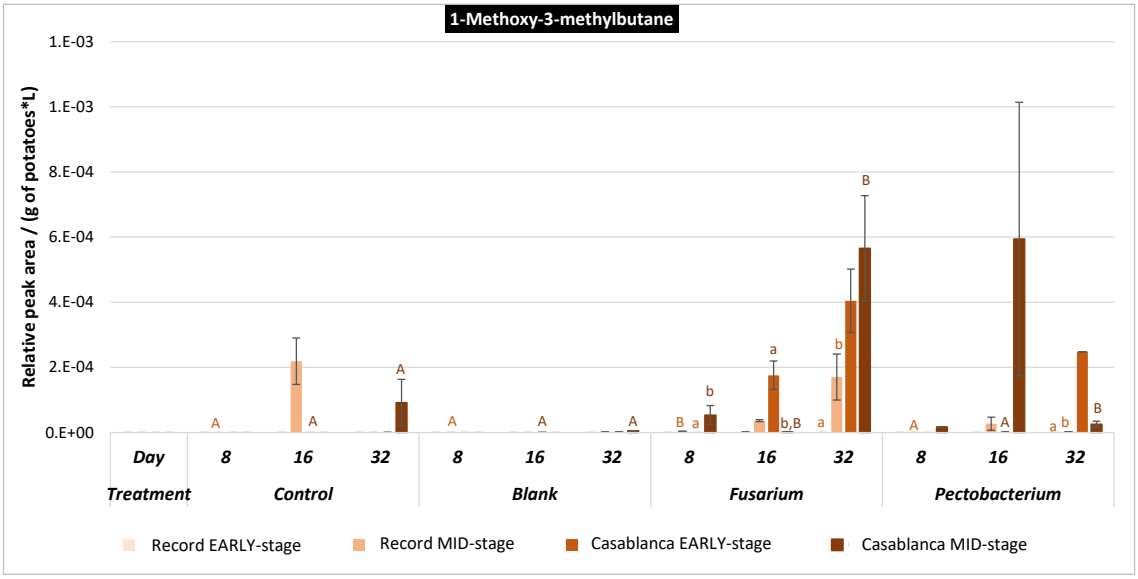


Figure 5-18 Temporal evolution of 1-methoxy-3-methylbutane on each of the treatments (Control, Blank, *Fusarium, Pectobacterium*) at two different stages of the storage period (Early and Mid-stage). a,b: Significant differences between stages at each specific treatment, day and cultivar (Record and Casablanca) (Kruskas-Wallis p< 0.05). A, B: Significant differences between treatments at each specific day and stage (Record and Casablanca) (Wilcoxon test, p-value<0.05).

Methyl acetate temporal evolution was compared between the two different stages of the storage (Figure 5-19). Its detection was higher at an Early-stage of the storage compared to the Mid-stage. However, significant differences were only detected after 8 days of incubation in presence of *F. sambucinum* in cv. Casablanca. As it is shown, its abundance was higher at an early point of the incubation time compared to the end. Significant differences were detected between treatments after 8 days of incubation at cold temperature, presenting higher methyl acetate abundance in presence of soft rot in cv. Casablanca compared to the rest of the treatments.

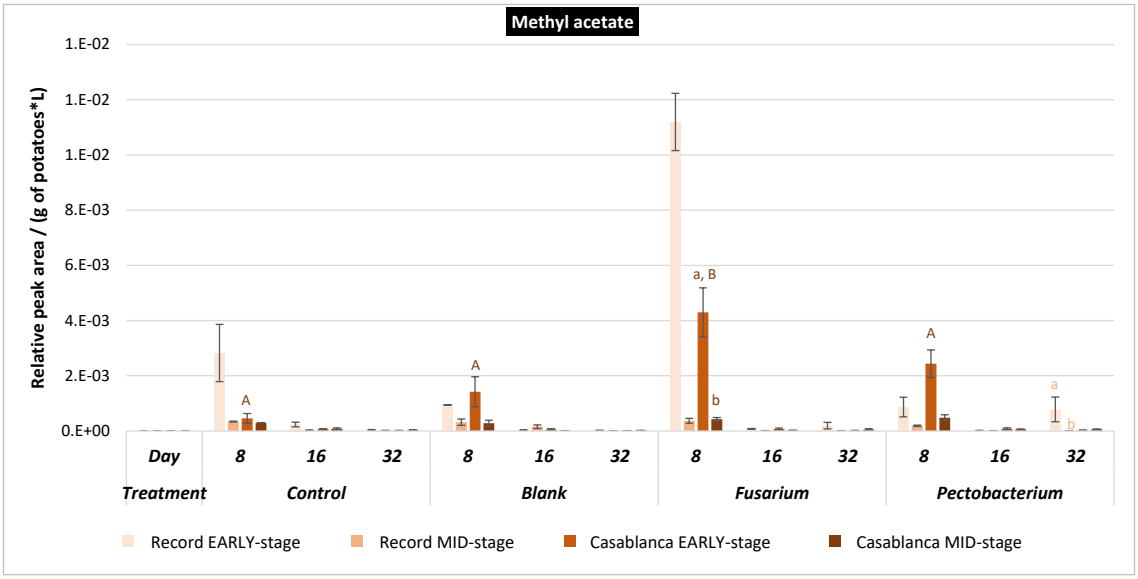


Figure 5-19 Temporal evolution of methyl acetate on each of the treatments (Control, Blank, *Fusarium, Pectobacterium*) at two different stages of the storage period (Early and Mid-stage). a,b: Significant differences between stages at each specific treatment day and cultivar (Record and Casablanca) (t-test, p-values<0.05). A, B: Significant differences between treatments at each specific day and stage (Record and Casablanca) (t-test, p-value<0.04).

A different VOC that was detected through all the time points in presence of soft rot is 2-butanone. Its temporal evolution and comparison between the two different stages is shown in Figure 5-20. Its abundance in the control and the blank was very low compared to its detection in presence of both diseases, dry rot and soft rot. In presence of *F. sambucinum,* it was detected in higher abundance after 16 days of storage while for *Pectobacterium c. atrosepticum* it

was detected from day 8 until day 32 with a higher abundance. Significant differences (p-value< 0.05) were detected between treatments at 16 days of incubation for cv. Casablanca in presence of dry rot and after 16 and 32 days in presence of dry rot, at a Mid-stage of the storage period. The abundance of 2-butanone was significantly higher at a Mid-stage of the storage compared to the Early-stage in cv. Casablanca.

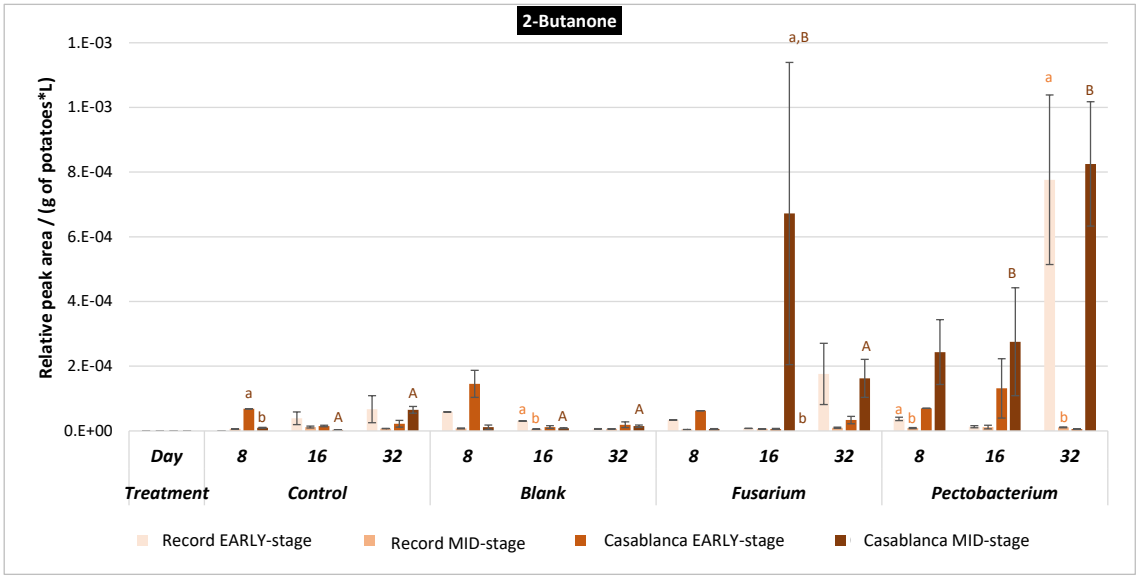


Figure 5-20 Temporal evolution of 2-butanone on each of the treatments (Control, Blank, *Fusarium, Pectobacterium*) at two different stages of the storage period (Early and Mid-stage). a,b: Significant differences between stages at each specific treatment, day and cultivar (Record and Casablanca) (Kruskal-Wallis, p-values< 0.05). A, B: Significant differences between treatment at each specific day and stage (Record and Casablanca) (Wilcoxon test, p-values<0.05).

Dimethyl disulfide temporal evolution is shown in Figure 5-21. Its detection was mainly detected in presence of dry rot and soft rot, and at a late stage of the incubation in the control and the blank. Dimethyl disulfide is detected after 32 days of storage in cv. Casablanca at both storage stages in every treatment, being higher in presence of dry rot and soft rot. While significant differences (p-values<0.008) between treatments were only detected for those Casablanca potato tubers at a Mid-stage inoculated with *Fusarium sambucinum* and *Pectobacterium c. atrosepticum,* separately. When the comparison was carried out between stages of the storage, cv. Casablanca presented a significantly

higher abundance of dimethyl disulfide at a Mid-stage than at an Early-stage of the storage period after 32 days of storage in those potato tubers inoculated with *Pectobacterium.*

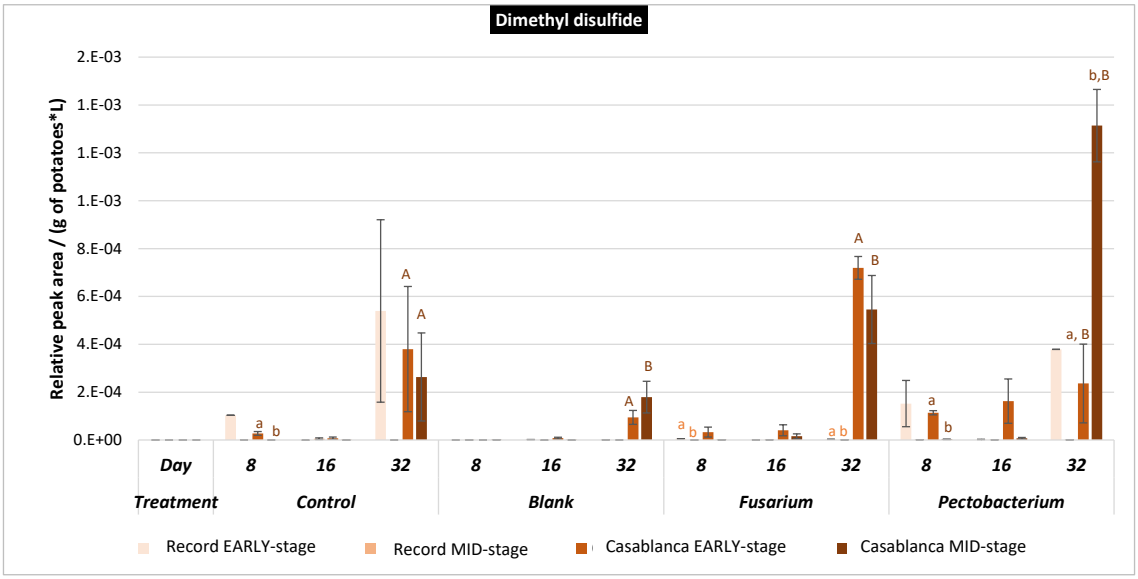


Figure 5-21 Temporal evolution of dimethyl disulfide on each of the treatments (Control, Blank, *Fusarium, Pectobacterium*) at two different stages of the storage period (Early and Mid-stage). a,b: Significant differences between stages at each specific treatment, day and cultivar (Record and Casablanca) (Kruskal-Wallis, p-values<0.03). A, B: Significant differences between treatments at each specific day and stage (Record and Casablanca) (Wilcoxon test, p-values<0.03).

The last VOC studied was hydrogen cyanide, shown in Figure 5-22. It was detected mainly in presence of dry rot and soft rot after 16 and 32 days of incubation. Significant differences (p-value<0.05) were detected in cv. Casablanca between stages of the storage period, being higher hydrogen cyanide abundance at a Mid-stage of the storage compared to an Early-stage.

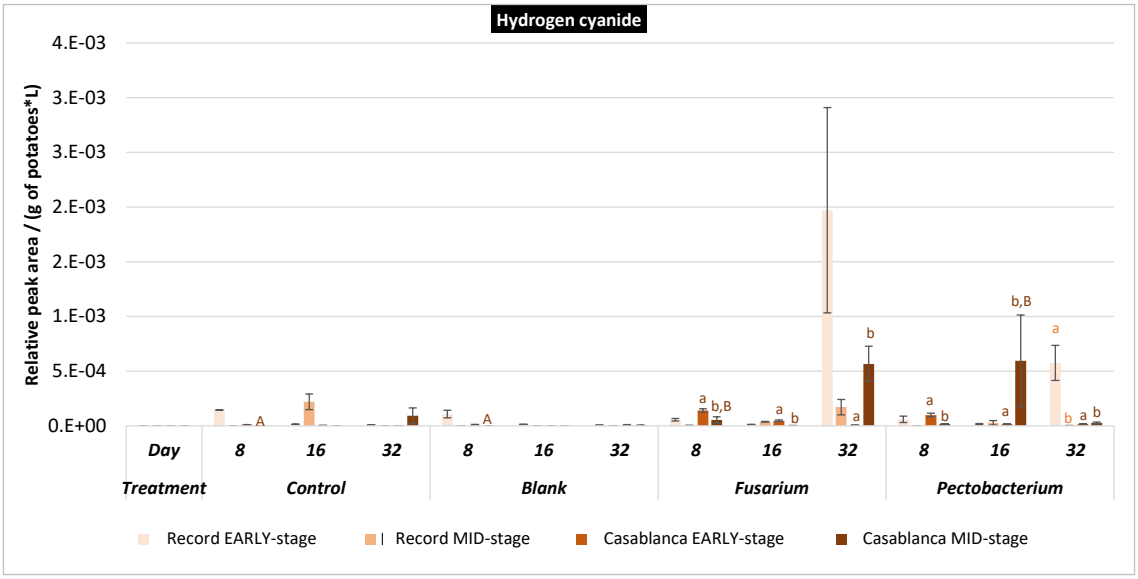


Figure 5-22 Temporal evolution of hydrogen cyanide on each of the treatments (Control, Blank, *Fusarium*, *Pectobacterium*) at two different stages of the storage period (Early and Mid-stage). a,b: Significant differences between stages at each specific treatment, day and cultivar (Record and Casablanca) (Kruskal-Wallis, p-values<0.05) A, B: Significant differences between treatments at each specific day and stage (Record and Casablanca) (Wilcoxon test, p-values<0.03).

5. 4 Discussion

5.4. 1 Disease severity

One of the objectives of this work was to study the severity of dry rot and soft rot in two different cultivars (Record and Casablanca) of potatoes at two different stages of storage.

The severity of soft rot and dry rot was assessed based on the external and internal area of the lesion produced in presence of each pathogen. Two parameters, infection rate and lag time, were also determined for the external infection, providing information about the rate of development of the disease and the time needed for the pathogen to start growing on the potato tuber.

5.4.1. 1 Dry rot (*Fusarium sambucinum)* severity

The severity of the lesion produced by dry rot was studied based on the external and internal area affected at an Early and Mid-stage of the storage period. Potato tubers at an Early-stage presented similar severity of lesion on the two cultivars studied (Record and Casablanca). This might be due to the different categories of potato tubers, Record is a maincrop cultivar, while Casablanca is an earlier cultivar. This is supported by two different studies where higher susceptibility to dry rot was observed in earlier cultivars compared to maincrop cultivars (Mckee, 1954; Boyd, 1967). However, the severity of the disease differed between cultivars at the Mid-stage of storage. Both cultivars were probably experiencing physiological changes during their storing at cold temperature. Those changes were allowing *Fusarium sambucinum* to produce higher lesions than before being stored.

The disease severity of dry rot on potato tubers at an Early-stage and Mid-stage of the storage period were compared. The storage time was directly affecting the infection rate of *Fusarium sambucinum,* a higher infection rate was observed on those potato tubers at a Mid-stage of the storage period compared to the Early-stage. Besides, the lag time was also affected by the storage time, with less days needed for *F. sambucinum* to start growing at a Mid-stage. Therefore, those potato tubers that were stored for a longer period of time presented higher

disease severity based on their infection rate and lag time. Potato tubers from Mid-stage of the storage period were rotting quickly and the time needed for *F. sambucinum* to start producing an external lesion was lower than in those potato tubers from the Early-stage. Storage time of potato tubers prior to their inoculation was directly affecting the external development of dry rot.

This increase in the disease severity of dry rot is probably due to the different changes that occur in potato tubers during storage. During storage different physiological processes take place as respiration, water loss, sprouting and Low-Temperature Sweetening (LTS) among others. Potato tubers continue their respiration in storage, those sugars produced during the starch hydrolysis are used for respiration, producing a loss in the dry matter content, therefore a weight loss of the potato tubers (Pinhero and Yada, 2016). Sprouting affects the quality of the potato tubers during storage, increasing the respiration, water loss and the increase level of glycoalkaloids (Peterson, Barker and Howarth, 1985; Burton, 1989; Spychalla and Desborough, 1990; Ross and Davies, 1992; Pinhero and Yada, 2016). Besides, the greater abundance of free sugars might be reducing the lag time and accelerating the lesion expansion. All the mentioned processes involve changes in the biochemical content of potato tubers that are directly affecting the growth of both pathogens after the storage of potato tubers. This can result in more waste towards the end of the storage period.

The previous results were in accordance with McKee et al. 1954 whose statement was that resistance to dry rot infection decreases as the storage season advance (Mckee, 1954). In a previous study, the effect of maturity in potato tubers on the development of dry rot was studied and it was elucidated that immature tubers presented high sucrose content, low dry matter content and poor skin set, increasing their susceptibility to dry rot (Heltoft *et al.*, 2015). In a prior study, it was already elucidated an association between the increase of sugar content (reducing sugars) in mature tubers and the increase in susceptibility (Boyd, 1967).

As part of this study, the internal lesion of dry rot was also assessed. In accordance with our external lesion results, similar internal development was

observed in both cultivars (Record and Casablanca). However, the storage time did not affect the internal development of dry rot. Therefore, while based on the visual lesion, dry rot severity was higher in cv. Casablanca from an Early-stage compared to the Mid-stage, the internal lesion remained the same at both stages of the storage period.

Most of the studies on the severity of dry rot were based on internal lesion. A previous study, where different cultivars were inoculated with *Fusarium* spp., showed 8-1000 mm^2 of internal infected area when potato tubers were stored at 20ºC (Manici and Cerato, 1994). The average internal infected area in our experiment was between 50 and 200 mm^2 around one-fifth of what was reported by Manici (1994). Those differences could be due to the temperature of incubation as at higher temperatures the development of dry rot increases.

Similar research has been carried out on the depth of the internal lesion produced by *F. sulphureum* and *F. solani* var. *coeruleum* at room temperature (Percival, Karim and Dixon, 1998) and at 13ºC (Percival, Karim and Dixon, 1999). In the second study, they reported values of 27.6 ±3.15 mm of depth after 21 days of incubation, substantially higher than our observations of 3.24 ± 0.27 and 6.05 ± 0.20 mm of depth after 10 and 30 days of incubation at 8.5ºC (Figure 5-11), respectively. The difference was probably due to the experimental methodology and the temperature that was used (Percival, Karim and Dixon, 1999).

Two previous studies were carried out at cold temperatures with potato tubers inoculated with *Fusarium sambucinum* and stored at 10ºC. Both studies detected higher values of depth of the internal lesion produced by dry rot, however, differences in the sampling day, cultivars and concentration of the inoculum could account for those differences (R. D. Peters *et al.*, 2008; Estrada *et al.*, 2010). There have been different methods in assessing the internal lesion of dry rot. To date, most of the studies mentioned have measured the depth of the internal rot. However, the volume of rotten tissue has also been used as a measure of assessment for disease severity. This has been achieved by considering the potato tuber lesion as conical and calculating the volume of a cone with the depth and the length of the internal lesion (J. C. Peters *et al.*, 2008). Those results

cannot be compared with ours as we calculated the internal lesion as depth and area affected, using an image analysis software. Comparing the results from the three different *Fusarium* spp. that were tested (*F. avenaceum, coeruleum, culmorum and sambucinum*), *F. sambucinum* presented a higher internal infected volume (J. C. Peters *et al.*, 2008).

5.4.1. 2 Soft rot (*Pectobacterium c. atrosepticum*) severity

In our study, the severity of soft rot based on the external lesion was at both stages of the storage period higher in cv. Casablanca compared to Record. As it was shown in the results (Figure 5-2 and Figure 5-3), the external development of soft rot in cv. Record was very low. This could be related to the resistance presented by cv Record. to soft rot. There are few studies where Record was used, in those cases, the susceptibility or resistance to soft rot was studied based on internal lesion (Lapwood, Read and Spokes, 1984; Wastie, Stewart and Brown, 1989). According to the Potato Variety Database created by AHDB, Record presented a resistance of 2 of 9, being 9 the highest level of resistance. While Casablanca presented an 8 of 9 in resistance to soft rot (AHDB, 2016). This data is not in accordance to what was observed in our work. This could be related to the methodology used by AHDB for the assessment of susceptibility of the two cultivars to soft rot and dry rot respectively.

The effect of storage time on the development of soft rot externally could only be studied in cv. Casablanca. It was shown that the storage time did not affect the infection rate neither the lag time of soft rot in potato cultivars. The main hypothesis would be that *Pectobacterium c. atrosepticum* external development on potatoes is not affected by the storage time. Further studies will need to be carried out including more potato cultivars to confirm this hypothesis. However, those potato tubers from a Mid-stage of the storage presented a drier skin than the ones from the Early-stage. This could be the reason of these results, as the dryness of the potato skin has been previously related with a decrease in susceptibility to soft rot, produced by *Pectobacterium c. atrosepticum* (Pringle and Robinson, 1996).

The results from the internal development of soft rot in cv. Record from an Early-stage of the storage were interesting. Although the external lesion results support that cv. Casablanca presented higher lesion severity of soft rot than Record, the internal lesion assessment supports that both cultivars presented similar lesion severity of soft rot. This might be explained with the behaviour of *Pectobacterium c. atrosepticum,* a bacterium whose growth rate is favoured with higher a_w values, as it was elucidated in previous studies (Lyew *et al.*, 2001; Moh *et al.*, 2011). The a_w of the internal part of the potato would be higher than the skin, therefore that could explain the low external development of soft rot in cv. Record.

This is providing an alarming information for the farmers, as according to our results regarding cv. Record, the part of the potato that is visible to the farmer will not show any soft rot symptoms, while its development might be taking place in the inside of the potato tuber. Therefore, those potato tubers infected with *Pectobacterium c. atrosepticum* will not show any external symptoms until they are completely rotten. These results support the use of different methods for the disease assessment, as VOC detection, for the early detection of potato diseases in storage, without the need of visual signs in the potato tubers.

Several studies were carried out on the susceptibility of soft rot, however, most of them were carried out at high temperatures (18-30^{o}C) (Webb and Wood, 1974; Bourne, Mccalmont and Wastie, 1981; Lapwood, Read and Spokes, 1984; Tzeng, McGuire and Kelman, 1990; Lebecka, Flis and Murawska, 2018). In one of those previous studies, cv. Record as a maincrop cultivar and it appeared to be more resistant to soft rot than first earlier potato cultivars (Lapwood, Read and Spokes, 1984). Those results are in agreement with ours, as cv. Record presented lower internal lesions than Casablanca (first earlier cultivar) at a late stage of the development of the disease.

The storage time affected the severity of soft rot based on the internal lesion only after 10 days of incubation, with higher internal lesions at a Mid-stage of the storage. However, similar limitations to the ones mentioned for dry rot need to be considered: only three sampling points were selected for the assessment of the

internal lesion and a high variability was observed between replicates. Therefore, the effect of storage time on the internal lesion could not be properly evaluated.

The effect of the storage time on soft rot has been previously studied. Those potato tubers that were more resistant to soft rot did not change their resistance during the six months of storage, while it did change in the known susceptible cultivars (Chung *et al.*, 2013). However, disease severity was assessed after incubation at 23ºC, while the real conditions during storage are lower temperatures.

5.4. 2 Volatile Organic Compounds (VOC) detection

The second main objective of this work was to determine the VOC fingerprint of potato tubers in presence of soft rot and dry rot at three different time points during the development of the disease, in two potato cultivars and at two stages of the storage period (Early and Mid-stage). The fingerprint of potato tubers was achieved based on an untargeted and targeted VOC analysis.

Different fingerprints were detected at the two stages of the storage period. The results are discussed separately at each of the stages of the storage, followed by a section comparing the detection of those specific VOC as 1-methoxy-3-methyl-butane, methyl acetate, 2-butanone, dimethyl disulfide and hydrogen cyanide that were detected in both stages.

5.4.2. 1 VOC detection at an Early-stage of the storage

The severity of dry rot and soft rot on potato tuber was different between cultivars (Record and Casablanca) as it was shown in Section 5.3. 1. This needs to be considered when discussing the VOC results. The extent of dry rot infection was very similar between cultivars, while for soft rot cv. Casablanca presented higher lesion severity compared to cv. Record. Although the growth of the fungi or bacteria is important, the VOC fingerprint will also depend on the physiology and ability of the potato tuber to respond in presence of an infection. In presence of *Pectobacterium c. atrosepticum*, the VOC fingerprints might differ also due to the difference in the development of the disease at the different VOC sampling points.

The results from the untargeted analysis are presented in Table 5-1 and Table 5-2. Due to the sampling strategy (Section 2.5. 2), the number of VOCs detected from the Bio Monitoring TD tubes was higher than the ones detected from Air Toxics. Therefore, the VOC detected in the Biomonitoring TD tubes are the one that will be discussed. The low development of soft rot in cv. Record could be the reason why a lower number of VOC were detected in presence of *Pectobacterium c. atrosepticum* compared to *F. sambucinum.*

Several VOCs were detected in both cultivars in presence of *F. sambucinum* as methyl formate, methyl acetate, trichloromethane and 1-methoxy-3-methylbutane. This last compound 1-methoxy-3-methylbutane was also detected in presence of *Pectobacterium c. atrosepticum* in cvs. Record and Casablanca. A similar study where VOCs were collected from *Fusarium coeruleum* inoculated potato tubers stored at 10^{o}C, was previously performed (de Lacy Costello *et al.*, 2001). However, within a large list of VOCs detected in presence of dry rot, only acetone and ethanol were detected in common with our results. This difference might be due to the different VOC sampling procedure. They used handmade Tenax TA TD tubes followed by GC-MS, therefore different VOC would be retained in this sorbent compared with Bio Monitoring. Besides, the incubation conditions were different, as potato tubers were incubated for 42 days, while our sampling points were 0, 8, 16 and 32 days (de Lacy Costello *et al.*, 2001). Ethenyl acetate, acetone, ethanol and dimethyl disulfide were also detected at low temperature (4-10^{o}C) in presence of potato tubers inoculated with soft rot (de Lacy Costello *et al.*, 1999; Sinha *et al.*, 2018). Ethenyl acetate was considered as a potential VOC indicator of the presence of soft rot in potato tubers (Lui, Vikram, Abu-Nada, *et al.*, 2005).

The variability of the results presented in the untargeted analysis was due to the variability between replicates. In some cases, the VOCs were not detected in the three stages of the incubation period. An intermediate VOC analysis was carried out considering the selected VOCs for the Total Targeted Volatile Production (TTVP). The calculation of the Total Peak Area was previously used as an indicator of the quantity of VOC detected in presence and absence of different

crop diseases (Varns and Glynn, 1979; Waterer and Pritchard, 1984b; Lyew *et al.*, 2001; Wang, Luca and Edelenbos, 2019). However, its main limitation is that in those cases where one of those selected VOCs is highly produced in presence of the disease, the Total Targeted Volatile Production will increase, therefore it will be considered that those 15 or 20 VOCs are directly related to the presence of the disease. When in fact, this increase is coming from one or two single VOCs. In our study, the TTVP results from the Early and Mid-stage of the storage experiment (Figure 5-13 and Figure 5-15) showed differences between cultivars, therefore, those 15 to 20 VOCs were produced differentially in presence of both pathogens in both cultivars. This could be explained with the differential growth of both pathogens in the two different cultivars.

Therefore, a more in-depth analysis of those 15-20 specific VOCs was carried out, to study the detection of each of the VOC in presence of soft rot and dry rot at the three different stages of the development of the disease. Acetic acid, ethanol and sulfur dioxide were selected as the three VOCs that were detected in significantly higher abundance in presence of soft rot and dry rot in both abundance in cv. Record. Ethanol was already detected in presence of soft rot in potato tubers (Lui, Vikram, Abu-Nada, *et al.*, 2005), and acetic acid in presence of *Fusarium* spp. in potatoes (de Lacy Costello *et al.*, 2001) and onions (Wang *et al.*, 2018, 2019). At this point, ethanol could be considered as potential biomarkers of the presence of a disease in potato tubers stored at 8.5^{o}C. However, further studies including more potato cultivars and different pathogens would need to be carried out to confirm these results.

5.4.2. 2 VOC detection at a Mid-stage of the storage

At Mid-stage of the storage period differences in severity of soft rot and dry rot were detected between cultivars. Casablanca. presented higher severity of both diseases compared to Record. Again, this needs to be considered when discussing and understanding the VOC fingerprint in presence of both diseases. Therefore, in this case, differences in VOC fingerprint are expected for both diseases between cultivars as the development of the diseases were different between cvs. Record and Casablanca at the same sampling points. For future

studies the normalisation of the data based on the lesion size at each VOC sampling point could be considered.

The results from the untargeted analysis were shown in Table 5-3. Different VOCs were detected between both cultivars and the ones in common were detected at different stages of the development of the disease. In the intermediate analysis of the VOCs, the calculation of the TTVP, the evolution of cvs. Record differed from Casablanca. A decreasing tendency was observed in Record, while in cv. Casablanca, where the development of both diseases was higher, an increasing tendency was observed. Higher TTVP was detected in presence of both pathogens compared to their absence. Therefore, some of those 15 specific VOCs detection might be directly related to the presence of those two specific potato pathogens.

To elucidate which VOCs are the responsible for this previous increase, an individual evaluation of those specific VOCs was carried out. Dimethyl sulfide, butan-2-yl formate, 2-methyl-1-propanol and 2-methyl-1-butanol were selected as the four VOCs that were detected in higher abundance in presence of soft rot and dry rot. Dimethyl sulfide is an organosulfur VOC that presented an increasing tendency with time in its abundance. However, at a late stage of the incubation, it was observed with a very high abundance in the control treatment. It has been previously detected in samples from different food matrixes as wild rocket (Luca, Mahajan and Edelenbos, 2016; Luca, Kjær and Edelenbos, 2017), truffles (Diaz 2003) cooked potatoes (Self, Rolley and Joyce, 1963; Gumbmann and Burr, 1964) and cultures of different bacteria as *Streptococcus* spp. (Hertel *et al.*, 2016). Its production in cooked potatoes was associated with the degradation of the methionine (Ballance, 1961), however, the production of VOC is different in cooked and fresh potatoes. Although it could not be considered as an interesting VOC to be selected as biomarkers of any of the two diseases (dry rot and soft rot), it could be a VOC naturally released by the potato tuber. Further studies will be needed to confirm the production of this VOC in fresh potato tubers.

Butan-2yl formate, 2-methyl-1-propanol and 2-methyl-1-butanol were mainly detected in presence of soft rot and dry rot and with higher abundance in

Casablanca. 2-Methyl-1-butanol was previously detected in presence of soft rot in potato tubers (Lui, Vikram, Abu-Nada, *et al.*, 2005) and 2-methyl-1-propanol in onions with dry rot (Wang *et al.*, 2019). 2-Methyl-1-propanol was also detected in potato tubers inoculated with soft rot, however it was also detected in the control (Waterer and Pritchard, 1984b).

5.4.2. 3 Effect of storage time on five selected VOCs

Before discussing the effect of storage time on the VOC detection in presence of soft rot and dry rot on potato tubers, its effect on the development of soft rot and dry rot needs to be considered. Storage time increased the severity of dry rot in both cultivars (Record and Casablanca) based on the external lesion, while no effect on the severity of soft rot was detected.

Five VOCs, that were detected in common at both stages of the storage, were studied.

5.4.2.3. 1 1-Methoxy-3-methylbutane

1-Methoxy-3-methylbutane is an ether that was detected with higher abundance in presence of *F. sambucinum* and *Pectobacterium c. atrosepticum* compared to their absence. Its detection was higher in cv Casablanca compared to Record. In cv. Record was only detected in the control and in presence of *Fusarium sambucinum* at the Mid-stage of storage.

In a previous study, where the VOCs from potato tubers inoculated with *Pectobacterium c. atrosepticum* were analysed, 1-methoxy-3-methylbutane was detected in higher abundance compared to the non-inoculated potatoes (Lui 2005). 1-Methoxy-3-methylbutane was also detected in presence of the same pathogen responsible for soft rot in carrots (Vikram *et al.*, 2006). It has also been associated with Plant Growth-Promoting Rhizobacteria (PGPR) as *Bacillus amyloliqufaciens* growing at 37^{o}C on Trypticase Soy Agar (TSA) and potato slices (Farag *et al.*, 2006; Lee *et al.*, 2012). The origin of this VOC was associated withthe presence of 3-methyl-1-butanol and 2-methyl-1-butanol, that via a methyltransferase activity can produce the corresponding ether: 1-methoxy-3-methyl-butane. However, in our experiment, 3-methyl-1-butanol was not

detected, and 2-methyl-1-butanol was only detected in cv. Casablanca at a late stage of the development of soft rot in those potato tubers that were just harvested (Table 5-1). 3-Methyl-1-butanol and 2-methyl-1-butanol are products of the degradation of amino acids as leucine, isoleucine and valine amino acids via Ehrlich Pathway (Marilley and Casey, 2004; Farag *et al.*, 2006). Ehrlich Pathway is part of the catabolism of branched-chain amino acids, aromatic and sulfur-containing amino acids resulting in α-keto acid excreted to the media or food matrix and convert into fusel alcohols (Hazelwood *et al.,* 2008).

Therefore, the detection of 1-methoxy-3-methylbutane might be directly related to the degradation of amino acids in the presence of soft rot or dry rot. Further studies will need to be carried out to confirm this hypothesis, including an analysis of the content of leucine, isoleucine and valine.

5.4.2.3. 2 Methyl acetate

Methyl acetate is a carboxylate ester that can be produced by the acetylation of methanol with acetyl CoA. However, methanol needs to be present in the environment. In plants, the transfer of the acetyl group from the AcetylCoA to an alcohol is carried out by an alcohol acetyltransferase enzyme (Shalit *et al.*, 2003; Jardine *et al.*, 2014).

The profile of its detection in our experiment was different to the previous VOC studied. It was detected with higher abundance at the start of the development of the disease and in those potatoes at the Mid-stage of the storage period. This result could suggest that methyl acetate is a wound induced volatile. Its abundance was significantly higher in presence of *Fusarium sambucinum* compared with the rest of the treatments. The detection in presence of *Pectobacterium c. atrosepticum* was similar to the absence of the pathogen. However, opposite results were achieved in a previous experiment where *P. c. atrosepticum, P. c. carotovorum* and *F. sambucinum* were inoculated on potato tubers, methyl acetate was not detected in presence of dry rot and it was detected in presence of both subspecies of *P. carotovorum.* (Varns and Glynn, 1979; Lui, Vikram, Abu-Nada, *et al.*, 2005). Therefore, the detection of methyl acetate might also be related to the presence of bacteria in potato tubers.

Methyl acetate was also detected in blueberries treated with ethanol, to avoid the appearance of microbial contamination. Its presence was associated with the accumulation of ethanol in blueberries, which when reacting with acetic acid, an intermediate product in the ethanol metabolism forms methyl acetate (Ji *et al.*, 2019). Its presence has also been reported in samples of truffles and green and white garlic, its role however in these species was not elucidated (March, Richards and Ryan, 2006; Koca, Karadeniz and Tekguler, 2016).

Further studies will need to be conducted to check if the alcohol acetyltransferase enzyme is present in potato tubers or any of the pathogens, allowing the formation of methyl acetate via acetylation of methanol. And also, checking the presence of an esterifying catalyst able to esterify ethanol with acetic acid, both VOC were in our experiment in presence of soft rot and dry rot.

5.4.2.3. 3 2-Butanone

2-Butanone is a methyl ketone, intermediate metabolite in the production of 2-butanol. It is normally produced via the decarboxylation of fatty acids. Acetoin (3-hydroxy-2-butanone) and its oxidized form 2,3-butanedione are derived from the pyruvate fermentation under anaerobic conditions (Audrain *et al.*, 2015).

The abundance of 2-butanone was higher in presence of *Fusarium sambucinum* and *Pectobacterium c. atrosepticum.* The detection of 2-butanone in presence of *Pectobacterium c. atrosepticum* was observed through all the stages of the incubation period. 2-Butanone has been previously detected in presence of soft rot inoculated potato tubers (Varns and Glynn, 1979; Waterer and Pritchard, 1984b; de Lacy Costello *et al.*, 1999; Lui, Vikram, Abu-Nada, *et al.*, 2005; Sinha *et al.*, 2018). However, in some of these studies traces of 2-butanone were also detected in the non-inoculated potato tubers (Vikram *et al.*, 2006; Sinha *et al.*, 2018). 2-Butanone was also detected in presence of PGPR bacteria, such as *Bacillus subtilis* and *Bacillus amyloliquefaciens,* when they were subjected to anaerobic conditions (Ryu *et al.*, 2003; Lee *et al.*, 2012). 2-Butanone has also been detected in a wide range of matrixes as beef samples and truffles (Mauriello *et al.*, 2004; Splivallo *et al.*, 2012; Pavlidis *et al.*, 2019) and in presence of different

fungal and bacterial pathogens (Sunesson *et al.*, 1995; Wheatley *et al.*, 1997; Beck, Hansen and Lauritsen, 2002; Bruce *et al.*, 2004; Mitchell *et al.*, 2010).

The presence of 2-butanone is mainly associated with the presence of bacteria, it might be possible that *Pectobacterium c. atrosepticum* under anaerobic conditions produces acetoin and 2,3-butanedione as products of the fermentation of glucose. In presence of a diol dehydratase, 2-butanone can be produced (Audrain *et al.*, 2015).Therefore, 2-butanone can be considered as a potential VOC biomarker of the presence of a bacterial disease in potato tuber, although it was detected in presence of both diseases.

5.4.2.3. 4 Dimethyl disulfide

Dimethyl disulfide is the simplest disulfide and known to be produced by the degradation of methionine and its subsequent oxidation to methanethiol (Bonnarme *et al.*, 2001; Arfi, Landaud and Bonnarme, 2006).

Dimethyl disulfide was detected mainly at the late stage of the development of *Fusarium sambucinum* and *Pectobacterium c. atrosepticum.* However, it was also detected at a late stage, after 32 days, in the control (non-inoculated potatoes). This might be due to a contamination at the end of the experiment and the appearance of some fungal or bacterial pathogens. Dimethyl disulfide could be related to the decaying process of potato tubers, once they are at the latest stage of this decaying. It could be considered as a potential VOC biomarker of a late stage of dry rot and soft rot, although it can be generalised and be considered as a biomarker of decay in potato tubers due to the presence of a fungal or bacterial pathogen.

Similar results were obtained in presence of soft rot and dry rot in potato tubers, where dimethyl disulfide was detected in potato tubers inoculated with *Pectobacterium carotovorum* and *Fusarium sambucinum* (Ouellete *et al.*, 1990; de Lacy Costello *et al.*, 1999; Lui, Vikram, Abu-Nada, *et al.*, 2005). However, its detection was not directly attributed to the presence of those pathogens, but as a consequence of the rotting tissues once they are invaded by other microorganisms (Ouellete *et al.*, 1990). In a recent study in 2018, a contradictory

result was reported where the release of VOCs from onions infected with *Burkholderia cepacian* (sour skin) was studied and dimethyl disulfide was detected in presence of sour skin but also traces in healthy controls (Sinha *et al.*, 2018). This was probably due to the differences in crops, as it might be that onions produce some traces of dimethyl disulfide (Choi *et al.*, 2017; Wang, Luca and Edelenbos, 2019).

This VOC has also been widely detected in different matrixes; truffle (Mauriello *et al.*, 2004; Gioacchini *et al.*, 2005; Culleré *et al.*, 2010; Splivallo *et al.*, 2012), in presence of different fungal (Sunesson *et al.*, 1995) and bacterial (Schöller *et al.*, 2002; Bruce *et al.*, 2004; Gu *et al.*, 2007; Blom *et al.*, 2011; Huang *et al.*, 2012; Lee *et al.*, 2012; Tenorio-Salgado *et al.*, 2013; Spraker *et al.*, 2014; Tyc *et al.*, 2017) pathogens grown *in vitro*. One of these bacteria was *Ralstonia solanacearum,* responsible of brown rot in potato tubers (Spraker *et al.*, 2014).

5.4.2.3. 5 Hydrogen cyanide

Hydrogen cyanide (HCN) is considered a secondary metabolite of cyanogenic microorganisms. The cyanogenesis in microorganisms such as bacteria and fungi is thought to be similar to the plant cyanogenesis, where cyanogens, are produced from amino acids. The hydrolysis of those cyanogens produces hydrogen cyanide. Therefore, hydrogen cyanide is a product of the catabolism of glucosidic cyanogens, where a glucosidase and oxynitrilase enzymes are involved (Knowles, 1976; F. Zhang *et al.*, 2014; Zdor, 2015). There was demonstrated that some of those cyanogenic microorganisms can inhibit the growth of other microorganisms due to their capability of producing HCN. There are many studies where cyanogenic microorganisms have been studied as biocontrol agents of different crop diseases (Zhang *et al.*, 2014; Zdor, 2015).

Hydrogen cyanide abundance in our experiment was very selective It was detected with higher abundance at a late stage of the development of dry rot and with less abundance at a mid and late stage of the development of soft rot. Hydrogen cyanide could be considered as VOC biomarker of a late stage of the development of a disease, once potato tubers are completely rotten.

HCN was detected in several studies where *Pseudomonas spp.,* isolated from potato fields, were studied as a biocontrol agent against different crop diseases, such as those produced by *Rhizoctonia solani, Helminthosporium solani* and *Fusarium oxysporum* in potatoes (Bailly and Weisskopf, 2017; Anand *et al.*, 2020; Varatharaju *et al.*, 2020). This result is interesting and contradictory, as it has been elucidated that some *Pseudomonas* spp. detected in the field where potato tubers are grown, produce HCN, a metabolite able to inhibit the growth of pathogens that are directly affecting potatoes. However, HCN was detected in those potato tubers that were at a late stage of the development of soft rot and dry rot. This might be due to the presence of other pathogens co-infecting the potato tubers at a late stage of the incubation period.

Further studies will need to be carried out to elucidate the *Pectobacterium c. atrosepticum* and *Fusarium sambucinum* capability of producing HCN, as to date, there no literature regarding the production of HCN by those two pathogens.

5. 5 Conclusions

The main conclusions from these experiments were:

- Storage time affects the **severity of dry rot** symptoms. Potato tubers that were stored for 22 weeks presented higher severity of dry rot compared to tubers that were stored for ten weeks.
- Storage time did not affect the **severity of soft rot** in potato tubers. Those potato tubers that were stored for a longer period of time presented similar severity to soft rot than the one that were stored for ten weeks.
- Dry rot and soft rot resulted in a different **VOC fingerprint** of potato tubers and these are cultivar-specific.
- Storage time affects the **VOC fingerprint** of potato tubers in presence of soft rot and dry rot.
- **1-Methoxy-3-methyl-butane** and **2-butanone** could be considered as potential indicators of the presence of dry rot and soft rot at any stage of the development of the diseases.
- **Methyl acetate** could be considered as a potential indicator of the presence of soft rot or dry rot at an Early-stage of the development of the diseases.
- **Dimethyl disulfide** and **hydrogen cyanide** could be considered as potential indicators of the presence of dry rot and soft rot at a late stage of the development of the diseases.

Those VOC detected and selected as potential biomarkers of the presence of soft rot and dry rot in potatoes will need to be confirmed. Further studies will need to be carried out to confirm these conclusions.

6 GENERAL DISCUSSION

This work was focused on the identification of Volatile Organic Compounds (VOCs) as biomarkers of potato rots at cold storage and the ecophysiological study of those pathogens involved. The identification of VOCs as biomarkers of potato rots has already been studied on several occasions (Varns and Glynn, 1979; Waterer and Pritchard, 1984b, 1985; Ouellete *et al.*, 1990; Jones, Ewen and Ratcliffe, 1998; de Lacy Costello *et al.*, 2001; Lui, Vikram, Abu-Nada, *et al.*, 2005; Lui, Vikram, Hamzehzarghani, *et al.*, 2005; Stinson, Persaud and Bryning, 2006), however, very few studies have focused their attention on the cold storage conditions of potato tubers in the commercial facilities (Waterer and Pritchard, 1985; de Lacy Costello *et al.*, 2001; Blasioli *et al.*, 2013; Sinha *et al.*, 2018).

In a first instance, it was necessary to identify which were the most important potato diseases during storage in the UK. In conversations with industry, it was clear that the bacterial disease, soft rot, had to be included in our work because of the lesions it produces. Concerning the fungal rots, we isolated different fungal pathogens from potato tuber rots as described in Chapter 2 (Section 2. 2). Interestingly, seven of our isolates were identified as *Fusarium* spp., and all of them have been previously related with dry rot in potato tubers. Consequently, dry rot was selected as one of the diseases to be studied. Another fungal disease, gangrene, was selected after discussions with Dr. Glyn Harper from Sutton Bridge Crop Storage Research (AHDB Potato Council, UK), as one of the diseases that was mainly affecting storage potatoes during the 2016-2017 season.

As potato tubers are stored for up to 10 months at 3-10^{o}C and high relative humidity (95-99%), the identification of new VOC biomarkers of potato rots under cold storage industrial conditions was needed. Different methodologies (GC-MS, SPME-GC-MS) have been previously used for the study of VOCs (Varns and Glynn, 1979; de Lacy Costello *et al.*, 1999; Savelieva *et al.*, 2016; Sinha *et al.*, 2018). The high relative humidity and the low temperatures of the potato storage facilities were complicating the use of those technologies. The high quantity of water presented in the environment is detected in the VOC sample, saturating

the samples and reducing the detection of the VOCs. Furthermore, low temperature reduces the volatility of the VOCs, reducing the number and concentration of VOCs that will be detected. For this reason, part of this work was focused on the use of a pre-concentration method, Thermal Desorption (TD), coupled with Gas Chromatography-Mass Spectrometry, with a method fully developed in Chapter 2 (Section 2. 5). This combination of technologies allowed the pre-concentration of the sample, the reduction of the amount of water and finally, the detection and identification of VOCs. An evaluation of the most appropriate TD tubes to be used for this work was carried out in Chapter 2 (Section 2.5. 1), where Bio monitoring and Air toxics were selected as they detected the highest number of VOCs between two and five carbons, VOCs that were expected at low temperatures due to their low volatility.

Once the VOC detection methodology was optimised, exhaustive research was carried out on the untargeted VOC analysis, as described in Chapter 2 (Section 2.5. 4). An online-based informatics platform, XCMS (https://xcmsonline.scripps.edu) was found as an interesting option as it was an open platform, for the analysis of our samples. It allowed the comparison between features, where the VOC were categorised based on their retention time, without the need for their identification. However, some issues in the alignment of the GC peaks and a lack of information regarding the algorithms used for the comparisons forced us to decide that this was not a suitable option and thus change this analysis approach. Finally, the untargeted analysis was performed using MPP (*MPP 12.6.1*, Agilent Technologies), a software that allowed the comparisons between GC-MS data, coupled with a targeted analysis using AMDIS (AMDIS 2.72, Automated Mass Spectral Deconvolution and Identification System). Although a license was needed for their use, it allowed us the best analysis of the VOC data.

The first step carried out for the detection of potential VOC biomarkers of potato rots was to study the ecophysiology and VOC production of the pathogens responsible *in vitro.* Studying the growth of the fungi with an exhaust control of the different environmental conditions allowed the determination of how

temperature and relative humidity will affect the fungi when growing in a semi-synthetic media (described in Chapter 2, Section 2.3. 1). Furthermore, regarding the VOC detection, this provided information regarding the origin of the VOC, as the fungi is growing *in vitro* and there is no other factor than temperature and a_w affecting its VOC production.

Studying the growth and VOC production of pathogens as *Boeremia foveata* or *Fusarium* spp. at different environmental conditions (temperature and a_w) provided useful information regarding their potential lesion size and VOCs produced during storage (Chapter 3 & 4). The study of *Boeremia foveata,* responsible for gangrene, *in vitro*, described in Chapter 3, showed that once the infection is present, an increase in temperature and relative humidity will increase the growth rate of *B. foveata* and a reduction in the number of days needed to start growing. Similar results were achieved for *Fusarium sambucinum* and *Fusarium oxysporum*, responsible for dry rot in potato tubers, both studied *in vitro* in Chapter 4. Therefore, considering the growth of those fungi in a potato-based media (NPDA), suggested that a similar behaviour will occur in potato tubers from a storage facility when they are exposed to higher temperatures and relative humidity.

The detection of VOCs *in vitro* was also studied in Chapters 3 and 4, providing useful information regarding those volatiles produced by the fungi and the type of VOC that might be expected from potato rots. VOCs as methyl acetate, acetone, ethanol, ethyl acetate and 2-methylpropan-1-ol were detected in common *in vitro* in presence of *Boeremia foveata* and both *Fusarium* spp.

The gained knowledge in the previous experiments was then used in experiments carried out *in vivo* with both dry rot and soft rot and fully presented in Chapter 5. The selection of cultivars and the development of the methodology for potato surface sterilisation was needed and presented in Chapter 2 (Section 2. 4). This *in vivo* study allowed to further develop the understanding of the behaviour of *Fusarium sambucinum* and *Pectobacterium c. atrosepticum* on potatoes under cold storage conditions. The effect of the storage time or potato “age” in the severity of each disease was also considered. A higher disease progression of

dry rot was observed in those potato tubers that had spent more time in storage prior to their commercialisation, while no effect was observed for soft rot. Therefore, potato tubers that are stored for a longer period of time and are infected with dry rot, will present a higher disease progression than potato tubers that are stored for a shorter time, prior to their commercialisation.

The detection of VOCs was also studied *in vivo,* as presented in Chapter 5. Ethanol, methyl acetate and 2-mehylpropan-1-ol were detected in the two different experiments, *in vivo* and *in vitro.* Therefore, these VOCs were directly related to the presence of dry rot in potato tubers. The detection of ethanol was higher in potato tubers with dry rot and soft rot compared to the non-inoculated potato tubers, and similar results were observed with methyl acetate. In one of the experiments where potato tubers were stored for a longer period, 2-methylpropan-1-ol was detected in higher concentrations in presence of dry rot and soft rot. Therefore, these three VOCs, ethanol, methyl acetate and 2-methylpropan-1-ol could be considered as potential biomarkers of potato rots at cold storage. However, further studies will need to be carried out considering different potato cultivars and fungal and bacterial diseases.

Those three VOCs were detected in potato-based media and in potato tubers in presence of dry rot (Chapter 4 & 5), therefore, this provided information regarding the origin of those VOCs. Therefore, ethanol, methyl acetate and 2-methylpropan-1-ol were probably produced by the pathogen and not as a defence mechanism of the potato tuber. An *in vitro* study will need to be carried out for *Pectobacterium carotovorum atrosepticum* to unveil its VOC fingerprint in potato-based media.

Ethanol, methyl acetate and 2-methylpropan-2-ol could be considered as potential biomarkers of spoilage or a potato disease in potato tubers during storage at cold temperatures (8.5^{o}C). The results indicate that a high level of any of those three VOCs will be related to the presence of gangrene, dry rot or soft rot in the storage room. Furthermore, it is interesting to mention that VOCs as dimethyl disulfide or 2-butanone were exclusively detected in the *in vivo* study and mainly in presence of soft rot (Chapter 5). Although those two VOC have

been previously related with bacterial infection (Sinha *et al.*, 2018), as no *in vitro* study was carried out for *Pectobacterium* spp., it cannot be directly attributed to the presence of *Pectobacterium* spp. As previously mentioned, an *in vitro* study will need to be carried out for *Pectobacterium c. atrosepticum* to confirm those results.

Lastly, this thesis is the first scientific report describing 1-methoxy-3-methylbutane, as an interesting VOC and potential biomarker, that was detected in higher abundance in presence of dry rot and soft rot in potato tubers. Although it was not detected in the *in vitro* study where the same pathogen was studied in potato-based media, it might be related to the number of amino acids available in the potato tuber compared to the media As it was previously mentioned, the production of 1-methoxy-3-methylbutane has been previously related to the degradation of amino acids (Marilley and Casey, 2004; Lui, Vikram, Abu-Nada, *et al.*, 2005; Vikram *et al.*, 2006).

The appearance of potato diseases in commercial storage facilities leads to an increase in waste generation, but also it supposes a potential food safety risk. Some of the fungal pathogens responsible of dry rot, as *Fusarium sambucinum* and *Fusarium oxysporum* can produce mycotoxins, which can be accumulated in potato tubers and then consumed. The effect of temperature and relative humidity on the production of some of these mycotoxins (T-2, HT-2, Diacetoxyscirpenol, Neosolaniol, Beauvericin and 15-Acetoxyscirpenol) were studied *in vitro* and presented in Chapter 4. At higher temperature and relative humidity, the production of mycotoxins was higher. Therefore, potato tubers infected with *Fusarium* spp., exposed to higher temperatures and RH will present a higher risk of accumulation of mycotoxins. Once the potato tubers are sold, the consumer will store them at room temperature, temperature that, as it has been described in Chapter 4, will increase the accumulation of mycotoxins.

Furthermore, it needs to be considered the diffusion of the mycotoxins through the potato tissue as it has been previously studied by Delgado et al. (Delgado *et al.*, 2010). Although they observed a very low diffusion at storage temperature (10-12^{o}C), the diffusion will need to be studied at higher temperatures. That will

mean that just removing the part of the potato tuber that is rotten might not be enough to remove the toxins. There are very few reports on mycotoxins in potato tubers (El-Banna, Scott and Lau, 1984; Ellner, 2002; Delgado *et al.*, 2010; Xue *et al.*, 2013, 2014). A recent study focused on the accumulation of mycotoxins on potato tubers in Algeria is taking place at Cranfield University in collaboration with ENS Kouba (Algiers, Algeria).

6.1 Development of real-time VOC sensor for better store management

The VOC data collected during these four years of study have contributed to the design of a new sensing technology developed by Cranfield University and other partners as part of the QCAP project ('Real-time interactive storage quality control in fresh agro products', funded by Interreg NWE). This sensor is a multi-species trace gas sensor based on a Mid-Infrared (MIR) supercontinuum light source. The sensor is able to detect the concentration of up to five different VOCs in real time (Jahromi *et al.*, 2019). Once installed in a potato storeroom, it should be able to quantify the VOCs that we have previously selected. Increases in those VOCs could be an indicator of the presence of a potato disease somewhere in the storage facility. Two different approaches can be carried out in that point depending on the farmers and their store management procedures. A first option could be to immediately proceed to empty that storeroom and sell the potatoes. Alternatively, if possible, an inspection could be carried out to identify which part of the room affected. Either way, the sensor would provide a valuable early warning system that will allow better potato storage management and contribute to the reduction of waste.

Our role in this project was to identify and quantify the VOCs that would be expected to be detected by the new sensor in the presence of potato pathogens at cold temperatures (storage conditions). The first prototype built and tested at Cranfield highlighted some areas for further improvement. These include: a) the level of detection, which currently is not low enough for the VOCs expected to be found in a potato storage room, b) the high humidity found in cold storage rooms can interfere with the sensor, and a new system has been implemented to

address this problem, and c) the system saturation when a single VOC appears in very high quantities, that compromises the sensors ability to detect and quantify other VOCs present in lower abundance (this was the case of ethylene in some experiments). The improvement of the sensor will take it closer to commercialisation and it is envisaged that it could be available in the market within the next 5 years.

Different sensors based on VOCs have been previously developed for the detection of potato diseases (de Lacy Costello *et al.*, 2000; Rutolo *et al.*, 2016, 2018; Sinha *et al.*, 2018), however, they were only achieving the discrimination between the presence and the absence of a disease based on the response produced by the sensor. In this case, the QCAP sensor will be able to detect specific selected VOCs as ethanol or methyl acetate among others, and in some cases, it will be able to differentiate between pathogens.

The main objectives of this PhD have been achieved with the selection of the optimal VOC methodology, TD-GC-ToF-MS, the elucidation of the positive effect of temperature and relative humidity on the ecophysiology and mycotoxin production of the different pathogens responsible for potato diseases and the effect that storage time has on the development of soft rot and dry rot under cold storage conditions.

Overall, the achievement of those objectives allowed the fulfilment of the main aim of this PhD, the identification of VOC as potential biomarkers of potato rots and the ecophysiological study of the pathogens involved. VOCs as methyl acetate, ethanol, 2-methylpropan-1-ol and 1-methoxy-3-methylbutane could be considered as potential biomarkers of potato rots, although further studies will need to be carried out.

7 CONCLUSIONS

Overall, the present work has been able to address the original objectives presented at the beginning of the research.

- The exposure of potato tubers to slightly higher temperatures and relative humidity than the ones in storage conditions will result in an increase in the development of dry rot and gangrene and in a reduction in the time needed for its development.
- The mycotoxin accumulation (T-2, HT-2, Diacetoxyscirpenol, Neosolaniol, Beauvericin and 15-Acetoxyscirpenol) on potato tubers infected with dry rot and stored at higher temperatures and relative humidity can be a risk for the food safety of the consumers.
- The two different potato cultivars studied (Record and Casablanca) presented different disease severity of dry rot and soft rot. Therefore, the development of dry rot and soft rot under cold storage conditions is linked to the genetic background of the potato tubers.
- The “age” of the potato tubers, the time they spend in storage prior to its commercialisation, increases, once they are infected, the disease progression of soft rot and dry rot.
- VOC fingerprints of potato tubers infected with dry rot and soft rot have been established. Similar VOC fingerprints were detected in presence of soft rot and dry rot.
- Acetone and ethyl acetate could be considered as potential biomarkers of the presence of dry rot or gangrene *in vitro.*
- Ethanol, methyl acetate and 2-methylpropan-1-ol have been identified as potential VOC biomarkers of the presence of a fungal or bacterial disease during the cold storage of potato tubers.
- Dimethyl disulfide and hydrogen cyanide could be considered as potential indicators of the presence of dry rot and soft rot at a late stage of the development of the diseases.

8 FUTURE RECOMMENDATIONS

Based on the findings that have been achieved in this work, future research should be focused on:

- Study the behaviour and the VOC production of *Pectobacterium carotovorum* spp. *in vitro*, to be able to compare those results with what was observed *in vivo.* The incubation of *Pectobacterium c. atrosepticum* at three different temperatures (5,10 & 15^{o}C) and at two different a_w (0.97,0.99) in Natural Potato Dextrose Broth (NPDB). The study of its growth at the different temperatures using spectrophotometry and the VOC production will be analysed by TD-GC-ToF-MS.
- Study the ecophysiology and the VOC production of different *Fusarium* spp. responsible for dry rot in potato tubers. *Fusarium solani coeruleum, Fusarium culmorum* and *Fusarium avenaceum* will be studied *in vitro.* This could provide information regarding the kind of VOC produced in presence of dry rot pathogens. At the same time, this can confirm some of our results.
- Achieve the quantification of the VOCs selected. The quantification of the VOCs will be carried out by the preparation of standard curves of each of the VOCs. This will allow the confirmation of the identity of each VOCs and their real quantification.
- Study the VOC detected on different potato cultivars in presence of soft rot and dry rot following a similar procedure as the one followed in Chapter 5. This will help establish the genotype-specificity of the VOC detected.
- Validate the VOC results under commercial storage conditions. Different cultivars will be infected with dry rot and soft rot, stored in a commercial facility and VOC will be sampled after different periods of time. These results will allow the validation of our results, providing information about those VOC and their concentrations in storage conditions.
- Investigate the mechanisms involved in the production of some of the VOC identified in this study. Once the mechanisms are known and understood, a temporal analysis of some of the precursors of those VOC could be

studied. Furthermore, researching which genes are responsible of the production of those VOCs and studying their expression *in vitro* and *in vivo,* could provide useful information.

- Study the production of mycotoxins in potato tubers infected with different *Fusarium* spp. responsible of dry rot using a similar methodology as the one described in Chapter 2, using Liquid Chromatography coupled with Mass Spectrometry. Potato tubers will be inoculated with different *Fusarium* spp. and stored at different temperatures, samples of potato tubers will be taken every five days, mycotoxins extracted and analysed by LC-MS. Furthermore, a gene expression study could also be considered, regarding those genes involved in the metabolic pathway of the production of mycotoxins.

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APPENDICES

Appendix A. Fungal and bacterial strains

Table A-1 Different potato pathogens and the culture collection where they have been purchased. CBS: Culture collection, Westerdijk Institute, Netherlands. CECT: Spanish Collection of type cultures, Spain. DSMZ: Culture collection, Leibniz Institute, Germany.

Disease	Pathogen	Culture collection	ID
Silver scurf	*Helminthosporium solani*	CBS	275.30
Black dot	*Colletotrichum coccodes*	CECT	2853
Skin spot	*Polyscytalum pustulans*	CBS	336.54
Dry rot	*Fusarium solani coeruleum*	CBS	113.23
Gangrene	*Boeremia foveata*	CECT	20797
Late blight	*Phytophthora infestans*	CBS	430.90
Early blight	*Alternaria solani*	DMSZ	62028
Black scurf	*Thamatephorus cucumeris* (Teleomorph of *Rhizoctonia solani)*	CECT	2813
Soft rot	*Pectobacterium carotovorum*	CECT	225
	Pectobacterium atrosepticum	CECT	314
Ring rot	*Clavibacter michiganensis sependonicus*	CECT	4886

Appendix B. Molecular identification results

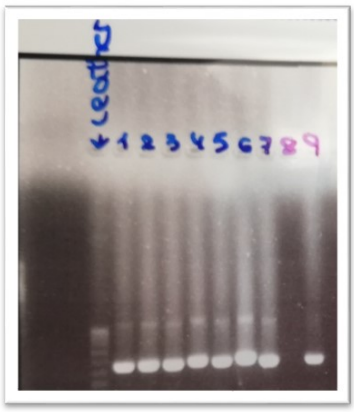

Figure B-1 UV A visualization of PCR products after their amplification for a region of the ITS. Different bands number correspond from left to right: 1. Isolate 1, 2. Isolate 2, 3: Isolate 3, 4: Isolate 4, 5: Isolate 5, 6: Isolate 6, 7: *Fusarium sambucinum,* 8: Negative control (MiliQ water) and 9: Positive control (*Fusarium graminearum*).

Figure B-2 UV A visualization of PCR products after their amplification for a region of the EF-1α. Different bands number correspond from left to right: 1. Isolate 1, 2. Isolate 2, 3: Isolate 3, 4: Isolate 4, 5: Isolate 5, 6: Isolate 6, 7: *Fusarium sambucinum,* -: Negative control (MiliQ water) and +: Positive control (*Fusarium langsethiae*).

Score	Expect	Identities	Gaps	Strand
948 bits(513)	0.0	513/513(100%)	0/513(0%)	Plus/Plus

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Query  1    GGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGCCT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  43   GGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGCCT  102

Query  61   CGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  103  CGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTG  162

Query  121  TTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  163  TTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCT  222

Query  181  CTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  223  CTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAA  282

Query  241  TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  283  TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATG  342

Query  301  CCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  343  CCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTC  402

Query  361  GCGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  403  GCGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTC  462

Query  421  GTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  463  GTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGA  522

Query  481  TCAGGTAGGAATACCCGCTGAACTTAAGCATAT  513
            ||||||||||||||||||||||||||||||||
Sbjct  523  TCAGGTAGGAATACCCGCTGAACTTAAGCATAT  555
```

Figure B-3 Sequence alignment from *Isolate 1* carried out using BLAST (NCBI). A 100% of identities were observed with *Fusarium oxysporum* clone SF_967 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (MT530243.1)

Score	Expect	Identities	Gaps	Strand
1269 bits(687)	0.0	687/687(100%)	0/687(0%)	Plus/Minus

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Query  1    ATCATGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGGAAAACATGTTAGCATGAGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  61   AGGTCATGAGTGTAAGTGATGACAACATACCAATGACGGTGACATAGTAGCGAGGAGTCT  120
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Sbjct  634  AGGTCATGAGTGTAAGTGATGACAACATACCAATGACGGTGACATAGTAGCGAGGAGTCT  575

Query  121  CGAACTTCCAGAGAGCGATATCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  574  CGAACTTCCAGAGAGCGATATCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGT  515

Query  181  CAAGAACCCAAGCGTACTTGAAGGAACCCTTACCGAGCTCGGCGGCTTCCTATTGTCGGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  514  CAAGAACCCAAGCGTACTTGAAGGAACCCTTACCGAGCTCGGCGGCTTCCTATTGTCGGG  455

Query  241  TGGTTAGTGACTGATTGACACGTGACGCGCAAGAGGTGATTTTGTGGGAAGAGGGCAAGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  454  TGGTTAGTGACTGATTGACACGTGACGCGCAAGAGGTGATTTTGTGGGAAGAGGGCAAGC  395

Query  301  GCCTGTCACTCGAGCAGCGGGGTATGAGCCCCACCAAAAAATTGCGGTTGAACCGCAAAA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  394  GCCTGTCACTCGAGCAGCGGGGTATGAGCCCCACCAAAAAATTGCGGTTGAACCGCAAAA  335

Query  361  TTTTGTATTCGAGCGGGGTGACAGGCGCATATTCAGTCATCGAGGCTGATTCGAGTGATG  420
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Sbjct  334  TTTTGTATTCGAGCGGGGTGACAGGCGCATATTCAGTCATCGAGGCTGATTCGAGTGATG  275

Query  421  GATCGGTGTGCAGAGGGCGTGCGATCGAGGAAAATGTAAACCAACCTTCTCGAACTTCTC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  274  GATCGGTGTGCAGAGGGCGTGCGATCGAGGAAAATGTAAACCAACCTTCTCGAACTTCTC  215

Query  481  GATGGTTCGCTTGTCGATACCACCGCACTGGTAGATCAAGTGACCGGTCTATGCAATTTT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  214  GATGGTTCGCTTGTCGATACCACCGCACTGGTAGATCAAGTGACCGGTCTATGCAATTTT  155

Query  541  GTCAGCAAATGTGTAAGTTGATATTACCCCACCACGTATGGCGGGGTTGATGACTGCTGA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  154  GTCAGCAAATGTGTAAGTTGATATTACCCCACCACGTATGGCGGGGTTGATGACTGCTGA  95

Query  601  TAAGCAGGTCATTGAGGGTAGTACTCACAGTGGTCGACTTGCCAGAGTCGACGTGGCCGA  660
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Query  661  TGACGACGACGTTAAGGTGAGTCTTGT  687
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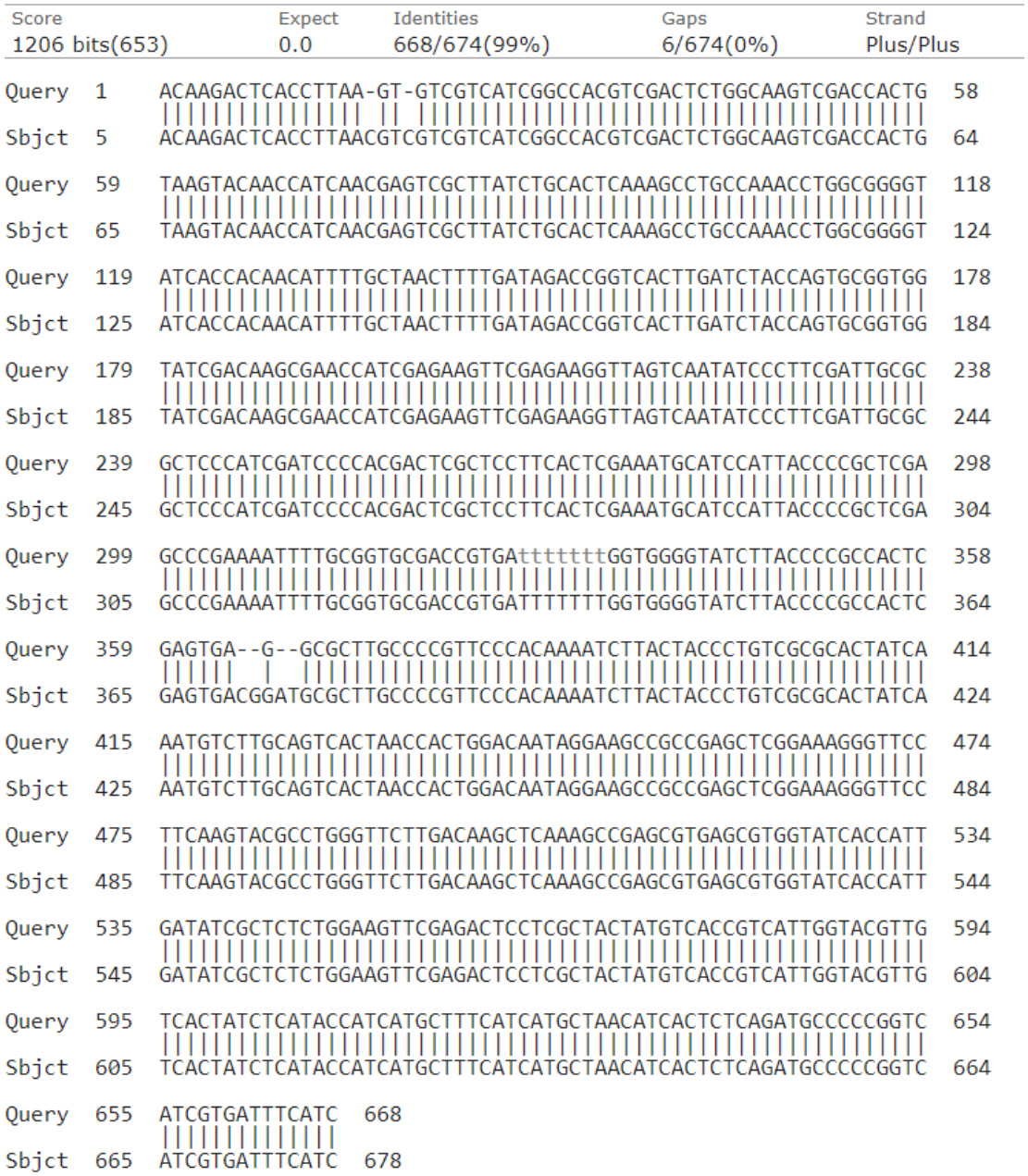
Sbjct  34   TGACGACGACGTTAAGGTGAGTCTTGT  8
```

Figure B-4 Sequence alignment from *Isolate 2* carried out using BLAST (NCBI). A 99% of identities were observed with *Fusarium equiseti* strain HGUP17361.2 translation elongation factor 1-alpha gene, partial cds (MK069605.1)



Score	Expect	Identities	Gaps	Strand
1206 bits(653)	0.0	668/674(99%)	6/674(0%)	Plus/Plus

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Sbjct  5    ACAAGACTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTG  64

Query  59   TAAGTACAACCATCAACGAGTCGCTTATCTGCACTCAAAGCCTGCCAAACCTGGCGGGGT  118
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Query  119  ATCACCACAACATTTTGCTAACTTTTGATAGACCGGTCACTTGATCTACCAGTGCGGTGG  178
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125  ATCACCACAACATTTTGCTAACTTTTGATAGACCGGTCACTTGATCTACCAGTGCGGTGG  184

Query  179  TATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCAATATCCCTTCGATTGCGC  238
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185  TATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCAATATCCCTTCGATTGCGC  244

Query  239  GCTCCCATCGATCCCCACGACTCGCTCCTTCACTCGAAATGCATCCATTACCCCGCTCGA  298
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245  GCTCCCATCGATCCCCACGACTCGCTCCTTCACTCGAAATGCATCCATTACCCCGCTCGA  304

Query  299  GCCCGAAAATTTTGCGGTGCGACCGTGAttttttGGTGGGGTATCTTACCCCGCCACTC  358
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305  GCCCGAAAATTTTGCGGTGCGACCGTGATTTTTTTGGTGGGGTATCTTACCCCGCCACTC  364

Query  359  GAGTGA--G--GCGCTTGCCCCGTTCCCACAAAATCTTACTACCCTGTCGCGCACTATCA  414
            ||||||  |  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365  GAGTGACGGATGCGCTTGCCCCGTTCCCACAAAATCTTACTACCCTGTCGCGCACTATCA  424

Query  415  AATGTCTTGCAGTCACTAACCACTGGACAATAGGAAGCCGCCGAGCTCGGAAAGGGTTCC  474
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425  AATGTCTTGCAGTCACTAACCACTGGACAATAGGAAGCCGCCGAGCTCGGAAAGGGTTCC  484

Query  475  TTCAAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATT  534
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  485  TTCAAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATT  544

Query  535  GATATCGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTACGTTG  594
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  545  GATATCGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTACGTTG  604

Query  595  TCACTATCTCATACCATCATGCTTTCATCATGCTAACATCACTCTCAGATGCCCCCGGTC  654
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  605  TCACTATCTCATACCATCATGCTTTCATCATGCTAACATCACTCTCAGATGCCCCCGGTC  664

Query  655  ATCGTGATTTCATC  668
            ||||||||||||||
Sbjct  665  ATCGTGATTTCATC  678
```

Figure B-5 Sequence alignment from *Isolate 3* carried out using BLAST (NCBI). A 99% of identities were observed with *Fusarium venenatum* culture ICMP:8997 translation elongation factor 1 (EF1a) gene, partial cds (MG857304.1)

Score	Expect	Identities	Gaps	Strand
1206 bits(653)	0.0	668/674(99%)	6/674(0%)	Plus/Plus

```
Query  1    ACAAGACTCACCTTAA-GT-GTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTG  58
            |||||||||||||||| || ||||||||||||||||||||||||||||||||||||||
Sbjct  5    ACAAGACTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTG  64

Query  59   TAAGTACAACCATCAACGAGTCGCTTATCTGCACTCAAAGCCTGCCAAACCTGGCGGGGT  118
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  65   TAAGTACAACCATCAACGAGTCGCTTATCTGCACTCAAAGCCTGCCAAACCTGGCGGGGT  124

Query  119  ATCACCACAACATTTTGCTAACTTTTGATAGACCGGTCACTTGATCTACCAGTGCGGTGG  178
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125  ATCACCACAACATTTTGCTAACTTTTGATAGACCGGTCACTTGATCTACCAGTGCGGTGG  184

Query  179  TATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCAATATCCCTTCGATTGCGC  238
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185  TATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCAATATCCCTTCGATTGCGC  244

Query  239  GCTCCCATCGATCCCCACGACTCGCTCCTTCACTCGAAATGCATCCATTACCCCGCTCGA  298
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245  GCTCCCATCGATCCCCACGACTCGCTCCTTCACTCGAAATGCATCCATTACCCCGCTCGA  304

Query  299  GCCCGAAAATTTTGCGGTGCGACCGTGAttttttGGTGGGGTATCTTACCCCGCCACTC  358
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305  GCCCGAAAATTTTGCGGTGCGACCGTGATTTTTTTGGTGGGGTATCTTACCCCGCCACTC  364

Query  359  GAGTGA--G--GCGCTTGCCCCGTTCCCACAAAATCTTACTACCCTGTCGCGCACTATCA  414
            ||||||  |  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365  GAGTGACGGATGCGCTTGCCCCGTTCCCACAAAATCTTACTACCCTGTCGCGCACTATCA  424

Query  415  AATGTCTTGCAGTCACTAACCACTGGACAATAGGAAGCCGCCGAGCTCGGAAAGGGTTCC  474
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425  AATGTCTTGCAGTCACTAACCACTGGACAATAGGAAGCCGCCGAGCTCGGAAAGGGTTCC  484

Query  475  TTCAAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATT  534
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  485  TTCAAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATT  544

Query  535  GATATCGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTACGTTG  594
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  545  GATATCGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTACGTTG  604

Query  595  TCACTATCTCATACCATCATGCTTTCATCATGCTAACATCACTCTCAGATGCCCCCGGTC  654
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  605  TCACTATCTCATACCATCATGCTTTCATCATGCTAACATCACTCTCAGATGCCCCCGGTC  664

Query  655  ATCGTGATTTCATC  668
            ||||||||||||||
Sbjct  665  ATCGTGATTTCATC  678
```

Figure B-6 Sequence alignment from *Isolate 4* carried out using BLAST (NCBI). A 99% of identities were observed with *Fusarium flocciferum* culture ICMP:12131 translation elongation factor 1 (EF1a) gene, partial cds (MG857472.1)

Score	Expect	Identities	Gaps	Strand
1075 bits(582)	0.0	582/582(100%)	0/582(0%)	Plus/Plus

```
Query  1    GGTGACATAGTAGCGAGGAGTCTCGAACTTCCAGAGAGCGATATCAATGGTGATACCACG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  55   GGTGACATAGTAGCGAGGAGTCTCGAACTTCCAGAGAGCGATATCAATGGTGATACCACG  114

Query  61   CTCACGCTCGGCTTTGAGCTTGTCAAGAACCCAGGCGTACTTGAAGGAACCCTTACCGAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  115  CTCACGCTCGGCTTTGAGCTTGTCAAGAACCCAGGCGTACTTGAAGGAACCCTTACCGAG  174

Query  121  CTCGGCGGCTTCCTATTGACAGGTGGTTAGTAACTGATTGACACGTGATGGTGCGCGCCT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  175  CTCGGCGGCTTCCTATTGACAGGTGGTTAGTAACTGATTGACACGTGATGGTGCGCGCCT  234

Query  181  AGGGAATGGTTTGTGGGAAGAGGGCAAGCGCCTGTCGCTCGAGTGGCGGGGTATGAGCCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  235  AGGGAATGGTTTGTGGGAAGAGGGCAAGCGCCTGTCGCTCGAGTGGCGGGGTATGAGCCC  294

Query  241  CACCAGAAAAATTACGACAAAGCCGCAAAATTTTTGACCTCGAGCGGGGTAACAGGCGCG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  295  CACCAGAAAAATTACGACAAAGCCGCAAAATTTTTGACCTCGAGCGGGGTAACAGGCGCG  354

Query  301  TATCGAGTCGTCGTGTGAGGGCGATTCGAATGATGTTTCGAAAGGGAAAAGGGCGCGCGA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355  TATCGAGTCGTCGTGTGAGGGCGATTCGAATGATGTTTCGAAAGGGAAAAGGGCGCGCGA  414

Query  361  TCGAGGAAAATGAGACCAACCTTCTCGAACTTCTCGATGGTTCGCTTGTCGATACCACCG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  415  TCGAGGAAAATGAGACCAACCTTCTCGAACTTCTCGATGGTTCGCTTGTCGATACCACCG  474

Query  421  CACTGGTAGATCAAGTGACCGGTCTATCAAAGTATGTCAGCACATTGGAAATTTGAAACT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  475  CACTGGTAGATCAAGTGACCGGTCTATCAAAGTATGTCAGCACATTGGAAATTTGAAACT  534

Query  481  ACCCCGCCAAGTATCGGCGGGGTTGGGATGCAGTGGTACTCACAGTGGTCGACTTGCCAG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  535  ACCCCGCCAAGTATCGGCGGGGTTGGGATGCAGTGGTACTCACAGTGGTCGACTTGCCAG  594

Query  541  AGTCGACGTGGCCGATGACGACGACGTTAAGGTGAGTCTTGT  582
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  595  AGTCGACGTGGCCGATGACGACGACGTTAAGGTGAGTCTTGT  636
```

Figure B-7 Sequence alignment from *Isolate 5* carried out using BLAST (NCBI). A 99% of identities were observed with *Fusarium culmorum* strain Fu-BI8 translation elongation factor 1-alpha (EF1-alpha) gene, partial cds (MF431609.1)

Score	Expect	Identities	Gaps	Strand
1253 bits(678)	0.0	683/685(99%)	2/685(0%)	Plus/Plus

```
Query  1    ACAAGACTCACCTTAA-GT-GTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTG  58
            |||||||||||||||| || ||||||||||||||||||||||||||||||||||||||
Sbjct  2    ACAAGACTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTG  61

Query  59   TAAGTACAACCATCAGCGAGTCGCTTATCTGCACTCGGAACCCGCCGAACCTGGCGGGGT  118
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62   TAAGTACAACCATCAGCGAGTCGCTTATCTGCACTCGGAACCCGCCGAACCTGGCGGGGT  121

Query  119  ATCACCACGACATCTTGCTAACTCTTGACAGACCGGTCACTTGATCTACCAGTGCGGTGG  178
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122  ATCACCACGACATCTTGCTAACTCTTGACAGACCGGTCACTTGATCTACCAGTGCGGTGG  181

Query  179  TATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCAATATCCCTTCGATTACGC  238
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182  TATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCAATATCCCTTCGATTACGC  241

Query  239  GCGCTCCCATCGATTCCCACGACTCGCTCCCTCATTCGAAACGCATTCATTACCCCGCTC  298
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242  GCGCTCCCATCGATTCCCACGACTCGCTCCCTCATTCGAAACGCATTCATTACCCCGCTC  301

Query  299  AAGTCCGAAAATTTTGCGGTGCGACCGTGAtttttttGGTGGGGTATCTTACCCCGCCA  358
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302  AAGTCCGAAAATTTTGCGGTGCGACCGTGATTTTTTTTGGTGGGGTATCTTACCCCGCCA  361

Query  359  CTCGAGTGACGGATGCGCTTGCCCTGTTCCCACAAAACCTCACCACACTGTCGCGCACTA  418
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  CTCGAGTGACGGATGCGCTTGCCCTGTTCCCACAAAACCTCACCACACTGTCGCGCACTA  421

Query  419  TGTCTTGCAGTCACTAACCACTGGACAATAGGAAGCCGCCGAGCTCGGAAAGGGTTCCTT  478
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  TGTCTTGCAGTCACTAACCACTGGACAATAGGAAGCCGCCGAGCTCGGAAAGGGTTCCTT  481

Query  479  CAAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATTGA  538
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  CAAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATTGA  541

Query  539  TATCGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTC  598
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542  TATCGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTC  601

Query  599  ACTGTCTCACACCACCATGCCTTCATCATGCTAACATCCCTCTCAGATGCCCCCGGTCAT  658
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602  ACTGTCTCACACCACCATGCCTTCATCATGCTAACATCCCTCTCAGATGCCCCCGGTCAT  661

Query  659  CGTGATTTCATCAAGAACATGATCA  683
            |||||||||||||||||||||||||
Sbjct  662  CGTGATTTCATCAAGAACATGATCA  686
```

Figure B-8 Sequence alignment from *Isolate 7* carried out using BLAST (NCBI). A 99% of identities were observed with *Fusarium avenaceum* isolate Z337 translation elongation factor 1-alpha (TEF1) gene, partial cds (KP400709.1)

Score	Expect	Identities	Gaps	Strand
1182 bits(640)	0.0	648/651(99%)	3/651(0%)	Plus/Plus

```
Query  1    GACTCACCTTAA-GT-GTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTAAG  58
            ||||||||||||| || ||||||||||||||||||||||||||||||||||||||||||
Sbjct  7    GACTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTAAG  66

Query  59   TTGACCCAAATCTAAGCTCGCCTACAATTGGCGGGGTAGCCTCAAGATACGCTTGTGCTG  118
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67   TTGACCCAAATCTAAGCTCGCCTACAATTGGCGGGGTAGCCTCAAGATACGCTTGTGCTG  126

Query  119  ACATACATCATAGACCGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCAT  178
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  ACATACATCATAGACCGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCAT  186

Query  179  CGAGAAGTTCGAGAAGGTTGGTCTCATTTTCCTCGATCGCGCGCCCTACTTTCCATCGAT  238
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  CGAGAAGTTCGAGAAGGTTGGTCTCATTTTCCTCGATCGCGCGCCCTACTTTCCATCGAT  246

Query  239  CCATCATTCGAATCGCTCTGATACGACTCGACACACGCCTGCTACCCCGCTCGAGTTCAA  298
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  CCATCATTCGAATCGCTCTGATACGACTCGACACACGCCTGCTACCCCGCTCGAGTTCAA  306

Query  299  AAATTTTACGACTTTGTCGTAAttttttttGGTGGGGCTCATACCCCGCCACTTGAGCGAC  358
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  AAATTTTACGACTTTGTCGTAATTTTTTTTGGTGGGGCTCATACCCCGCCACTTGAGCGAC  366

Query  359  ATGCCCTTCCTCCAAAGCCACGGGCGCGCATCATCACGTGTTGATCAGTTACTAACAACC  418
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  ATGCCCTTCCTCCAAAGCCACGGGCGCGCATCATCACGTGTTGATCAGTTACTAACAACC  426

Query  419  TGTCAATAGGAAGCCGCCGAGCTCGGTAAGGGTTCTTTCAAGTACGCTTGGGTTCTTGAC  478
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  TGTCAATAGGAAGCCGCCGAGCTCGGTAAGGGTTCTTTCAAGTACGCTTGGGTTCTTGAC  486

Query  479  AAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATCGATATCGCTCTCTGGAAGTTCGAG  538
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  AAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATCGATATCGCTCTCTGGAAGTTCGAG  546

Query  539  ACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCACTACCACCTCCATCACATTCC  598
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  ACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCACTACCACCTCCATCACATTCC  606

Query  599  CGCACTAACTCACCTATCA-ACGCTCCCGGTCACCGTGATTTCATCAAGAA  648
            ||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  607  CGCACTAACTCACCTATCAGACGCTCCCGGTCACCGTGATTTCATCAAGAA  657
```

Figure B-9 Sequence alignment from *Fusarium sambucinum* strain carried out using BLAST (NCBI). A 99% of identities were observed with *Fusarium sambucinum* isolate 2sidE1C3 translation elongation factor 1-alpha (TEF1) gene, partial cds (MK752453.1)

Appendix C. Optimisation of mashed potato percentage

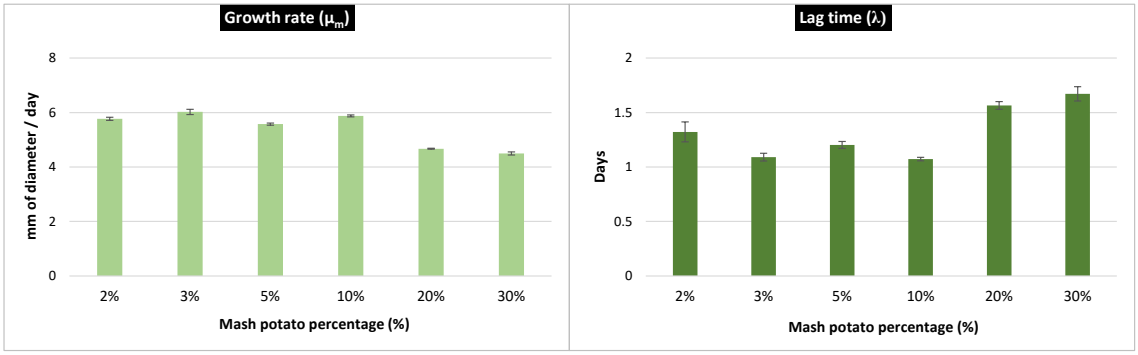

Figure C-1 Effect of mashed potato percentages on *Fusarium sambucinum* growth rate (μ_m) and lag time (λ). Data shows means of four replicates with bars indicating standard deviation (SD) (ANOVA, p-values<0.05).

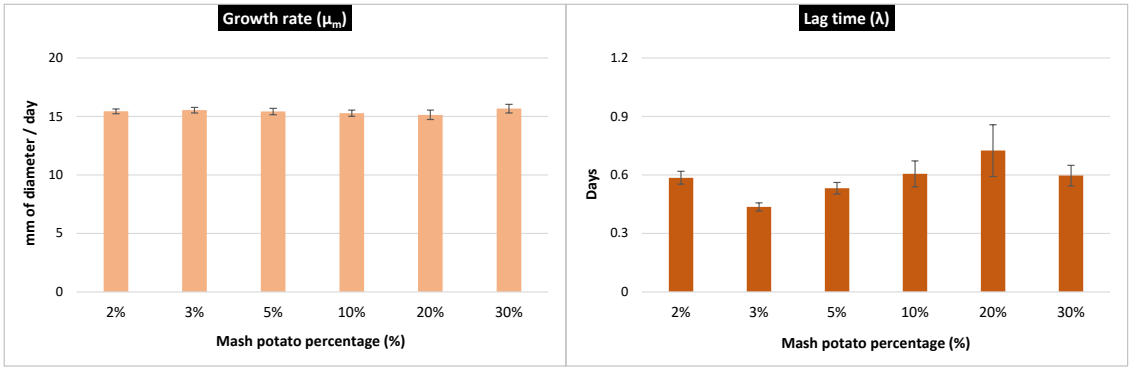

Figure C-2 Effect of mashed potato percentages on *Boeremia foveata* growth rate (μ_m) and lag time (λ). Data shows means of four replicates with bars indicating standard deviation (SD) (ANOVA, p-values<0.05).

Appendix D. Natural Potato Dextrose Agar (NPDA) a_w curve

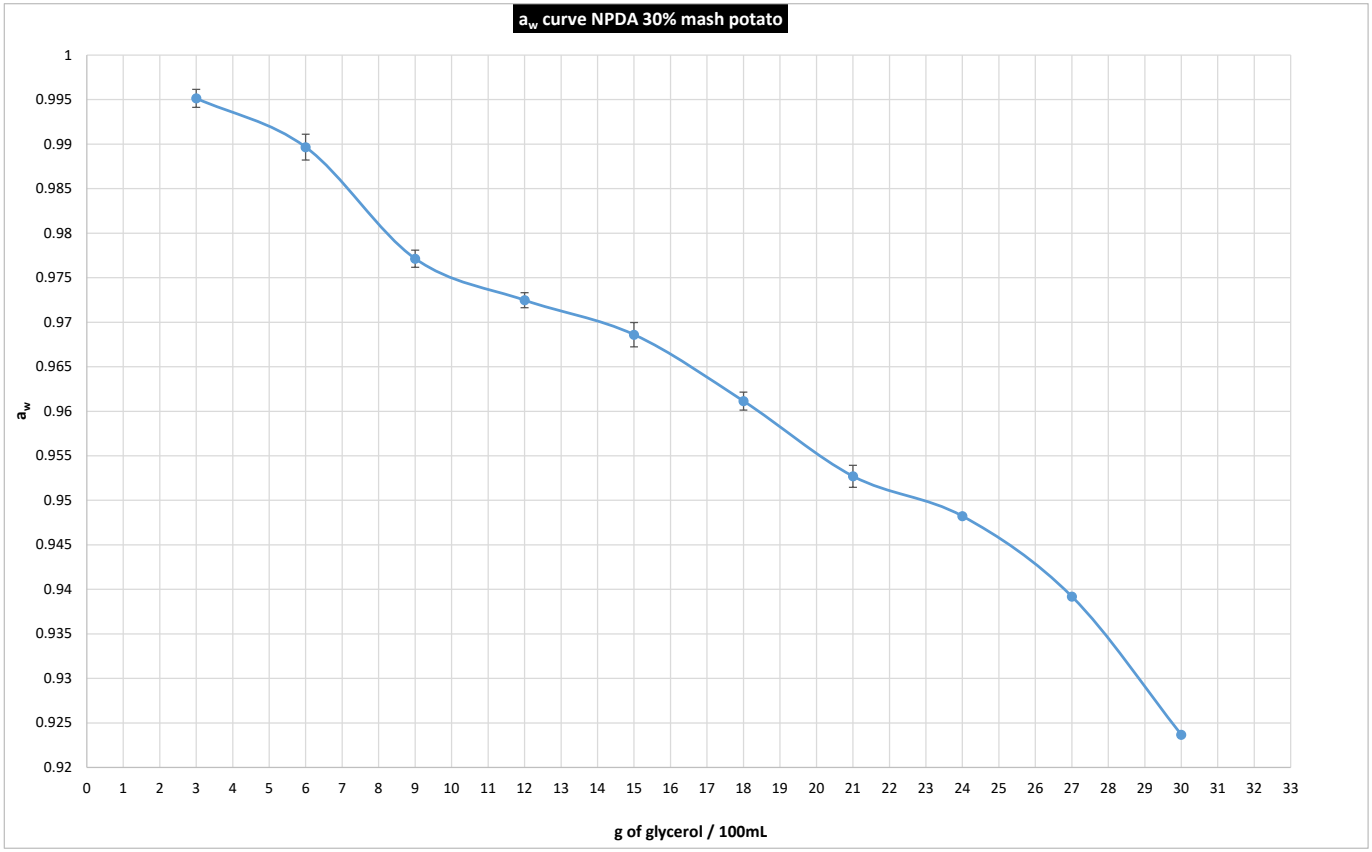

Figure D-1 a_w curve of Natural Potato Dextrose Agar (NPDA) with a 30% of mashed potato. The quantity of glycerol per 100 mL of solution are presented for its correspondent a_w.

Appendix E. Surface sterilisation trials

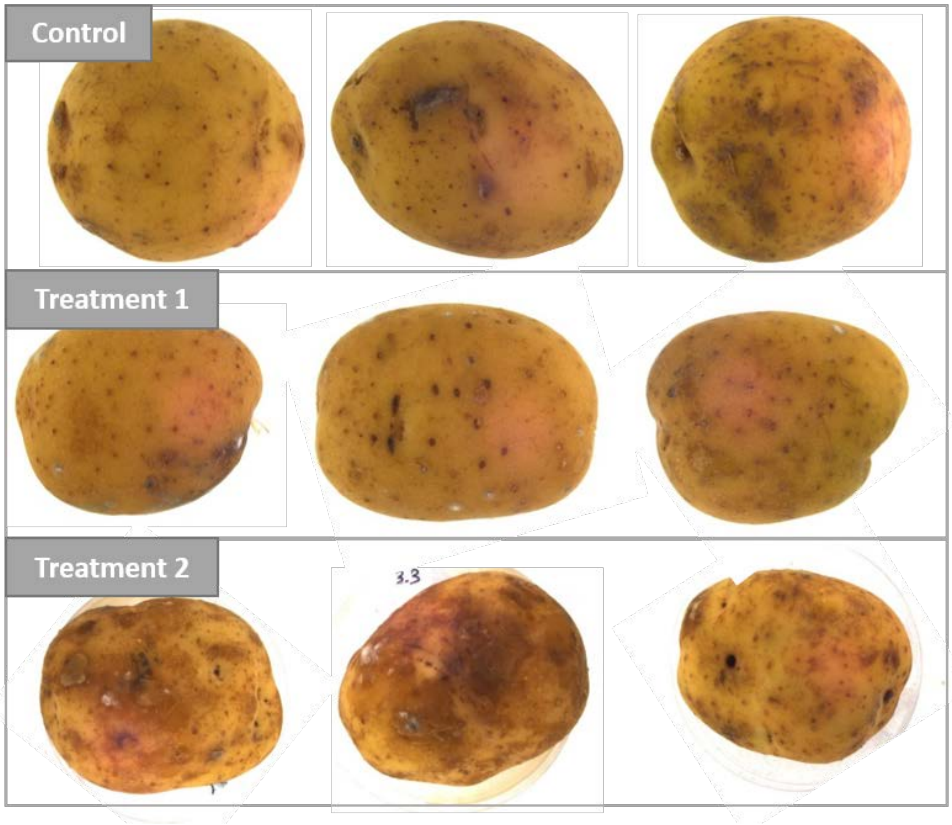

Figure E-1 Trial 1. Potato tubers non-treated (Control), treated with ethanol 70% for 3 minutes followed by their soak in a 1.5% of NaClO (Treatment 1) and a 3% of NaClO (Treatment 2) for 10 minutes. All potato tubers were washed with sterile distilled water for 10 minutes prior to their surface sterilisation treatment.

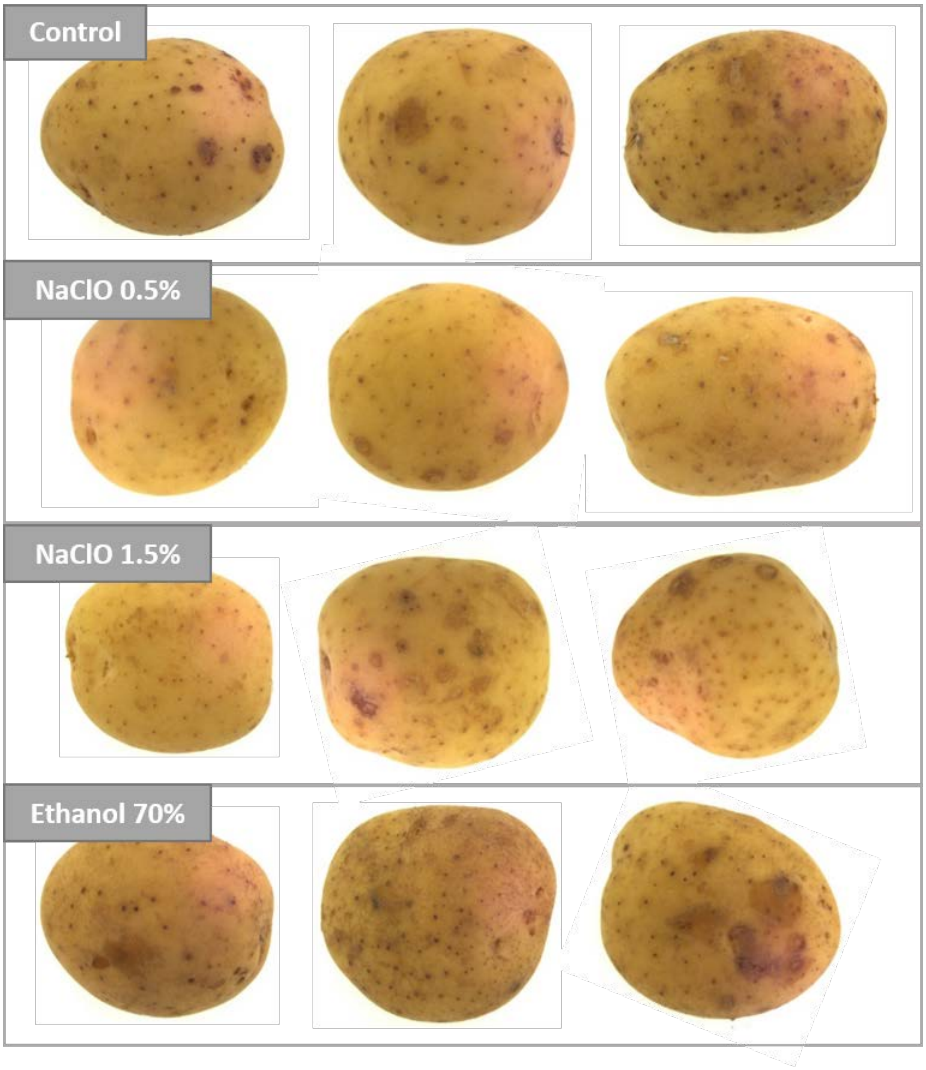

Figure E-2 Trial 2. Potato tubers non-treated (Control), treated with a 0.5% of NaClO for 15 minutes, treated with 1.5% of NaClO for 15 minutes and treated with ethanol 70% for 3 minutes. All potato tubers were washed with sterile distilled water for 10 minutes prior to their surface sterilisation treatment.

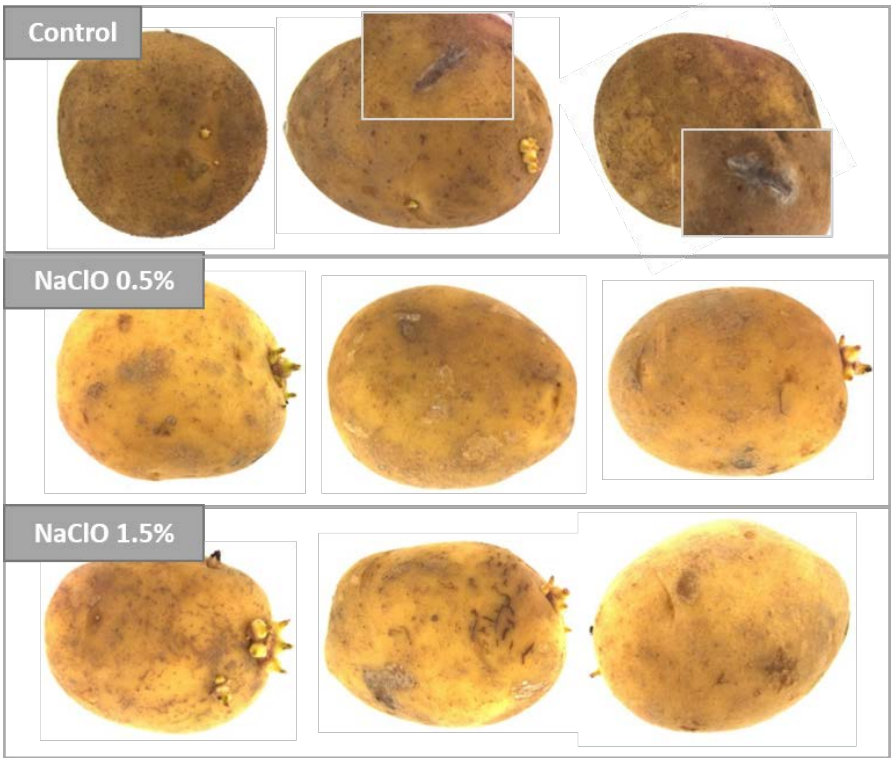

Figure E-3 Trial 3. Potato tubers non washed and non treated (Control), treated with a 0.5% of NaClO for 15 minutes and treated with 1.5% of NaClO for 15. In both treatments, with the exception of the control all potato tubers were washed with sterile distilled water for 10 minutes prior to their surface sterilisation treatment.

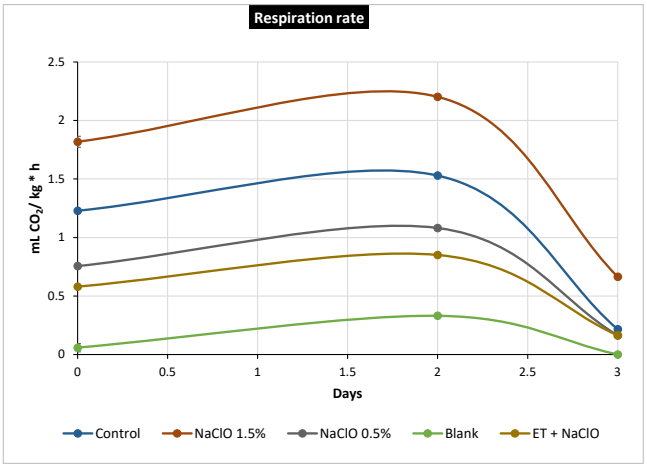

Figure E-4 Trial 3. Respiration rate of potato tubers during three days stored at room temperature and surface sterilised with the different treatments. The control consisted of potato tubers non washed, the blank, potato tuber washed with sterile distilled water, washed with NaCl 0.5% and 1.5% and those potato washed with both ethanol 70% and NaCl 0.5%.

Appendix F. Thermal Desorption tubes conditioning

Table F-1 Temperature and time used for the conditioning of the five different Thermal Desorption (TD) tubes.

TD tube	Time and temperature
Tenax TA®	15-20 minutes at 330ºC
Bio Monitoring®	30 minutes at 330ºC
Air Toxics®	15 minutes at 100ºC 15 minutes at 200ºC 15 minutes at 300ºC 15 minutes at 380ºC
Universal®	15 minutes at 100ºC 15 minutes at 200ºC 15 minutes at 300ºC 15 minutes at 330ºC
Tenax Sulficarb®	15 minutes at 100ºC 15 minutes at 200ºC 15 minutes at 300ºC 15 minutes at 330ºC

Appendix G. GC-MS standards

Table G-1 VOC standards, supplier, form and retention time in minutes in Bio Monitoring TD tubes of some of the VOCs detected.

VOC standard	Supplier	Form	RT (min)
2-Propanol	Sigma Aldrich, UK	Liquid	9.21
2-Methylpentane	Sigma Aldrich, UK	Liquid	10.26
3-Methylpentane	Sigma Aldrich, UK	Liquid	10.9
2-Methylpropan-1-ol	Sigma Aldrich, UK	Liquid	15.60
Acetone	Sigma Aldrich, UK	Liquid	9.03
Ethanol	Sigma Aldrich, UK	Liquid	8.05
1-Propanol	Sigma Aldrich, UK	Liquid	11.83
Methyl acetate	BOC limited, UK	Gas	9.15
Dimethyl disulfide	BOC limited, UK	Gas	20.15
Methyl formate	BOC limited, UK	Gas	6.78
Ethyl acetate	BOC limited, UK	Gas	10.48

Appendix H. Mycotoxin analysis by LC-MS

Table H-1 Mycotoxins, retention time, qualifier (Q1) and quantifier (Q3) included in the negative mode of the LC-MS method.

RT (min)	Name	Q1/Q3
4.32	3-Acetyldeoxynivalenol	397.3/59.2
		397.3/307.1
1.70	Deoxynivalenol	355.1/59.2
		355.1/265.2
1.01	Nivalenol	371.1/59.1
		371.1/281.1
6.90	Zearalenone	317.1/175.0
		317.1/121.1
3.50	Fusarenon X	413.1/352
		413.2/59.1
7.17	Alternariol methyl ehter	271.0/255.7
		271.0/227.8
5.66	Alternariol	257.0/213.0
		257.0/215.0

Table H-2 Mycotoxins, retention time, qualifier (Q1) and quantifier (Q3) included in the positive mode of the LC-MS method.

RT (min)	Name	Q1/Q3
6.10	HT-2	447.4/345.1
		442.2/323.2
6.75	T-2	484.3/215.2
		484.3/185.1
6.39	Fumonisin B1	722.5/352.3
		722.5/336.4
7.80	Fumonisin B2	706.5/336.4
		706.5/318.4
5.50	Diacetoxyscirpenol	384.2/307.0
		384.2/246.9
3.92	Neosolaniol	400.2/215.0
		400.2/185.0
11.07	Beuvericin	801.5/244.2
		806.5/384.4
4.97	15-Monoacetoxyscirpenol	342.2/265.1
		342.2/307.2
4.30	15-Acetyldeoxynivalenol	339.1/321.2
		339.1/137.2

Appendix I. *Fusarium* spp. (*F. sambucinum* & *F. oxysporum*) colony growth in potato-based media

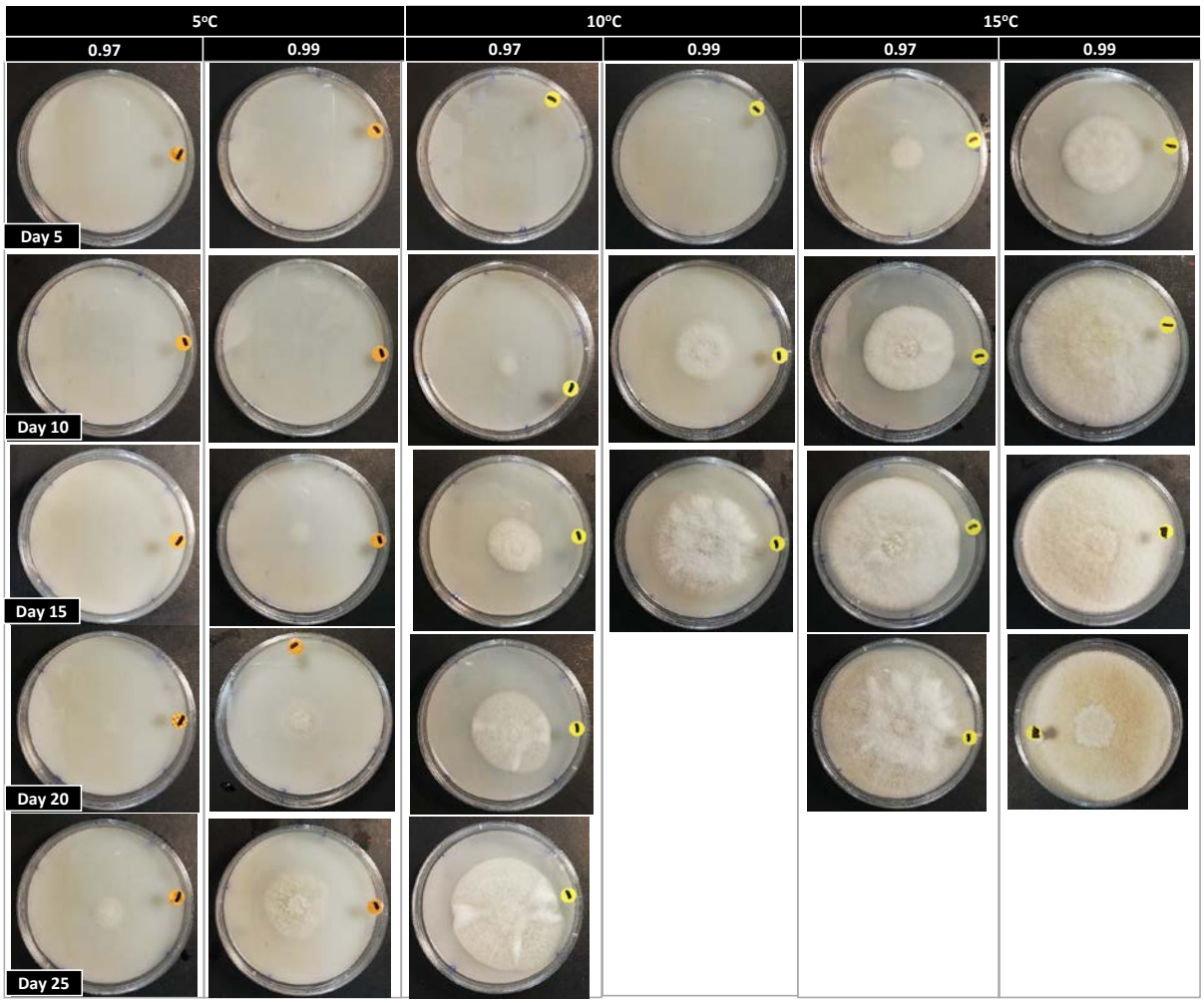

Figure I-1 *Fusarium sambucinum* colony growth after 5, 10, 15, 20 and 25 days of incubation at 8.5°C.

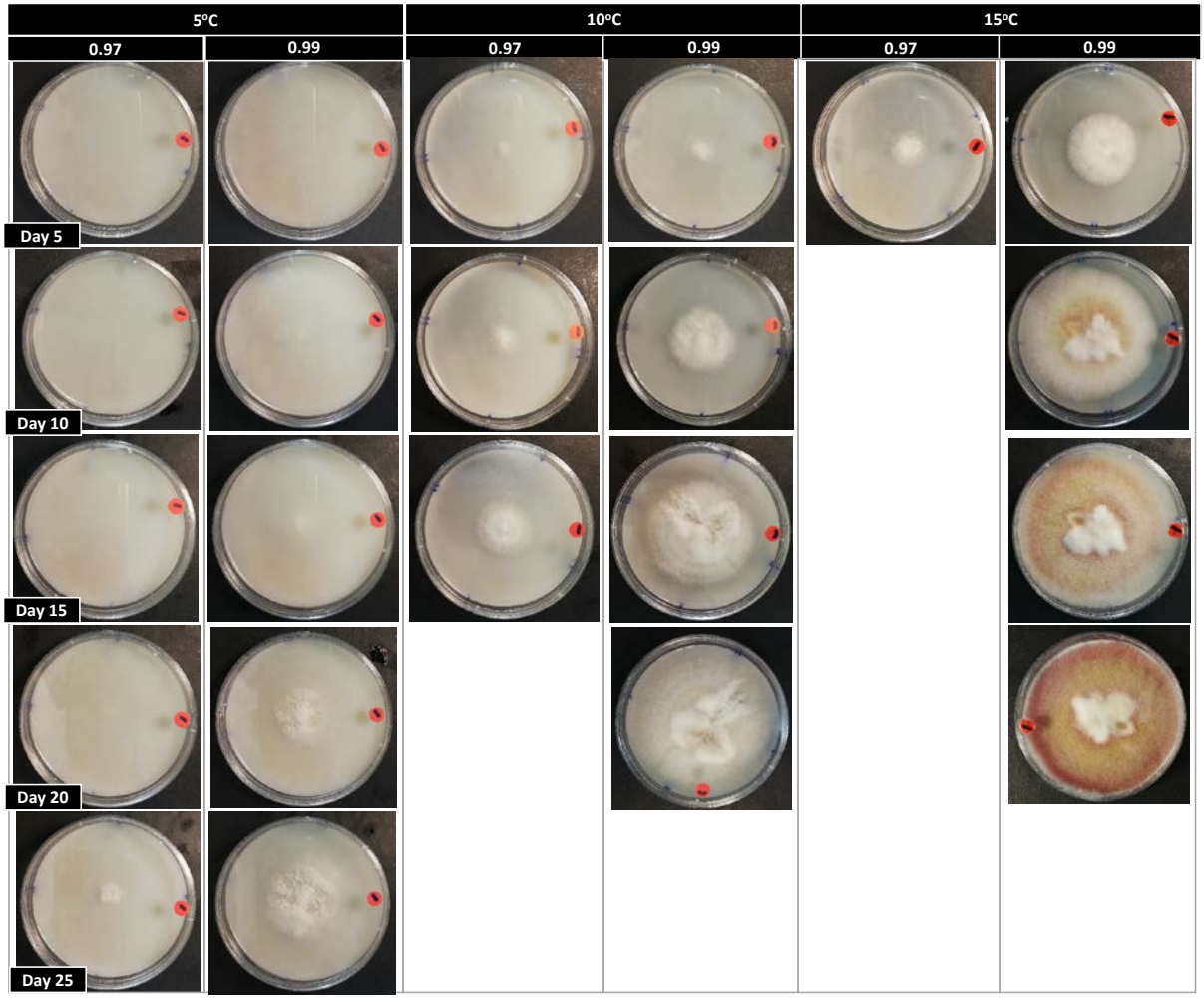

Figure I-2 *Fusarium oxysporum* colony growth after 5, 10, 15, 20 and 25 days of incubation at 8.5°C.

Appendix J. VOC untargeted analysis of *Fusarium* spp. *in vitro* study

Table J-1 VOC selected for the calculation of the Total Targeted Volatile Production (TTVP).

RT	VOC
5.17	Carbon dioxide
5.79	Sulfur dioxide
5.88	Isobutane
6.48	Acetaldehyde
7.86	Ethanol
8.77	Acetone
8.92	Isopropyl alcohol
9.11	Ethyl formate
9.47	Methyl acetate
10.08	2-Methylpentane
10.66	4-Methyl-1-hexene
11.47	1-Propanol
13.16	Ethyl Acetate
14.10	Trichloromethane
15.17	2-Methylpropan-1-ol
15.79	Isopropyl acetate
16.18	3-Methylbutanal
18.83	n-Propyl acetate
21.4	2-Methyl-1-butanol
22.12	Isobutyl acetate

Appendix K. Linear regression of external lesions on potato tubers (cvs. Record and Casablanca)

K-1 Early-stage of the storage

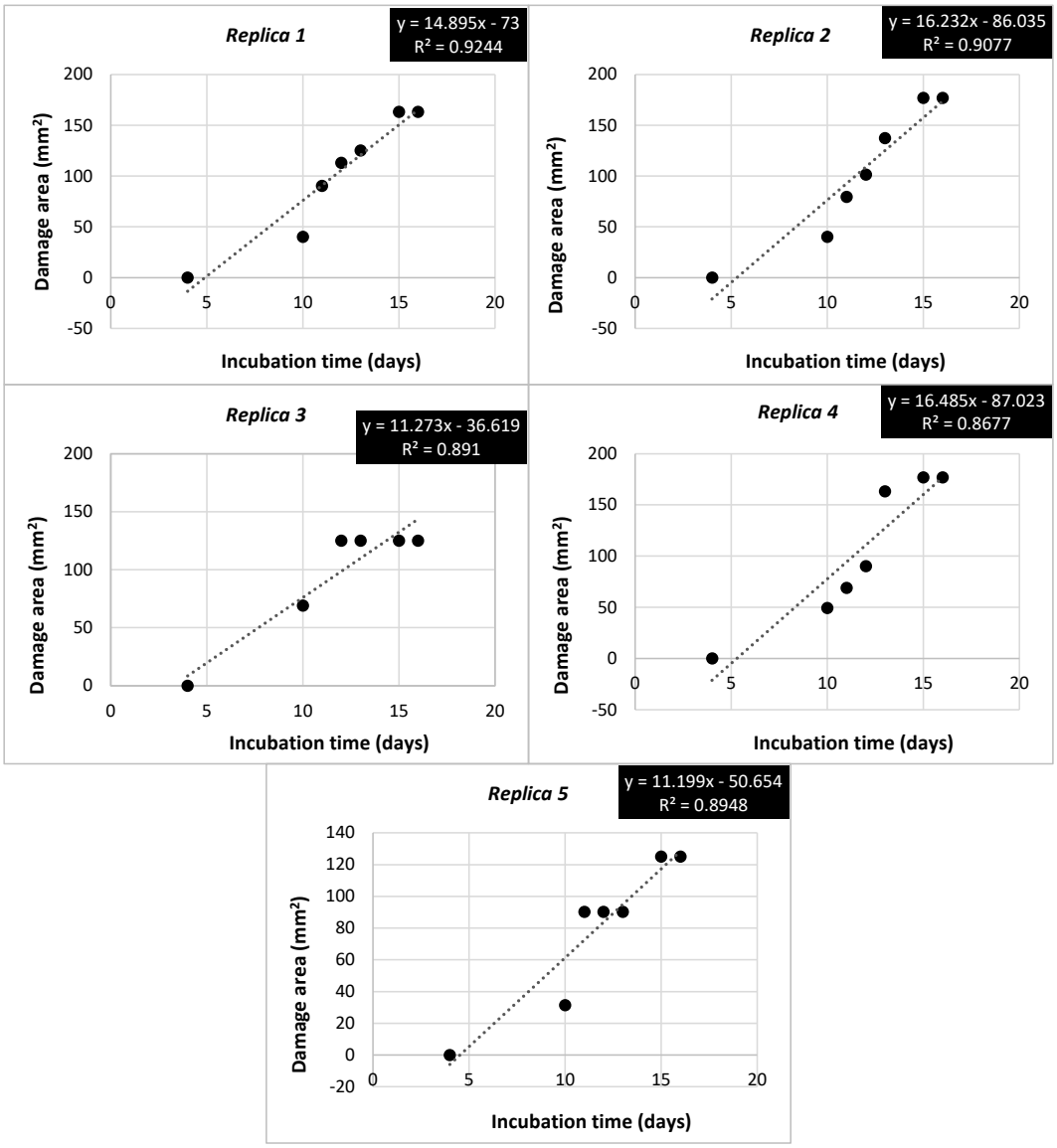

Figure K-1 Lesion area of potato tubers cv. Record from an Early-stage of the storage inoculated with *Fusarium sambucinum* at different incubation time points (days). Five different replicas are shown. A linear model was applied for the calculation of the infection rate and lag time.

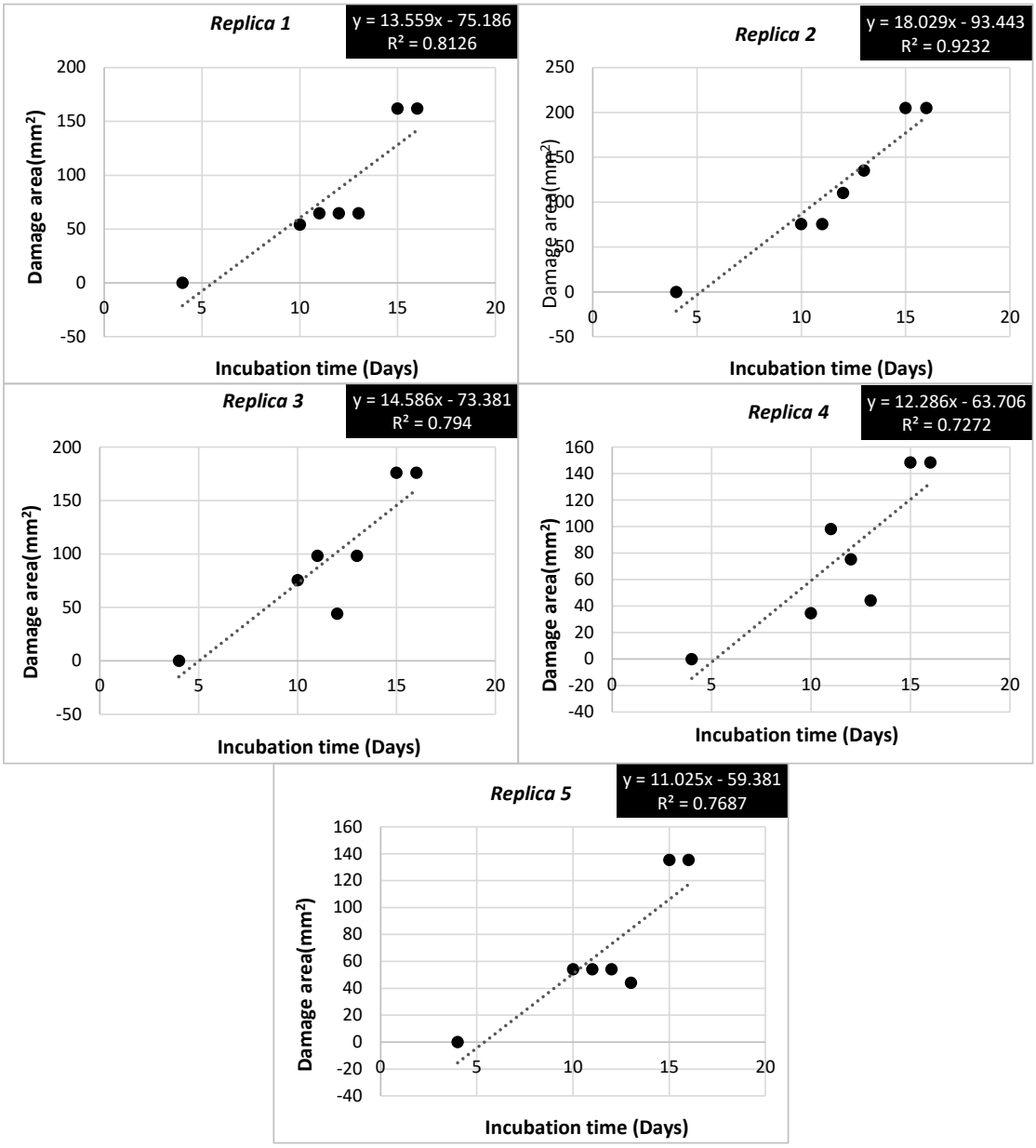

Figure K-2 Lesion area of potato tubers cv. Casablanca from an <u>Early stage of the storage</u> inoculated with *Fusarium sambucinum* at different incubation time points (days). Five different replicas are shown. A linear model was applied for the calculation of the infection rate and lag time.

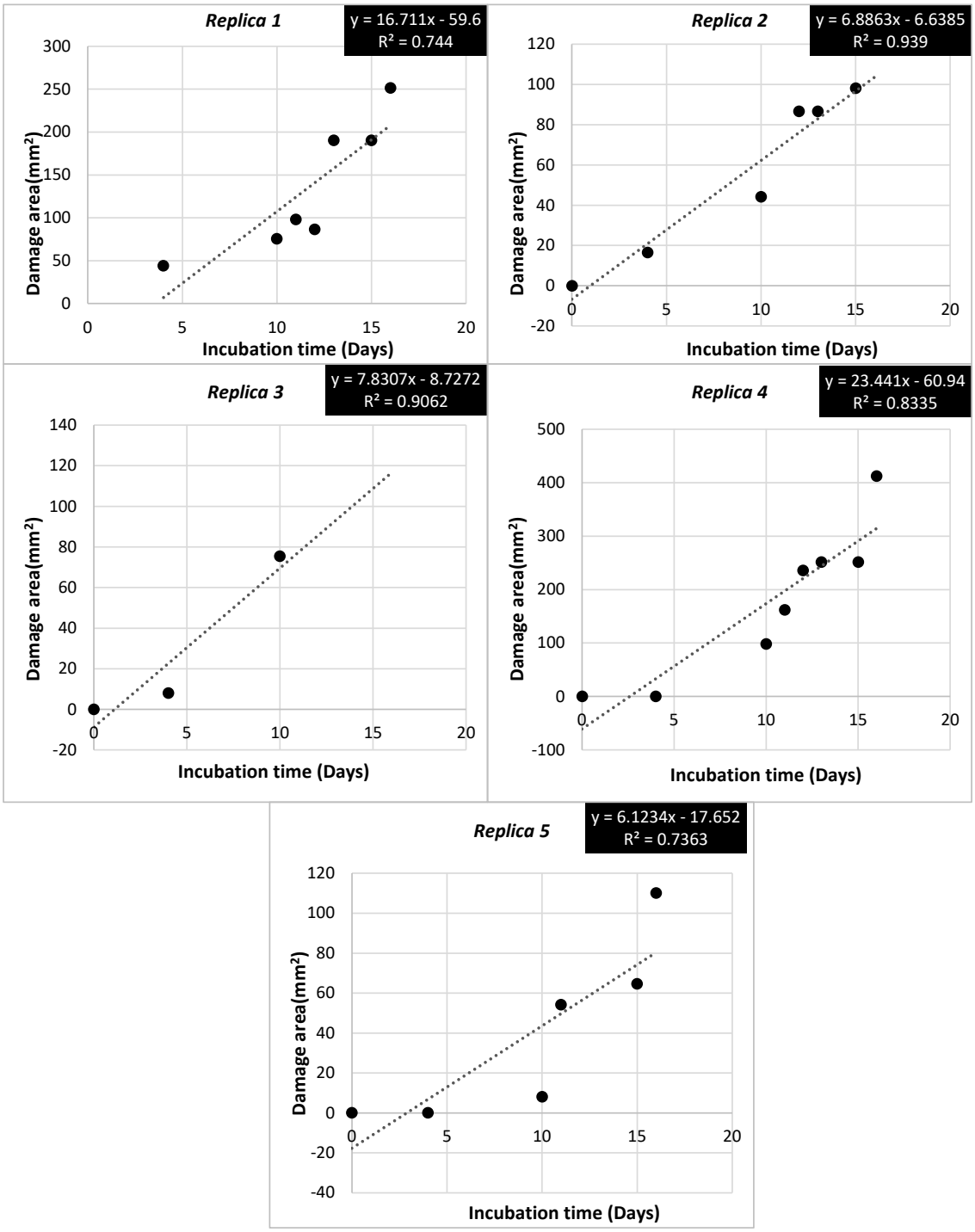

Figure K-3 Lesion area of potato tubers cv. Casablanca from an <u>Early-stage of the storage</u> inoculated with *Pectobacterium c. atrosepticum* at different incubation time points (days). Five different replicas are shown. A linear model was applied for the calculation of the infection rate and lag time.

K-2 Mid-stage of the storage

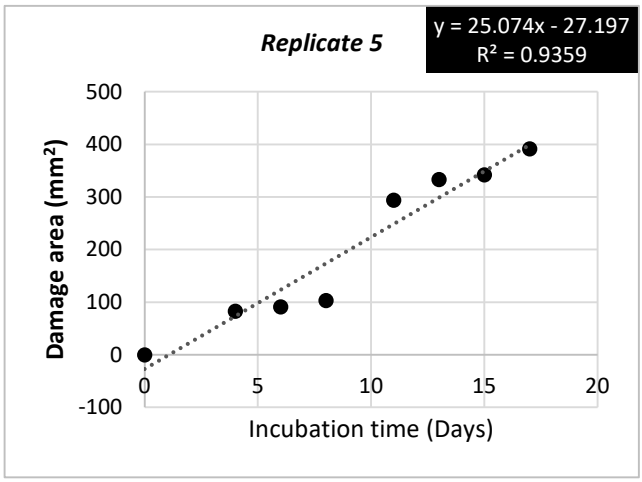

Figure K-4 Lesion area of potato tubers cv. Record from a Mid-stage of the storage inoculated with *Fusarium sambucinum* at different incubation time points (days). One replica from five were selected. A linear model was applied for the calculation of the infection rate and lag time.

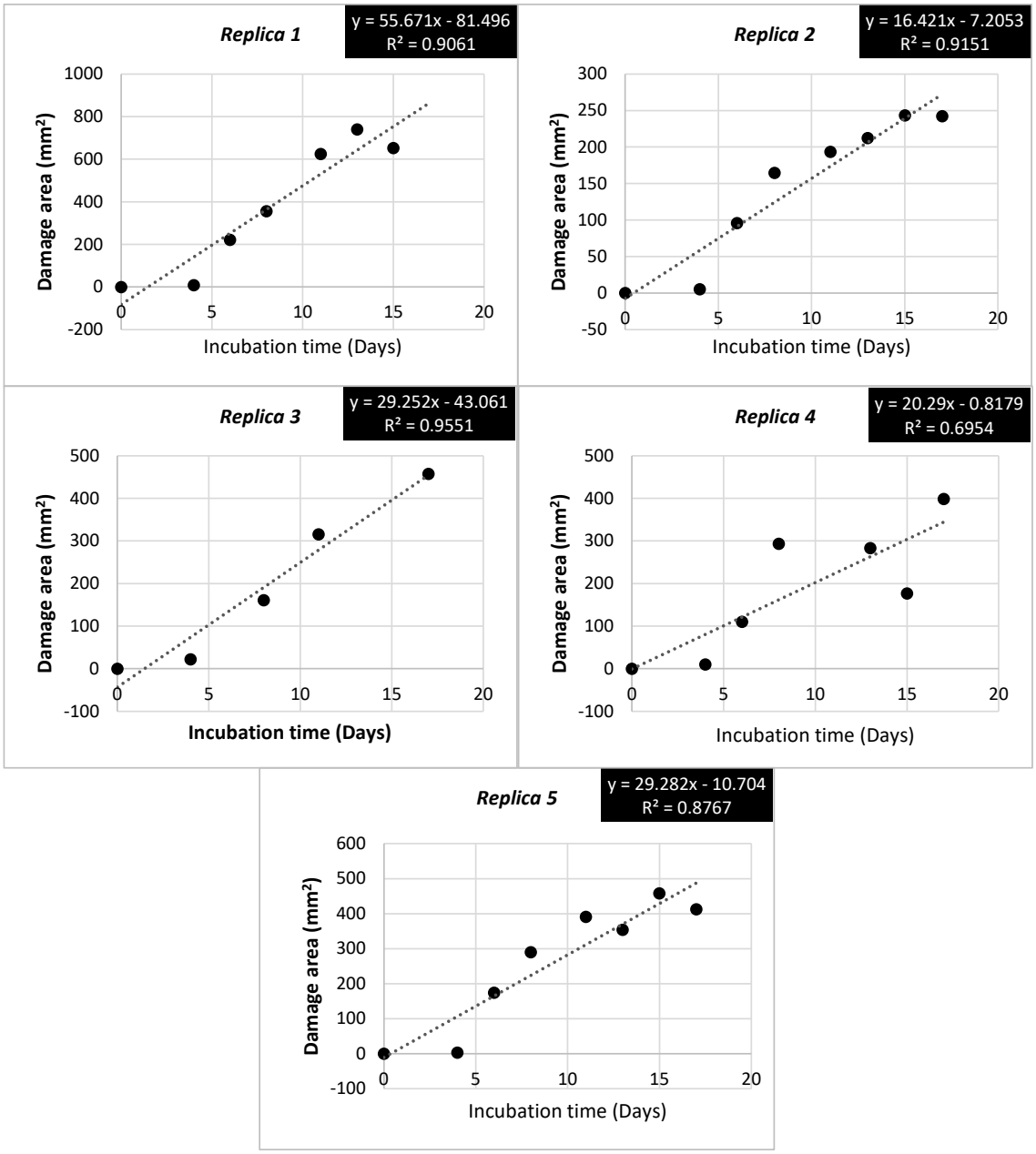

Figure K-5 Lesion area of potato tubers cv. Casablanca from a <u>Mid-stage of the storage</u> inoculated with *Fusarium sambucinum* at different incubation time points (days). Five different replicas are shown. A linear model was applied for the calculation of the infection rate and lag time.

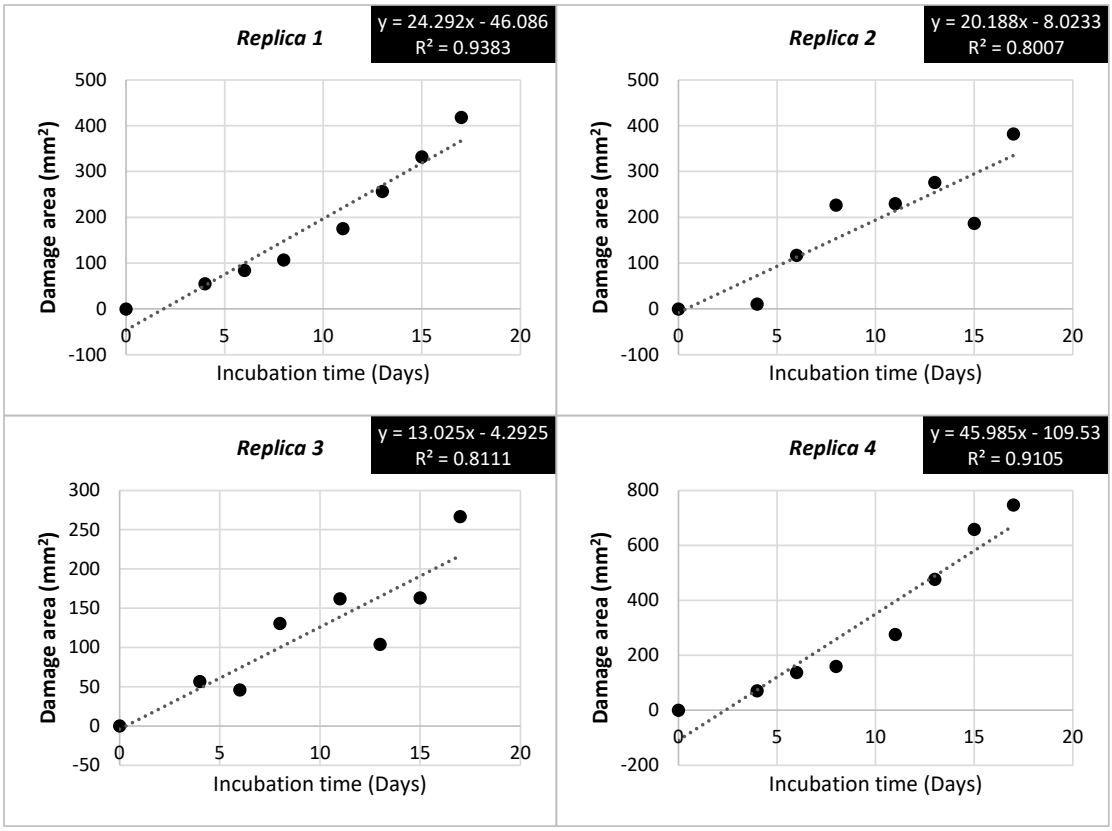

Figure K-6 Lesion area of potato tubers cv. Casablanca from a <u>Mid-stage of the storage</u> inoculated with *Pectobacterium c. atrosepticum* at different incubation time points (days). Four different replicas from five were selected. A linear model was applied for the calculation of the infection rate and lag time.

Appendix L. Principal Component Analysis (PCA) of VOCs results

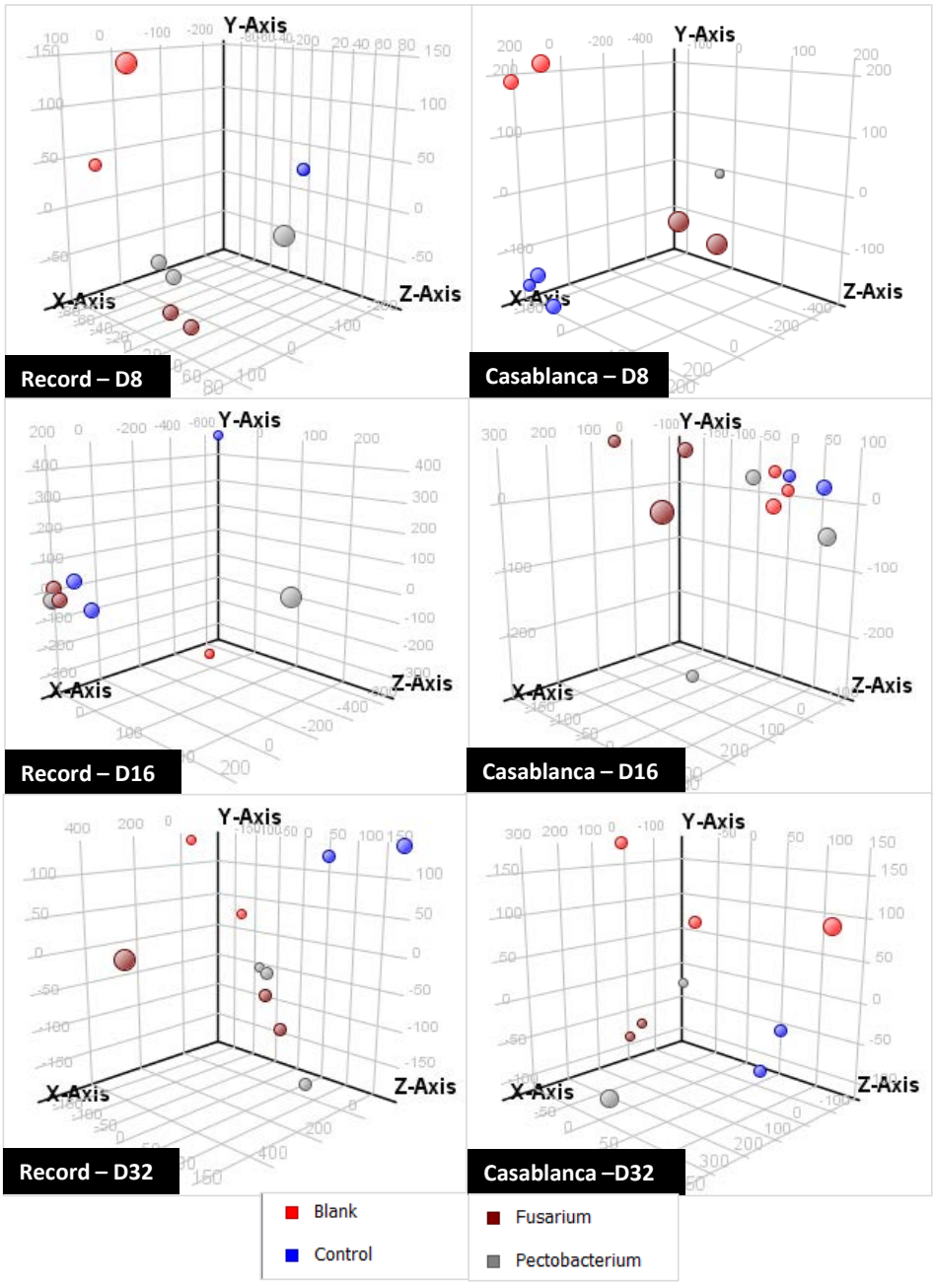

Figure L-1 Early-stage of storage in Bio Monitoring tubes. Principal Component Analysis (PCA) in two different cultivars (Record and Casablanca) at three different stages of the development of the diseases, after 8, 16 and 32 days of incubation at 8.5ºC.

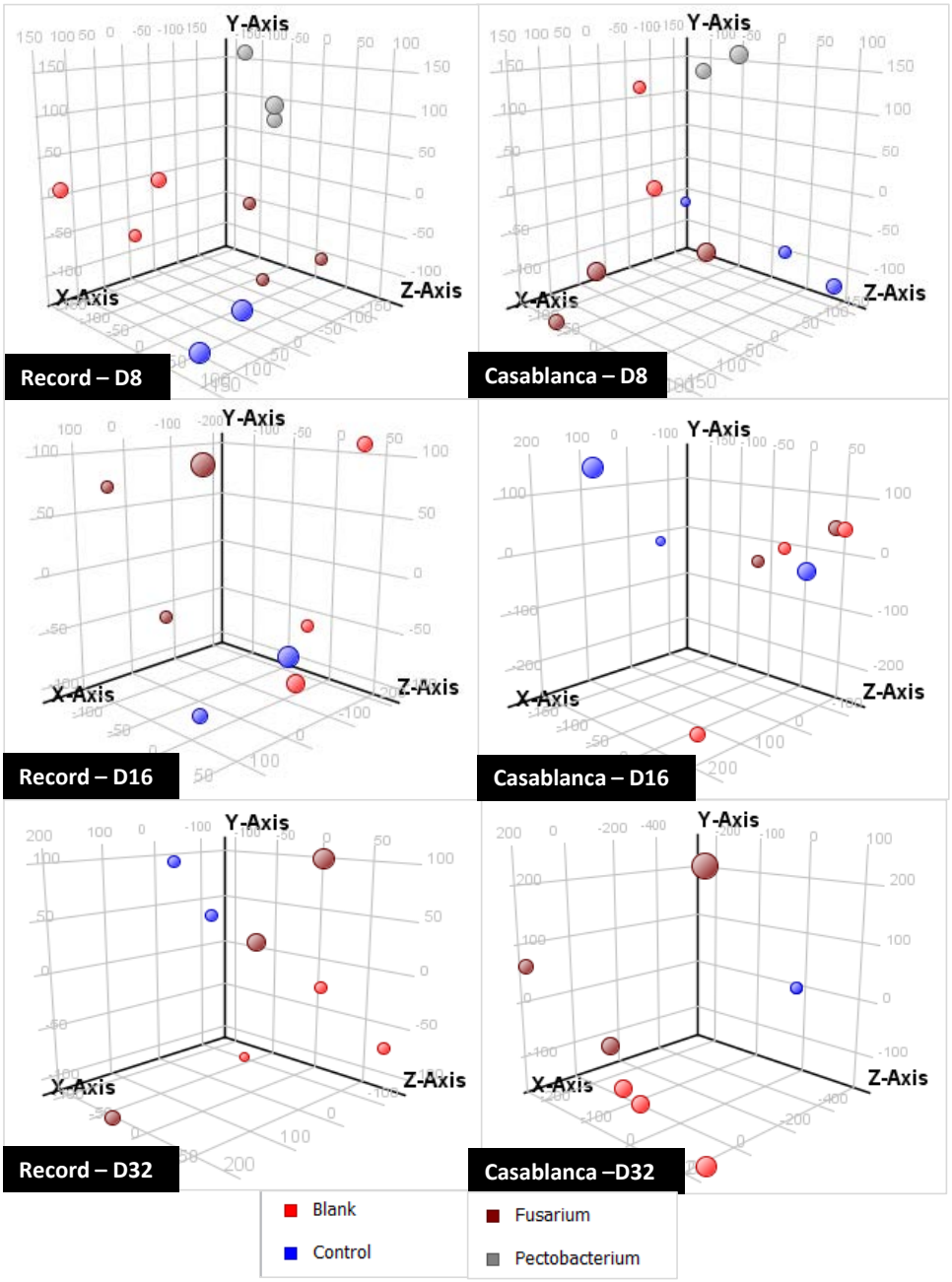

Figure L-2 Early-stage of storage in Air toxics tubes. Principal Component Analysis (PCA) in two different cultivars (Record and Casablanca) at three different stages of the development of the diseases, after 8, 16 and 32 days of incubation at 8.5ºC.

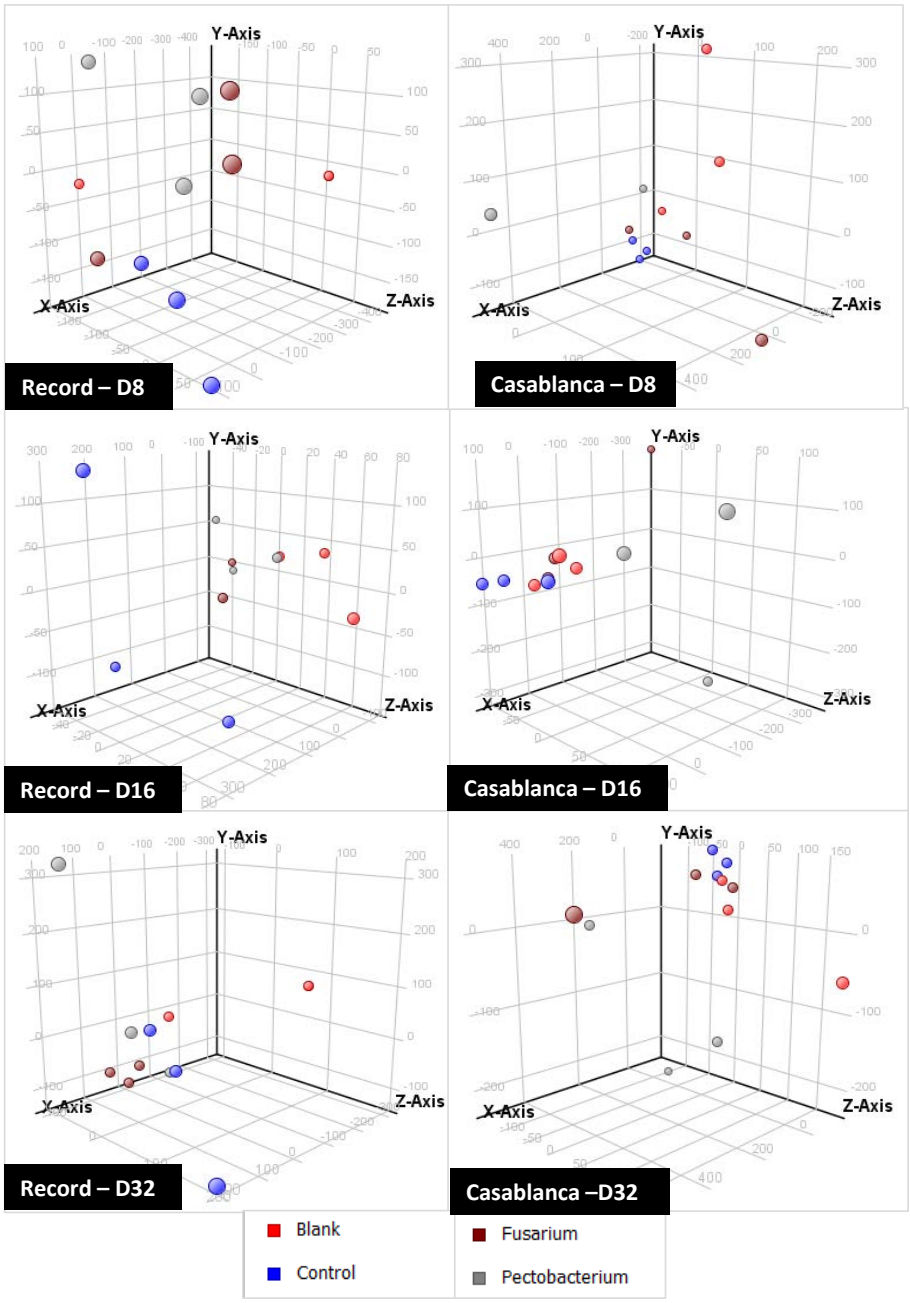

Figure L-3 Mid stage of storage in Air toxics tubes. Principal Component Analysis (PCA) in two different cultivars (Record and Casablanca) at three different stages of the development of the diseases, after 8, 16 and 32 days of incubation at 8.5ºC.

Appendix M. Untargeted VOC analysis for dry rot and soft rot *in vivo* study

Table M-1 VOC selected for the calculation of the Total Targeted Volatile Production (TTVP).

Early-stage storage - BM	Mid stage storage -BM
Sulfur dioxide	Sulfur dioxide
1-Propene, 2-methyl-	1-Propene, 2-methyl-
Hydrogen cyanide	Hydrogen cyanide
Methyl formate	Methyl formate
Ethanol	Ethanol
Acetone	Acetone
Isopropyl alcohol	Dimethyl sulfide
Methyl acetate	Isopropyl alcohol
2-Butanone	Methyl acetate
2-Butanol	2-Butanone
Trichloromethane	Formic acid, 1-methylpropyl ester
Acetic acid	2-Butanol
Butane, 1-methoxy-3-methyl-	Trichloromethane
Dimethyl disulfide	Acetic acid
2,4-Dimethyl1-1heptene	1-Propanol, 2-methyl-
	Butane, 1-methoxy-3-methyl-
	Dimethyl disulfide
	1-Pentanol
	1-Butanol, 2-methyl-
	2,4-Dimethyl1-1heptene