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Polymères de chitosane et extraits de plantes pour développer des biofongicides

Anne Loron

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Anne Loron. Polymères de chitosane et extraits de plantes pour développer des biofongicides. Autre. Université de Bordeaux, 2021. Français. NNT : 2021BORD0002 . tel-03199568

HAL Id: tel-03199568

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE

**DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX**

ÉCOLE DOCTORALE DES SCIENCES CHIMIQUES DE BORDEAUX
SPÉCIALITÉ POLYMÈRES

Anne LORON

**Chitosan polymers and plant extracts
to develop biofungicides**

Sous la direction de Véronique COMA

Soutenue le 26 janvier 2021

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What we know is a drop, what we don't know is an ocean.

Isaac NEWTON

REMERCIEMENTS

Je souhaiterais remercier Sébastien Lecommandoux ainsi que Henri Cramail de m'avoir permis d'effectuer ma thèse au Laboratoire de Chimie des Polymères Organiques au sein de l'équipe 2. Je remercie Véronique Coma de m'avoir permis de réaliser ce travail de thèse pendant trois ans, de m'avoir laissé de la liberté dans le projet tout en restant disponible lorsque j'en avais besoin. Merci d'avoir pris le temps de minutieusement préparer les présentations orales, même si ces répétitions n'étaient pas mes moments favoris. Un grand merci à Christian pour le soutien technique, précis, discret mais considérable. Je tiens à remercier Frédérique Ham-Pichavant pour son attention et son efficacité particulièrement appréciable au N0. Je remercie également Nicolas Tabary de m'avoir accueillie au laboratoire UMET à Lille et accompagnée. Mes remerciements vont aussi à Vessela Atanasova pour son aide, ses conseils et les discussions scientifiques ou personnelles tout au long de la thèse. J'ai beaucoup apprécié travailler dans d'autres laboratoires, où j'ai toujours été bien accueillie et encadrée. *I also thank Ramunė Rutkaitė, Vesta Navikaitė-Šnipaitienė and Deimantė Rosliuk for their help and the interesting discussions we had. I particularly thank Ramunė for her understanding and kindness.*

J'aimerais remercier les « anciens » : Coralie, David, Élodie, Fiona, Hélène, Imane, Lauriane, Leila, Marie, Michèle, Nadia, Quentin S., Stéphanie et Sylwia et plus particulièrement Christopher, Martin C., Erwan, Fiona, Florian B., Guillaume et Pauline... et les « actuels » : Anouk, Assia, Chloé, Clémence, Diana, Dijwar, Fanny, Hanaé, Laurine, Léa, Martin F., Megi, Mostafa, Nadia, Pablo, Paul, Pedro, Péroline, Peter, Pierre, Romane, Samir, Thaïssa, Tingting, Tim, Vaïana et Valentin. Merci à Anderson pour sa constante gentillesse, à Victor et Margaux pour l'escalade et leur sympathie :) et à Cédric C., l'oreille attentive. Je remercie Yunhui pour son aide et son endurance ainsi que Sylvain, le semi-permanent.

Je remercie particulièrement le groupe unique (et leur +1 respectifs) : Bobi, Jamais203, Mashur, Gavroch, Chaton-Guerrier et Le Maître Pokémon. Boris, pour sa compréhension et son ouverture d'esprit. Flo, pour son attention et sa perspicacité. Manon, pour sa franchise et son dynamisme. Jamie, pour sa générosité et son soutien. Jessie, pour sa capacité à s'émerveiller et sa force de caractère. Jérémy, pour son abnégation et son exemplarité.

Je remercie également toute ma famille, en particulier mon frère et ma sœur, pour la bienveillance spontanée de notre relation et mes parents, pour leur confiance, leur réconfort et leur soutien constant.

Je remercie Romain du fond du cœur.

TABLE OF CONTENTS

REMERCIEMENTS	V
TABLE OF CONTENTS	VII
TABLE OF ABBREVIATIONS	XV
TABLE OF SYMBOLS.....	XVII
RESUME EN FRANÇAIS	XIX
GENERAL INTRODUCTION.....	1
CHAPTER I – BIOFUNGICIDES: NECESSITY & PRODUCTION.....	5
1 Fungal diseases of maize and wheat and their treatments.....	6
1.1 General diseases caused by <i>Fusarium</i> spp.....	6
1.2 <i>Fusarium graminearum</i> infection.....	6
1.3 Mycotoxins produced by <i>F. graminearum</i>	8
1.3.1 Chemical structure	8
1.3.2 Effects on animals and plants.....	9
1.3.3 TCTs in the food chain.....	9
1.4 Management of <i>F. graminearum</i> on crops	10
1.4.1 Agronomic practices	10
1.4.2 Use of antagonistic microorganisms	11
1.4.3 Traditional synthetic or bio-based fungicides	11
1.4.3.a Traditional synthetic fungicides	11
1.4.3.b Bio-based options suitable for organic farming.....	12
1.4.4 Global management.....	13
1.5 Concluding remarks	14
2 Chitosan: an active biopolymer with antifungal and elicitor properties	14
2.1 Generalities on chitosans.....	14
2.1.1 Structure, origin and elaboration process.....	14
2.1.2 Specific characterisation of MW and DD	15
2.2 Specific antimicrobial properties of chitosans	16
2.2.1 Modes of action: what we know so far	16
2.2.2 Impact of some physicochemical parameters on antimicrobial properties...	18

2.3	Some applications of chitosan.....	19
2.3.1	Different formulations depending on the application.....	19
2.3.2	Specific applications for pest control on crops.....	20
2.3.2.a	Antifungal effects	20
2.3.2.b	Eliciting properties	22
2.3.2.c	Two other beneficial effects on the plant	24
2.4	Concluding remarks.....	24
3	Plant extracts: active molecules with antifungal and antioxidant properties ...	25
3.1	Plant extracts.....	25
3.1.1	Three principal classes of plant extracts.....	25
3.1.2	Extraction processes.....	26
3.1.3	Properties of plant extracts	27
3.1.3.a	Lipophilic properties linked with the antifungal action	28
3.1.3.b	Intrinsic antioxidant properties	29
3.2	Phenolic compounds from plant extracts.....	31
3.2.1	Structure of phenolic compounds.....	31
3.2.2	Properties of interest of phenolic compounds.....	32
3.2.2.a	Antifungal actions	32
3.2.2.b	Antimycotoxigenic action and antioxidant properties.....	33
3.3	Specific case of tetrahydrocurcumin.....	34
3.3.1	A curcumin derivative.....	34
3.3.2	Anti-microbial properties.....	36
3.4	Concluding remarks.....	37
4	Encapsulation techniques of poorly water-soluble compounds	37
4.1	What is encapsulation?.....	38
4.2	Characterization of particles.....	39
4.3	Physical entrapment.....	40
4.3.1	Spray-drying.....	40
4.3.2	Freeze-drying.....	42
4.3.3	Other physical techniques.....	42
4.4	Encapsulation through physicochemical interactions.....	43
4.4.1	Covalent interactions	43
4.4.2	Molecular interactions: the case of cyclodextrins	44
4.4.3	Ionic interactions	47
4.4.3.a	Ionic gelation	47
4.4.3.b	Coacervation	47
4.4.3.c	Layer-by-layer process	48

4.4.4	Hydrophobic interactions	48
4.4.4.a	Micelles	48
4.4.4.b	Liposomes	48
4.4.4.c	Polymersomes	49
4.4.5	Solvent evaporation from emulsion	49
4.4.6	Co-crystallization	50
4.5	Concluding illustrations	50
5	Strategy of the present study	51
CHAPTER II – MATERIAL & METHODS		53
1	Material.....	54
1.1	Chemical products and media for microbiology	54
1.2	Fungal strains	55
2	Extraction and characterisation of the plant extracts	55
2.1	Subcritical water extraction	55
2.2	Determination of the total phenolic content	56
2.3	Chemical characterisation by LC–MS	56
3	Elaboration and characterisation of chitosan solutions and films	57
3.1	Characterisation of chitosans	57
3.1.1	Determination of the deacetylation degree with ¹ H-NMR spectrometry or infrared spectroscopy	57
3.1.1.a	By NMR.....	57
3.1.1.b	By infrared spectroscopy	57
3.1.2	Determination of the molecular weight	57
3.1.3	Determination of the charge density.....	58
3.2	Chitosan solutions	58
3.2.1	Elaboration of the solution	58
3.2.2	Rheology study of a chitosan solution	58
3.3	Chitosan films	59
3.3.1	Elaboration of films.....	59
3.3.2	Characterisation of films	59
3.3.2.a	Determination of the film thickness.....	59
3.3.2.b	Determination of the contact angle and the surface tension energy	59
3.3.2.c	Dynamic water penetration analysis (DPA)	59
3.3.2.d	Determination of the water vapour transmission rate (WVTR).....	59
3.3.2.e	Impact of UV	60
4	Elaboration and characterisation of tetrahydrocurcumin-loaded systems	60

4.1	Characterisation of THC	60
4.1.1	Characterisation by mass spectrometry.....	60
4.1.2	Characterisation by NMR	61
4.2	Elaboration of THC-loaded systems.....	61
4.2.1	Elaboration of THC-loaded cyclodextrins	62
4.2.2	Elaboration of THC-loaded OSA-starch particles	62
4.2.3	Elaboration of THC-loaded chitosan particles	63
4.3	Characterisation of THC-loaded systems.....	64
4.3.1	Dynamic light scattering (DLS) of emulsions	64
4.3.2	Microscopic observations	64
4.3.3	Thermal analysis.....	64
4.3.3.a	Differential Scanning Calorimetry (DSC)	64
4.3.3.b	Isothermal Titration Calorimetry (ITC).....	64
4.3.4	THC-solubility study with UV-visible spectrophotometry.....	65
4.3.5	Characterisation by 1D- and 2D-NMR spectrometry	66
4.3.6	Determination of the THC loaded content in particles	66
4.3.7	Antioxidant properties: DPPH [•] technique	66
5	Antimicrobial properties.....	67
5.1	Preparation of the spore solution	67
5.2	Inhibitory properties against the fungal growth.....	68
5.2.1	Medium supplemented with the tested molecules	68
5.2.2	Medium coated with the tested formulation	68
5.2.3	Determination of inhibition percentages.....	69
5.2.4	Determination of the IC ₅₀	69
5.3	Inhibitory properties against the production of toxins	69
5.4	Statistical analysis.....	70
CHAPTER III – CHITOSAN-BASED FORMULATIONS		71
1	Characterisation of chitosans	73
1.1	Determination of the molecular weights.....	73
1.1.1	Determination of the specific refractive index increment	73
1.1.2	Molecular weights and polydispersity index	74
1.2	Determination of the deacetylation degree (DD)	75
1.2.1	By ¹ H-NMR.....	76
1.2.2	By Fourier Transform Infrared Spectroscopy	76
2	Antifungal and antimycotoxigenic activities of chitosans in solution	78
2.1	Selection of chitosans – Radial growth test.....	78

2.2	Additional study of antifungal properties of the selected chitosans.....	80
2.2.1	Antifungal properties	80
2.2.1.a	How to choose the working pH?	80
2.2.1.b	Impact of chitosans in the medium on the mycelial growth	81
2.2.1.c	Calculation of the IC ₅₀ after four days of incubation	83
2.2.1.d	Understanding relationships between structural & antifungal properties..	84
2.2.2	Antimycotoxigenic properties of selected chitosans.....	86
2.2.3	Concluding remarks.....	89
3	Study of chitosan films as a model for the coating of leaves	89
3.1	Antifungal properties	90
3.1.1	Effect of coatings on the development of the mycelium.....	90
3.1.2	Impact of the treatment moment.....	92
3.2	Physicochemical characterisations	95
3.2.1	Behaviour towards liquid water	96
3.2.1.a	Determination of the contact angle and the free surface energy.....	96
3.2.1.b	Dynamic penetration analysis (DPA).....	97
3.2.2	Moisture barrier properties	98
3.2.3	UV-radiations over the time.....	99
4	Concluding remarks	100

CHAPTER IV – ADDITION OF TETRAHYDROCURCUMIN TO CHITOSAN-BASED FORMULATIONS 101

1	Structure and properties of THC.....	103
1.1	Chemical characterisation of THC.....	103
1.1.1	Infra-red analyses.....	103
1.1.2	NMR characterisation.....	104
1.1.3	Mass spectrometry analyses.....	105
1.2	UV-visible absorption and stability	105
1.3	Antioxidant properties	106
1.4	Antifungal properties against <i>F. graminearum</i>	107
2	Association of THC to chitosan-based formulations.....	109
2.1	Study of chitosan-based solutions	109
2.1.1	Effect on the mycelial growth	109
2.1.2	Effect on the accumulation of TCTB.....	110
2.2	Study of films as a model of plant coating	112
2.2.1	Effect of chitosan and THC films on the mycelial growth.....	112
2.2.2	UV-absorption of films.....	114

2.3	Concluding remarks.....	115
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**CHAPTER V – ENCAPSULATION OF TETRAHYDROCURCUMIN FOR
FUTURE ADDITION TO CHITOSAN-BASED FORMULATIONS 117**

1	Encapsulation <i>via</i> cyclodextrins	119
1.1	UV-absorption of THC and CDs to study interactions	119
1.2	Selection of a suitable CD	120
1.3	Proof of the inclusion.....	121
1.3.1	¹ H-NMR.....	122
1.3.2	HSQC	123
1.3.3	ROESY	124
1.4	Study of the thermochemical equilibrium with Me β CD	124
1.4.1	Solubility study	124
1.4.2	Isothermal Titration Calorimetry (ITC).....	125
1.4.3	Concluding remarks.....	127
1.5	Characterisation of the THC-loaded Me β CD complex powder	128
1.5.1	Morphology of the complex powder by scanning electron microscopy	128
1.5.2	Differential Scanning Calorimetry (DSC) analysis	129
1.6	Activity against <i>F. graminearum</i>	130
1.6.1	Impact on the fungal growth.....	130
1.6.2	Impact on the production of toxins	131
1.7	Concluding remarks.....	132
2	Towards an encapsulation easy to implement industrially	133
2.1	Elaboration of emulsions with rapeseed oil	133
2.1.1	OSA-starch based emulsions.....	133
2.1.2	Chitosan-based emulsions	135
2.2	Drying processes: spray-drying and freeze-drying	136
2.3	Determination of the encapsulated amount of THC.....	136
2.4	Morphology of the powders by SEM	138
2.4.1	Freeze-drying.....	138
2.4.2	Spray-drying.....	139
2.5	Differential scanning calorimetry analysis of the particles	139
2.6	Microbiological activity of THC-loaded particles against <i>F. graminearum</i>	140
2.6.1	Antifungal activity of THC-loaded particles	140
2.6.2	Antimycotoxigenic activity of THC-loaded particles.....	142
3	Concluding remarks and prospects	144

CHAPTER VI – WOOD BY-PRODUCT EXTRACTS: CHARACTERISATION	
AND ADDITION TO CHITOSAN-BASED FORMULATIONS	145
1 Extraction, characterisation and antifungal properties of extracts	146
1.1 Specific method of extraction	146
1.2 Extraction	147
1.3 Preliminary characterisation – total phenolic content.....	148
1.4 Antimicrobial activity	149
1.4.1 Impact on the fungal mycelial growth	149
1.4.2 Impact on the TCTB production.....	151
2 Addition of wood extracts to chitosan-based formulations for antifungal applications.....	153
2.1 Effect on the mycelial growth	153
2.2 Effect on the TCTB accumulation.....	154
3 First industrial assay on fields	155
3.1 Viscosity of the chitosan solution.....	155
3.2 Full-scale assay on field.....	155
GENERAL CONCLUSION.....	157
SUPPLEMENTARY INFORMATION.....	163
REFERENCES.....	167

TABLE OF ABBREVIATIONS

15-ADON	15-acetyldeoxynivalenol
3-ADON	3-acetyldeoxynivalenol
AA	Acetic acid
BHT	Butyl hydroxytoluene
CD	Cyclodextrin
CMC	Carboxy methylcellulose
DAD	Diode array detector
DD	Deacetylation degree
DLS	Dynamic light scattering
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DPA	Dynamic penetration analyses
DPPH [·]	di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium <i>aka</i> 1,1-diphenyl-2-picrylhydrazyl radical
DSC	Differential scanning calorimetry
EE	Encapsulation efficiency
FHB	Fusarium Head Blight
FT-IR	Fourier transform infrared spectroscopy
HPLC	High pressure liquid chromatography
HPMC	Hydroxypropyl methylcellulose
HSQC	Heteronuclear single quantum correlation
IC ₅₀	Half maximal inhibitory concentration
ITC	Isothermic titration calorimetry
LA	Lactic acid
LC	Loading capacity
LC-MS	Liquid chromatography coupled with mass spectrometry
LCPO	Laboratoire de Chimie des Polymères Organiques
MALDI - TOF	Matrix assisted laser desorption ionization - Time of flight
MIC ₅₀ or MIC ₉₀	Minimal inhibitory concentration: minimum amount of substance to inhibit 50 % or 90 % of the growth
mRNA	messenger Ribonucleic acid
MS	Mass spectroscopy
MW	Molecular weight

NMR	Nucleic magnetic resonance
OSA	Octenyl succinic acid
OWRK	Owen, Wendt, Rabel, Kaelble
PDA	Potato dextrose agar
PEG-PTMC	Poly(ethylene glycol)–poly(trimethylene carbonate)
PFBA	Pentafluorobenzaldehyde
Poly-Me β CD	Polymer of randomly methylated β -cyclodextrins
Poly- β CD	Polymer of β -cyclodextrins
R.H.	Relative humidity
ROESY	Rotating frame Overhauser enhancement spectroscopy
ROS	Reactive oxygen species
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SP	Semi-polar
TCT	Trichothecene
THC	Tetrahydrocurcumin
UMET	Unité Matériaux et Transformations
UV	Ultraviolet
WVTR	Water vapour transmission rate

TABLE OF SYMBOLS

$[i]$	Concentration of the compound i
A_i	Absorbance of the compound i
$C_{m,i}$	Molar concentration of the compound i
$\frac{dn}{dc}$	Refractive index increment
Δm	Weight gain/loss
Δt	Time of the experiment
$\varepsilon_{i,j}$	Molar attenuation coefficient of the compound i in presence of j
G_i	Number of i groups
I_i	Intensity of the NMR signal for protons i
l	Length of the UV cell
λ	Wavelength
M_i	Molecular weight of the molecule i
\overline{M}_n	Number average molecular weight
\overline{M}_w	Weight average molecular weight
N_i	Amount of the compound i
ν_i	Stretching vibration of the compound i
ρ_i	Volume weight of the compound i
S_i	Area of the surface i
<i>slope</i>	Value of the slope of a linear regression
T_{pl}	Transpiration of a plant
V_i	Volume of the liquid i
x_i	Weight percentage of the compound i

RESUME EN FRANÇAIS

Première région agricole de France, la Nouvelle-Aquitaine cultive des nombreuses ressources, telles que les céréales. Trente pourcents de la production de maïs française provient de cette région. Malheureusement, les céréales comme le blé ou le maïs peuvent être contaminés par des champignons mycéliens pathogènes, provoquant l'apparition de maladies telles que de la fusariose de l'épi. Les grains des épis contaminés posent un problème à la fois économique et aussi de santé publique. Premièrement, les grains sont jetés, causant des pertes de rendement allant jusqu'à cinquante pourcents [1]. Deuxièmement, et de façon plus préoccupante, les grains touchés peuvent contenir des toxines produites par les champignons, appelées mycotoxines. La fusariose est principalement due aux moisissures du genre *Fusarium*, parmi lesquelles l'espèce *F. graminearum* est fréquemment isolée. C'est pourquoi ce travail de thèse a ciblé cette espèce, et plus particulièrement la souche modèle et séquencée, la souche CBS 185.32 de *F. graminearum*. Cette souche est capable de biosynthétiser des mycotoxines de la famille des trichothécènes B (TCTBs). La plus connue des TCTBs est la 4-déoxynivalenol, dont la toxicité aigüe, combinée à une stabilité jusqu'à ... °C, en font une des mycotoxines réglementées au niveau européen (CE 1126/2007). La biosynthèse des TCTBs étant influencée par de nombreux facteurs de stress et étant encore mal comprise à ce jour, il est difficile de trouver des produits efficaces pour lutter contre la fusariose et ses conséquences. Actuellement, l'emploi des fongicides synthétiques, pouvant être dangereux pour l'environnement et la santé humaine, est récurrente. Face à la prise de conscience de l'opinion publique des dangers pouvant être associés à leurs utilisation, des alternatives se sont développées, comme le recours à des solutions basées sur des produits non toxiques. Ce travail de thèse s'est inscrit dans cette lignée-là. Plus précisément, ce projet a eu pour objectif le développement de bioformulations actives à la fois contre la croissance fongique et la production de TCTBs de *F. graminearum*. Nous avons souhaité ajouter des propriétés filmogènes à cette formulation afin qu'elle couvre les plants traités et résiste davantage aux aléas climatiques. En plus de cette propriété, la formulation devait ne contenir qu'un solvant aqueux et des ressources renouvelables, qui ne soient pas modifiées chimiquement.

La stratégie de ce travail repose sur la formation d'une matrice active par contact englobant des composés, eux aussi actifs mais de plus petite taille. L'efficacité d'une telle formulation serait ainsi améliorée de façon immédiate, mais aussi à plus long terme, grâce au relargage potentiel des plus petits composés. La matrice est composée du biopolymère de chitosane, connu pour ses propriétés bioactives et filmogènes. Des extraits de plantes, en particulier la tétrahydrocurcumine (THC), déjà étudiée au laboratoire, ou bien des extraits de coproduits des filières forestières et viticoles aquitaines y ont été ajoutés. Tous ces composés possèdent des propriétés antifongiques et respectent les contraintes mentionnées précédemment – le chitosane est issu des crustacés, des

champignons ou des insectes. Le chitosane est actif contre de nombreux champignons [2–5]. Les activités antifongiques du chitosane sont liées à sa structure particulière. Ce polymère est composé d'unités *N*-acétyl-D-glucosamine et d'unités D-glucosamine, ces dernières pouvant être protonées lorsque le chitosane est mis en solution légèrement acide à un pH inférieur à 6.5 (pK_a du chitosane). Des interactions entre ce polyélectrolyte et les membranes fongiques ou les molécules d'ergostérols présentes en surface des champignons seraient délétères et constituent une hypothèse quant à l'action antifongique du chitosane. En ce qui concerne la THC, ce composé phénolique dérivé de la curcumine a déjà démontré des propriétés antifongiques contre *Fusarium proliferatum* [6], *Candida albicans* et *Aspergillus niger* [7]. La THC est également capable de réduire la production de mycotoxines chez *F. proliferatum*, et bien que les biosynthèses de toxines pour cette espèce et celle de notre étude soient différentes, il nous a semblé intéressant d'utiliser ce composé. Les extraits des coproduits de la filière « bois » ont été choisis afin de valoriser les ressources régionales et aussi parce que d'autres extraits similaires ont démontré des activités antifongiques intéressantes. Dans le travail de Mezni *et al.* [8], des extraits d'aiguilles de pin maritime ont réduit la croissance mycélienne chez *Aspergillus* spp. et *F. solani*. D'autres extraits, des extraits de pins maritime et d'Alep [9] ou encore des extraits d'écorces de pin maritime [10] ont prouvés posséder une action antifongique. À notre connaissance, aucune étude n'a encore porté sur des activités anti-TCTB pour des extraits issus d'arbres.

Les résultats de ce manuscrit de thèse sont présentés en quatre parties, portant respectivement sur les formulations de chitosane, l'étude de la THC et son ajout aux formulations, le développement d'un système d'encapsulation pour la THC et enfin sur l'étude des extraits et leur addition aux solutions de chitosane.

Quatre chitosanes industriels d'origine marine ont été employés et dissous grâce à des acides organiques : l'acide acétique ou l'acide lactique. Les solutions de chitosane et d'acide ont toutes été actives pour réduire la croissance fongique de *F. graminearum*. L'activité antifongique des solutions a été davantage prononcée lorsque le pH était plus faible. Cette propriété a pu être imputée aux acides, et notamment l'acide acétique, et au fait que le chitosane possède une densité de charges « + » plus élevée à plus faible pH, ce qui est en accord avec les mécanismes d'action décrits plus haut. Un chitosane en particulier s'est détaché aux vues de notre application. Il s'agit d'un chitosane de masse molaire 78 kg/mol et avec un degré de déacétylation de 92 %. Ce chitosane conserve ses propriétés antifongiques sous forme de revêtement qui nous a servi à modéliser le devenir d'une solution de chitosane pulvérisée sur des feuilles (simulées dans notre étude par le milieu de culture). Ce chitosane, toujours dissous grâce à de l'acide acétique, possède également une activité contre la production de TCTBs, avec une inhibition de 70 % à 25 mg/L.

Afin d'améliorer l'efficacité immédiate et prolongée de la formulation, des extraits de plantes ont été ajoutés. La THC possède des propriétés anti-TCTB intéressantes, car elle inhibe 20 % de la

production de toxines de *F. graminearum* à 10 μM . La biosynthèse de TCTBs est liée au stress oxydant perçu par le champignon [11–13]. L'inhibition de cette production pourrait ainsi mettre en jeu les propriétés anti-oxydantes de la THC, dont la capacité à piéger les radicaux libres est en effet 1.7 fois supérieure à celle de l'hydroxytoluène butylé, un antioxydant couramment utilisé dans l'industrie. Cependant, l'ajout de THC à une formulation de chitosane 78 kg/mol et d'acide n'a pas d'effet, voire est contre-productif. L'accumulation de TCTBs reste en effet inchangée par rapport à l'action de la formulation sans THC. Par ailleurs, la croissance du mycélium est significativement moins réduite en présence de formulations contenant 10 μM de THC, par rapport aux mêmes formulations n'en contenant pas, lorsque le pH initial du milieu nutritif est de 4.8. Ce résultat est inattendu car la THC seule à 10 μM n'a pas d'effet sur le développement du mycélium. De possibles interactions ions–dipôles induits entre le chitosane et la THC pourraient être à l'origine de ces observations. Afin d'empêcher ces interactions nuisibles pour notre application et en vue d'augmenter la solubilité en solution aqueuse de la THC, des voies d'encapsulation de la THC ont été explorées.

La THC a été encapsulée dans différents systèmes basés sur l'utilisation de biopolymères. La THC a été complexée dans des cyclodextrines (CDs), des macromolécules composées d'unités glucose assemblées en forme de cône creux. Les CDs sont particulièrement employées pour piéger des composés hydrophobes dans leur cavité elle aussi hydrophobe, tout en garantissant une solubilité dans l'eau au complexe grâce aux groupements hydroxyles des glucoses situés à la surface extérieure des CDs. Une CD composée de 7 unités glucose et méthylée aléatoirement, la Me β CD, a permis d'augmenter la solubilité apparente de la THC. Après une étude de solubilité, il a été prouvé que le ratio molaire du complexe était de 1, lorsqu'il y a quinze Me β CD pour une THC. Autrement dit, en solution on observe une CD en interaction avec une THC, et quatorze CDs de libres, dans la gamme de concentration étudiée. Cette étude a également permis de calculer la constante d'association du complexe, qui a une valeur de $591 \pm 46 \text{ M}^{-1}$. Cette valeur est de l'ordre de grandeur de celles couramment trouvées dans la littérature, par exemple pour des complexes CDs/curcumine [14–18] ou pour le complexe CD/THC [19]. Des études morphologiques par microscopie électronique à balayage et thermiques par calorimétrie différentielle à balayage ont été menées. Enfin, l'effet du complexe sur *F. graminearum* a été évalué. La croissance du champignon est malheureusement favorisée, probablement du fait de la dégradation des CDs en glucose. Afin de contrer cet effet, des polymères de CDs, mis à disposition par le laboratoire UMET, ont été employés. Ces polymères de CDs ont réduit la croissance mycélienne jusqu'à 25 % avec un polymère chargé en THC. Bien que prometteurs, ces polymères de CDs piégeant de la THC étaient difficilement industrialisables à court terme. Des alternatives ont alors été envisagées, grâce à une collaboration avec un laboratoire de l'Université de Technologie de Kaunas (Lituanie). La THC a donc été encapsulée dans des particules de chitosane ou bien d'amidon modifié obtenues par deux

méthodes de séchage. La méthode de séchage par atomisation (spray-drying) génère des particules sphériques alors que la lyophilisation conduit à des particules de forme variées mais plutôt planes. Les particules de chitosane, atomisées ou lyophilisées, possèdent des activités antifongiques et antimycotoxigéniques intéressantes.

Les deuxièmes extraits de plantes à avoir été additionnés aux solutions de chitosane sont des extraits des coproduits des filières forestière et viticoles. Les extraits utilisés dans ce travail ont été fournis par un partenaire industriel, le laboratoire Phenobio®. Ils ont été obtenus par extraction à l'eau sous-critique, qui est un procédé sans solvant organique. Des extraits de sciures ou d'écorces de pin maritime ont montré des activités antifongiques. L'extrait de sciures de pin maritime obtenu à une température d'extraction de 175°C a été particulièrement efficace pour lutter contre la production de TCTBs [20]. Cet extrait a été ajouté à une solution de chitosane (chitosane 78 kg/mol et acide acétique). La formulation est prometteuse au niveau de son action antifongique mais les résultats ne sont pas reproductibles au niveau de l'inhibition de la mycotoxinogénèse.

En perspectives de recherche, il serait intéressant d'étudier les mécanismes d'action entrant en jeu aussi bien contre la croissance du champignon que contre sa biosynthèse de TCTBs. Les effets du chitosane ou des composés phénoliques sur les parois fongiques à l'intérieur des cellules pourraient être observés par des techniques de microscopie. Il serait de plus judicieux d'étudier de façon approfondie les interactions entre le chitosane et la THC, notamment pour optimiser le système d'encapsulation de cette dernière. Les particules aussi bien de CDs que d'amidon ou de chitosane pourraient être additionnées aux formulations de chitosane et ainsi parachever cette étude sur l'association de THC et de chitosane.

La formulation la plus prometteuse semble être celle composée de chitosane 78 kg/mol dissous grâce à de l'acide acétique et contenant des extraits de sciures de pin maritime. Il faut néanmoins garder à l'esprit que l'utilisation d'extraits de plantes possède des contraintes qui seraient à étudier, comme la variabilité de sa composition ou sa stabilité. L'analyse de la compatibilité entre la formulation décrite plus haut et la croissance des plants de céréales serait également à conduire. Enfin, des essais sur champs, comme celui initié dans ce travail, pourraient être répétés et complétés.

GENERAL INTRODUCTION

The region *Nouvelle-Aquitaine* is one of the largest regions of France with an area of 84,000 km². Half of the territory is dedicated to agriculture, with a huge economic impact of 10.5 billion euros, corresponding to 15 % of the global farm incomes in France [21]. This agricultural region mainly produces maize, with an annual production of 3.7 million tonnes (Mt), soft wheat (3.1 Mt) and barley (0.7 Mt) [22].

Like some other agricultural products, cereals are naturally sensitive to fungal contaminations that can cause yield and quality losses with negative consequences, as an altered appearance, reduced technological properties of raw materials associated to the potential production and accumulation of toxic molecules. Many fungi can infest a plant during its growth, such as *Fusaria*, *Aspergilli* or *Penicillia* [23,24]. Among them, *Fusarium* spp. and *Microdochium* spp. are responsible for the wheat disease called Fusarium Head Blight and *Fusarium graminearum* causes Gibberella Ear Rot or Stalk Rot in maize fields.

The occurrence of post-harvest diseases is not to be neglected either. Grains can also be contaminated by *Aspergilli* or *Penicillia*, potential producers of aflatoxins and ochratoxins, respectively. The grains are especially vulnerable if the moisture content locally reaches a value that enables the development of mould [25–27].

As above, *Fusaria* represent a major health issue as they can produce toxins as secondary metabolites. Among these mycotoxins, trichothecenes or fumonisins are potential carcinogenic compounds. Levels of mycotoxins in Europe are regulated and fixed to a certain threshold, for example, at 750 µg/kg in pasta and cereals intended for direct human consumption for deoxynivalenol, one major toxin of *F. graminearum* (EU Regulation CE No 1126/2007). Over-contaminated harvests are either classified as animal feedstuff, with a limit of 10,000 µg/kg for beef and chicken and 5,000 µg/kg for swine in the United States [28], or discarded. The economic burden for farmers is huge as they have to invest in treatments and preventive measures and suffer from lower-than-expected incomes. To have an idea of the impact of fungal contamination, in 2015–2016 in the United States, the cost related to *Fusarium* attacks on wheat was over \$4 billion [29]. For maize, the yield loss was up to 30 % for example in 2015 and 2016 in a region of Brazil [30].

To tackle this both economic and public health issue, current strategies of pest control rely on different strategies including the use of synthetic fungicides. Unfortunately, they are frequently not sufficient to eradicate such fungal diseases and can lead to the development of resistant strains, like in commercial wheat fields in China, an isolated *F. graminearum* strain has been found to be resistant to a widely used fungicide [31]. In addition, synthetic fungicides have also received special attention because some of them are suspected to impact the endocrine system, thus potentially

damaging the reproduction and development of mammals [32]. Finally, the European regulation REACH and the French Ecophyto plan have implemented a reduction of the use of synthetic fungicides in the coming years, due to deleterious impacts on the environment.

As a consequence, there is an urgent need for sustainable and safe alternatives to synthetic fungicides. The use of biomolecules to create bio-based antifungal formulations is gaining in importance, as shown by the rise of the biocontrol industry in France. Biocontrol can be defined as the use of natural enemies to reduce the populations of invasive species. These biological control agents include living organisms such as insects or microorganisms, pheromones, kairomones and phytopharmaceuticals from plant, animal or mineral origin. They cover a wide range of products and means of action. In France, some of them are already authorised, such as microorganisms like *Bacillus* spp. or *Trichoderma* spp. Products based on mineral salts such as potassium hydrogen carbonate or phosphonate, iron phosphate, sulphur, organic acids like acetic or pelargonic acid, natural extracts like garlic extracts or orange essential oil are also available on the market, but not necessarily for a clear antifungal effect.

In this context, a project involving numerous academic and industrial partners has emerged in 2017. It notably aimed to develop biocontrol solutions against toxigenic cereal pathogenic fungi, while enhancing the value of local resources in *Nouvelle-Aquitaine*. More specifically, the objective was to control the growth of *Fusarium* spp. and their mycotoxin production. The actors of the project came from various horizons including small industrial groups and public laboratories and we had a particularly close collaboration with Phenobio® (Lubrizon), CR distribution, and the MycSA laboratory at INRAE (Figure 1).

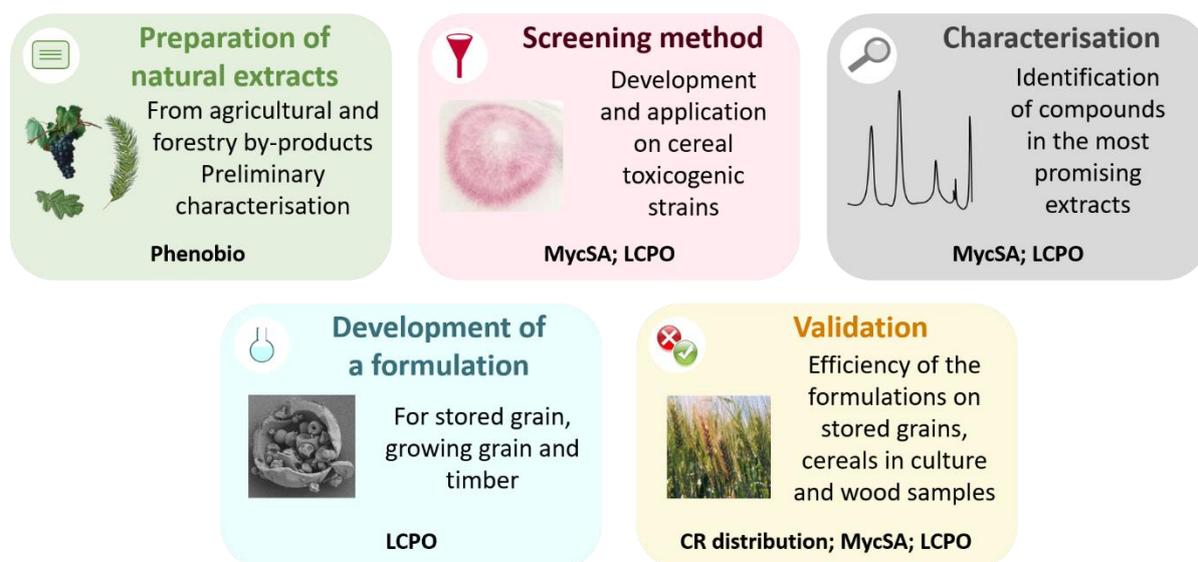


Figure 1: Overview of the steps of the project.

Chitosan – a chitin derivative – was first selected as the main component of such antifungal formulations. Indeed, chitosan is well-known for its antimicrobial and elicitor properties, stimulating the defence mechanisms of plants, and, depending on its molecular weight, it has potentially interesting film forming properties to create active biocoatings on plants. It has been studied on an ongoing basis at the LCPO laboratory [33] to form antimicrobial films [34,35] or coatings [36]. Briefly, chitosan is a non-toxic biopolymer, with a median lethal dose of 16 kg/day in mice [37] and no demonstrated adverse effects on human [38]. Some biopesticides based on chitosan are already commercialised, such as chitosan hydrochloride [39] and chitosan and galacturonic oligosaccharides [40]. The Expert Group for Technical Advice on Organic Production concluded that both meet the criteria to be used in organic farming [41]. Chitosan and galacturonic oligosaccharides would act as an elicitor and demonstrated a reduction of the severity of powdery mildew on grapevines and cucumbers in several trials in France, Spain and Belgium [42].

To increase the antifungal activity both on the fungal growth and the mycotoxin production, one strategy is to combine molecules with complementary properties. As a result, this study focuses on the combination of chitosan with plant biomolecules and extracts from agricultural and wood by-products in order to finally create biocoatings active by contact and through the migration of components. The phenolic compound tetrahydrocurcumin (THC) was selected as an active biomolecule, because it has shown a great potential against fungi and especially led to a reduction of the *Fusarium proliferatum* growth by 70 % *in vitro* and an inhibition of 75 % of the accumulation of mycotoxins [6]. On the other hand, since our region is one of the largest producer of wood and wood derivatives in France, the valorisation of silviculture by-products for the development of biopesticides is a very active field of research. Indeed, in the recent literature, various studies highlighted the antimicrobial or anti-parasite potential of wood extracts. For example, two studies showed that extracts of Norway spruce barks could control *Pseudomonas aeruginosa* and *Alternaria alternata* [43] as well as the species responsible of downy mildew [44]. In the same vein, extracts of barks from Indonesian trees were shown to have antifungal properties [45]. In the work of Rodrigues *et al.* [46], extracts from six Amazonian woods were evidenced as reducing the development of soft-rot fungi. Therefore, in the present work, some wood by-products are studied as promising source for sustainable alternatives to control *F. graminearum* and its production of mycotoxins.

In this framework, this present research study aims to create a formulation compatible with biocontrol, based on renewable products, efficient at preventing the growth of *F. graminearum* – the selected target model species – and its production of mycotoxins notably for maize or wheat protection. With the goal to create an innovative and high-performance formulation, we explore the possibility of combining both chitosan and THC or chitosan and wood by-product extracts,

simultaneously to benefit from their complementary and synergistic antifungal and antimycotoxigenic actions.

This work is divided into six parts.

Chapter I is devoted to the literature review highlighting general considerations on the target fungi *F. graminearum*, the biopolymer chitosan and then plant extracts and especially THC. A succinct state of the art on the encapsulation processes of phenolic compounds have also been established.

Then, Chapter III focuses on the feasibility and the efficiency of chitosan-based formulations. It is dedicated to the physicochemical characterisation and antifungal or antimycotoxigenic activities of chitosan solutions to understand the structure–antifungal properties relationships.

Chapter IV, after demonstrating the potential of THC as an inhibitor of the toxin production, is devoted to the association of this pure phenolic compound to chitosan solutions and films. An emphasis is done on the interactions between the biopolymer and THC. Afterwards, to solve some problems of THC, Chapter V studies the encapsulation in different carriers. The obtained particles are characterised and the effect on fungal growth and accumulation of mycotoxins is detailed.

Finally, in Chapter VI, the characterisation of some extracts from wood by-products and the results of their addition to chitosan formulations on the antifungal efficiency are presented. The last section deals with industrial trials of chitosan and extracts on wheat fields.

CHAPTER I – BIOFUNGICIDES: NECESSITY & PRODUCTION

FUSARIUM AND PROMISING BIO-BASED ANTIFUNGAL AGENTS

1	Fungal diseases of maize and wheat and their treatments.....	6
1.1	General diseases caused by <i>Fusarium</i> spp.....	6
1.2	<i>Fusarium graminearum</i> infection.....	6
1.3	Mycotoxins produced by <i>F. graminearum</i>	8
1.4	Management of <i>F. graminearum</i> on crops.....	10
1.5	Concluding remarks.....	14
2	Chitosan: an active biopolymer with antifungal and elicitor properties	14
2.1	Generalities on chitosans.....	14
2.2	Specific antimicrobial properties of chitosans	16
2.3	Some applications of chitosan	19
2.4	Concluding remarks.....	24
3	Plant extracts: active molecules with antifungal and antioxidant properties ...	25
3.1	Plant extracts.....	25
3.2	Phenolic compounds from plant extracts.....	31
3.3	Specific case of tetrahydrocurcumin.....	34
3.4	Concluding remarks.....	37
4	Encapsulation techniques of poorly water-soluble compounds	37
4.1	What is encapsulation?.....	38
4.2	Characterization of particles.....	39
4.3	Physical entrapment.....	40
4.4	Encapsulation through physicochemical interactions.....	43
4.5	Concluding illustrations	50
5	Strategy of the present study	51

1 Fungal diseases of maize and wheat and their treatments

1.1 General diseases caused by *Fusarium* spp.

Classed recently as the fourth most important fungal plant pathogen regarding scientific and economic criteria [47], *Fusarium graminearum* belongs to the genus *Fusarium*, whose species infect cereal fields causing huge grain losses and a public health concern. Indeed, *Fusaria* can produce mycotoxins such as trichothecenes, enniatins or fumonisins (Figure I.1) which are toxic compounds for humans.

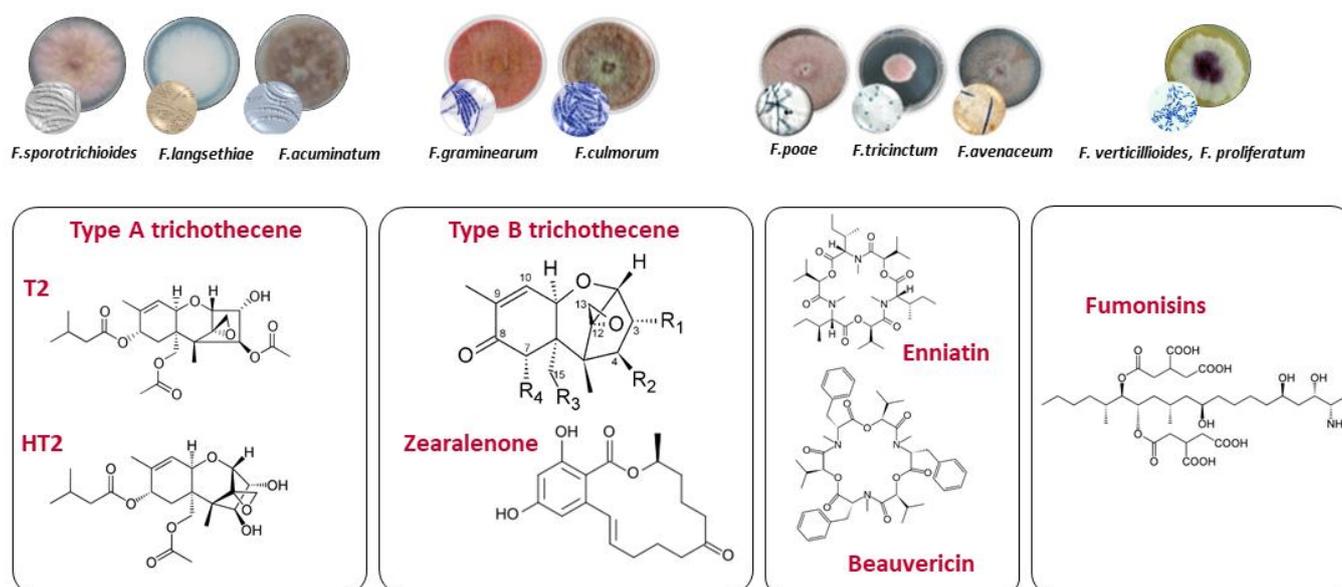


Figure I.1: Mycotoxins produced by different *Fusarium* species.

1.2 *Fusarium graminearum* infection

F. graminearum is an ascomycete fungus, which possesses an asexual form and a sexual form. *F. graminearum* develops from spindle-shaped spores called macroconidia, composed of five to seven septa (Figure I.2).

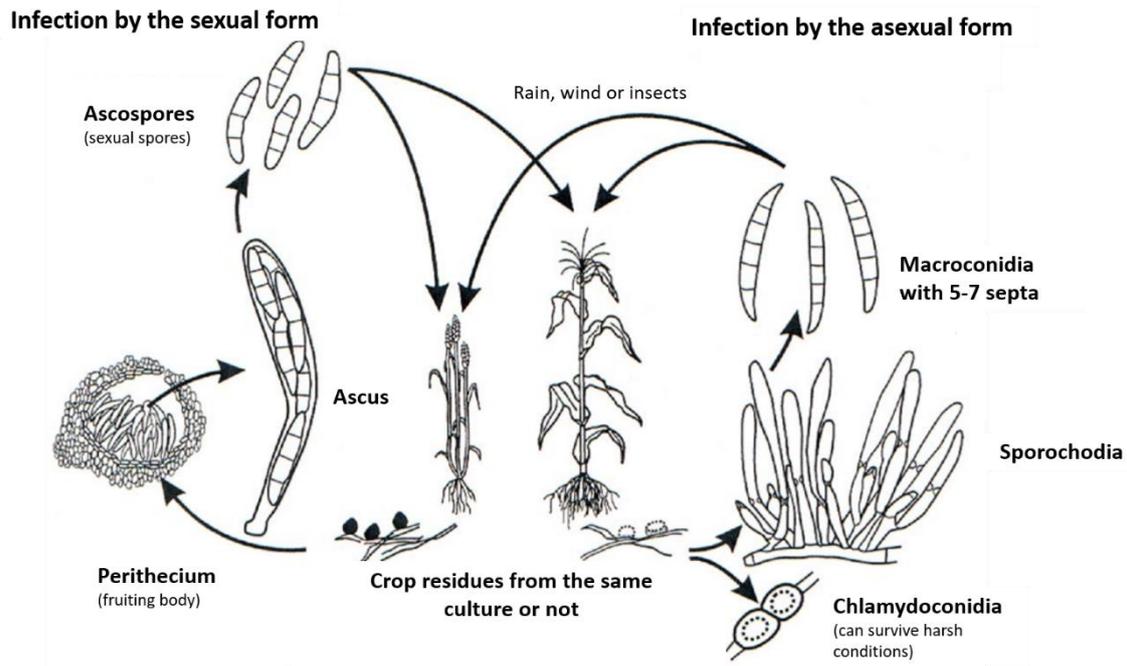


Figure I.2: *F. graminearum* infection cycle, adapted from A. Bily [25].

Hyphae are growing from a macroconidium, ingesting nutrient through an apex, forming a dense and radial network of mycelium. New macroconidia are produced at the end of a hypha in a structure called sporochium. For the sexual form, spores are called ascospores, gathered and protected by an ascus, which is included in the perithecium [1,25,48,49]. Both forms of the fungus can be found in natural conditions and both can infest plants, even if the first one seems to be at first present on the host [25]. Plants are infested by spores (either macroconidia or ascospores), which are carried by the wind, insects and the rain to the infection site. They are coming from crops residues on the soil, where chlamydospores (chlamydoconidia) or saprophyte mycelium can overwinter. The fungi usually infect plants through wounds or during anthesis, the flowering period. Moist and warm (28 °C–29 °C) conditions favour fungal infection [48]. The different diseases that can occur are summarized in Table I.1. For FHB, the anthesis is the stage where the plant is the most sensitive to fungal attack, as flowers are wide open and pollens are rich in saccharides, which could favour fungal development. If the infection occurs in the anthers, the flowers' pollen producing part, the development of kernels is reduced. If florets are infected, the plant will produce shrivelled kernels, named "tombstones", florets themselves will bleach and sporochion (pink mould) can be seen. If kernels are attacked, their appearance will not be affected but there could still be accumulated mycotoxins [50].

Table I.1: Diseases on maize and wheat with pictures of symptoms for Maize Ear Rot and Fusarium Head Blight.

Maize			Wheat		
Gibberella Root Rot	Gibberella Stalk Rot	Gibberella Ear Rot	Fusarium Seedling Blight	Fusarium Foot Rot	Fusarium Head Blight (FHB)
					
		[51]			[52]

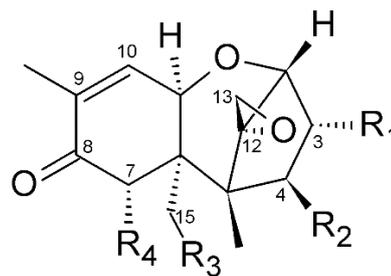
1.3 Mycotoxins produced by *F. graminearum*

1.3.1 Chemical structure

F. graminearum, like other fungi from the order *Hypocerales* [53], produces trichothecenes (TCTs). TCTs are sesquiterpenes with a double bond in the C₉-C₁₀ position, an epoxide in C₁₂-C₁₃ and possible hydroxyl, ketone or acetate moieties in the C₃, C₄, C₇, C₈ or C₁₅ position (Table I.2). Ueno and Hsieh [54] classed them into four categories: TCTA which do not have a ketone in the C₈ position; TCTB with a ketone in the C₈ position; TCTC bearing 2 epoxides and TCTD with an additional macrocycle. TCTC and TCTD are less represented in natural production conditions, whereas TCTB are the most frequently found. The most isolated TCTB are nivalenol, 4-deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), fusarenone X and 3-acetyldeoxynivalenol (3-ADON) in maize ears [55]. The ability of *F. graminearum* to produce certain toxins is genetically determined and is called the chemotype. Three chemotypes can be distinguished: 15-ADON, 3-ADON or nivalenol producers. The strains used for this work possess a 15-ADON chemotype.

Table I.2: Structure of the various TCTB.

Name of the TCTB	R1	R2	R3	R4
4-Deoxynivalenol	OH	H	OH	OH
3-Acetyldeoxynivalenol	OAc	H	OH	OH
15-Acetyldeoxynivalenol	OH	H	OAc	OH
Nivalenol	OH	OH	OH	OH
Fusarenone-X	OH	OAc	OH	OH



1.3.2 Effects on animals and plants

TCTs are toxic compounds, particularly if ingested; the T2-toxin, a TCTA, is classified as *Fatal if swallowed, Fatal in contact with skin, Fatal if inhaled, Cause skin irritation* (Globally Harmonized Standard of Security Data Sheet). The toxicity is due to the epoxide ring in C₁₂-C₁₃ and the double bond in C₉-C₁₀ and the level of toxicity varies with the substituents [56].

Exhaustive reviews of Pestka [57] and Rocha *et al.* [58] demonstrated effects of TCTs or DON. An acute TCTs intake is associated with vomiting, especially DON, which is also known as “vomitoxin”. Symptoms like feed refusal, anorexia and growth retardation have been observed in pigs or mice fed with grains containing DON. TCTs could affect the serotonin production, which is linked to the intestinal activity. TCTs may dysregulate the immune system and reproduction and development on experimental animal models. At the cellular level, TCTs inhibit protein synthesis, DNA and RNA synthesis and can affect cell membranes and mitochondria. 15-ADON is said to be more deleterious on the intestinal barrier than 3-ADON and DON, when tested on human intestinal cell line [59], on piglet intestinal cell line or *in vivo* in piglets [60].

TCTs are also phytotoxic compounds. Indeed, DON provokes growth retardation, inhibition of seedling and green plant regenerations [58].

1.3.3 TCTs in the food chain

TCTs are stable compounds, especially during food processing, which is the reason why so many efforts are deployed to deal with fungal infection and toxin accumulation. Duffy et Reid [61] showed that the T2-toxin is stable one year in deuterated phosphate-buffered saline solution for a pH range 5-12. The epoxide ring, which is essential for the toxicity of TCTs [62], is stable to nucleophilic additions [63]. Hazel et Patel [64] reviewed the effect of processing on the level of mycotoxins. They appear to be stable at 120 °C and not even fully degraded at 210 °C. As kernels can look shrunk, sorting is a preliminary barrier to the presence of DON with a reduction of 74 %, but it is not always the case, and the process efficacy can decrease to 20 %. Milling and baking lead to diverse DON reduction. The levels of mycotoxins are twice as less in flour than in clean wheat but two times higher in bran. The authors also report that 50 % of DON are eliminated in boiling water when cooking pasta. Beer is not an exception to DON presence and impact of brewing, especially fermentation with yeast, on DON transformation or degradation is still equivocal.

Levels of mycotoxins are regulated by the European Commission in the Commission Regulation No 1126/2007, which are summarised in Table I.3. Regulations are evolving and other mycotoxins of *Fusarium* like enniatins may soon be targeted, as well as derivative products of DON, especially 15-ADON.

Table I.3: Maximum levels for DON in foodstuffs.

Type of grains	Limitation (µg/kg)
Unprocessed cereals other than durum wheat, oats and maize	1 250
Unprocessed durum wheat and oats	1 750
Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling	1 750
Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption	750
Pasta (dry)	750
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
Processed cereal-based foods and baby foods for infants and young	200
Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	750
Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	1250

1.4 Management of *F. graminearum* on crops

Different strategies are available to reduce the infection of *F. graminearum* and mycotoxin accumulation in grain. Wegulo *et al.* [65] and Torres *et al.* [66] mentioned agronomic practices, the use of genetic resistant varieties, the use of chemicals, biological control, forecasting and management strategies during harvest.

1.4.1 Agronomic practices

Tillage is one of the most efficient way to reduce contaminant sources as old crop residues are buried. Indeed, residues can be used as substrates for perithecia (containing spores) development under light conditions. Crop rotation is also a way to decrease *Fusarium* infection, for example by cultivating soybean, rape, cabbage, flax or peas before planting maize or wheat. As moisture boosts fungal development, it is recommended to reduce irrigation during anthesis and early grain filling [26,65–67].

The screening and understanding of resistant cultivars is an active research topic, as it is an arduous and long-term task. Sometimes, the physiological resistance determined in the laboratory is not linked with less infection in the fields. Moreover, five types of resistance are known, from resistance to primary infection to resistance to the mycotoxin accumulation and are described in details by Champeil *et al.* [67]. In natural conditions, tall wheat and wheat without awns seemed to be more resistant [68].

1.4.2 Use of antagonistic microorganisms

Biocontrol is another strategy to reduce *Fusarium* occurrence in crops. It is defined by the use of an antagonistic microorganism, called the biocontrol agent. The microorganism is assumed to have an antibiosis action, or to parasitize the targeted species, to induce plant resistance or to compete for nutrients on the plant. Numerous options are promising *in vitro* with *Trichoderma* sp., *Paecilomyces* sp. [69] or the bacterium *Bacillus subtilis* [70]. Tests in greenhouses and fields have also been performed, with *Clonostachys rosea* which inhibits the formation of new spores on maize residues [71]. Biocontrol on fields is often not sufficient to reduce the fungal development [1,65,66]. Biocontrol commercial products represent 5 % of the total market of crop protection [72].

1.4.3 Traditional synthetic or bio-based fungicides

1.4.3.a Traditional synthetic fungicides

Chemical inputs can also be used to control fungal attacks [65,66,73]. Although a large variety of fungicides are available on the market [74], they are not suitable for organic agriculture (OA). The period of application is crucial for optimal efficiency, which is usually during flowering or some days after. However, in a field, all plants do not flourish at the same time. Fungicides often do not translocate inside plant tissues, which makes them less efficient. Moreover, good weather for spraying is not the likely time for fungi to develop. It was also proven that fungi acquire some resistance to fungicides as it was the case in China with carbendazim [75]. The association of two molecules like tebuconazole + prothioconazole (Prosaro®, Figure I.3) is therefore often used. However, tebuconazole is harmful if swallowed and is suspected of damaging the unborn child, and it is not the only example that could be cited here. Gupta described their potential impact on endocrine system, for some of them can bind with receptors (*e.g.* oestrogen receptors) or interact with hormones [76].

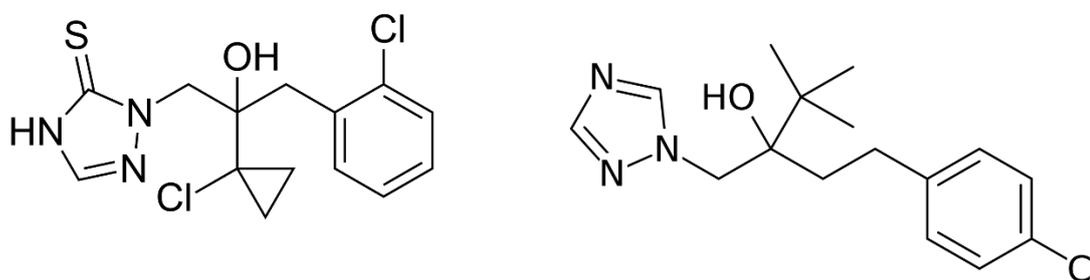


Figure I.3: Prothioconazole and tebuconazole used in Prosaro®.

Public concern about the use of potentially toxic compounds is rising. Although synthetic compounds degrade in the environment within some weeks [66], their application leads sporadically to a high dose on the plant. Some authors found alternatives by encapsulating

traditional fungicide to control the release of the active ingredient and thus limiting the applied dose at a given time. For example, tebuconazole was encapsulated in cyclodextrins, enhancing its solubility by 265 times [77]. Polysaccharides [78] or cyclodextrins [79] are often chosen because they are modifiable, bio-based, non-toxic and easily available. Encapsulation can thus reduce the environmental toxicity, as well as improve the shelf-life of a product.

1.4.3.b *Bio-based options suitable for organic farming*

Although synthetic chemicals are powerful tools for farmers, they cannot be used in organic agriculture to control fungal pests since they are not environmental-friendly. For this reason, options suitable for organic farming have been developed for decades. Addition of biofumigants like cabbage or broccoli waste, which are rich in sulphur, or other amendments like compost and specific nutrients which help the plant to be healthy, and therefore to be more resistant to fungal attacks, are more employed in organic agriculture than in conventional agriculture. A lot of innovative techniques are reviewed by Panth *et al.* [80] and van Bruggen *et al.* [81], but they are mostly applied to fruits and vegetables.

In fact, cereals in organic farming do not suffer major growth problems [82], which could probably explain why specific solutions in case of pests are less developed for cereals than for more sensitive plants. One serious risk of disease in organic farming comes from the untreated seeds possibly containing pathogens [83], risk that could be reduced by hot water or plant extract [81]. Foliar or head diseases are not as severe as we can imagine in organic farming compared to the traditional one. Nitrogen levels and a high crop density associated with conventional agriculture are said to favour the development of fungi [83]. Góral *et al.* [83] studied 30 wheat cultivars, in the same environment under conventional agriculture (two fertilizers, herbicide, insecticide, *no* fungicide) and organic agriculture conditions. With a low Fusarium Head Blight severity, they observed that fungal mass detected by DNA measurement in grain was three times bigger for organic farming than for conventional agriculture. These results yielded more dead or non-germinating kernels in organic agriculture. The TCTB level in conventional agriculture was measured at 100 µg/kg in grain *versus* 76.2 µg/kg on organic farming produced grains. The absence of treatment in organic agriculture may favour the biomass development of both pathogenic and non-pathogenic microorganisms on plant and soil. Pathogenic mycobiotes competed with non-pathogenic ones and existing microbiota, thus limiting their development. On soil, living microorganisms could strengthen the plant's defensive responses. Birzele *et al.* [84] collected data from wheat cultivars in conventional or organic farming of neighbouring fields for two years. It appeared that, for a medium contamination, organic agriculture gave the best results with the lowest infected grainy proportion and DON content. However, when the contamination was very high, with more than 50 % of contaminated kernels for conventional agriculture without fungicide, DON production was the lowest for conventional agriculture with fungicide, followed by organic

agriculture and then the conventional one without fungicide. These results suggest that organic farming is a reliable way to grow wheat, with similar to less pest occurrences, except for exceptionally high disease severity, where no efficient remedy is available.

To overcome this issue, some treatments had been developed, such as copper- and sulphur-based fungicides or bicarbonate salts [85]. The Bordeaux mixture, composed of copper sulphate and calcium hydroxide has phytotoxic and bad effects on soil microbiota [86]. The European Commission recently decreased the maximal amount of any kind of copper containing product by stating that “only uses resulting in a total application of a maximum 28 kg of copper per hectare over a period of 7 years shall be authorised” [87]. The need for an alternative is now a subject of great concern. Brunel *et al.* [88] synthesized chitosan nanogels loaded with copper (II) ions. Nanogels confers a good handling and stability meanwhile amine moieties of chitosan and Cu^{2+} formed complexes. Remaining ammonium moieties on chitosan brought some additional antifungal properties (see 2.2 for details). The authors observed a synergistic effect between copper and the biopolymer, with a three times enhanced efficiency of copper (II) in the presence of 15 $\mu\text{g}/\text{mL}$ of chitosan dispersion, concentration at which the chitosan dispersion did not even possess any activity against *F. graminearum*.

1.4.4 Global management

Forecasting is a tool available for farmers, which predicts with a 60 % to 80 % accuracy the risk of FHB outbreaks and, for the more powerful ones, DON accumulation. Systems take into account weather forecasts and data from previous weeks, such as rainfall, temperature, etc., and integrate agricultural parcel data, like crop rotation, tillage, resistant cultivars, sowing date, etc. Programs are based on empirical figures from previous years. It enables a targeted application of fungicide, with an adapted amount regarding the risk. Each harvest from different plots are cleaned, sorted and designated to a certain market in a relevant way [26,65,66,89]. ARVALIS - *Institut du végétal* proposes its tool Myco-LIS ® in France for example [89].

Integrated management was proven to be much more efficient than a single strategy to prevent fungal infection. Blandino *et al.* [90] showed that the combination of ploughing, moderately resistant variety and triazole application at the heading reduce by 97 % the DON level compared to the case where none of these strategies was used. Moreover, the means to fight the disease work in synergy. Many other authors studying the impact of resistant cultivar and fungicide obtained similar results [65,73]. Unfortunately, no study on integrating management involving biocontrol, which is still at the early stage, interactions with other treatment (herbicides) or other agricultural practices (irrigation, nitrogen supply) can be found.

1.5 Concluding remarks

The control of diseases caused by *F. graminearum* is a key issue nowadays, as they lead to yield decrease and the accumulation of mycotoxins in kernels. Contaminated cereals are wasted, because trichothecenes are stable during food processing. A lot of strategies have been implemented, but none of them seems really effective on their own. That is why new multi-approach bio-based options have to be developed. Farmers in organic agriculture are especially seeking for solutions in case of high disease severity. In this context, the promising active biopolymer chitosan is presented in the next part.

2 Chitosan: an active biopolymer with antifungal and elicitor properties

2.1 Generalities on chitosans

2.1.1 Structure, origin and elaboration process

Chitosan is a biopolymer resulting from the deacetylation of chitin, a β -(1 \rightarrow 4)-linked *N*-acetyl-D-glucosamine biopolymer. Structures of chitin and chitosan are represented in Figure I.4.

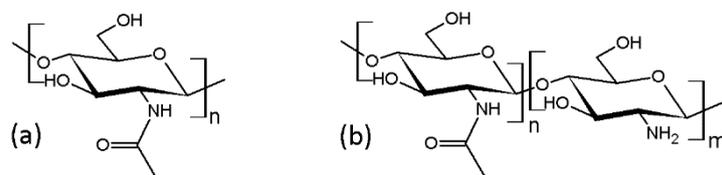


Figure I.4: Structures of (a) chitin and (b) chitosan.

One specific characteristic of chitosan is its degree of deacetylation (DD). Rigorously speaking, the DD of chitosan should be 100 %, but it is commonly accepted that 50 % are enough to consider the polymer as chitosan [91]. The DD is the molar ratio of D-glucosamine units. It is calculated according to equation (I.1), where G_{NH_2} is the number of D-glucosamine and G_{NHAc} is the number of *N*-acetyl-D-glucosamine.

$$DD = \frac{G_{NH_2}}{G_{NH_2} + G_{NHAc}} * 100 \quad (I.1)$$

Chitosan was first isolated by Rouget in 1859 from residues of chitin boiled in potassium hydroxide [92]. Nowadays, chitin is mainly extracted from crustacean exoskeleton like shrimp or crab waste residues from the industry. Crustacean waste shells are complex structures made of chitin, proteins, calcium carbonate and pigments. They usually undergo a demineralisation with NaOH, a deproteination with HCl and a bleaching in oxidant medium. Chitin is then deacetylated in a NaOH solution at 40-50 %, between 80-150 °C for several hours. The process is not environmentally friendly, but it is the most used industrially [93]. Some techniques using lactic acid-producing bacteria and protease for the first two steps and chitin deacetylase for the deacetylation have been reported, but the long processing time makes them non attractive [94]. A deacetylation process in the microwave lasting a few minutes was recently developed [95].

Some fungi, algae or yeasts also contain chitin in their cell wall. Chitosan extraction from fungal sources presents numerous advantages. The demineralisation step is suppressed because they naturally have a low amount of minerals. Mild alkaline and mild acidic conditions are sufficient to extract chitin. The resulted chitosan is naturally free of allergens and heavy metals, and the molecular weight is more controlled [96]. Moreover, this resource is available all over the year. Unfortunately, this process is not widespread in the industry [93,97] and chitosans from fungal resources were shown to be less active for targeted applications [98].

Regarding the impact of the chitosan origin on some physicochemical characteristics, it seems that the molecular weight (MW) from shrimps is higher than those obtained from fungi [93]. Small chitosans with a polymerisation degree less than 100 are referred as chitooligosaccharides in this work. It corresponds approximately to a MW of less than 16 kDa [99].

Chitosans possess protonable amine functions, whose pK_a are close to 6.3 [36]. As a consequence, contrary to chitin, chitosan can be solubilised in aqueous solutions by lowering the pH to obtain NH_3^+ soluble functions. Thus, solutions of chitosan are usually prepared under mild acidic conditions. Acetic, formic, lactic, hydrochloric or citric acid solutions are commonly employed. As expected, chitosans with a high MW are less soluble than small ones.

2.1.2 Specific characterisation of MW and DD

MW and DD are key parameters for the development of active solutions based on chitosan, as explain below in 2.2. Unfortunately, in scientific articles, these two parameters are determined using different techniques and therefore results become difficult to compare.

The MW can be measured by several techniques. Size Exclusion Chromatography (SEC) is a powerful tool to set the number average molecular weight (\overline{M}_n), the weight average molecular weight (\overline{M}_w) and the polydispersity index. A first issue comes from the denomination of the MW. In fact, scientific studies based on chitosan frequently omitted to precise if the MW they are talking about corresponds to the \overline{M}_n or the \overline{M}_w , which may be important knowing that the chitosan is often

polydisperse. A second difficulty occurs when the MW is found with some less accurate techniques. As said just before, chitosans are soluble under acidic conditions and the viscosity is MW-dependent. The measurement of the intrinsic viscosity is therefore a very commonly employed way to approximate the MW. An automatic viscometer equipped with a capillary was used for this purpose. Calibration curves showed a linear dependency between the concentration of the chitosan and its viscosity. However, precautions must be taken with low MW chitosans [100]. All of that leads to non-accurate measurements.

As for the DD, it can be determined by ¹H-Nuclear Magnetic Resonance (NMR) spectroscopy, *via* Fourier Transform Infra-Red (FT-IR) spectroscopy or other methods. By NMR, the relative intensity, *I*, of protons of the acetyl groups are located at approximately 2 ppm and those from hydroxyl groups between 3 and 6 ppm. So, the DD can be expressed as follows (I.2) [95,101]:

$$DD = 100 - \frac{I_{Ac} / 3}{I_{H2-6} / 6} * 100 \quad (I.2)$$

By FT-IR, different formulae involving the absorbance of the amide band and the hydroxyl band are described in literature [95,98]. X-ray powder analysis [95] as well as elemental analysis [98] can also be employed to estimate the DD.

2.2 Specific antimicrobial properties of chitosans

2.2.1 Modes of action: what we know so far

Chitosan under its cationic form can interfere externally with the cell walls of the microorganisms, as well as internally, causing damages at the DNA level. The mechanism is not totally understood yet and is still being studied. It was said to be dependent according to the microorganisms which was affected by chitosan.

For Gram-positive bacteria, chitosan would act externally, through non-covalent interactions with teichoic acid, an essential molecule for cell development present throughout the cell wall. The alteration of such molecules could be fatal for cells. This mode of action on teichoic acid is said to be predominant for Gram-positive bacteria [80,84] (Figure I.5 (a)).

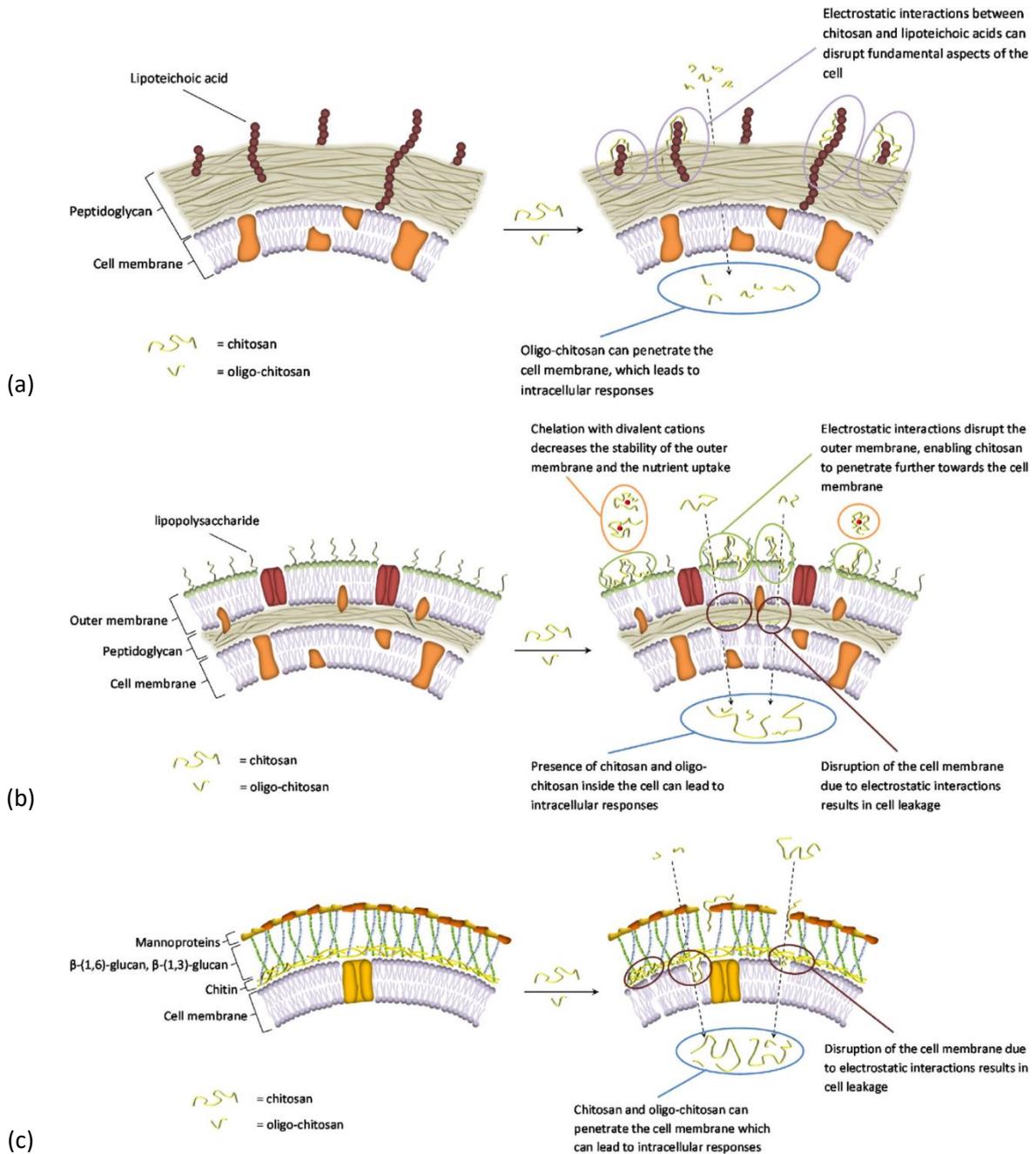


Figure I.5: Effect of chitosan and chitooligosaccharide (oligo-chitosan here) on (a) Gram-positive bacteria; (b) on Gram-negative bacteria; (c) fungi. These three schemes are from Verlee et al. [99].

Mechanisms would be more varied for Gram-negative bacteria and fungi and would include external actions, like electrostatic interactions with membranes, changes in nutrients or gas uptake as well as internal actions. The cell wall of Gram-negative bacteria is made of two negatively charged membranes of phospholipids, separated by peptidoglycans (Figure I.5: (b)).

The fungal cell wall possesses a cell membrane of phospholipids, coated by a layer of chitin, then glucans and finally mannoproteins (Figure I.5: (c)).

Due to electrostatic interactions with positively charged chitosan, cell wall functions would be disturbed, leading to a cell leakage and a loss of intracellular material [70,99,102–105]. These electrostatic interactions are also said to deactivate viruses [102,106]. Chitosan could provoke an efflux of K^+ and an internalisation of H^+ through the inhibition of H^+ -ATPase activity [106]. At higher concentration, chitosan could form a film on bacterial surface. The nutrient [106] and oxygen [104] flux could be blocked, leading to cell death. The coating power may also mask negative charges of cell walls. Newly neutralised microorganisms would aggregate, as no repulsive forces keep them any further away. This flocculation was observed for algae *Microcystis aeruginosa* [97]. The ability of chitosan to chelate metals hinders the development of bacteria and fungi. Complexation of Mg^{2+} and Ca^{2+} is particularly detrimental to cells [103,106].

In addition, low MW chitosans or oligosaccharides might enter the cell and cause intracellular damages. It could flocculate some components and stop their operation. Some authors claimed that a low MW chitosan could enter the fungal nucleus. For both Gram-positive and Gram-negative bacteria, and fungi, it is argued that chitosan can disturb DNA transcription thus blocking mRNA production and protein synthesis [102–106].

2.2.2 Impact of some physicochemical parameters on antimicrobial properties

Chitosan activity strongly depends on the DD but also on the MW and the polydispersity index.

The higher the DD, the more free NH_2 groups compared to acetylated groups there are. Knowing that NH_2 groups are involved in electrostatic interactions when protonated, three Gram-positive and three Gram-negative bacteria [107], *Staphylococcus aureus* [108], *Stenotrophomonas maltophilia*, *Enterobacter cloacae* and *Bacillus subtilis* [109] were more affected by a chitosan with a high DD than by one with a smaller DD.

In addition, the MW strongly influences the antimicrobial properties of chitosan. However, it is still an unpredictable task to correlate the MW and antimicrobial actions. Within fungi, a low MW can be more efficient on a species but less efficient against another [104]. Generally speaking, it seems however that chitosans with a low MW are more efficient towards fungi because some chains act electrostatically at the surface and other chains could enter the cell membrane and act internally [99]. However, it is often difficult to compare the results from different studies, because most MW are those given by the suppliers and therefore they correspond to a MW range and are subject to batch variations. And when the MW is evaluated by the authors of the publications, some problems are generally encountered as said in 2.1.2.

As the charge density is higher at lower pH, all cited antimicrobial properties are accentuated under acidic conditions. Some works are focused on the production of chitosan with a higher positive charge density, for example through the formation of permanent quaternary ammonium groups [110] (not dependent on pH), but these cases are not discussed here.

2.3 Some applications of chitosan

2.3.1 Different formulations depending on the application

The form of chitosan varies according to its application. It can be used as a gel [111–113], especially for biomedical purposes, as a film, particularly for the food/packaging industry, as a complex in solution for detoxification issues and as a matrix of particles in the pharmaceutical, food or agronomical field.

Chitosan is biocompatible and biodegradable, which makes it an excellent candidate for biomedical applications [114–121], but these applications will not be discussed here.

Chitosan solutions can be cast and dried to form films. Chitosan films show hydrophilic properties with a relatively high water permeability [35]. Contrary to vapour, the oxygen transfer is low through chitosan at low relative humidity [102]. Chitosan films may be cross-linked or mixed with fibres (nanocellulose [122], chitin [123], pectin or methylcellulose [102]) to enhance their mechanical properties and to prevent dissolution in water. The widespread cross-linker glutaraldehyde [124,125], not attractive due to its toxicity, can be replaced by divalent metal ions or polyelectrolytes [102]. Properties of chitosan films can be improved thanks to cross-linking with organic acids like citric [126], genipin [127] or tannic acid [128]. These film-forming properties are particularly appealing in the food industry for they can protect fruits from postharvest pathogens, moisture loss and oxidation [93,102,129–131]. They can be used as packaging agent for grapes and honey melons [132] or nectarines [133]. The coating is often enriched with natural extracts, such as an essential oil or a phenolic compound for more antioxidant properties to increase the shelf life of potatoes [134], blueberries [135], peaches [136], kumquats [137] or mangoes [138]. Chitosan treatment of fruits can preserve freshness longer, with a contribution of antimicrobial activities (2.2), film-forming properties and eliciting activities (further described in 2.3.2.b) of 35-45 %, 20-30 % and 30-40 % respectively [139].

The chelation of metals must be added to this already abundant list of promising properties. In the soil, chitosan complexes preferentially $\text{Cu}^{2+} > \text{Hg}^{2+} > \text{Zn}^{2+} > \text{Cd}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+}$ and Ca^{2+} . Chelation occurs between $-\text{NH}_2$ non-binding doublets or sometimes with OH and metals [140], which is useful to clean water from heavy metals for example, such as Cr from the leather industry [97], or to create metal-loaded nanoparticles. The absorption of organic pollutants was also noticed [93], with a chitosan composite to remove toxic dye from the textile industry for example [97].

Chitosan can form particles useful for the encapsulation of a wide variety of compounds (see 4 for other aspects). A special attention is paid to chitosan as a matrix in agriculture, although it is widespread in the pharmaceutical or food [141] industry. Herbicide 3-hydroxy-5-methylisoxazole was encapsulated in chitosan through a microencapsulation in emulsion process. The release was delayed up to 160 hours [142]. Chitosan (120 kDa) was employed to coat beads of atrazine, an herbicide, and urea, on fertiliser. After an initial burst, atrazine was released at 0.02 %/h and urea at 20 %/h extending the release at 6 months. Films of chitosan and alginate were also able to reduce the release rate of atrazine to 1 %/h [143]. In another study, carbendazim, a synthetic fungicide, was encapsulated in cyclodextrin polymers. The resulting complex was further used in composite films of chitosan (106 kDa, DD = 85.6 %). 76 % of carbendazim was released after 48 h from the composite. A seedling oilseed rape stem was 100 % protected from *Sclerotinia sclerotiorum* (Lib.) de Bary until 21 days and 49.4 % after 28 days. The fungicide alone was efficient the first week only [144]. Moreover, nanogels of cashew gum and chitosan (180 kDa, DD = 75 %) were prepared for *Lippia sioides* oil encapsulation. After one day, only 35 % of the initial oil content was released with one system. These nanogels induced a mortality rate of 90 % of mosquito larvae after 3 days [145]. A three in one material, developed by Narayanan and Dhamodharan [146] and made of chitosan and ethylenediaminetetraacetic acid, is capable to release slowly water, urea and complexed metal ions. Other examples of microelements delivery are summarized by Malerba and Cerana [147].

2.3.2 Specific applications for pest control on crops

Chitosan is a very promising agent for pest control. In addition to its direct impact on phytopathogenic microorganisms, chitosan can have eliciting properties by mimicking an infection of pathogens. Other intrinsic effects of chitosan on plant are described.

2.3.2.a Antifungal effects

As a reminder, the modes of action of chitosan on fungi include electrostatic interactions at the surface, chelations of metals such as magnesium or calcium and intracellular mechanisms. Some fungi are less sensitive than others to chitosan. Chitosan-sensitive fungi possess a high-fluid membrane, rich in polyunsaturated free fatty acids, whereas chitosan-resistant fungi membranes are low-fluid and rich in saturated free fatty acids [106,148]. Some examples are summarized in Table I.4. Some authors studied *Fusarium oxysporum*, a pathogenic fungus of mostly fruits and vegetables, responsible of banana wilt or *Fusarium* wilt of tomato. With chitosan at 8 g/L (DD 88 %) dissolved in an agar medium on the Petri dish at pH 5.6, an inhibition of 76 % after 8 days of incubation was obtained [3]. Chitosan at 30 g/L (DD 85 %) also in the medium at pH 5.6 gave an inhibition of 63 % after 7 days of inoculation [132].

Table 1.4: Literature review on antifungal effects of chitosan.

Publication	Chitosan		Form	Added substance	Fungi	Growth reduction
	MW (kDa)	DD (%)				
Alburquenque [149]	70	>75 %	In solution	None	<i>Candida</i> spp.	Inhibition of 72 isolates out of 105 with 4.6 mg/L at pH 4.0
Hernández-Lauzardo [150]	Low, Medium, High	Not mentioned	In solution	None	<i>Rhizopus stolonifer</i>	Partial inhibition for all chitosans at pH 5.6. Best is low MW.
Al-Hetar <i>et al.</i> [3]	Not mentioned	88	In solution	None	<i>Fusarium oxysporum</i>	76 % at 8 g/L, MIC ₅₀ = 1.4 g/L
Ing <i>et al.</i> [4]	70, 310, N-trimethyl chitosan	75-85, 85, Not mentioned	Nanoparticles	None	<i>Candida albicans</i> , <i>Fusarium solani</i> , <i>Aspergillus niger</i>	MIC ₉₀ : MIC _{70kDa} = 0.25-0.86 g/L and MIC _{310kDa} = 0.6-1.0 g/L, MIC _{70kDa} = 0.86-1.2 g/L and MIC _{310kDa} = 0.5-1.2 g/L, No inhibition
Irkin et Guldás [132]	Not mentioned	85	In solution	None	<i>Penicillium chrysogenum</i> , <i>Fusarium oxysporum</i> , <i>Aspergillus parasiticus</i> , <i>A. fumigatus</i> and <i>A. niger</i>	58 %, 63 %, 73 %, 0 %, 0 % at 3 % of chitosan
Bilbao-Sainz <i>et al.</i> [134]	50-190, 310-375	Not mentioned	Film	Alginate and fludioxonil (fungicide)	<i>Fusarium solani</i>	56 % (fungistatic effect)
Ilk <i>et al.</i> [5]	Low MW	75-85	Particles	Lecithin and kaempferol	<i>Fusarium oxysporum</i>	67 %
Sansone <i>et al.</i> [151]	100	95	Particles	<i>Faenonia rockii</i> extract	<i>Candida albicans</i>	MIC ₅₀ : 0.6-0.8 mg/L (twice less than raw extract)
Yang <i>et al.</i> [152]	240	85	Grafted thiosemicarbazide chitosan	Coumarin	<i>Alternaria solani</i> Sorauer, <i>Fusarium oxysporum</i> f.sp. <i>vasinfectum</i> and <i>Fusarium moniliforme</i>	53 %, 77 %, 66 % at 1 g/L (chitosan 10 %)

2.3.2.b *Eliciting properties*

When applied on plant, chitosan is known to elicit plant defences, meaning that it triggers a response in the plant similar to that caused by a pathogen attack.

Chitosan can enter the leaf and reach the cell wall. It is said that chitosan could bind to lecithin receptors and maybe other receptors, known as pattern recognition receptors. Chitosan can also enter the nucleus and bind to chromatin and change DNA conformation. Whether one or both mechanisms occur, and whether they are concomitant or successive is still undefined [93,147]. At a certain point, the arrival of chitosan is transduced into a signal through several messengers, leading to a cascade of events. Different pathogen-related genes are hence activated.

At the cellular level, a calcium influx is observed, probably due to electrostatic interactions with phospholipids of the membrane. Such Ca^{2+} accumulation is linked with callose deposition. Callose are β -(1→3)-D-glucan necessary in plant development, that can be accumulated in case of bacterial, fungal or viral intrusion [106,147]. Callose brakes pathogenic progression between two cells, as it can be seen on Figure I.5. Ca^{2+} are also needed to produce H_2O_2 , a reactive oxygen species known to be induced during plant stress [153]. H_2O_2 is very important for defence-related gene activation as well as for slowing down pathogen progression by stressing it [49]. Chitosan application also provokes a nitric oxide accumulation. Moreover, some pathogen-related phytohormones like organic acids and ethylene are produced. Among others, salicylic acid induces resistance in all the plant and not only where the intrusion occurred. Jasmonic acid regulates defence-response genes. Abscisic acid is for its part linked to callose deposition [72,106,147].

A lot of defence related enzymes are activated, such as phenylalanine ammonia-lyase. It converts L-phenylalanine into trans-cinnamic acid, which is later on transformed into different phenolic compounds. Phenolic compounds are major defence-related secondary metabolites. They are powerful antioxidant [154] and precursors. They can be converted into quinones *via* oxidase to produce H_2O_2 , or into monolignols through polyphenol oxidase. Monolignols are assembled to form lignin, to reinforce cell wall and make it more difficult for a pathogen to enter the plant.

Phytoalexins are also synthesized by a complex enzyme pathway [72,106,147]. Phytoalexins do not correspond to a specific class of molecules, but gather all secondary metabolites associated to plant defences. Playing the role of phytoalexins, phenylamide can be found for wheat [155] and diterpenoids for maize [156].

Enzymes targeting directly pathogens are activated. Chitinase and β -(1→3)-glucanase are able to degrade fungi cell wall components. They are accumulated in grapevine leaves within a few hours [157], in pears [158] or peaches [159], and many other plant tissues [93,106].

To summarise, chitosan eliciting properties are described in Figure I.5. The response to a fungal attack is represented in Figure I.6. Captions for the two figures are presented below:

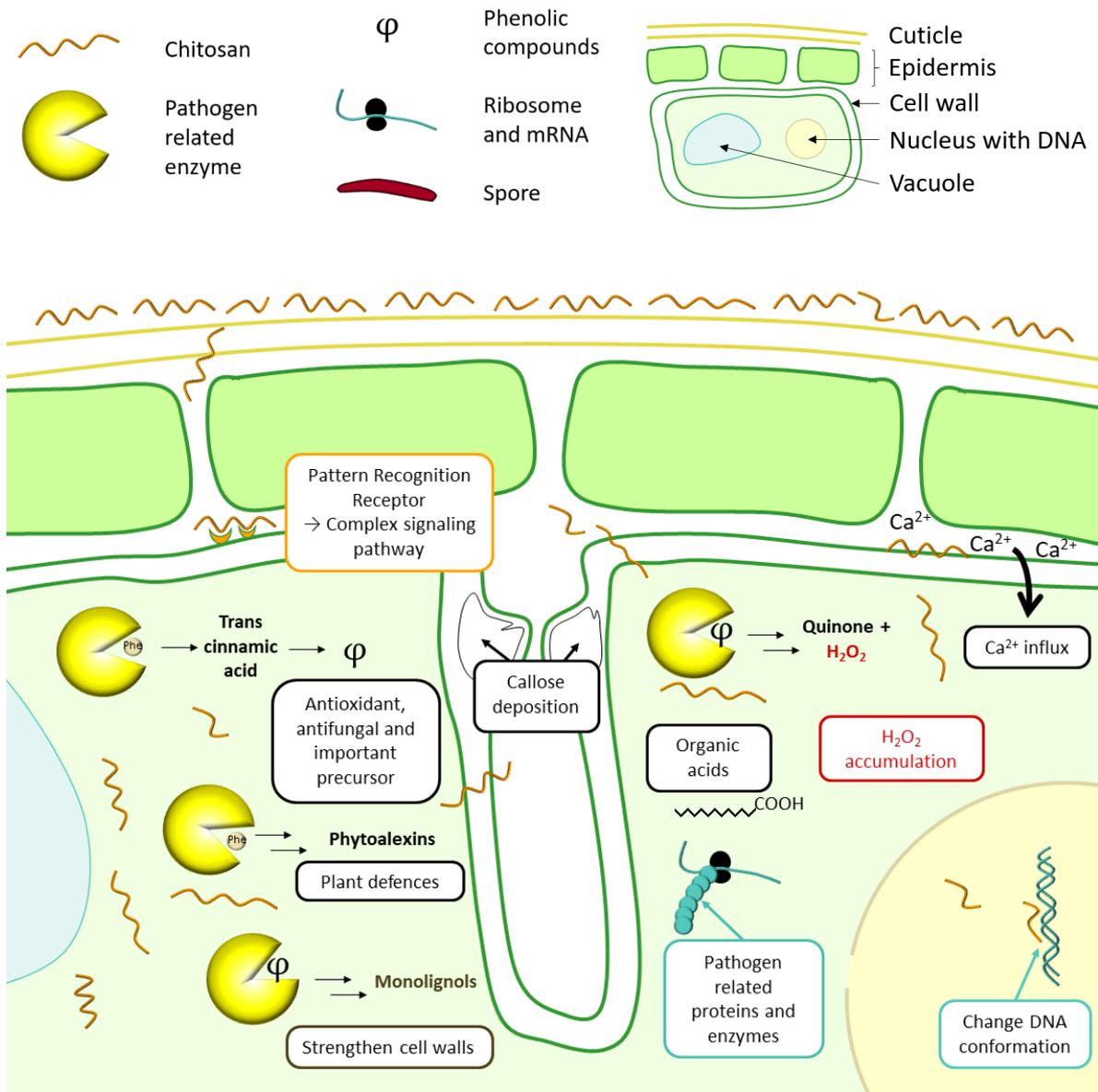


Figure I.5: Possible plant response to chitosan and chitooligosaccharide treatment.

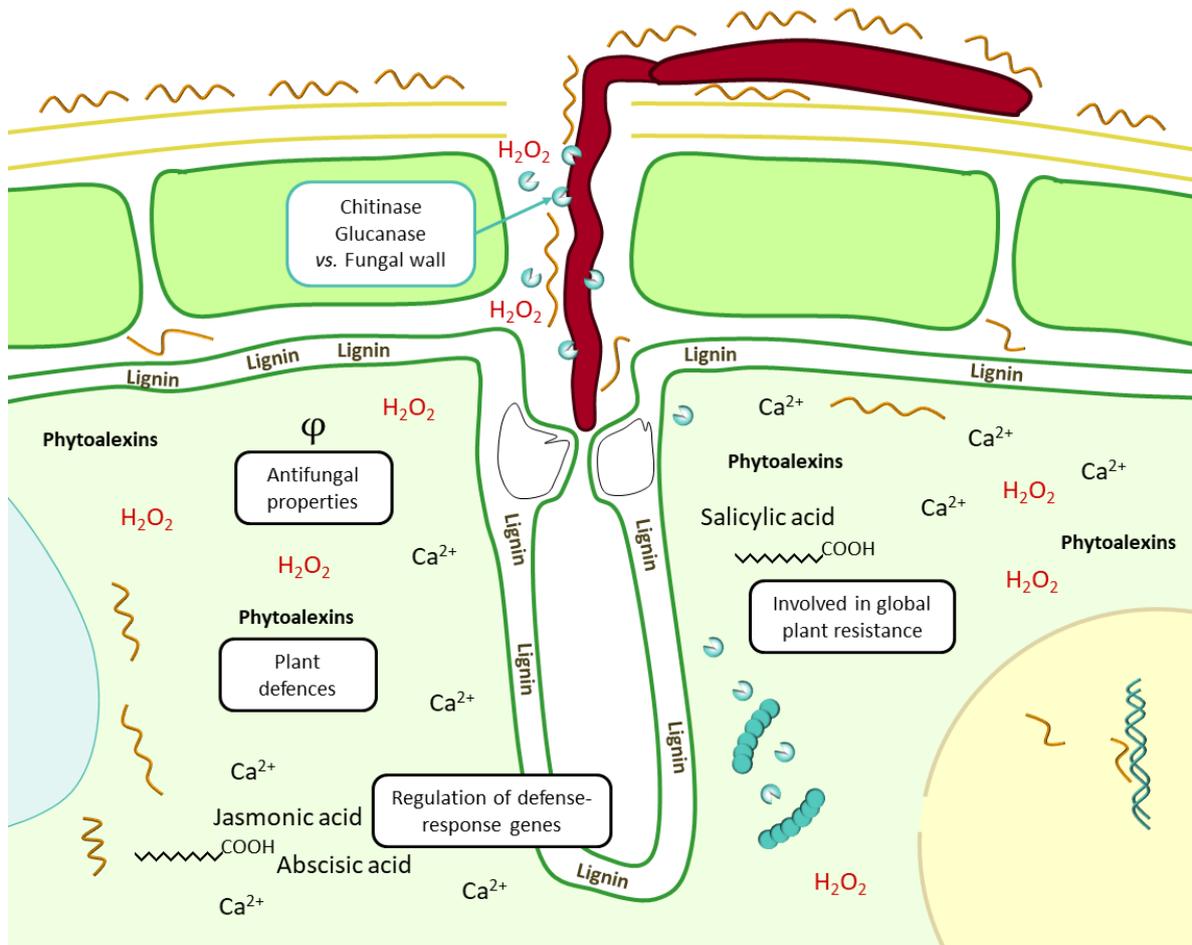


Figure I.6: Possible mechanisms of plant resistance to fungi after chitosan and chitoooligosaccharide application.

2.3.2.c Two other beneficial effects on the plant

In addition to previous properties, chitosan could help the plant to overcome drought stress. It was proved that a prevention spraying of chitosan on potato leaves was beneficial for osmotic regulatory substances and strengthened the recovery after the drought period [160]. Chitosan possesses anti-transpirant properties, due to the partial H_2O_2 -mediated stomata closure [93], monitored by the phytohormone abscisic acid [161]. It leads to a better water use and a reduction of transpiration.

Growth and yield improvements were shown for cereals, vegetables and fruits [93,147]. For example, four varieties of wheat were treated with chitoooligosaccharides, either rain-watered or irrigated. The yield and number of grains per spikelet were enhanced for irrigated crops but no effect was seen on non-irrigated fields. Change in proteins and gluten content was noticed [162].

2.4 Concluding remarks

Chitosan is an easily-available polymer potentially produced from bio-wastes and generally recognized as safe. No major risk, except digestive discomfort, were found in Humans at a dose

below 2 g/j [163]. Physicochemical properties, such as the possibility to form aqueous solutions, electrolytes or films, make it suitable for a wide range of formulations. In addition, antimicrobial and eliciting activities make chitosan a very promising biopolymer for biopesticide formulations in agriculture.

Beside chitosan, some other biomolecules may also be of interest for such applications. Particularly, some natural products extracted from plants have shown activity against microorganisms and their production of toxins. Thus, the next part deals with natural extracts, especially with phenolic compounds, and a specific molecule called tetrahydrocurcumin.

3 Plant extracts: active molecules with antifungal and antioxidant properties

Plants naturally possess antipathogenic compounds acquired throughout the evolution. These active compounds demonstrate direct damaging effects on the pathogens as well as global protecting effects on the cells and thus, could be used in antifungal formulations.

Because it is such an important and vast topic, this part is devoted to active compounds from natural extracts, with a particular attention given on phenolic compounds and tetrahydrocurcumin (THC), a curcumin derivative. For each class of active compounds, a focus is made on antifungal and antimycotoxigenic properties, as well as on general antioxidant properties which are linked to direct damaging actions on fungi.

3.1 Plant extracts

3.1.1 Three principal classes of plant extracts

Plant extracts are a mixture of plant secondary metabolites. They differ from primary metabolites, which are found in almost every living cells and whose deficiency would immediately lead to cell death. Their definition remains unclear because some necessary compounds are considered to be secondary and vice versa. Biosynthetic pathways of primary and secondary metabolites are intertwined. Secondary metabolites are unequally distributed into living organisms; some are biosynthesised by only one species while others are found in different orders. They have a defence and signalling role for interaction, symbiosis or competition between organisms as well as a role of metal transport [164]. They are divided into three principal categories: alkaloids, terpenes and phenolic compounds, which are presented in the following paragraphs.

According to Pelletier [165], alkaloids are “cyclic organic compounds containing nitrogen in a negative oxidation state which is of limited distribution among living organisms”. This definition derived from the initial meaning of “alkali-like compounds” (basic compounds), but is still very wide. They can be produced by plants, fungi, bacteria or animals. The group of alkaloids is very heterogeneous, from nicotine to caffeine or the anti-cancer vinblastine.

Terpenes are molecules derived from one or more isoprene unit, which has a five carbon backbone (Figure I.7 (a)). They are classed according to the number of terpene units, corresponding to two isoprenes [166]. Terpenes are only composed by carbons and hydrogens, whereas terpene derivatives have oxygen atoms and can be linear or cyclic. They possess numerous and various medical applications. For example, some essential oils are mainly composed of terpenes, especially monoterpenes such as menthone, citral, citronellal, limonene, pinene... They are also useful as flavour or perfume in food or cosmetic industry. Sesquiterpenes, which possess three isoprene units, *i.e.* one and a half terpene units, can demonstrate antibacterial and medicinal properties. Vitamin A and all carotenes are diterpenes (Figure I.7 (b)). Triterpenes represent a high percentage of plant lipids [167].

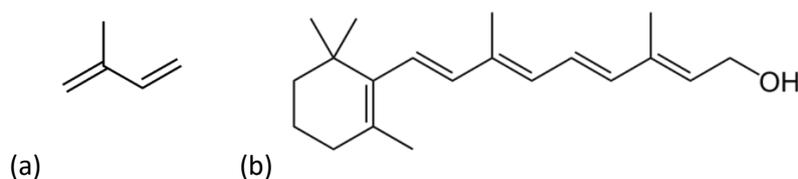


Figure I.7: Structure of (a) isoprene and (b) Vitamin A (diterpene).

Finally, phenolic compounds are synthesized by every vascular plants [168]. They are organic compounds composed of at least one aromatic ring, with at least one hydroxyl group attached to one ring. They are responsible for plant odour, flavour and pigmentation [169]. They are cellular sentinels against oxidative species.

A detailed paragraph will be dedicated to phenolic compounds (3.2) further in this chapter.

3.1.2 Extraction processes

Plant extracts are used, and therefore extracted, for centuries. Techniques rely on different solvents, processes, time of extraction, etc. and have evolved over the decades.

Various well-known extraction processes are commonly found. Maceration is when finely cut plant parts are mixed with a solvent in a close reactor. The plant extract is recovered after filtration and removal of any residual solvent [170]. Percolation is almost like maceration but with

change of solvent during the extraction. Decoction is also similar to maceration but the solvent is heated [171]. Enfleurage is not used anymore, but used to give a fragrance to an animal or a vegetable fat. Plants were put on the surface of a fat and extracts were diffusing into it.

More recently, techniques like reflux extraction, with organic solvent or water, have been developed. Steam extraction is also a widespread method [170]. For the solvent or steam extraction, extracts are driven by vapours, which are later condensed. The processes can be long (several days) and consume solvent. Pressurized liquid extraction overcomes these drawbacks. Soxhlet extraction can also reduce time and always requires pure solvent.

To replace liquid organic solvents, which are problematic regarding environmental aspects, supercritical fluids are being used more and more. For instance, CO₂ supercritical extraction is used to extract components from wood [172,173], extracting apolar to slightly polar compounds, under relatively mild conditions. Liquid or solid extracts are recovered directly after extraction, as the gas expands. Similarly, a promising technique, the extraction by subcritical water, can also be applied to polar and slightly apolar compounds [174]. This is much more cost-effective and has less impact on the environment than CO₂-subcritical extraction [175]. This represents a safe and solvent-free alternative [176], where the water is heated between 100 °C and 374 °C in a reactor pressurised between 10 bars and 220 bars. For example, phenolic compounds have been extracted with this method from various natural sources, at an optimal extraction temperature around 200 °C. To name a few tannins and flavonols were extracted from pistachio [177], sugar, proteins and phenolics from ginseng [178], non-polar flavonoids from orange peels and phenolic products from green kiwifruits [179].

Other techniques like microwave, ultrasound, enzyme assisted extractions or pulsed electric field extraction may also be employed [171].

3.1.3 Properties of plant extracts

Natural compounds, such as terpenes, terpenoids or phenolic compounds are efficient both against the fungal growth and the production of toxins. Their actions can be explained by direct lipophilic or electrostatic interactions, with enzymes, cellular membranes or organelles. Antioxidant properties of such compounds, especially phenolic ones, may be directly linked to the reduction of toxins. The modes of action of plant extracts against fungi are summarised in Figure I.8 and will be described in the next parts.

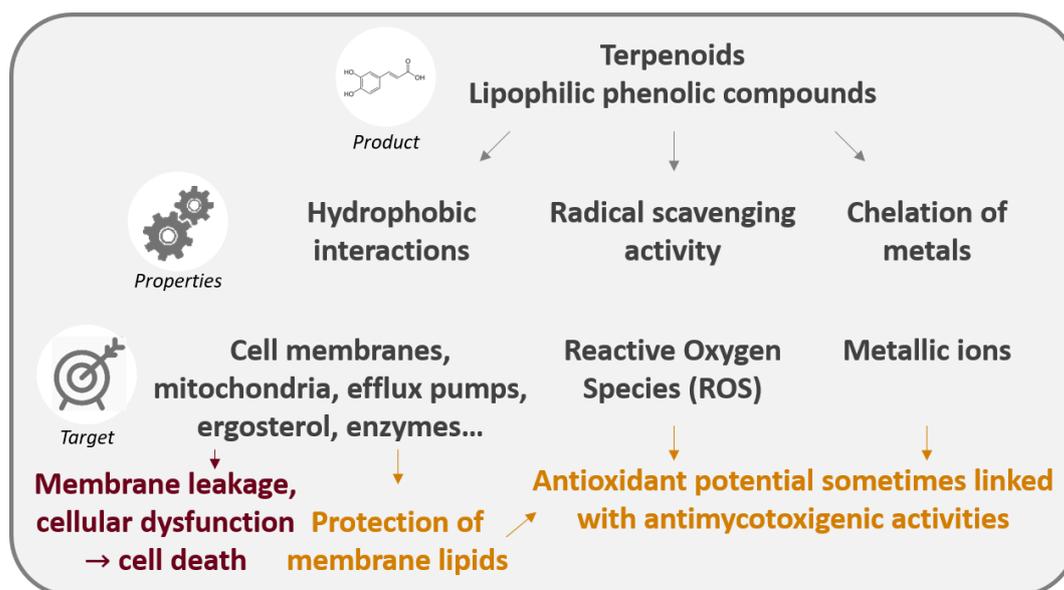


Figure I.8: Lipophilic and antioxidant properties of natural compounds against the fungi.

3.1.3.a Lipophilic properties linked with the antifungal action

A broad range of essential oils were proved to inhibit the growth of fungi [180,181]. For example in [182], eight essential oils inhibited the growth of *F. graminearum* and *F. culmorum*. In other works, the essential oil of *Eucalyptus camaldulensis* was efficient against five *Fusaria* (MIC from 7–8 mL/L) [183]. Phenols are suggested to be the most active components of essential oils, followed by aldehydes, ketones, alcohols, ethers and hydrocarbons [180].

The lipophilic nature and the low molecular weight of terpenes, terpenoids and some phenolic compounds in essential oils are known to be responsible of their antifungal properties. The cell membrane of fungi can be disrupted, leading to cell death, inhibition of sporulation or germination. In addition, some morphological changes had been observed [181]. For example, anethole caused a growth reduction of *Mucor mucedo*, with a MIC at 0.625 mM, and swollen hyphae at the tip, linked to inhibition of chitin synthase [184]. Membrane damages were also reported for *Candida albicans* treated with a tea tree oil [185] or for *Aspergillus niger* grown with a *Citrus sinensis* essential oil [186]. As another example, the phenolic compound (–)-nortrachelogenin caused the dissipation of the cytoplasmic membrane or the cell apoptosis, *via* the depolarisation of mitochondrial membranes of *C. albicans* [187]. One other mode of action on cell walls can be the interaction with ergosterol, a fundamental molecule for fungi located at the membrane surface. The essential oil of *Curcuma longa* [188] and eugenol and methyleugenol [189] were shown to decrease the ergosterol synthesis by *Aspergillus flavus* and *Candida* spp., respectively. In addition, essential oils can disrupt organelle function, such as mitochondria, or modify proton pumps, leading to acidification [181,188]. Other transporter pumps like the efflux pump of drugs can be affected too, leading to an accumulation of damaging compounds within the cells [190]. To summarise, Peng *et*

al. [191] hypothesised possible chain reactions by *Penicillium italicum* treated with pinocembrin extracted from propolis. They observed that pinocembrin disturbed the mycelial respiration and the mitochondrial respiration. This mitochondrial deficiency led to an energy deficit, marked by a lower content of ATP. This latter could cause lipid peroxidation [192], further leading to membrane disruption and later to the leakage of cellular contents, accelerating cell death.

3.1.3.b *Intrinsic antioxidant properties*

Secondary metabolites found in extracts are well-known to exhibit strong antioxidant properties. One might wonder why plants need a stock of antioxidant molecules in cells. In fact, they scavenge reactive oxygen species (ROS), which are synthesized in small amounts during normal functioning [193]. However, they are also produced during an oxidative burst, in response to a pathogen attack, like bacteria, fungi, viruses or insects, or a change in environmental conditions such as heat, drought, UV-radiations or presence of metal ions. ROS gather oxygen radicals like $O_2^{\cdot-}$, HO^{\cdot} and even H_2O_2 , which is a non-radical molecule [194]. H_2O_2 is well known to contribute to the production of oxidizing species through the Fenton's reaction (I.3):



In the case of pathogen attacks, enzymes located inside the cell wall can produce ROS, which are directly released in intercellular domains to fight undesirable intruders. However, an excessive amount of ROS, which is called "an oxidative stress", leads to proteins cuts or dimerization, DNA damages as well as oxidations of polyunsaturated fatty acids of membranes causing cell leakage and death [194]. Therefore, plants need antioxidant molecules to counterbalance the possible over-production of ROS.

Phenolic compounds are considered to be the most active antioxidant class of secondary metabolites. For example in [195], although yellow-flesh nectarines and peaches contain ten times more carotenoids than white-flesh ones, they have less radical scavenging activity. This antioxidant potential is even stronger for plums, which possess the highest content of phenolics among all tested fruits. The same correlation between radical scavenging activity and phenolic compounds content was found in barley, especially with flavan-3-ol [196]. In some cases, the antioxidant ability can be attributed to other compounds. Almost all antioxidant properties of non-citrus based beverages are due to phenolic compounds, but these are driven by vitamin C (organic acid) in citrus-based juice [197]. Essential oils have antioxidant properties but only some of them contain phenolic compounds in significant quantities [198]. Basil, clove, nutmeg, oregano or thyme essential oil for example, are not primarily made of phenolic compounds [199]. *Thymus* spp. oils displayed a strong antioxidant activity, although they are composed of terpenoids, like linalool or 1,8-cineole (eucalyptol) [200].

Baschieri *et al.* [201] highlighted two distinct modes of action for phenolic compounds and terpenes. These mechanisms are summarised on Figure I.9 (b) and (c), along with the mechanism of substrate degradation by radicals (a).

Phenolic compounds react easily with radicals, to form more stable phenolic radicals. These latter will come across other radicals, thus terminating the propagation chain and protecting the substrate (DNA, proteins...). This mechanism remains possible while there are still enough phenolic compounds (Figure I.9). On the other hand, terpenes were shown to act differently. Limonene, linalool and citrate reacted in a smaller extent with radicals and were actually oxidised. Even in presence of terpenes, the degradation of the substrate happens since reactions (c) and (a) occur simultaneously. We speak of an antioxidant effect when reaction (c) is predominant over reaction (a). One counter-part of this kind of mechanism is that terpenes can act as prooxidant, if they are present in a too large quantity. It can be understood since the reaction (c) increases the dioxygen uptake in the medium. Moreover, their efficiency is difficult to predict, as it evolved in a non-linear way.

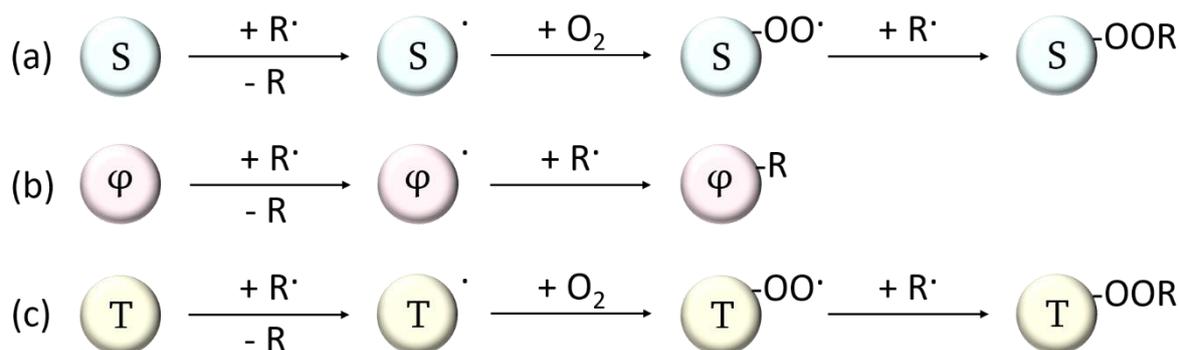


Figure I.9: Effect of a free radical on (a) substrate that must be protected (S), (b) phenolic compound (φ) and (c) terpene (T).

What is important for us is that these antioxidant properties are suspected to contribute to antifungal and antimycotoxigenic actions of plant extracts. The antioxidant properties has been shown to be linked with antifungal effects of essential oils [181]. For example, the bioactivity of phenols and flavonoids in maritime pine bark extracts [202] and of phenolic compounds in three *Pinus* extracts [203] was correlated to their antioxidant activity.

As far as TCTBs are concerned, it has been highlighted that the oxidative stress induces radical changes in the fungal phenotype and influences genes responsible for the production of toxins [11,12,204]. Antioxidant molecules could therefore help to counterbalance an oxidative stress, thus contributing to the reduction of the toxin accumulation. Many correlations between the radical-scavenging activity of a compound and the reduction of the production of toxins have been demonstrated [12,204]. For instance, 10 g/L of the antioxidant *Curcuma longa* essential oil inhibited the growth of *F. verticillioides* by 80 % and the production of fumonisins by more than 100 times

[205]. Culture filtrates of *Lentinula edodes* were found to be antioxidant and to inhibit the aflatoxin production by *Aspergillus parasiticus* [206]. Moreover, several essential oils reduced the production of zearalenone by *F. culmorum* and all of them inhibited the TCTB accumulation by *F. graminearum* [182].

A detailed paragraph in 3.2.2.b is dedicated to antioxidant properties of phenolic compounds on fungi.

3.2 Phenolic compounds from plant extracts

3.2.1 Structure of phenolic compounds

Phenolic compounds are synthesized *via* the phenylpropanoid acid pathway [207]. Phenylalanine is converted into cinnamic acid and then into phenolic acid as explained in 2.3.2.b.

Phenolic compounds are gathered into classes corresponding to their structures and functions (Figure I.10):

- Flavonoids are composed of at least 15 carbon atoms, forming two phenolic rings linked by a bridge. They are one of the most studied class of phenolic compounds for anti-microbial and therapeutic properties [208]. Flavonoids are found in vacuoles of plant epithelial cells and protect them from UV irradiation [194].
- Coumarins are used for bio-medical applications [169]. They are toxic for herbivores and possess a bitter taste, which is a natural repellent for insects and herbivores [194].
- Tannins are employed to convert hides into leather. They prevent hardening and bring mechanical resistance [209]. Powder from oak bark, chestnut or valonea are rich in tannins and can be used for vegetable-tanning [210].
- The most known stilbene is resveratrol. It is found in grapes and red wine, peanut, soy, Itadori tea [211] and berries. It is studied for its antioxidant properties [169] and applications in medicine too.
- Quinones are brown-coloured compounds. They can bind to membranes and are therefore toxic for micro-organisms and humans [169].
- Phenolic acids are hydroxycinnamic acids or benzoic acids, composed of one phenolic ring and a carboxylic moiety. In wheat for example, phenolic acids are usually bound in the cell wall to polysaccharides, such as hemicellulose, or to lignin by the mean of ester or ether bonds [207,212]. Free, they are found in the vacuole. The predominant phenolic acids are ferulic acid (60-65 % in maize for example), *p*-coumaric acid and vanillic acid. Ferulic acids can form dehydrodimers, which represent 20-30 % of phenolic acids in

maize [207]. They are sensitive to high pH and often exhibit a low solubility in water [169], and therefore requires encapsulation strategies to be used efficiently as additive.

- Lignans are precursors of lignin, but they are also used in medicinal applications and as enzyme inhibitors [168].

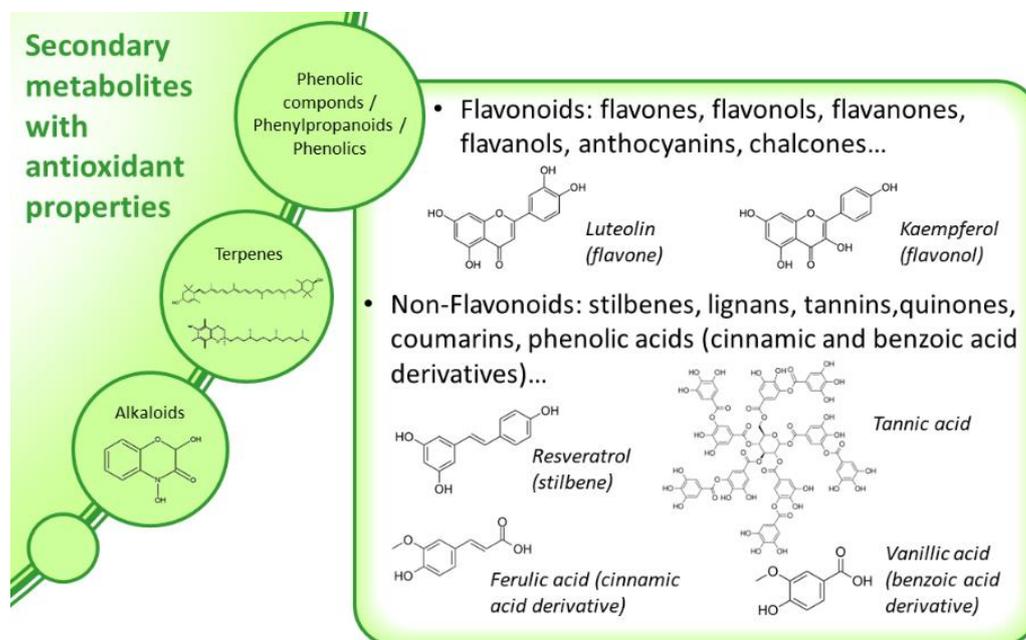


Figure I.10: Some examples of secondary metabolites.

3.2.2 Properties of interest of phenolic compounds

3.2.2.a Antifungal actions

Numerous studies have recently revealed the effects of phenolic compounds against the growth of fungi.

Fungal growth has been shown to be more efficiently reduced by phenolic acids than by flavonoids [207]. In the work of Ponts *et al.* [213], different strains of *F. graminearum* were inhibited by phenolic acids. The concentration at which the mycelial growth is reduced by 50 % is noted IC_{50} and is commonly used to quantify and compare the effectiveness of substances. The IC_{50} of ferulic acid, caffeic acid and *p*-coumaric against *F. graminearum* are respectively equal to 0.7–2.2 mM, 4.0–7.1 mM and 1.0–4.2 mM. In another work, IC_{50} of benzoic acid derivatives higher than those of cinnamic acid derivatives were evidenced, with values from 6.6 mM to up to 15 mM for *p*-hydroxybenzoic acid and 3.5–6.2 mM for syringic acid. Caffeic acid was also shown to decrease the mycelium growth of *F. graminearum* (IC_{50} = 6.7–10 mM) and *F. culmorum* (IC_{50} = 8.8–10 mM) [214]. Similarly, the growth of *Fusarium verticillioides* was inhibited by phenolic acids and non-flavonoids from wood in [215]. An IC_{50} of 50 mg/L was found for both caffeic and vanillic acids,

while the ones of maakianin and chlorophorin were respectively of 3.13 mg/L and 6.25 mg/L. Finally, in another work, the growth of six different species of *Fusarium* was reduced by phenolic acids, whose effectiveness followed this order: ferulic > *p*-coumaric > caffeic > chlorogenic [216].

Mechanisms involved in the growth inhibition are multiple. The action of phenolic compounds against the fungal growth is likely to be due to direct interactions, lipophilic or electrostatic. Indeed, phenolics can interact with exoenzymes produced by fungi to degrade their environment into nutrients and therefore disturb their food uptake [217]. Moreover, phenolic extracts of beans, containing chlorogenic and gallic acids, inhibit fungal α -amylase, a starch-degrading enzyme [218]. Perturbation of the fungal membrane was also observed [219], that can lead to a cellular leakage [207]. For example, ferulic acid and gallic acid can change the hydrophobicity and decrease the negative charges of the membrane [220].

3.2.2.b *Antimycotoxigenic action and antioxidant properties*

In plants, the main abilities of phenolic compounds as antioxidant species are to directly trap radicals, as described in 3.1.3.b, but also to regulate prooxidant proteins and to chelate metal ions, which is typical of phenolics.

Indeed, some phenolics can bind with proteins or phospholipids on membrane surface and inhibit membrane lipid peroxidation [194]. It is also well-known that interactions occur between phenolic compounds and proteins, with H-bonds or covalently by oxidation and condensation [221,222]. Molecular complexes enable a regulation of prooxidant proteins, such as xanthine oxidase, which catalyse superoxide radical production [168,223].

Phenolic compounds can also chelate metallic ions, especially when phenols are deprotonated [168]. Complexation of redox-active transition metals can reduce the occurrence of the Fenton's reaction (I.3) and therefore the production of ROS. Iron can be complexed by phenolic compounds such as catechins [224], sinapic acid or gallic acid [225]. Heavy metal ions or toxic ions can be chelated as well [194]. A study revealed that amounts of catechol, catechin, curcumin and quercetin increased in maize root tips in response to aluminium treatment. This accumulation was found higher in Al-resistant varieties, suggesting that these compounds constitute an efficient detoxification strategy [226].

As described earlier in 3.1.3.b, the accumulation of toxins can be part of the fungal stress response [11,12,204]. The quenching of ROS that helps reducing the stress is thus a potential mode of action contributing to the reduction of the biosynthesis of mycotoxins [13,207].

Many studies revealing the impact of phenolic compounds on the production of mycotoxins are available. By introducing 0.5 mM of caffeic acid, the TCTB accumulation in *F. graminearum* growth medium can be reduced by 74 % after 7 days and 52 % after 14 days [227]. The same

concentration of ferulic acid decrease by 90 % the toxin accumulation after 10 days. Finally, 2.5 mM of ferulic acid can inhibit totally the production of toxins, while 0.1 mM can already reduce it by 68 %. We can add that these results were found independent of whether the inhibitor product was applied on the day of inoculation or 3 days after. In addition when the product was applied twice, a cumulative effect was observed [13].

To cite few more works, Fumonisin B1 accumulation in *F. verticillioides* was shown to be inhibited by 90 % with 1 mg/L (~6 µM) of ferulic, caffeic or vanillic acid and by 94 % with chlorophorin [215]. Fumonisin production from *F. sporotrichioides* was also reduced with 10 mM of chlorogenic acid [216].

In [207], the change in the accumulation of mycotoxins was linked to a modification on the gene expression. A lower expression of a cluster of genes responsible of TCTB biosynthesis by *F. graminearum* was also observed in the presence of ferulic acid in [13]. Finally, as the production of mycotoxins occurs during a stress, the H₂O₂ addition in [228] also led to an increased gene expression related to the TCT production.

Despite all these studies, the efficiency of phenolic compounds regarding the accumulation of mycotoxins remains controverted. For example, Ferruz *et al.* [216] did not observe any significant change in the fumonisins or TCTB production in different *Fusarium* species with four different tested phenolic acids. In another work, they observed two opposite trends, with an inhibition of the TCTA production with ferulic acid and an activation with *p*-coumaric acid [229]. Different responses was also seen for *Fusarium* species, depending on the class of the phenolic compounds (flavonoids [230,231] or non-flavonoids [230]) and the toxins.

3.3 Specific case of tetrahydrocurcumin

3.3.1 A curcumin derivative

Curcumin (Figure I.12 (a)) is a diarylheptanoid found in *Curcuma longa* L. rhizomes (turmeric), a plant cultivated in Southeast Asia. It is commonly found in powder form which results from the rhizome grinding and is used as a spicy in Indian food. This yellowish powder is also employed in traditional medicine, as well as in cosmetic and in the food industry [232]. Powders extracted from turmeric are essentially composed of curcumin, but also of bisdemethoxycurcumin and demethoxycurcumin (Figure I.11).

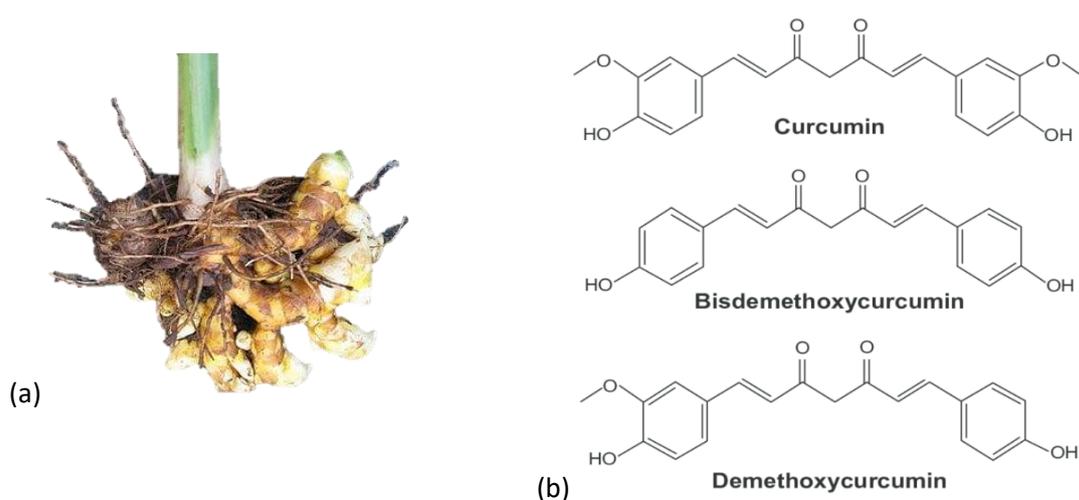


Figure I.11: Rhizomes of (a) turmeric and (b) molecules extracted from it.

The tetrahydrogenated product of curcumin is tetrahydrocurcumin (THC) (Figure I.12 (b)), which is naturally produced by *Zingiber officinale* in small quantities [233–235]. Since curcumin is not stable, it can spontaneously degrade into THC. Therefore, when curcumin is ingested, THC is then found in the plasma [236], and has also been found in the intestinal tract of humans, rats [237] or mice [238].

THC can be obtained as a result of the degradation of curcumin by fungi, like *Alternaria alternata*, *Cunninghamella* spp. or *Penicillium brevicompactum* [239], by yeast like *Pichia kudriavzevii* [240] and even by a cultured cell plant *Marchandia polymorpha* [241].

THC can be commonly synthesized thanks to a two-step process. The first step consists in the synthesis of curcumin from vanillin and boron protected 2,4-pentanedione by a Knoevenagel addition, as described by Krackov and Bellis [242] (which follows older procedures [243,244]). The second step consists in the reductions of the two alkene functions with either platinum dioxide [245], platinum on carbon [246], palladium on carbon [247] or activated Raney nickel [248].

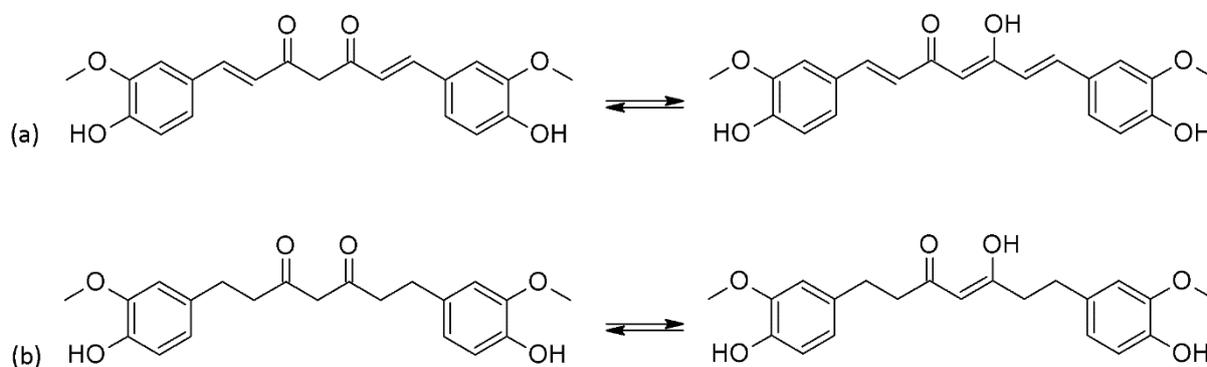


Figure I.12: Keto-enol forms of (a) curcumin and (b) tetrahydrocurcumin.

THC in its solid state is stabilised with H-bonds and interactions between CH and phenolic rings. THC molecules are twisted so that both rings are orthogonal [249]. In solution, THC can be found under a diketone form or a keto-enol form, which is stabilised by an internal H-bond. The tautomeric equilibrium can be shifted towards the ketone or the keto-enol form depending on the solvent. This equilibrium can be reached after 48 h for example in CDCl_3 [232], with a predominance of the enol form.

Regarding the physicochemical properties, the melting point of THC is $98\text{ }^\circ\text{C}$. Unlike curcumin, which is orange, THC crystals, in their keto-enol forms, have the appearance of a white to slightly yellowish powder. This property is particularly interesting and used in the food industry.

THC exhibits antioxidant properties, which can be measured by the ability to scavenge 2,2-diphenyl-1-picrylhydrazyl radical (DPPH \cdot). The “half maximal inhibitory concentration”, noted $\text{IC}_{50, \text{DPPH}}$ and used to quantify this antioxidant strength, corresponds to the concentration for which the number of radicals is reduced by 50 %. The lower this value, the stronger the antioxidant effect. THC was found to be as antioxidant as curcumin but other studies suggested even stronger antioxidant activities [250,251]. In reference [252], THC was also compared to zingerone, a molecule extracted from *Z. officinale*, whose structure is approximately that of half the THC (Figure I.13). For 1 g/L of DPPH \cdot ($\sim 2.5\text{ mM}$), the IC_{50} was found to be $17\text{ }\mu\text{M}$ for THC and $42\text{ }\mu\text{M}$ for zingerone, emphasizing the antioxidant strength of the THC molecule.

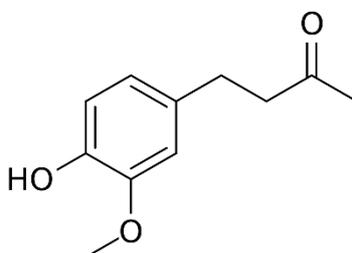


Figure I.13: Structure of zingerone.

3.3.2 Anti-microbial properties

As THC is a phenolic compound, the mechanisms involved against bacteria and fungi development are expected to be the same, namely a direct membrane or metabolites disturbance, antioxidant properties through radical scavenging, chelation of metal, change in ROS-related protein expression. Antimicrobial properties of THC have not been detailed in the literature. However, turmeric showed antifungal properties against human-pathogenic fungi or bacteria, such as *Staphylococcus* spp., *Escherichia coli*, *Pseudomonas* spp. [253] or plant fungi like *Botrytis cinerea* or

Rhizoctonia solani [254]. Curcumin potential against various *Candida* strains was evaluated by different authors, who reported a reduction of the cell viability in biofilms and an inhibition of the fungal growth depending on the concentrations [255–257]. It can also be highlighted that two curcumin-based formulations were also recently patented. The first one is composed of curcumin and solvent and targets pests, insects and fungi on seeds or plants [258]. The second one is made of chitosan, biological gelatin, citric acid, curcumin, chitosan oligosaccharide and water and was designed for preventing fruit rotting [259].

On the other hand, the growth of *Fusarium proliferatum* was shown to be inhibited by 67 % as a result of the addition of 14 μM of THC in the culture medium. Moreover, fumonisin accumulation was reduced by up to 75 % with 2 μM [6].

Nevertheless, despite this promising above-mentioned studies, no application of THC in agriculture have been reported to this day. Biomedicine remains the field where the THC is the more widely used, with a proven anti-inflammatory, anti-cancer, anti-diabetic and neurologic effect [251].

3.4 Concluding remarks

Natural extracts are highly valuable plant components, which frequently possess antioxidant properties as well as antifungal and antimycotoxigenic effects. The particular classes of phenolic compounds or terpenes present a great potential for controlling pest in agriculture although the scientific literature on this topic is rather limited. In this context, the curcumin derivative THC is a promising substance, taking into account its positive action to control the production of mycotoxins *in vitro*.

However, the hydrophobic character of molecules such as THC leads to a major drawback that is their poor bioavailability due to their very low solubility in water. Encapsulation is the traditional way to overcome this issue. This is why the next part focuses on the encapsulation of poorly water-soluble compounds for improving their solubility and stability in aqueous-based medium.

4 Encapsulation techniques of poorly water-soluble compounds

To deal with scarcely water-soluble active components, dispersion techniques have been developed. Reduction of drug size, nanosuspension, surfactant addition, salt formation and encapsulation can solve the solubility problem [260,261]. This part is only dedicated to

encapsulation processes and the following examples are mainly focused on systems encapsulating phenolic compounds.

4.1 What is encapsulation?

Encapsulation is a process whereby a solid, a liquid or a gas is entrapped in a matrix material. Particles, capsules, spheres or aggregates that are obtained can range from the micro- to the nano-scale. Both coated and coating materials can be pure or not, natural or synthetic.

Two different natures of particles can be distinguished and are shown on Figure I.14 [168,262–264]. The core-shell structure is characterized by a mononuclear distribution of the active compounds coated by the matrix. The wall material itself can be composed of a multi-layered assembly. The second organisation corresponds to the case where the encapsulated compounds are homogeneously dispersed in the matrix corresponding to the host material.

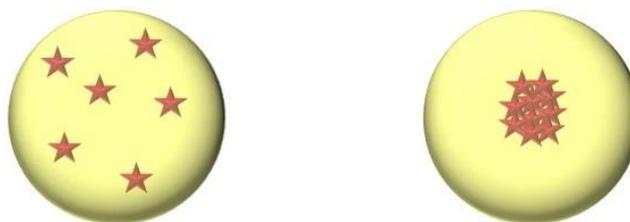


Figure I.14: Schematic representation of the two main morphologies of particles (left) Matrix structure (right) Core-shell structure.

Encapsulation is mostly used in the pharmaceutical field and the food industry. The active ingredient is protected by encapsulation from the oxidation or irradiation that can occur during the processing and storage. For health applications, the bioavailability of active compounds can be raised. The first benefits are the protection from degradation by enzyme or from pH conditions. It is also possible to control the release of the active material under specific conditions. The products can for example be delivered in a targeted site of the body. Encapsulation can also help the absorption of an active compound by overcoming the raw material low permeability or low water-solubility.

Encapsulation is also a way to change physicochemical properties of the guest compound. As already mentioned, the solubility in water can be raised, the state (liquid → solid) can be modified, as well as the density and the hydrophobicity. An unpleasant taste can also be screened by a coated compound, which is used in the food industry. Finally, the separation of two incompatible products can also be made possible by encapsulating one of them.

4.2 Characterization of particles

The efficiency of a process is assessed by different parameters. The morphology and the size of particles are one of the first features to be verified. Microscopic techniques are performed to determine the particle shapes, sizes and distributions as well as the presence of undesired materials [119,141,145,151,265–267]. This technique is often combined to the determination of the average charge of particles with ζ -potential [120,262]. To determine if synthesized particles are crystalline or amorphous, X-Ray Diffraction is carried out [17,119,265,268]. Once these particle parameters determined, one can look for a proof of the proper encapsulation of the compound.

Regarding the encapsulation, the trapped compound undergoes a modification of its interaction with its environment due to the presence of the matrix around it. NMR or FT-IR Spectroscopy can be therefore used to track changes in properties. NMR is often used to see a shift that protons undergo when the particle is formed [5,268]. Especially for inclusion complexes (see 4.4.2), rotating-frame Overhauser enhancement spectroscopy (ROESY) can detect interactions between the encapsulating agent and the encapsulated molecule very accurately [14,266,269]. Raman spectroscopy can also be employed [17,266,270].

This qualitative information is completed by precise measurements of the quantity of material encapsulated within the particles. For this purpose, Ultraviolet (UV)-visible spectroscopy is almost always used, because products subject to encapsulation often possess aromatic ring(s), which confer them UV or even visible light absorption. High pressure liquid chromatography (HPLC) was also performed in various works [121,151,268].

Encapsulation Efficiency (EE) and loading capacity (LC) can be defined as follows:

$$EE \% = \frac{N}{N_0} * 100 \quad (I.4)$$

$$LC \% = \frac{N}{m_0} * 100 \quad (I.5)$$

N represents the amount of loaded component determined by dosage (UV or HPLC), N_0 is the amount of ingredient put at the beginning, in other words, the maximal amount that can be loaded [267]. m_0 is the total mass of the particles at the end [271]. During a process with a high EE value, a huge proportion of compound initially introduced is encapsulated. That means that only a small quantity of product is lost. Indeed, EE represents the percentage of initial material that is successfully entrapped. LC on the other hand indicates how rich in active compound the synthesized particles are, in terms of mass. EE is usually high (>85 %) but LC is more difficult to increase and can remain very low (<10 %).

We provided a short description of how particles can be characterised. Different encapsulation processes will now be presented in detail. The distinction between physical entrapment and entrapment through chemical interactions will be discussed.

4.3 Physical entrapment

Physical encapsulation is characterized by a one-step process, which creates particles directly from the raw materials, or a simple solution mixing both materials. A representation is given in Figure I.15, where coating components are represented by beige spheres and active ingredients by red stars.

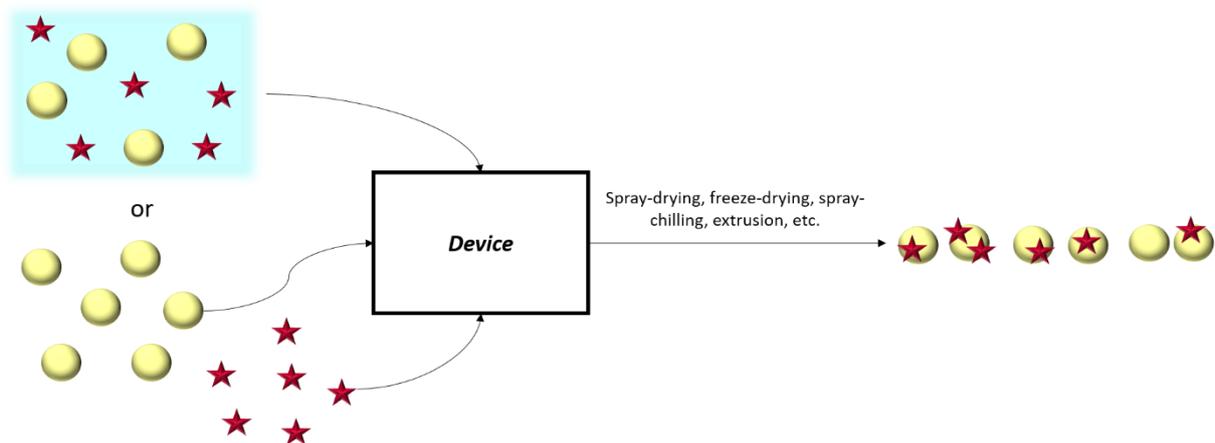


Figure I.15: Schematic representation of a one-step process of encapsulation.

4.3.1 Spray-drying

Spray-drying is a technique to form spherical beads from a solution, a dispersion or an emulsion of embedding and active materials [272]. It is the most employed technique by the industry, due to its flexibility, its possibility for continuous process and its economic viability [263,273]. It is particularly used in the food industry for the preparation of dry food additives and flavours [264] (milk, egg powder...). As the solvent is usually water, modified starch, maltodextrin, gums or chitosan diluted in acid are often employed as matrix. The huge quantity of water required is the main drawback of this technique.

The solution flows to the column (Figure I.16) and needs to be diluted to be pumped through the spraying system. The liquid is sprayed through an atomizer, often called the head, which can be a centrifugal atomizer, a piezoelectric atomizer or nozzle. Meanwhile, a gas, the ambient air or an inert gas when needed, is heated and filtrated. It penetrates the column from the top (co-current configuration) or from the bottom of the column (counter-current configuration). Sprayed droplets fall down through the column. Their small size, leading to high tension surface, and the contact with the hot air flux provoke the instantaneous evaporation of the solvent. Resulting particles are collected at the bottom of the column. A further filtration of the outlet air is often needed to retrieve all particles. A cyclone or bag filter can be used for this purpose. An electrostatic precipitator composed of two electrodes can attract the particles by Coulomb forces. Wet scrubbers can perform

a final cleaning of the particles of the outlet air. An optimized spraying is obtained by playing with parameters such as the flow rate and the temperature of the inlet gas, the temperature and pore size of the head, as well as the pump flow rate. Inlet gas temperature can be set between 100 °C and 200 °C, which determines the outlet gas temperature, which is approximately half of the inlet gas one. Head pores are usually 4 µm to 7 µm large for nano spray-dryer and in the mm scale for an industrial spray-dryer used in food application for example. Examples of reported encapsulation of natural extracts and especially phenolic compounds are gathered in Table I.5. We can see that starch and maltodextrins are often used as coating agents.

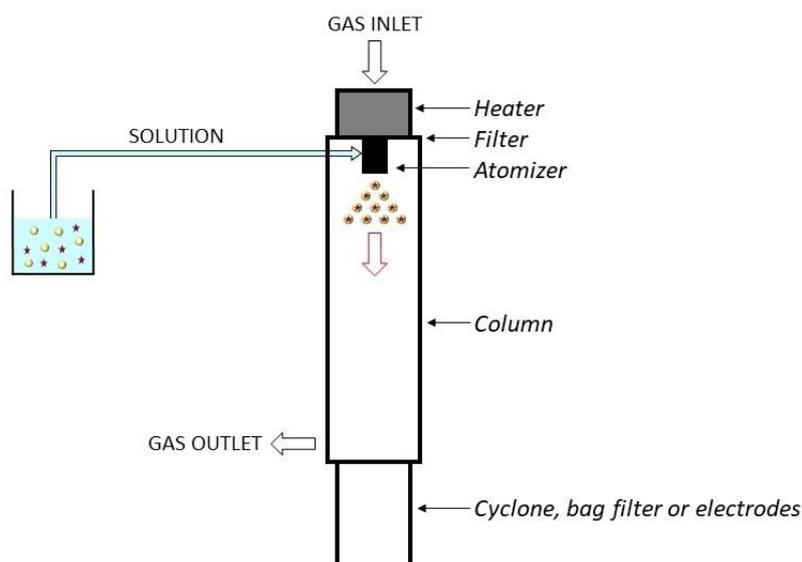


Figure I.16: Schematic representation of a spray-drying apparatus.

Table I.5: Examples of spray-drying for natural extracts encapsulation.

Publication	Matrix	Active compound	EE	Remark
Fang and Bhandari [262]	Maltodextrin	Bayberry juice	Not specified	Powder must be stored under 25 °C and water activity of 0.33 to prevent high degradation of phenolics and anthocyanins
García-Tejeda <i>et al.</i> [274]	Succinylated normal and waxy starches	Anthocyanin	Up to 98 %	Spherical particles with cavities; better retention for normal starch
Gómez-Aldapa <i>et al.</i> [275]	Achira starch	<i>Hibiscus sabdariffa</i> extract	Up to 69 %	Spherical particles with cavities
Sun-Waterhouse <i>et al.</i> [276]	Alginate, Methylated-βCD, Hydroxopropylmethylcellulose, Inulin	Quercetin, Vanillin	8.9-19 % for quercetin; 37-53 % for vanillin	Spherical particles, especially with Methylated-βCD
Wang <i>et al.</i> [277]	Starch and gelatin at different ratio	Curcumin	Up to 98 %	
Zhang <i>et al.</i> [278]	Gum Arabic, maltodextrin	Cranberry phenolics	Not specified	Powder must be stored under 25 °C to retain phenolics

4.3.2 Freeze-drying

Similar to spray-drying, lyophilisation starts from a solution or emulsion of dispersed coating material with active ingredients. Various encapsulants can be used such as biopolymers or smaller molecules [267]. Maltodextrin [279–284] and gum Arabic [282] in a smaller extent, are widely employed. The aqueous solution is frozen before being placed in the freeze-dryer. A reduced pressure, usually 0.1 mbar, allows the solid → vapour transition of water once when the temperature is rising. The condenser is working at -50 °C or -80 °C. It is an equipment commonly found in laboratory, and it constitutes a simple way for producing particles. Although spray-drying is more convenient, quicker and four to five times less energy consuming [264], freeze-drying can lead to a better encapsulation efficiency or less moisture content [283,284].

This technique is usually used to dry solution, as it is the case in food industry, rather than to encapsulate compounds. But it is also widely used to remove the water from any encapsulation system in aqueous solution, as it will be later described. For example, red beet root juice was freeze-dried with maltodextrin (100 % or 99.5 %) and xanthan gum (0 % or 0.5 %), to prevent the yellowing of the red colour [281]. In reference [283], in order to protect the antioxidant activity of phenolic compounds from spent coffee grounds, these compounds were encapsulated in a matrix of maltodextrin and/or gum Arabic, in the following quantities: 20 g of matrix (ratio of biopolymers 1:0, 0:1, 1:1) in 100 mL of extracts. Freeze-dried particles formed with maltodextrin retained the phenolic compounds the most efficiently, compared to those with the others wall material or spray-dried. In another work, maltodextrin was again able to protect anthocyanin from blackberry by-products, with 76 % of retention [279]. Papoutsis *et al.* [284] added soybean proteins or carrageenan to maltodextrin to encapsulate lemon by-product extracts.

4.3.3 Other physical techniques

Spray-chilling consists in atomizing a solution of matrix and active material mixed with a fat. Polysaccharides like sodium alginate [285] or other gelifiants like stearic acid can be used as matrix and hydrogenated vegetable oil as fat [286]. The droplets are formed through a nozzle and cooled below the melting temperature of the oil, provoking the solidification of the particles [287].

Extrusion can be used for encapsulation. This one-step organic solvent-free technique consumes a small quantity of water and energy compared to other processes. One of its drawback however is its relatively low encapsulation efficiency of 15-20 % at maximum [288].

Encapsulation with supercritical fluids is also possible. Supercritical CO₂ is employed as anti-solvent, meaning that the active compound and the matrix are first dissolved in an organic solvent where the supercritical CO₂ is later added. A change in the solubility of the mixture of the two solvents occurs, leading to the co-precipitation of the matrix and the guest [289].

4.4 Encapsulation through physicochemical interactions

Physicochemical interactions between active and coating compounds occurring in solution can also be exploited to produce encapsulated particles. After formation of the structure, the solvent can be removed leading to stabilized solid particles. Solvent removal is performed *via* one of the above-mentioned physical processes, usually by freeze-drying, by simple evaporation of the solvent under ambient atmosphere, or by infrared drying, filtration, vacuum or other drying process (see Figure I.17). This approach based on physicochemical interactions creates stable droplets, complexes or aggregates in solution which can be used without further drying, if the targeted application is compatible.

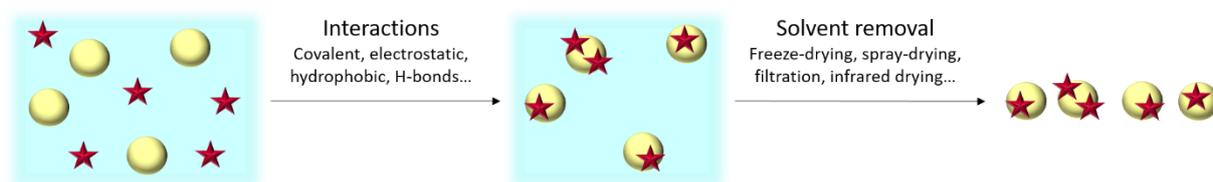


Figure I.17: Schematic representation of encapsulation through chemical interactions and drying.

4.4.1 Covalent interactions

A covalent bond can be created between the active material and the matrix, leading to spheres, capsules or grafted oligosaccharide. In interfacial polycondensation, an emulsion made of water droplets, containing the active material and an oil continuous phase with dissolved monomers is formed. The reverse situation, where the guest compound is soluble in an organic phase, can also occur. Reactions between monomers and active compounds happen at the interface, forming spheres if the synthesized oligomer is soluble in the dispersed phase, and capsules otherwise.

A second technique, called interfacial cross-linking, is defined as the condensation of an active material with an oligomer or a polymer [168]. Chitosan is often cross-linked with a cross-linker or through carbodiimide formation. For example, Liu *et al.* [290] cast chitosan grafted films with gallic acid, gentisic acid, protocatechuic acid, syringic acid and vanillic acid. Chitosan nanoparticles grafted with eugenol or carvacrol were synthesized by Chen *et al.*, exhibiting antibacterial effect similar to chitosan particles but better antioxidant properties [291]. Chitosan was also grafted with chlorogenic acid for food packaging applications [136]. Finally, chitosan grafted curcumin was also proposed to increase the solubility and stability of curcumin in [292]. In this work, the antioxidant properties of curcumin remained after its release when its covalent bonds to the glutaric anhydride linker were broken.

4.4.2 Molecular interactions: the case of cyclodextrins

Cyclodextrins (CDs) are a ring-shaped biopolymers composed of α -(1 \rightarrow 4)-linked glucose units. They were first discovered in degradation products of bacteria of the *Bacillus* genus. Nowadays, they are still produced by the action of cyclodextrin glucanotransferase enzyme on starch degradation products through an intramolecular transglycolysation [293]. Three CDs, named α -cyclodextrin (α CD), β -cyclodextrin (β CD) and γ -cyclodextrin (γ CD) and respectively composed of six, seven and eight glucose units are commonly found (Figure I.18). The size of these CDs is detailed in Table I.6 according to Del Valle *et al.* [293]. Their structure is a funnel-shaped torus with a hydrophilic outside, since all hydroxyl moieties of glucose are located outside the ring, and a hydrophobic cavity, since the axial hydrogens of the glucose units are pointing inside. β CD is the most widely used CD, encountered in various applications.

Table I.6: Characteristics of Cyclodextrins (data from Del Valle *et al.* [293]).

Property	α -cyclodextrin	β -cyclodextrin	γ -cyclodextrin
Number of glucopyranose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water at 25 °C (% , w/v)	14.5	1.85	23.2
Outer diameter (Å)	14.6	15.4	17.5
Cavity diameter (Å)	4.7–5.3	6.0–6.5	7.5–8.3
Height of torus (Å)	7.9	7.9	7.9
Cavity volume (Å ³)	174	262	427

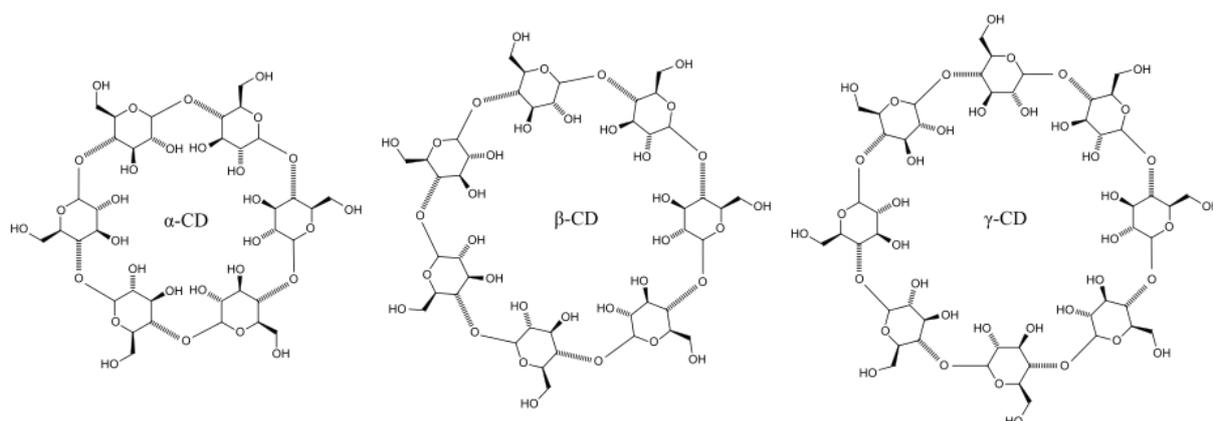


Figure I.18: Structures of α CD, β CD and γ CD.

This specific structure gives them the ability to trap hydrophobic compounds inside their cavity while remaining water-soluble. Applications in pharmaceutical and food industry are numerous, as CDs are approved by the Food and Drug Administration [169] and are Generally Regarded As Safe [294]. Like any other encapsulation technique, the solubility and stability of trapped molecules can be enhanced and the colour, the taste or an undesirable property of a

molecule can be hidden. They were also employed to remove cholesterol [295] or other undesirable compounds from food products [296].

The release of the encapsulated compound is particular, as it relies on the complexation constant of the inclusion complex formed by the guest molecule and the CD, and not on diffusion, solubilisation or crumbling of the encapsulating matrix. The higher the complexation constant, the higher the concentration of trapped guests in a CD solution.

Various CDs have been modified in order to increase their ability to form inclusion complexes. Alkylation of hydroxyl groups were mostly used, with hydrophobic or hydrophilic groups being either anionic, cationic or neutral. For example, the methylated- and hydroxyalkylated-CDs are widely employed since they confer to the β CD a better water solubility [169]. Inclusion complexes can be formed with the kneading method, in which the guest is mixed in a mortar with a CD slurry, leading to high encapsulation yields. Alternatively, coprecipitation is a method where the active component is dissolved in an organic solvent and the CD in water. The two solvents are mixed and complex crystals precipitate under cooling. Another method consists in dissolving the guest within a CD solution at an appropriate concentration. This solution is either further freeze-dried or spray-dried. Examples, where the encapsulated compound were closed to THC, are summarized in the following Table I.7. A lot of examples with THC and curcumin have been reported and surprisingly, some authors found a 1:1 ratio [14,19] and the others a 1:2 ratio, corresponding to two CDs for one curcumin [14,16,270].

Table I.7: Examples of CDs use for encapsulation of aromatic compounds.

Publication	Type of cyclodextrin	Active compound	Method	Stoichiometry; complexation const. (EE %)
Aksamija <i>et al.</i> [297]	β CD	Rosmarinic acid	Study in aqueous solution	1:1 ; K 445-465 M ⁻¹
Anselmi <i>et al.</i> [268]	α CD	Ferulic acid	Co-precipitation	1:1 ; 1162 \pm 140 M ⁻¹
Çelik <i>et al.</i> [298]	β CD , Methylated β CD, Hydroxyethyl β CD, Hydroxypropyl β CD, Heptakis(2,6-di-O-methyl) β CD, Sulfobutylether β CD	Rosmarinic acid	Study in aqueous solution	1:1 ; 82 \pm 7 M ⁻¹ with α CD to 328 \pm 39 M ⁻¹ with Methylated β CD
Duarte <i>et al.</i> [299]	Methylated β CD	Rersveratrol	Solution then freeze-drying	1:1 ; 1483 M ⁻¹
Goto <i>et al.</i> [19]	Hydroxypropyl β CD	THC	Kneading	1:1 ; 1200 M ⁻¹
Jahed <i>et al.</i> [14]	β CD	Curcumin	Solution then freeze-drying	1:1 or 1:2 ; 167 M ⁻¹
López-Tabar <i>et al.</i> [270]	β CD and γ CD	Curcumin	Solution then freeze-drying	1:2
Mangolim <i>et al.</i> [17]	β CD	Curcumin	Co-precipitation, solution then freeze-drying or solvent evaporation	Undetermined, (74 %)
Marcolino <i>et al.</i> [18]	β CD	Curcumin, bixin	Co-precipitation	1:2 with curcumin, 1:1 with bixin ; 4 *10 ⁴ mol.L ⁻² with curcumin, 334 M ⁻¹ with bixin
Tønnesen <i>et al.</i> [15]	Methylated β CD, Hydroxypropyl α CD, Hydroxypropyl β CD, Hydroxypropyl γ CD, Sulfobutylether β CD, Hydroxytrimethylammoniumpropyl β CD	Curcumin	Study in aqueous solution	1:1 ; 3.6 \pm 0.5 *10 ⁵ M ⁻¹ with Methylated β CD, 2.1 \pm 0.4 *10 ⁵ M ⁻¹ with Hydroxypropyl β CD, 4.7 \pm 0.9 *10 ⁵ M ⁻¹ with Sulfobutylether β CD,
Wang <i>et al.</i> [144]	β CD	Carbendazim	Kneading	1:1 IC ; 330 M ⁻¹
Yadav <i>et al.</i> [16]	β CD, Methylated β CD, Hydroxypropyl β CD , γ CD	Curcumin	Kneading and solvent evaporation	1:2 ; env. 400 M ⁻¹
Zhang <i>et al.</i> [269]	Hydroxypropyl β CD	Caffeic acid	Study in aqueous solution	1:1 ; 580 \pm 56 at pH 3.0
Zhao <i>et al.</i> [300]	β CD	Chlorogenic acid	Solution then drying at low pressure	1:1

4.4.3 Ionic interactions

4.4.3.a Ionic gelation

The ionic gelation method is based on electrostatic interactions between a charged polymer and ions in order to form a gel. A solution of aqueous polymer is dropped thanks to a syringe or a nozzle into an ionic solution under stirring to create beads. The most common system is the calcium alginate association. When a solution of sodium alginate is poured dropwise in a CaCl_2 solution, Ca^{2+} replaces Na^+ counter-ion of alginate, leading to polymer chain huddling. Some examples are available in Table I.8.

Table I.8: Examples of ionic gelation encapsulation.

Publication	Polymer and counter-ion	Active ingredient	Particles recovery	Encapsulation efficiency
Belščak-Cvitanović <i>et al.</i> [301]	Sodium alginate, calcium chloride and chitosan	Raspberry leaf, hawthorn, ground ivy, yarrow, nettle and olive leaf extracts		80–89 %
Cho <i>et al.</i> [119]	Chitosan in lactic acid and sodium tripolyphosphate	Resveratrol	Filtration 110 mm	Up to 99 %
Deladino <i>et al.</i> [141]	Sodium alginate and calcium chloride and optional chitosan	Yerba mate <i>Ilex paraguariensis</i>	Ambient condition or in oven or freeze-drying	87.1 % and 48.5 %
Lai <i>et al.</i> [265]	Alginate, calcium chloride and chitosan in acetic acid	Ascorbic acid	Filtration, air-dried	90 %
Liang <i>et al.</i> [120]	Carboxymethyl chitosan or chitosan hydrochloride	Tea polyphenols		80–83 %
Loquercio <i>et al.</i> [302]	Sodium alginate, calcium chloride and chitosan	<i>trans</i> -cinnamaldehyde	freeze-drying	58 %

4.4.3.b Coacervation

A coacervate is a neutral phase, miscible in water, composed of two polymers bearing opposite charges. Basically, the active material is dissolved in oil and the two polymers are dispersed in an aqueous solution. After mixing both solutions, the coacervate is formed and progressively coats the oil droplets. Particles present a core-shell structure with an unclear shape. The pH, the ionic strength, the temperature and the concentration of polymers determine the success of the coacervation. Biopolymers such as gelatin, whey proteins, vegetal proteins isolate, chitosan, carboxymethyl cellulose, alginate, pectin, etc. can be used. Each association possesses its own optimal pH, ranging from 2.0 to 9.0, as it is well summarized in a recent review [303]. For a better stability, particles can be further cross-linked with glutaraldehyde. This process is used for the production of highly sensitive products and high added value products [168,262,303–305].

Core-shell particles consisting of dextran outside and gelatin inside containing tea polyphenols were synthesized to enhance the cytotoxicity of phenolic compounds [306]. Pandan flavour had been recently encapsulated in Arabic gum and gelatin to form a powder with low moisture and water activity [307].

4.4.3.c *Layer-by-layer process*

The goal of this method is to create a multi-layer capsule trapping the active ingredient. The first step is to dissolve the host component in a substrate, which could be a synthetic polymer (poly(styrene sulfonate), poly(allylamine), etc.), a biopolymer (proteins or polysaccharide [308]), an ionic solution or an oil [309]. The substrate containing the active material forms the core of the capsule, on which each layer will be deposited. More precisely, droplets of substrate and compound are put in contact with a first solution of polymer. After rinsing and washing, capsules are soaked into a second solution of polymer. Polymers with different charges are typically chosen for solutions, called electrolytes. In addition to electrostatic interactions, hydrogen bonds or other weak interactions can also drive cohesiveness of the multi-layer assembly. Further rinsing and soaking are performed until desired wall thickness is achieved. The core can be dissolved to obtain hollow capsules loaded with a chosen active ingredient [168,310].

4.4.4 Hydrophobic interactions

4.4.4.a *Micelles*

Micelles are spherical self-assembled macro-molecular structures composed of amphiphilic molecules, where lipophilic molecules can be trapped inside. Micelles are often unstable and have a high polydispersity index, which can be overcome by cross-linking [168]. Surfactants are well known to form these kinds of structure. Sodium dodecyl sulphate was used to solubilize curcumin and artemisinin for health application. Micelles can provide a drug solubility enhancement of up to 50 times [311]. Amphiphilic polymers, such as di-block polymers can also be very efficient for encapsulation into micelles. Monomethyl poly (ethylene glycol)-poly (ϵ -caprolactone)-poly (trimethylene carbonate) can lead to curcumin-loaded nano-particles for the development of anticancer systems [312]. Modified biopolymer can also play the role of surfactant, as it is the case with modified starch. Caraway essential oil was stored for 8 months in potato starch substituted with hydroxypropyl and 1 % to 2.6 % of octenyl succinic acid (OSA) chains in [313]. Both particles with the highest ratio of OSA, with or without hydroxypropyl substitution, were approximately 343 nm large. Hydroxypropyl and OSA grafting on starch offer up to 84 % of essential oil retention.

4.4.4.b *Liposomes*

Liposomes are spherical vesicles delimited by one or more lipid bilayers. Their wall is 3–5 nm thick. They can encapsulate lipophilic materials in the wall and hydrophilic ones inside the

cavity. They are easily produced, often by hydration of a dried phospholipid film, but also with various techniques like sonication, reverse phase evaporation, melting, and freezing-thawing. Their main application domain is drug delivery. They protect the encapsulated ingredient from digestion in the stomach, enabling a better absorption in the gastrointestinal tract, resulting in an improved bioavailability [262]. The release rate and the efficiency of the system is controverted in the literature and the stability is often said to be low [168,262,314]. Hasan *et al.* [315] successfully encapsulated curcumin in nanometer scale liposomes from rapeseed, soya or salmon lecithins. In the pharmaceutical field, Fang *et al.* [316] added deoxycholic acid and 15 % of ethanol to liposome to maintain the stability of polyphenols from green tea extracts and enhance the tumour uptake. To overcome the issue of stability, liposome can be coated through a layer-by-layer system. Chen *et al.* [317] successfully trapped paclitaxel, a powerful anti-cancer molecule, in liposomes composed of lecithin, cholesterol and stearyl amine, covered by layers of polyacrylic acid and chitosan. Another drug, an epidermal growth factor, was released over a longer period thanks to an encapsulation within dioctadecyldimethylammonium bromide liposome coated by xanthan and galactomannan [318].

4.4.4.c Polymersomes

Polymersomes are vesicles composed of synthetic biocompatible di-block polymers, one lipophilic block and one hydrophilic block. Polymer chains self-assemble in solution leading to supermolecular structures of various shapes like cylindrical micelles, conical micelles or vesicles. Controlling the formation process as well as ratio between the two blocks is essential. Polymersomes, like liposomes, can trap lipophilic or hydrophilic drugs. However, the thickness of their wall is ten times bigger than the one of liposomes, decreasing their mobility, but increasing their stability [319].

4.4.5 Solvent evaporation from emulsion

The solvent evaporation of an emulsion is performed in several steps [168]. A polymer in the role of coating material is dissolved in a hydrophobic solvent. The active ingredient is dispersed in this solution, which is then turned into an emulsion. Different protocols, such as stirring, extrusion or baffles (circuit to create turbulence in a flow) can be used to form droplets in the solution [320]. This emulsion is heated or put under vacuum to evaporate the solvent and make particles precipitate. The polymer needs to be soluble in the solvent and not in water and the active component must have a better affinity with the oil phase than with the water phase.

Contrary to the previous method, a perfect miscibility between the solvent and water is required for emulsion extraction. First, a polymer and the active material are dissolved in the solvent. This solution is added to an aqueous solution of surfactant. As time goes by, the solvent

diffuses into the water phase causing the precipitation of particles made of polymers, surfactants and the ingredient.

Many examples in pharmaceutical field could be listed. Polymers used for this application are biocompatible, like poly(lactic acid) or poly(lactic-*co*-glycolic acid) [321–324].

4.4.6 Co-crystallization

Co-crystallization is a process where the active ingredient is enclosed in-between matrix aggregates of microcrystals. This technique is used in pharmaceutical and candy industry, as the aggregates are easily produced and directly suitable for further formulation [262]. Deladino *et al.* [325,326] started from sucrose syrup at 71.5 % (w/w) heated up to 140 °C. When the syrup became turbid, the active compound is quickly added. After cooling under stirring and drying, a powder is obtained. Its moisture is below 0.5 % and its hygroscopicity is low. The EE is compound dependent, as this method is not adapted for volatile or heat sensitive products. The main advantage results in the maintain or gain of stability even under humid conditions. The loading of encapsulated calcium lactate, magnesium sulphate and lyophilized Yerba mate extract varied from 2.52 mg/g to 43.5 mg/g.

4.5 Concluding illustrations

For physical entrapment, the two main techniques are summarised in Figure I.19. Since they are suitable for the industry, freeze-drying and spray-drying can be used for agricultural purposes.

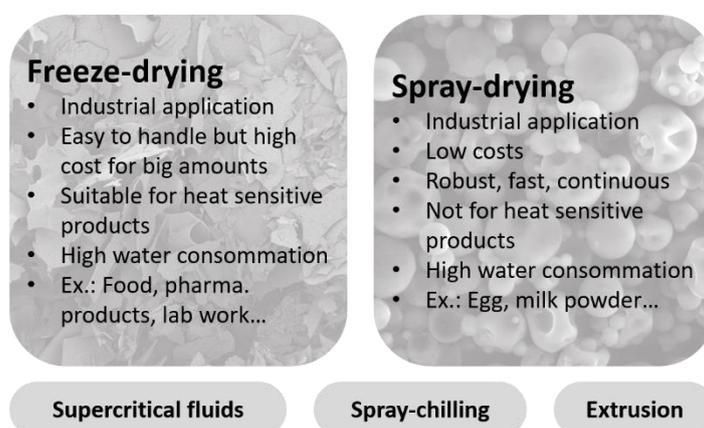


Figure I.19: Main techniques of physical entrapment of active compounds.

The techniques involving physicochemical interactions are represented in Figure I.20. For each process, the kind of molecule that can be trapped is specified. Some advantages/drawbacks and one example of matrix are also indicated. Processes involving many steps, like the solvent evaporation techniques or all processes involving ionic interactions, do not seem appropriate for

an application in agriculture. The scheme includes the abbreviation PEG-PTMC, which means poly(ethylene glycol)–poly(trimethylene carbonate).

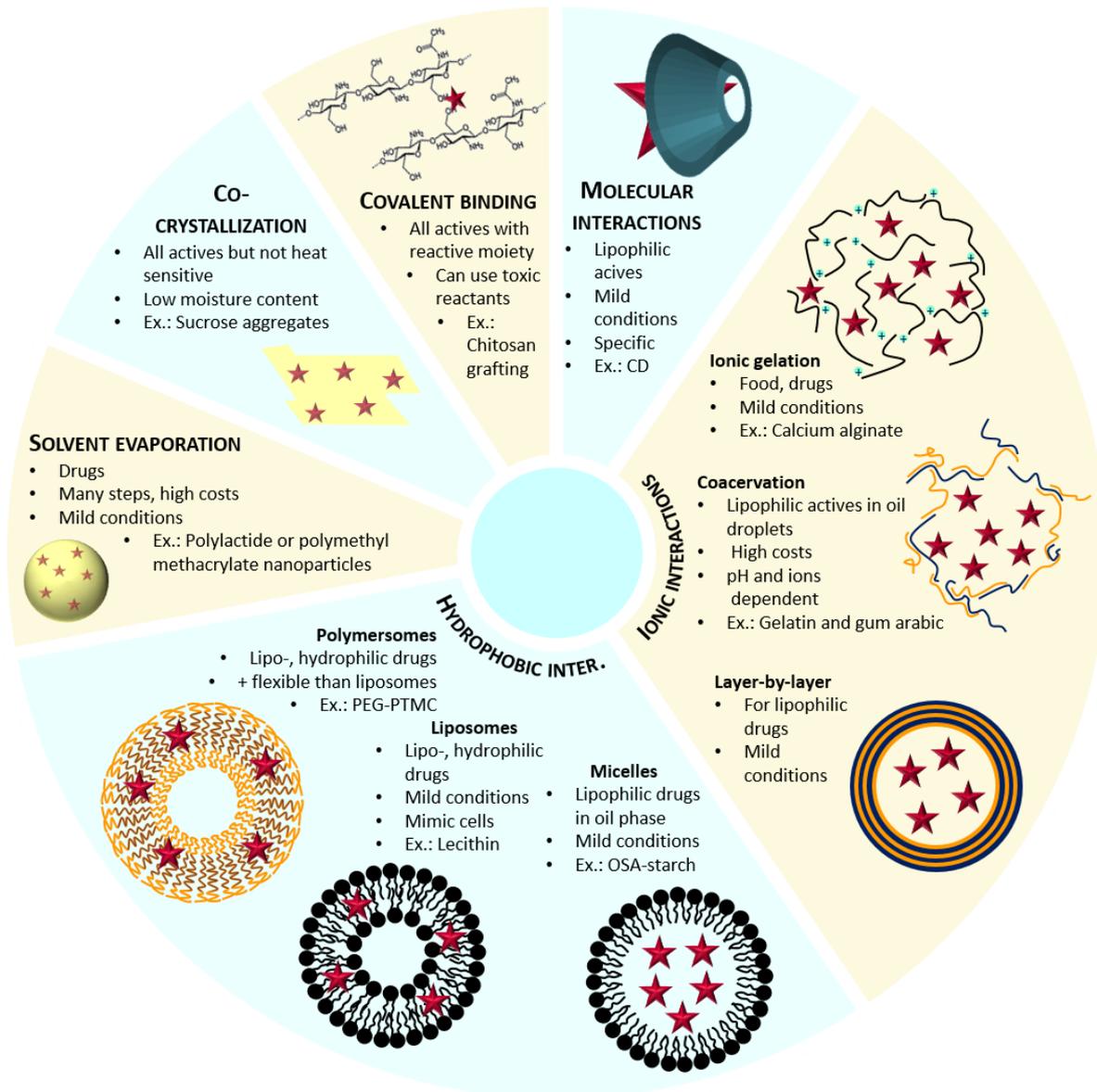


Figure I.20: Main techniques of encapsulation involving physicochemical interactions.

5 Strategy of the present study

As already mentioned, the aim of the project is to develop a fungicide based on renewable products and active against both the fungal growth and the production of toxins. The formulation must be suitable for organic farming, where the need for alternative treatments to synthetic fungicide is high, especially in the case of high occurrences of diseases.

Firstly, to see if chitosan is a good candidate for being the matrix of our formulation, its efficiency against the target strain is assessed. A comprehensive characterisation of the

biopolymers, notably by the determination of the MW and the DD, helps us to understand the link between structure and antifungal properties. Since chitosan can form a film when a chitosan solution is left at the open air, a fungicide sprayed on plant leaves will probably form a coating at the surface of the plant. For this reason and thus to get closer to the target application, the antifungal potential of chitosan as a coating on plant leaves is modelled.

Secondly, we want to create a synergy between both bio-based components in the formulation and benefit from the direct antifungal action of chitosan and the long-lasting release of smaller active molecules; that is why THC or extracts are then added to the chitosan-based solution. The effect on the growth and the production of toxins of such blends is studied and we emphasise the role of the interactions between compounds.

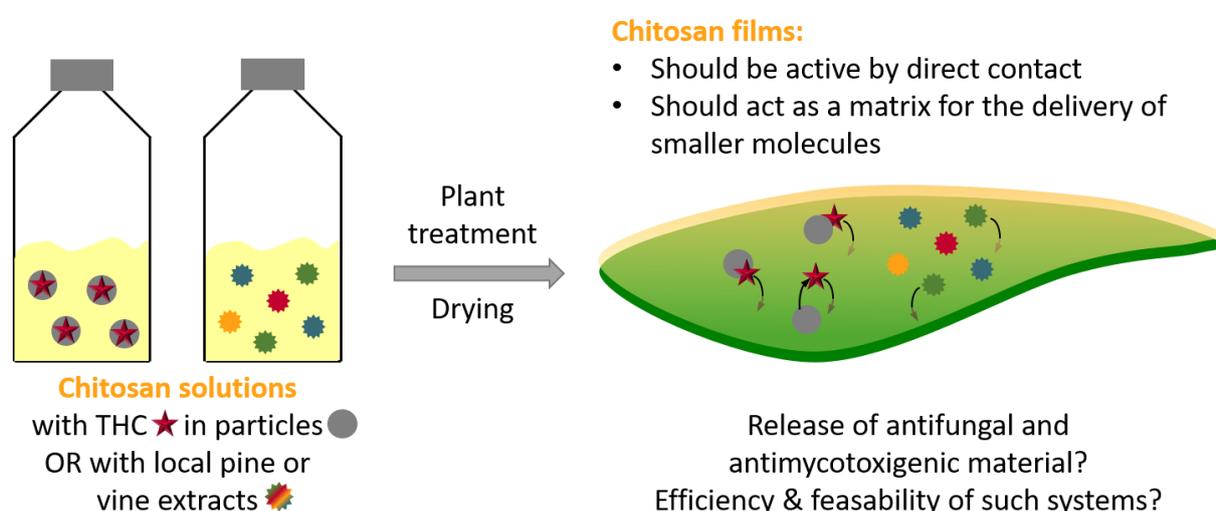


Figure I.21: Strategy of the work.

A deeper study on THC is needed to increase the solubility of THC and avoid deleterious interactions with chitosan along with increasing the release time. THC is thus encapsulated in different systems. Bio-based ones are chosen to act as an encapsulated matrix for THC. The encapsulation into cyclodextrins is focused on the improvement of THC water solubility and the demonstration of the formation of a complex. Other biopolymers, starch and chitosan, are used to form THC-loaded particles. This industry-oriented approach involves freeze-drying and spray-drying as encapsulation techniques. For each encapsulation system, the action on the mycelial growth and on the production of mycotoxins are evaluated, to check if the encapsulation brings more efficiency.

As far as extracts are concerned, we choose local resources of the region *Nouvelle-Aquitaine*. Vine and pine by-products are then extracted with the subcritical water process, which is a solvent-free and reliable technique.

CHAPTER II – MATERIAL & METHODS

1	Material	54
1.1	Chemical products and media for microbiology.....	54
1.2	Fungal strains	55
2	Extraction and characterisation of the plant extracts	55
2.1	Subcritical water extraction	55
2.2	Determination of the total phenolic content	56
2.3	Chemical characterisation by LC–MS.....	56
3	Elaboration and characterisation of chitosan solutions and films	57
3.1	Characterisation of chitosans.....	57
3.2	Chitosan solutions	58
3.3	Chitosan films	59
4	Elaboration and characterisation of tetrahydrocurcumin-loaded systems	60
4.1	Characterisation of THC.....	60
4.2	Elaboration of THC-loaded systems.....	61
4.3	Characterisation of THC-loaded systems	64
5	Antimicrobial properties.....	67
5.1	Preparation of the spore solution	67
5.2	Inhibitory properties against the fungal growth.....	68
5.3	Inhibitory properties against the production of toxins	69
5.4	Statistical analysis.....	70

1 Material

1.1 Chemical products and media for microbiology

Tetrahydrocurcumin (THC) was purchased from SABINSA EUROPE GmbH. Ferulic acid and caffeic acid (Sigma), butylated hydroxytoluene (BHT, Acros), 2,2-diphenyl-1-picrylhydrazyl (DPPH[•], Sigma-Aldrich) and potassium bromide were used in analytical grade.

HPMC was a sample provided by Hercules Culminal. HPC (MW = 100 kDa) was from Aldrich. Chitosan low molecular weight, lot #STBH2613 was obtained from Aldrich. One other chitosan was obtained from France chitine® (Orange, France) and Chitosan 20 cps and Chitooligosaccharide 3000 form SPN Agrobio® (Varades, France).

β -cyclodextrin (β CD) was purchased from Sigma-Aldrich, γ -cyclodextrin from Tokyo Chemical Industry and Randomly methylated β CD from Roquette (Kleptose Crysmeb, Batch E0002).

Anionic water-soluble polymers of β CD (Poly- β CD) and Me β CD (Poly-Me β CD) were kindly donated by the UMET laboratory (Université de Lille, F-59655, Villeneuve d'Asque, France). They were synthesised according to a method proposed by Junthip, Tabary, Leclercq and Martel [327] by solubilisation of citric acid as crosslinking agent, sodium hypophosphite as catalyst and CD in respective weight ratio 10 g/3 g/10 g in 100 mL of water. Briefly after water removal, the solid mixture was then cured at 140 °C during 30 min under vacuum. Water was then added and the resulting suspension was filtered and the filtrate dialyzed during 72 h against water using 6–8 kDa membranes (SPECTRAPOR 1, Spectrumlabs). Finally, the water soluble anionic CD polymer was recovered after freeze drying. The number average molar mass (M_n) were 11.8 kg/mol and 15.6 kg/mol, respectively for Poly- β CD and Poly-Me β CD (Polydispersity index=1.7). Based on the integrations values of H_1 (CD) and citrate methylene signals (CH_2 -citrate) by proton NMR studies, Poly- β CD and Poly-Me β CD were estimated to contain 72.0 wt% in β CD moieties and 63.4 wt% in Me β CD moieties respectively. This percentage corresponds to what will be later called the number of cavities of CDs.

Octenylsuccinic acid modified starch (OSA-starch) was a sample named Capsul® (Ingredion E1450 Batch No BJH35). Rapeseed oil was from UAB “Rokiškio aliejinė”, Lithuania. Tween 80 was purchased from Sigma.

Acids used were glacial acetic acid (≥ 99 %) and lactic acid (80-85 % w/w). All solvents were used in analytical grade.

Potato Dextrose Agar (PDA) medium (potato starch 4g/L, dextrose 20g/L, agar 15g/L) was provided from Biokar. Carboxymethylcellulose (CMC) was provided by Sigma-Aldrich. All salts used in media were in analytical grade.

TCTB standard solution was provided by Romer Labs (Baulkham Hills, Austria).

1.2 Fungal strains

The following strains of *Fusarium graminearum* were used during this study: Fg 156, Fg 164, Fg 215, CBS 185.32, Fg 605 and Fg 812. All strains present DON/15-ADON chemotype and belong to INRA-MycSA's laboratory collection (Villenave d'Ornon, France). INRA-MycSA's strains are deposited in the International Center for Microbial Resources - Filamentous Fungi [328]. The strain CBS 185.32 is sequenced and was used as a reference strain in this work.

2 Extraction and characterisation of the plant extracts

2.1 Subcritical water extraction

Extractions were performed by the industrial partner Phenobio (Martillac, France) from vine shoots, maritime pine sawdust or maritime pine barks. Maritime pine sawdust came from stem (sapwood + heartwood) without crown and branches.

Briefly, the extractions were performed using a dynamic subcritical water device that included a High Performance Liquid Chromatography (HPLC) pump, a 300 mL extraction vessel, a heating device, a pressure controller and a collector. For extraction of each raw material, 50 g were loaded into the high-pressure vessel (Figure II.1). The vessel was placed in an oven set at 120 °C, and water was heated with a heating jacket at the chosen temperature (125 °C for “polar extracts” or 175 °C for “semi-polar extracts”). The outlet valve of the extraction vessel was then closed and the system was pressurized to the desired pressure of 15.105 Pa at a constant flow rate of 15 mL/min. The liquid-to-solid ratio was maintained at the value of 5, to obtain 250 mL of extract. After the extraction vessel, the extract passed through a cooling system, was collected in sampling vessel and then stored at -20 °C for further analysis.

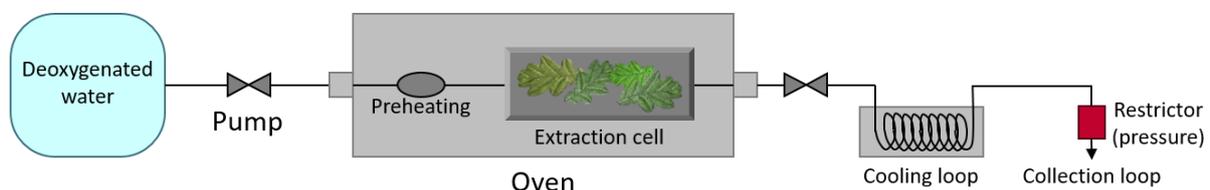


Figure II.1: Representation of the extraction process carried out at Phenobio®.

The concentration of natural extracts was determined by lyophilizing an aliquot of 50 mL of each extract and weighing dry sample. Extract concentration was expressed as gram dry matter per litre of extract. Extract yields were calculated using the following formula: extract yield % = (weight of extracted plant / weight of plant raw sample) x 100.

Extracts were received liquid and sterilised through a 0.22 μm filter (Thermo Scientific Nalgen Rapid-Flow 90 mm Filter Unit – 500 mL). Filtered extracts were then lyophilized and used as a powder.

2.2 Determination of the total phenolic content

The total phenolic content was spectrophotometrically measured according to a 96-well microplates modified Folin-Ciocalteu method as previously described [329].

2.3 Chemical characterisation by LC–MS

Analyses were performed using a 1290 Series UHPLC from Agilent Technologies (Santa Clara, CA, USA) equipped with an auto sampler module, a binary pump with degasser, a column heater/selector and an UV-Vis diode-array detector. The UHPLC apparatus was coupled to an Esquire 6000 ion trap mass spectrometer using an electrospray ionization source (Bruker Daltonics, Billerica, MA, USA).

Chromatographic elution was carried out on a 100 mm x 2.1 mm i.d., 1.8 μm , Zorbax SB-C18 column, with a 2.1 mm x 5 mm i.d. guard column (Agilent Technologies, Santa Clara, CA, USA). A binary solvent system composed of water acidified with 0.1 % formic acid (solvent A) and acetonitrile acidified with 0.1% formic acid (solvent B) was used at a flow rate of 0.4 mL/min with the following gradient: 0-1.7 min, 10 % B; 1.7-5.1 min, 10-30 % B; 5.1-6.8 min, 30 % B; 6.8-8.5 min, 30-35 % B; 8.5-11.9 min, 35-60 % B; 11.9-15.3 min, 60-100 % B; 15.3-17 min, 100 % B; 17-17.3 min, 100-10 % B. Parameters of mass spectrometry were set in negative mode with a range of m/z 100–1200 with nitrogen as the drying gas at 10 L/min, nebulizer pressure of 40 psi at 365 °C, capillary voltage at 3100 V, capillary exit voltage at –118.3 V, skimmer voltage at –40 V, and the trap drive at 58.1.

Maritime pine sawdust SP extract was dissolved in methanol/water (3:7, v/v) at 10 mg/mL, filtered on 0.45 μm and injected at 1 μL . The polyphenolic compounds were characterized by using commercial standards and the polyphenol library developed in Molécules d'Intérêt Biologique laboratory (ISVV, Villenave d'Ornon, France) [330,331]. Identification was performed by comparing retention time, UV data (λ_{max}) as well as mass spectrometry data with pseudomolecular ion and product ions. Quantification was carried out for each compound with its own calibration curve at the maximum wavelength. Pure standards were injected at several concentrations (0, 1, 5, 20, 50, 100 $\mu\text{g/mL}$) in independent triplicate to obtain calibration and equation curves. All data were processed with Bruker Data Analysis 3.2 software.

3 Elaboration and characterisation of chitosan solutions and films

3.1 Characterisation of chitosans

3.1.1 Determination of the deacetylation degree with $^1\text{H-NMR}$ spectrometry or infrared spectroscopy

3.1.1.a *By NMR*

$^1\text{H-NMR}$ spectrometry was performed using a Liquid-state 400 MHz NMR spectrometer (Bruker AVANCE I). Chitosans were dissolved in D_2O with 1 % (v/v) of DCl . The DD was calculated according to the equation (II.1) [332], where I_{Ac} is the integration of acetyl groups protons and I_{H2-6} the integration of protons of a glucose unit:

$$DD = 100 - \frac{I_{Ac} / 3}{I_{H2-6} / 6} * 100 \quad (\text{II.1})$$

3.1.1.b *By infrared spectroscopy*

Infrared spectroscopy was performed with a Fourier transform infrared spectrometer (FTIR) from Bruker (PIKE technologies, France). Samples (2 %, w/w) were mixed in a mortar with extra dried potassium bromide. The powder was pressed under ten bars for 10 minutes in a mould of 1 cm in diameter to form a pellet. Analysis was performed in a transmission mode through the pellets, from 400 to 4000 cm^{-1} , every 4 cm^{-1} , with an accumulation of 128 scans. The DD was calculated according to the equation (II.2) with specific baselines as described in [333]:

$$DD = 100 - \frac{A_{1655}}{A_{3450}} * 115 \quad (\text{II.2})$$

3.1.2 Determination of the molecular weight

Polymer molar masses were determined by Size exclusion chromatography (SEC), which was performed on an Ultimate 3000 system from ThermoScientific equipped with a diode array detector (DAD). The system also includes a multi-angle light scattering detector (MALS) and a differential refractive index detector (dRI) from Wyatt Technology. The mobile phase consisted of a buffer solution (acetic acid 0.3 M and sodium acetate 0.2 M) Measurements in water were Polymers were separated on two Tosoh PWXL G3000 and G4000 (7.8*300) columns (exclusion limits from 5000 Da to 300 000 Da) at a flow rate of 0.6 mL/min. Column temperature was held

at 25 °C. Dextran from PSS was used as the standard. Samples were prepared in the buffer at 5 mg/L and filtered (0.22 µm) before being injected.

3.1.3 Determination of the charge density

The potentiometric titration of chitosan was performed using an automatic pH titrator (TitroLine 7800, SI Analytics, Mainz, Germany) equipped with a microelectrode. The chitosan solutions were prepared at 1 g/L in HCl 0.1 M to ensure the full protonation of glucosamine residues. The titrations were performed with 10 mL of chitosan solution using 0.2 M NaOH (prepared from NIST Standard Concentrate, for Volumetric Analysis, Fisher Chemical, Pittsburgh, PA, USA). The volume of each injection of NaOH was set to 10 µL at a rate of 5 mL/min and the time between each injection was set to 60 s.

3.2 Chitosan solutions

3.2.1 Elaboration of the solution

Solutions were prepared using acetic acid (AA) or lactic acid (LA). The ratio (w/v) of chitosan/acid was set at 1 (mg/µL). Distilled water was added very progressively. The mixture was let overnight under stirring until the total dissolution was achieved.

Thanks to the described analyses, the ratio of acid per unit of N-Glucosamine has been determined and is shown in Table II.1.

Table II.1: Mass and molar ratios of chitosan and acid.

	Ratio chitosan/acid (w/v)	Molar ratio acid/N-glucosamine
Ch. 78 and AA	1	3.1
Ch. 4.8 and AA	1	4.0
Ch. 78 and LA	1	2.1
Ch. 4.8 and LA	1	2.7

3.2.2 Rheology study of a chitosan solution

The measurement of the viscosity was performed with an Anton Paar MCR 302 apparatus. The configuration was the Pelletier configuration. A plan-cone of 50 mm in diameter and an angle of 1° was used. The gap between the surface and the plan-cone was 1 mm. The temperature was set at 25 °C and the shear rate varied from 0.001 to 1000.

3.3 Chitosan films

3.3.1 Elaboration of films

A solution was prepared from the Ch. 78 at 10 g/L (1 %, w/v of solution) with AA at 10 mL/L (1 %, v/v of solution). Typically, 2 g of Ch. 78 and 2 mL of AA were introduced in a bottle. Distilled water (198 mL) was added very progressively. The mixture was let overnight under stirring until the total dissolution was achieved. Gases from the solution were removed by putting the opened bottle under vacuum for one hour. Films were cast in Petri dishes (85 mm of diameter) from 20 mL of the solution and were put in a climatic chamber at 40 °C for 3 days. Films were peeled from the dishes and stored at 23 °C and 50 % of R.H. in the dark until further analysis.

3.3.2 Characterisation of films

3.3.2.a *Determination of the film thickness*

The thickness of films was measured with a Mitutoyo Digimatic Indicator.

3.3.2.b *Determination of the contact angle and the surface tension energy*

The surface tension energy was measured with the sessile drop method with a goniometer Krüss DSA 100. Chitosan films were previously stored at 23 °C and 50 % of R.H. The volume of drops was 2 µL with water and ethylene glycol and 1.3 µL with diiomethane. Measurements of contact angles were performed six times for each solvent. The software calculated the surface tension energy with the WORK method. A two-minute video was recorded with one drop of water to see the evolution of the drop on the surface.

3.3.2.c *Dynamic water penetration analysis (DPA)*

The behaviour of water in contact with the surface of a chitosan film was followed by DPA, with the device Emtec PDA C02 (Emtec Electronic) equipped with a 2 MHz source. The device consisted on a fix and sunken part where distillate water was added and on a mobile part where the film was carefully glued without air bubbles. The mobile part went down and made contact with the fix one. Ultrasounds were send through the water and the film and collected at a receptor for 30 seconds. The films were conditioned at 23 °C, 70 % of R.H. and in the dark before analysis. The measurement was performed in triplicate.

3.3.2.d *Determination of the water vapour transmission rate (WVTR)*

Calcium chloride (15–20 g) was put into a special aluminium pan (Figure II.2). Chitosan film was placed onto the pan. A ring of metal, as big as the inner diameter of the pan, was then positioned on the film. Wax was poured in the gully formed between the outer diameter of the pan

and the ring to seal hermetically the cavity of the pan. The whole system with its plastic cover was weighted. Pans were placed in a climatic chamber (23 °C and 50 % R.H.), and weighted every day. To remove the contribution of the mass gain due to the water absorbance of the film, a control pan was made without CaCl₂. The plot of the mass gain *versus* the time could be modelled by a linear regression, whose slope was equal to $\frac{\Delta m - \Delta m_{control\ pan}}{\Delta t}$. The WVTR, expressed in g.m⁻².day⁻¹, was calculated with (II.3), where S_f is the area of the surface of the film (radius of 4 cm). The measurement was performed in triplicate.

$$WVTR = \frac{(\Delta m - \Delta m_{control\ pan}) * 24}{\Delta t * S_f} \quad (II.3)$$

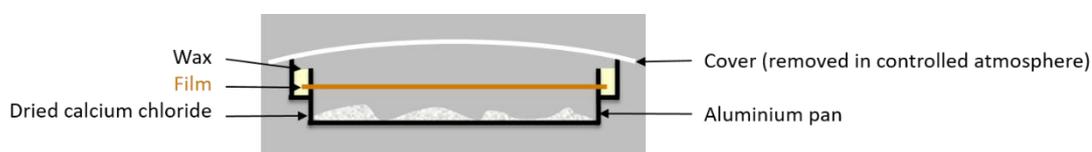


Figure II.2: Schematic representation of the device used to evaluate the WVTR of chitosan films.

3.3.2.e Impact of UV

Films were stored at 23 °C, 50 % R.H., in the dark for control films. Irradiated films were stored in an irradiation chamber equipped with four black light tubes (Mazdafluor TFWN18, maximum emission at 375 nm, irradiance of 0.45 mW.cm⁻² at 365 nm) and with a ventilation system. Film pieces of 3 cm x 1 cm were cut and placed in thin quartz cell for the analysis. Spectra were recorded on a UV-visible spectrophotometer (Agilent Cary 100) from 250 nm to 400 nm.

4 Elaboration and characterisation of tetrahydrocurcumin-loaded systems

4.1 Characterisation of THC

4.1.1 Characterisation by mass spectrometry

Mass spectroscopy was used to control the purity of the THC. MALDI-MS spectra were performed by the CESAMO (Bordeaux, France) on an Autoflex maX TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a frequency tripled Nd:YAG laser emitting at 355 nm. Spectra were recorded in the positive-ion mode using the reflectron and with an

accelerating voltage of 19 kV. Samples were dissolved in MeOH at 10 mg/ml. The trihydroxyacetophenone matrix solution was prepared by dissolving 10 mg in 1 ml of MeOH. A MeOH solution of cationisation agent (NaI, 10 mg/ml) was also prepared. The solutions were combined in a 10:1:1 volume ratio of matrix to sample to cationisation agent. One to two microliters of the obtained solution were deposited onto the sample target and vacuum-dried.

4.1.2 Characterisation by NMR

NMR was performed using a Liquid-state 400 MHz NMR spectrometer (Bruker AVANCE I).

^1H -NMR of THC was performed in deuterated dimethylsulfoxide (DMSO) and CDCl_3 , with 16 scans. ^{13}C -NMR was also performed but with 32 scans.

4.2 Elaboration of THC-loaded systems

THC have been encapsulated in different systems as shown in Figure II.3.

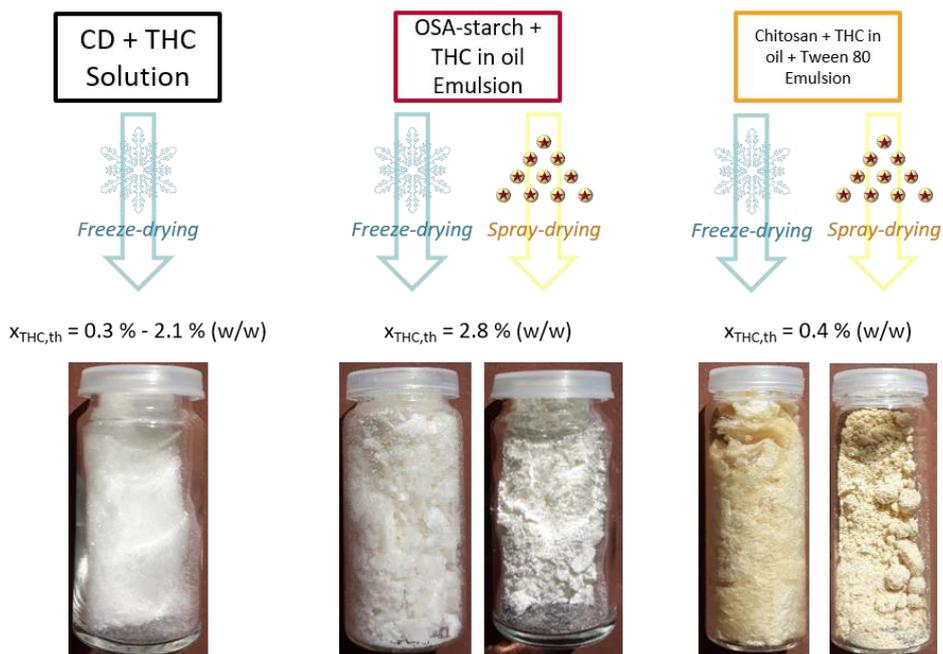


Figure II.3: Different types of THC-loaded particles synthesized in this work. $x_{\text{THC,th}}$ represents the theoretical mass fraction of THC inside each particle.

CD = cyclodextrins
 OSA-starch = Octenyl succinic acid modified starch
 $x_{\text{THC,th}}$ = theoretic amount inside particles

4.2.1 Elaboration of THC-loaded cyclodextrins

Solutions of THC and CD at the concentrations given in

Table II.2 were prepared at ambient temperature. The equation (II.4) was used to calculate the mass concentration of each CD or polymer of CDs. The solution was stirred overnight at ambient temperature in the dark and then frozen to be further freeze-dried. Dried particles from Me β CD, Poly-Me β CD or Poly- β CD should contain 2.1 %, 0.4 % and 0.5 % (w/w) of THC respectively.

$$C_{m,CD} = \frac{[CD's\ cavity]}{x * M} \quad (II.4)$$

Table II.2: Concentrations used to prepare THC-loaded CD particles.

Type of CD	Concentration of CD's cavity (mM)	x, percentage of cavities in the powder of CD (%)	Concentration of CD or polymer of CDs (g/L)	Concentration of THC (mM)
Me β CD	15	100	17.8	1.0
Poly-Me β CD	15	68.9	25.8	0.3
Poly- β CD	15	72.5	23.5	0.3

4.2.2 Elaboration of THC-loaded OSA-starch particles

Capsul® (Ingredion E1450) were weighted according to their moisture content to form an aqueous solution of 20% (w/w) or 30% (w/w). Moisture content was determined with a drying balance (Kern MRS 120-3). Distillated water was added and the solution was heated up to 70°C until the total dissolution of starch. After cooling, the solution was light yellowish. Water was added to the solution to compensate losses due to water evaporation. Solutions was stored in the fridge for maximum one week.

THC (5 %, w/v) was suspended in rapeseed oil at ambient temperature under magnetic stirring at 400 rpm for two days.

The Capsul® solution (20 g, 18 g and 16 g) and the oil solution (0 g, 2 g and 4 g) were poured in a narrow beaker, where they were homogenized with IKA® T25 digital ULTRA-TURRAX for 5 to 20 minutes, at ambient temperature, at 10,000 rpm or 13,000 rpm. Emulsions without THC added in the oil were produced as control. Both THC-loaded and control OSA-starch particles were produced from the emulsions containing 16 g of Capsul® solution at 20 % and 4 g of oil solution, corresponding to a ratio Capsul®/oil of 0.8.

Spray-drying: emulsion was diluted 10 to 20 times and run in a Nano spray-dryer (Büchi Nano Spray Dryer B-90, Figure II.4, left). The gas flow was 110 L/min, the inlet temperature was 100°C, the outlet temperature 45°C and the nozzle size 7 μ m.

Freeze-drying: emulsions were frozen and freeze-dried.

Dried THC-loaded particles contained theoretically 44.4 % of OSA-starch, 52.8 % of oil and 2.8 % of THC. Dried control capsules were made of 44.4 % of OSA-starch and 55.6 % of oil.

4.2.3 Elaboration of THC-loaded chitosan particles

A solution of Ch. 78-AA at 10 g/L was prepared as described in 3.2.1.

THC (5 %, w/v) was suspended in rapeseed oil at ambient temperature under magnetic stirring at 400 rpm for two days.

The chitosan solution (500 g), the oil solution (1 g) and Tween 80 (500 mg) were poured in a narrow beaker, where they were homogenized with IKA® T25 digital ULTRA-TURRAX for 20 minutes, at ambient temperature, at 10,000 rpm. Emulsions without THC added in the oil were produced as control. The corresponding ratio Ch. 78/oil/Tween 80 was 1/0.2/0.1.

Spray-drying: Emulsions were spray-dried on an apparatus from Lab-Plant (Figure II.4, right). The inlet temperature was 170 °C and the outlet temperature was 70 °C.

Freeze-drying: Emulsions were frozen and freeze-dried.

Dried THC-loaded particles contained theoretically 43.5 % of Ch. 78, 43.5 % of AA, 8.3 % of oil, 4.3 % of Tween 80 and 0.4 % of THC. Dried control capsules were made of 43.5 % of Ch. 78, 43.5 % of AA, 8.7 % of oil and 4.3 % of Tween 80.



Figure II.4: Spray-dryer from Büchi (left), with a zoom on the head and on collecting electrodes. Spray-dryer from Lab-Plant (right).

4.3 Characterisation of THC-loaded systems

Particles produced to encapsulate THC were analysed with different techniques, including analyses of powders and particles in solution.

4.3.1 Dynamic light scattering (DLS) of emulsions

Starch and chitosan emulsions were characterized using a Particle analyzer (Beckman Coulter Delsa Nano C Particle Analyzer), with three times 20 measurements, to determine the size of the droplets and the polydispersity index.

4.3.2 Microscopic observations

SEM observations of CD powders were performed at the Bordeaux Imaging Center with a ZEISS Gemini SEM 300 equipment. Stubs with double-face tape were used as supports for a thin layer of powder. Samples were metallized with Platine during 5 minutes and observed in high vacuum mode under a 2 keV electron beam.

SEM observations of starch and chitosan powders were performed in Kaunas University of Technology, Lithuania, on a FEI Quanta 200 FEG scanning electron microscope.

4.3.3 Thermal analysis

4.3.3.a *Differential Scanning Calorimetry (DSC)*

DSC was employed to study the behaviour of THC around its fusion temperature. It was performed with TA Instrument Q100 RCS. Approximately 4 mg of compound were placed in an aluminum pan and heated from 20 °C to 110 °C with a heating rate of 10 °C/min under nitrogen (25 mL/min). A first cycle with a plateau at 90 °C was necessary to eliminate water. THC-loaded particles were compared with a physical mixture of the control particles and THC. In this physical mixture, THC was put in the same amount (w/w) that found in the THC-loaded particle.

4.3.3.b *Isothermal Titration Calorimetry (ITC)*

Only for THC-loaded Me β CDs, ITC was performed on a Nano ITC (TA Instrument) at 25 °C. The solution of Me β CD at 4.136 mM was titrated against the THC solution at 10 μ M introduced in a cell of 950 μ L. The reference cell was filled with distilled and degassed water.

A 250 μ L syringe was used with a rotation of 250 rpm; the injection volume was set at 8 μ L every 240 seconds. However, neither trend was observed nor curve fit was possible following this procedure, due to small peaks. To improve the signal/noise ratio, it was needed to increase the quantity of reactant. However, the concentration was already set for THC at its maximum in water.

Instead of running a single experiment with multiple injections, several runs of single, but larger, injections were made (Figure II.5).

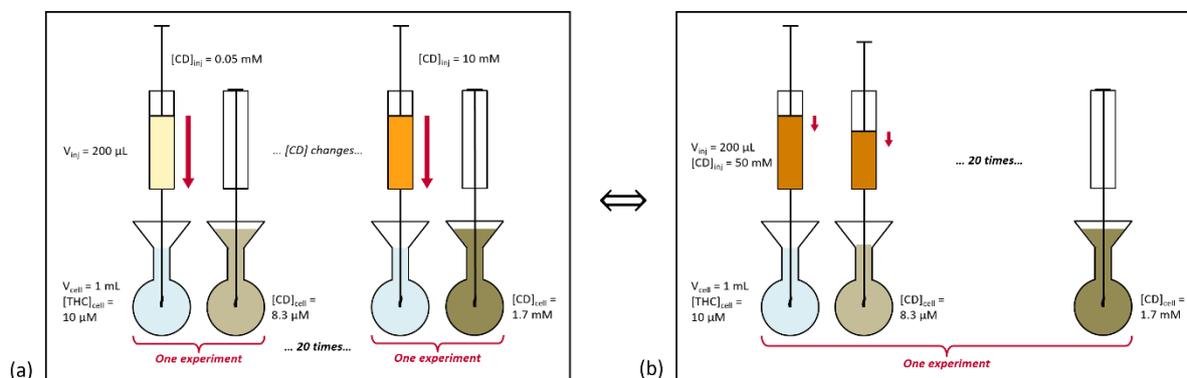


Figure II.5: Schematic representation of several single injections runs equivalent to one unique run with multiple injections.

Each experiment consisted on an injection of 200 μL of a MeβCD solution at variable concentrations (0.05 mM to 10 mM). The THC solution (10 μM) was in the cell. Temperature was set at 25 °C and the syringe rotation at 400 rpm. Each run was performed twice to assess the repeatability. Each run with a single injection corresponded to a specific concentration of MeβCD in the cell, *i.e.* a specific ratio between THC and MeβCD. The heat produced was plotted *versus* the molar ratio $[\text{Me}\beta\text{CD}]_{\text{cell}}/[\text{THC}]_{\text{cell}}$.

4.3.4 THC-solubility study with UV-visible spectrophotometry

Only for THC-loaded MeβCDs, information about the complexation constant have been obtained by a solubility study. An excess amount of THC was added to 5 mL of a MeβCD solution of varying concentrations (0, 5, 10, 15, 20, 30, 40 mM) in amber vials. After 24 hours of stirring, solutions were centrifuged for 5 minutes at 1200 *g* at room temperature. The supernatant was analysed at 280 nm, in quartz cell of 1 cm, at room temperature. A 10-times dilution was necessary for samples with highest concentrations of MeβCD to obtain an absorbance lower than 1.5. Solubility diagrams were obtained by plotting the concentration of THC *versus* the one of MeβCD, which should give an affine line if the stoichiometry of the inclusion complex is 1:1. The complexation constant, *K*, is given by the equation (II.5) described by Higuchi and Connors [334]. The measurements were performed twice for each concentration. The standard deviation of the complexation constant was evaluated through the calculation of the standard deviation of the regression coefficient obtained by the least squares method.

$$K = \frac{\text{slope}}{[THC]_0 * (1 - \text{slope})} \quad (\text{II.5})$$

4.3.5 Characterisation by 1D- and 2D-NMR spectrometry

Only for THC-loaded Me β CDs, NMR studies were performed using a Liquid-state 400 MHz NMR spectrometer (Bruker AVANCE I). NMR of CD and THC-loaded complex was performed in D₂O, with 16 scans. NMR of THC and THC-loaded complex were also performed in MeOD/D₂O 1:4 (v/v), with 1024 scans. HSQC was also performed in this solvent mix, and was useful to attribute signals of aromatic protons of THC.

A rotational Overhauser enhancement experiment (ROESY) was used to detect intermolecular nuclear Overhauser effects between Me β CD and THC. This technique enables the detection of interactions of non-covalently bounded protons, which is characteristic of inclusion complexes. The number of scans was fixed at 32, the temperature at 300 K and the dwell time at 120.00 μ sec.

4.3.6 Determination of the THC loaded content in particles

Only for THC-loaded starch particles, the determination of the THC amount was performed by NMR using a Liquid-state 400 MHz NMR spectrometer (Bruker AVANCE I). Starch particles were analysed in DMSO, with 20 μ L of 2,3,4,5,6-pentafluorobenzaldehyde (PFBA), with modified parameters (128 scans, D1 = 16 seconds). The quantity of THC was determined using the following equation (II.6):

$$x_{THC} = \frac{1 * I_{ar,THC} * M_{THC} * \rho_{PFBA} * V_{PFBA}}{2 * I_{PFBA} * m_{part} * M_{PFBA}} \quad (\text{II.6})$$

where $I_{ar,THC}$ and I_{TMS} are intensities of aromatic protons of THC and the proton of PFBA, M_{THC} and M_{TMS} are the molar masses of THC and PFBA (372.417 g/mol and 196.07 g/mol), m_{part} is the mass of particles, ρ_{TMS} is the volumic mass of PFBA (1588 g/L) and V_{PFBA} the volume of added PFBA. The experiment was performed in triplicate.

4.3.7 Antioxidant properties: DPPH \cdot technique

The DPPH \cdot technique was the one selected to determine antioxidant properties. The DPPH \cdot is reduced in the presence of an H-donor to form the entity DPPH-H. To follow the reaction, a UV-spectrophotometry is performed, as the DPPH \cdot is purple and the DPPH-H is yellow in solution.

The radical solution was freshly prepared for the day and stored in the fridge when not used. DPPH \cdot (1.9716 mg) was added in a 50 mL graduated flask. After adding methanol, the solution at 10⁻⁴ M was poured in an amber bottle. Solutions of active materials at 10⁻³ were prepared following the Table II.3. For particles, it corresponds approximatively to 1.86 mg of THC-loaded particles. The reaction was carried out in amber vials or in 2 mL tubes covered with aluminium paper. They were put under stirring for 25 minutes and removed to be centrifuged (3 minutes, 3000 rpm) for particles. 500 μ L of homogeneous reaction solution were quickly poured into a quartz cell and analysed with the spectrophotometer from 250 to 600 nm. The value of the absorbance at 517 nm compared to the one of the control solution gave the inhibition percentage as following (II.7), where A is the absorbance at 517 nm:

$$\%Inhibition = \frac{A(control) - A(antiox. molecule)}{A(control)} * 100 \quad (II.7)$$

Table II.3: Composition of antioxidant solutions and volumes needed for the DPPH \cdot test.

Preparation of solutions					Test	
Compounds	C (M)	M (g/mol)	V (mL)	m (mg)	V _{compound solution} (μ L)	V _{DPPH solution} (mL)
THC	10 ⁻³	372.4	10	3.72	30	1
BHT	10 ⁻³	220.35	15	3.31	150	5
Particles & CD	-	-	0.15	1.86	150	5

5 Antimicrobial properties

5.1 Preparation of the spore solution

Four agar cubes are cut from a Petri dish of newly grown mycelium. They are incubated in 20 mL of CMC medium (CMC 15 g/L, yeast extract 1 g/L, MgSO₄ 7H₂O 0.5 g/L, NH₄NO₃ 1 g/L, KH₂PO₄ 1 g/L) and put under agitation (180 rpm), in the dark and at 25°C. After 4 days of growth, the suspension of spores was filtrated with 100 μ m filter (Figure II.6). The spores were counted with a Malassez cell on a microscope X40 (Leica) and the desired concentration of the solution of spores was adjusted with distilled water.



Figure II.6: Solution of spores before filtration.

5.2 Inhibitory properties against the fungal growth

5.2.1 Medium supplemented with the tested molecules

The mycelial growth inhibition test was performed on Petri dish of 85 mm in diameter filled with 15 mL of PDA. The medium was supplemented with tested molecules, which had been mixed when the medium was hot and liquid (Figure II. 7). The control condition corresponded to the PDA medium. Approximately 10 μ L of the solution of spores, containing 100 spores, were inoculated on the centre of the dish. Petri dishes were incubated at 25 °C at 70% of relative humidity in the dark. Two perpendicular diameters were measured every day. All conditions were performed in triplicate.

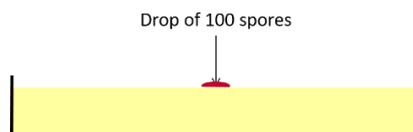


Figure II.7: Inoculation of the solution of spores on the PDA medium

5.2.2 Medium coated with the tested formulation

This test was also performed on Petri dish of 85 mm in diameter filled with 15 mL of PDA.

Three methods were used. The first one with a coating before inoculation was performed to model a preventive treatment (Figure II.8 left). The medium was coated with 2 mL of the product solution. The solutions were dried under the sterile hood for 2 hours. Approximately 10 μ L of the solution of spores, containing 100 spores, were inoculated on the centre of the dish.

The second one was employed to simulate a curative treatment (Figure II.8 right). The inoculation is then carried out first and the solution is cast two hours after the inoculation.

The third one corresponds to the casting of the coating 24 hours after the inoculation.

Control conditions corresponded to the PDA medium coated with 2 mL of distilled water.

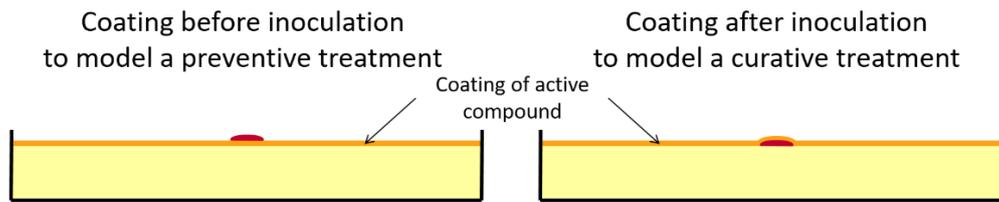


Figure II.8: Inoculation of the solution of spore solution on the coatings.

5.2.3 Determination of inhibition percentages

Petri dishes were incubated at 25 °C at 70% of relative humidity in the dark. Two perpendicular diameters were measured every day. All conditions were performed in triplicate.

Percentages of inhibition at day 4 were expressed as shown in (II.8).

$$\%Inh = \frac{\text{Average diameter of blank} - \text{Average diameter of condition } i}{\text{Average diameter of blank}} * 100 \quad (\text{II.8})$$

5.2.4 Determination of the IC₅₀

The determination of the IC₅₀ required that the product has been evaluated at several concentrations. Then, at day 4, percentages of inhibition were plotted *versus* the logarithm of the concentrations of the tested product. A linear regression was performed. The IC₅₀ was calculated according to the equation (II.9).

$$IC_{50} = 10^{\frac{50-b}{a}} \quad (\text{II.9})$$

b is the intersection at origin of the regression line and a the slope of the regression line.

5.3 Inhibitory properties against the production of toxins

Strains were grown in Minimal Synthetic liquid medium (glucose 20 g/L, KH₂PO₄ 0.5 g/L, K₂HPO₄ 0.6 g/L, MgSO₄ 17 mg/L, (NH₄)₂ SO₄ 1 g/L, Traces element of Vogel 10 µg/mL). The Minimal Synthetic solution (8 mL) was poured in small Petri dishes (diameter = 55 mm) and inoculated with 2.10⁵ spores/mL of the solution of spores. Petri dishes were incubated in the dark at 25 °C, with 70 % of relative humidity. After 15 days, fungal liquid cultures were transferred into beforehand weighted plastic tubes and then centrifuged at 3360 g during 5 min. Supernatants were poured into new tubes and stocked at -20 °C until analyses. Mycelia were placed at -80 °C and then lyophilised during 48 h. The mass of dry biomass was determined by weighing.

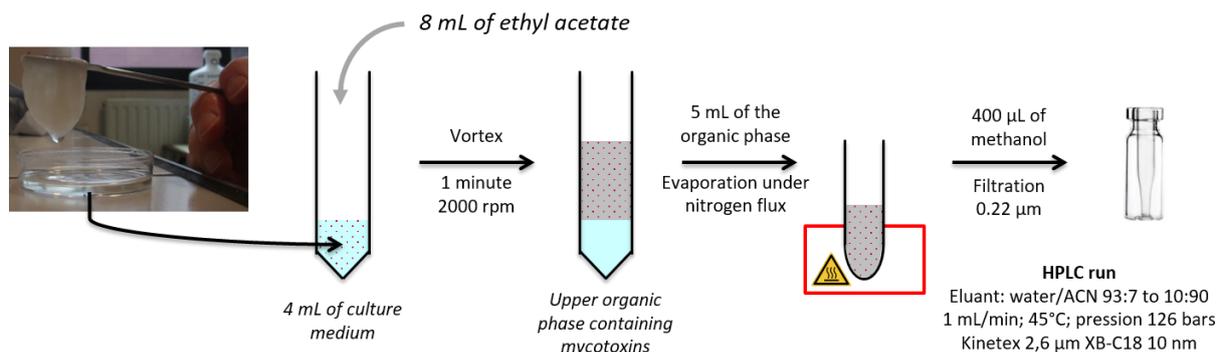


Figure II.9: Main steps of the extraction and analysis of TCTBs.

TCTBs were extracted by a liquid-liquid extraction following the MycSA's protocol (Figure II.9). The aqueous medium (4 mL) was vortexed with ethyl acetate (8 mL). The organic upper phase (5 mL) was then transferred into a new glass tube and evaporated under nitrogen flux. The different dry extracts were resolubilised in 400 µL of methanol/water (1:1). The extracted samples were filtrated through a 0.22 µm filter and then run into an Agilent Technology 1100 liquid chromatography system equipped an auto-sampler system, an Agilent photodiode array detector (DAD) and the ChemStation chromatography manager software (Agilent Technologies, Les Ulis, France). Separation was achieved on a Kinetex XB-C18 100 Å column (4.6 X 150 mm, 2.6 µm) (Phenomenex, France) maintained at 45 °C. The mobile phase consisted of water acidified at pH 2.6 with orthophosphoric acid (solvent A) and acetonitrile (solvent B). TCTBs were separated using a gradient elution as follows: 7 % to 30 % B in 10 min, 30 % to 90 % B in 5 min, 90 % B for 5 min, 90 % to 7 % B for 2 min, 7 % B for 5 min.

5.4 Statistical analysis

All experiments were done with at least 3 repetitions. Data for TCTB production and fungal growth were reported as mean values \pm standard deviation of three or four replications. Mean values were compared at the 5 % significance level using Conover-Iman test (control vs treated) with the XLSTAT software.

CHAPTER III – CHITOSAN-BASED FORMULATIONS

1	Characterisation of chitosans	73
1.1	Determination of the molecular weights.....	73
1.2	Determination of the deacetylation degree (DD)	75
2	Antifungal and antimycotoxigenic activities of chitosans in solution	78
2.1	Selection of chitosans – Radial growth test	78
2.2	Additional study of antifungal properties of the selected chitosans.....	80
3	Study of chitosan films as a model for the coating of leaves	89
3.1	Antifungal properties	90
3.2	Physicochemical characterisations	95
4	Concluding remarks	100

III. CHITOSAN-BASED FORMULATIONS

Let's reiterate that chitosans are chitin-derivative polymers composed of β -(1 \rightarrow 4)-linked *N*-acetyl-D-glucosamine and D-glucosamine, exhibiting numerous specific properties such as antibacterial and antifungal activities as it has been mentioned in the first chapter. In that respect, chitosans were here evaluated as an option to control both the *F. graminearum* mycelial growth and the accumulation of mycotoxins by the fungi.

After the characterisation of chitosans, the strategy developed was primarily to select some of them regarding the simple antifungal activity of chitosan-based solutions. Secondly, an in-depth study of the antifungal and antimycotoxigenic properties of selected chitosan solutions was carried out. At the end, to get closer to the targeted application – coatings on plant leaves –, chitosan solutions were cast and resulting films were studied regarding not only their antifungal activity but also their moisture, oxygen and carbon dioxide barrier properties, knowing that they could have an impact on the plant development. Global steps are summarised in Figure III.1.

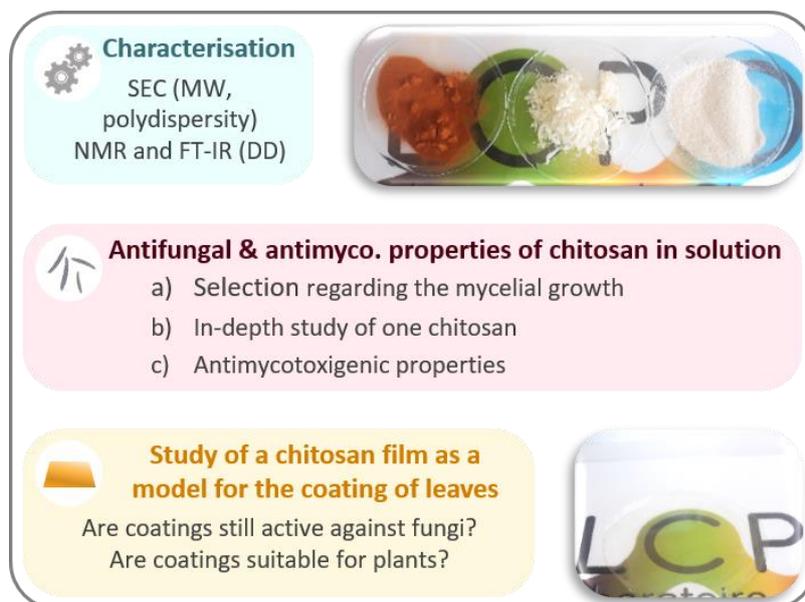


Figure III.1: Schematic representation of the strategy.

1 Characterisation of chitosans

Four chitosans from various suppliers were used (Table III.1) and were first characterised in terms of molecular weight and dispersity. Their DD was determined by NMR and FT-IR.

Table III.1: List of chitosan samples and their origin.

Chitosans	Origin
Industrial chitosan France chitine® SN	Crabs
Aldrich #STBH2613	Marine origin but no more precision
Chitosan 20 cps SPN Agrobio®	Shrimps
Chitooligosaccharide 3000 SPN Agrobio®	Shrimps

1.1 Determination of the molecular weights

The molecular weights of chitosans were determined with SEC, equipped with RI, UV and MALS detectors. First, the specific refractive index increment was evaluated to later access the MW from the signal of the refractive index detector.

1.1.1 Determination of the specific refractive index increment

The values of specific refractive index increment (dn/dc) were determined just for both chitosans from Agrobio through a calibration curve (Figure III.2 and Supplementary Information), which were 0.17 and 0.15 mL/g for the Chitosan 20 cps and Chitooligosaccharide 3000, respectively. Because the first value was within the range of 0.16-0.18, usually found in the literature [335,336], we used 0.17 mL/g for all the calculations of chitosan molecular weights excepted for the chitooligosaccharide.

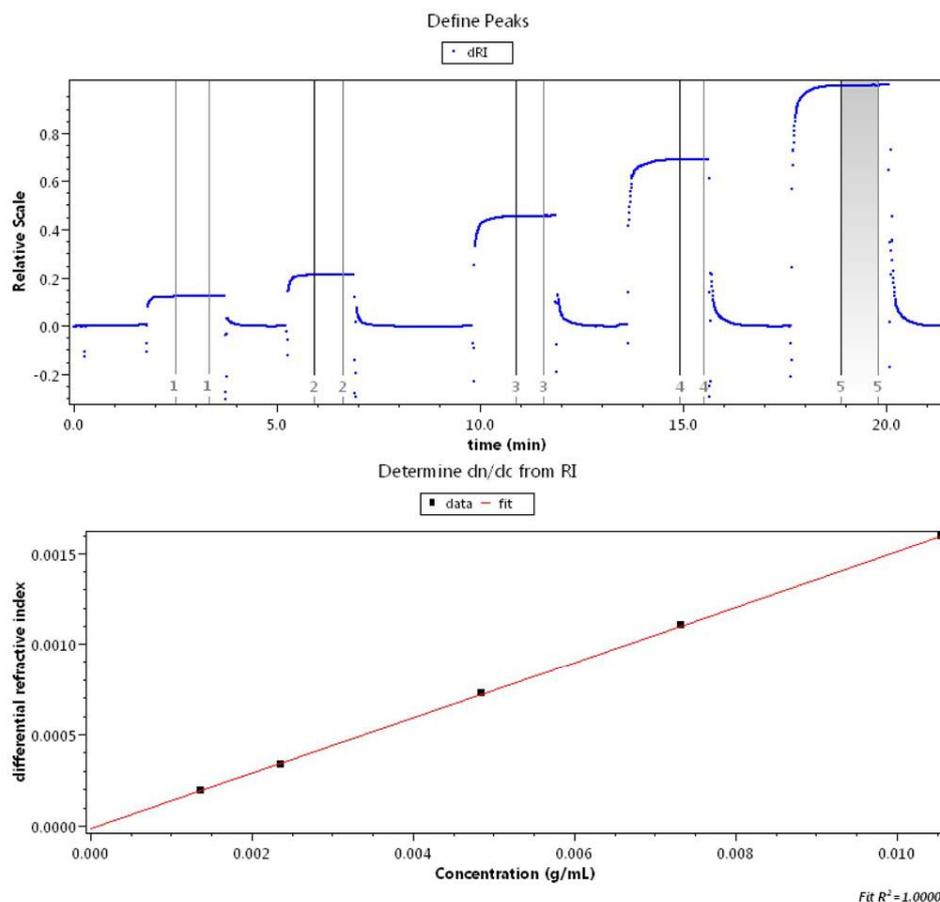


Figure III.2: Determination of the specific refractive index increment (dn/dc) for Chitooligosaccharide 3000 SPN Agrobio [®].

1.1.2 Molecular weights and polydispersity index

The resulting MW of the various chitosans are presented in Table III.2.: To simplify the rest of the manuscript, chitosans will be named using their abbreviation according to their \overline{M}_n . All chitosans can be considered as low molecular weight chitosans except the Ch. 4.8, which is a chitooligosaccharide. All samples are slightly to highly polydisperse, with a polydispersity index from 2 for Ch. 78, which is common for polysaccharides, to 12 and Ch. 4.8. The high polydispersity of the Ch. 4.8 should be due to the industrial process to depolymerise “native” chitosans. Particularly, the Ch. 127 and the Ch. 130 contained chains of very high molecular weight (> 800 kDa), as can be seen in Figure III.3.: The Ch. 4.8 also possessed a sizeable proportion of chains with low molecular weight.

Table III.2: Molecular weight, polydispersity index and abbreviations of the chitosan samples.

Chitosan	\overline{M}_n Peak 1 (kDa)	\overline{M}_n Peak 2 (kDa)	\overline{M}_n Peak 3 (kDa)	\overline{M}_n (kDa)	\overline{M}_w (kDa)	Polydispersity index	Abbreviations
Industrial chitosan France chitine® SN	862	93	-	130±3	823±8	6.3±0.1	Ch. 130
Aldrich #STBH2613	917	354	93	127±3	381±3	3.0±0.1	Ch. 127
Chitosan 20 cps SPN Agrobio®	434	68	-	78±1	160±2	2.0±0.1	Ch. 78
Chitoooligosaccharide 3000 SPN Agrobio®	206	23	3.8	4.8±0.1	58.6±0.4	12.0±0.1	Ch. 4.8

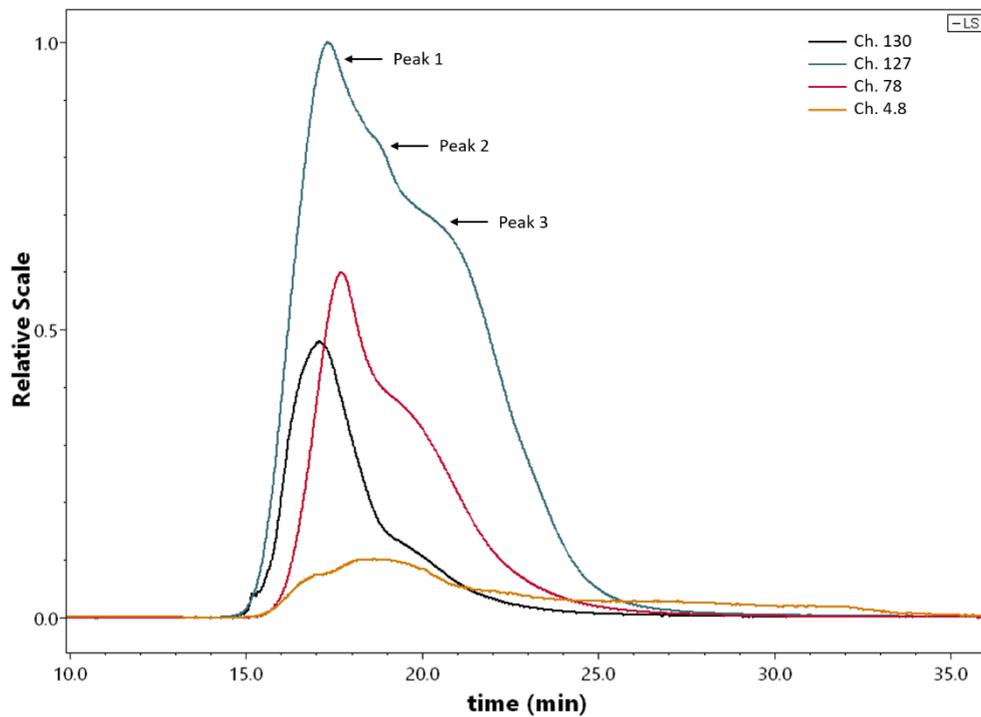


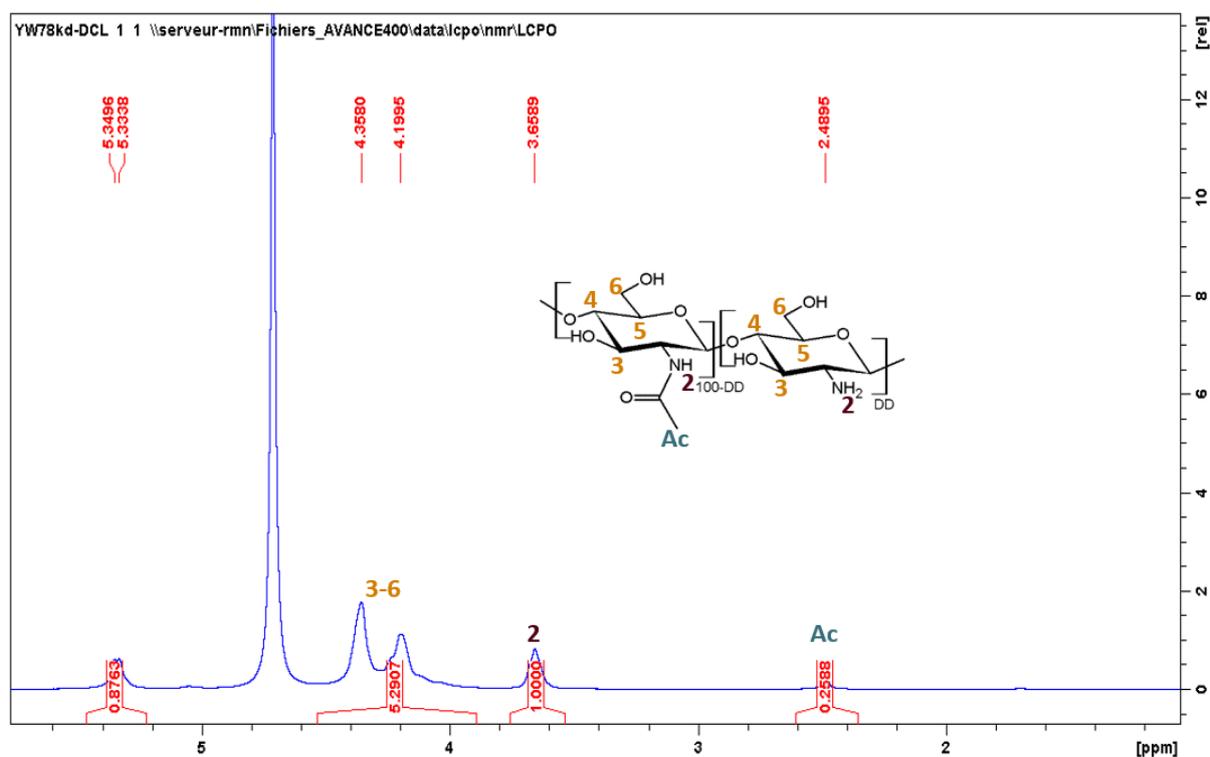
Figure III.3: SEC chromatogram of Ch. 130, Ch. 127, Ch. 78, Ch. 4.8 in acetate buffer.

1.2 Determination of the deacetylation degree (DD)

The antimicrobial properties of chitosans are mainly due to the cationic charges brought to the polymer chain by the protonation of amino groups. The higher the DD is, more amine groups on the chitosan backbone could be protonated. The determination of chitosan DD is thus a conventional characterisation that can be done by different techniques such as FT-IR, UV or $^1\text{H-NMR}$. $^1\text{H-NMR}$, one of the most accurate, and FTIR were used.

1.2.1 By $^1\text{H-NMR}$

The DD of chitosans was calculated from the relative integration of the signals of protons 2 to 6, $I_{\text{H}2-6}$, and of protons of acetyl functions, I_{Ac} . The example of Ch. 78 can be seen in Figure III.3. The results are presented in Table III.2.



All chitosans had relatively high DD values, which should be interesting for our application, as free protonated amine groups are obviously related to antifungal properties.

Table III.2: Intensity of signals and calculated DD.

Chitosan	I_{Ac}	$I_{\text{H}2-6}$	DD (%)	Supplier's data
Ch. 130	0.03	5.81	98	-
Ch. 127	0.57	6.69	83	75-85
Ch. 78	0.26	6.29	92	90
Ch. 4.8	0.57	8.38	86	85

1.2.2 By Fourier Transform Infrared Spectroscopy

To complete the chemical characterization of chitosans, and determine DD by another technique, FT-IR analyses were carried out on all chitosans.

The FT-IR spectra of the chitosans are given in Figure III.4. As expected, characteristic absorption bands were found, especially the ones at 3450 cm^{-1} attributed to OH moieties and the ones at 1655 cm^{-1} attributed to stretching of C=O of amide functions. The bands at 2920 cm^{-1} , 1583 cm^{-1} and 1180 cm^{-1} correspond to the stretching of C-H, the bending of N-H functions of amines and C-N, respectively [36,337].

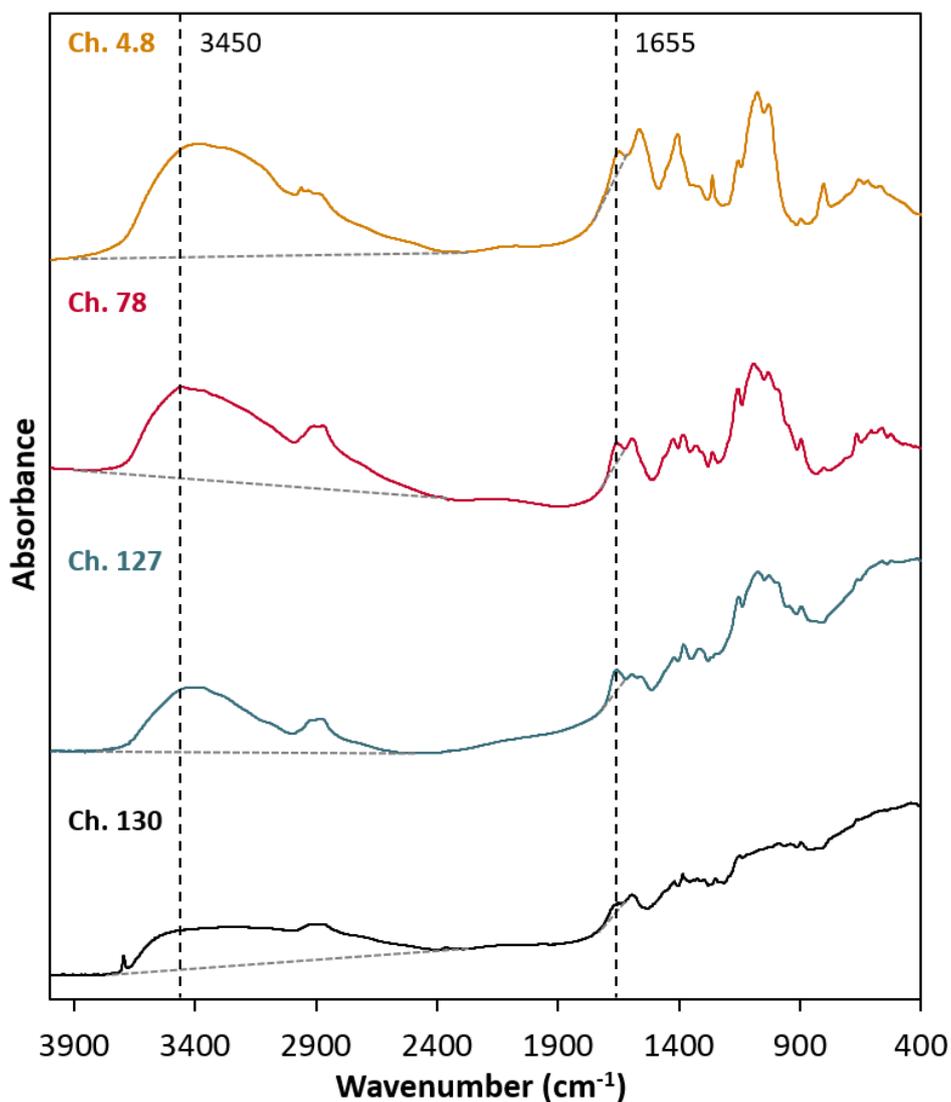


Figure III.4: FT-IR spectra of Ch. 130, Ch. 127, Ch. 78, and Ch. 4.8 in KBr pellet.

To estimate the DD of chitosan, the relative intensity of the bands was used. The baselines to measure the intensities were those used from Baxter *et al.* [333] and are plotted in grey in Figure III.4.

The values found for all chitosans (Table III.3) were far much lower than the DD calculated from NMR and also further away from the supplier's data. This should be discussed because this technique is usually employed to determine chitosan DD in literature [338,339] but it is complicate

to measure the absorbance of the band at 1655 cm^{-1} . Czechowska-Biskup *et al.* [340] compared different methods for the determination of the DD. They concluded that the FT-IR method was imprecise because some bands can be shifted due to humidity, the baseline determination is operator-dependent or some impurities could absorb in the same range as interesting bands.

Table III.3: Relative intensity of two characteristic bands and calculated DD.

Chitosan	A₃₄₅₀	A₁₆₅₅	DD (%)
Ch. 130	2.7	0.5	78
Ch. 127	3.9	1.4	59
Ch. 78	7.2	1.6	75
Ch. 4.8	7.4	1.2	81

2 Antifungal and antimycotoxigenic activities of chitosans in solution

The antifungal activity of chitosans was first evaluated to select the most promising chitosan with regards to our application. The antifungal activity was evaluated using the radial growth test, where the active compound was directly mixed with the agar-based medium. To dissolve chitosans, mild acidic conditions are needed. Acetic acid (AA) or lactic acid (LA) were thus compared and the pH was then adjusted to 5.6, which was the initial pH of the culture medium. After selecting the best chitosan, its activities against both the growth and the production of toxins were further studied and compared.

2.1 Selection of chitosans – Radial growth test

Chitosan solutions lead to strong growth inhibitions of *F. graminearum* in both acidic conditions, AA or LA (Table III.4).

The AA, *i.e.* the acid itself, totally inhibited the mycelial growth of the fungus. Its concentration was therefore too high to discriminate which chitosan was the most promising. On the contrary, the lactic acid alone promoted the fungal growth and chitosan solutions showed a strong growth inhibition easily attributed to chitosans. Finally, with LA as counter-ion, the antifungal effect of chitosan followed the following order: Ch. 78 > Ch. 130 = Ch. 127 > Ch. 4.8.

Table III.4: Percentage of the mycelial growth inhibition of *F. graminearum* on PDA medium at pH = 5.6 supplemented with chitosans at 5 g/L and acids at 5 mL/L after 4 days of incubation. Standard deviation on 3 Petri dishes. Different letters in superscript indicate significant differences, within the same experiment 1, 2 or 3.

Chitosans 5 g/L	Percentage of the growth inhibition at day 4 (%)	
	Acetic acid 5 mL/L	Lactic acid 5 mL/L
Ch. 130	100 ^{1,a}	82±3 ^{1,b}
Ch. 127	100 ^{1,a}	76±5 ^{1,b}
Ch. 78	100 ^{2,a}	100 ^{3,a}
Ch. 4.8	89±1 ^{2,b}	51±3 ^{2,c}
No chitosan	100 ^{1,a}	-24±2 ^{1,c}

In the literature, the influence of the MW on antimicrobial effects is not fully understood. Some authors found that low MW chitosan was more efficient than medium MW chitosan, which was itself more efficient than high MW chitosan to inhibit three strains of *Rhizopus stolonifera* at the same pH as ours [150]. In the same vein, chitooligosaccharides are also known to have antifungal properties [341,342]. In our experiment, a hypothesis is that the Ch. 4.8 was too small to modify the membrane permeability of the fungus after interaction with some fungal wall compounds, a mode of action generally admitted to explain chitosan’s antimicrobial activity, thus being less efficient than other chitosans with longer chains.

Regarding the influence of the DD, we compared Ch. 130 and Ch. 127 because their MW are close. The higher efficiency of Ch. 130 could be due to its higher DD, leading to a higher amount of amino groups.

Chitosan with AA are more efficient than chitosan with LA.

In the end, Ch. 78 was selected regarding its activity against the mycelial growth. In addition, the Ch. 4.8 was also selected because it is possible that very low MW could be efficient to control the production of toxins and could also serve as elicitor. Chitooligosaccharides demonstrated antioxidant properties [342], which are often linked with an antimycotoxigenic action.

2.2 Additional study of antifungal properties of the selected chitosans

2.2.1 Antifungal properties

2.2.1.a How to choose the working pH?

AA and LA were used to dissolve Ch. 78 and Ch. 4.8, and the addition of acid in the PDA medium changed the pH. Three working pH-possibilities were then considered.

In the first one, the acidic solution of chitosan was used without adjusting the pH of the culture medium. The resulting pH was 4.8 in chitosan solutions and 4.3 or 4.0 only with AA or LA respectively (no chitosan added). The advantage of this experiment is that a minimum of products is needed.

In the second one, the pH of the medium was adjusted to 5.6, the initial pH of the culture medium, to avoid an impact of the pH on the fungus. This action implied that varying amounts of soda were added to adjust all initial pHs, with the risk that sodium ions interfere with the fungi, thus affecting the results.

Finally, the third possibility is to adjust the pH of acid solutions to 4.8, which was the pH of the chitosan solutions.

The acid/ionic form for each acid is represented in Figure III 5.

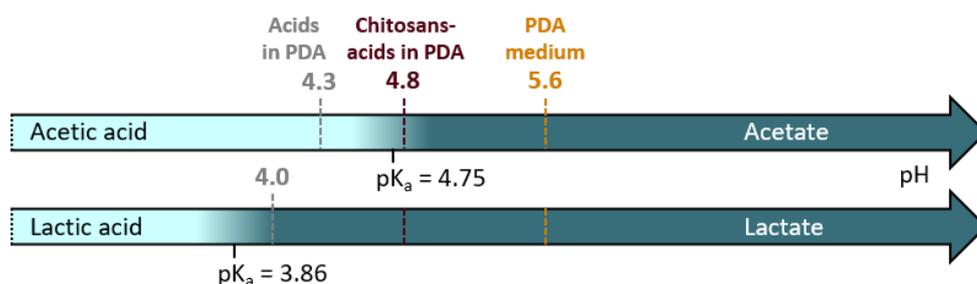


Figure III 5: pK_a -scale of acetic and lactic acids with working pHs.

The two acids did not have the same behaviour. We expect lactate ions in the medium whatever the pH we choose, whereas the acetic acid can be in its two forms depending on the pH. We chose to work with a non-adjusted pH, to add the less products as possible or with an initial pH of 5.6, pH at which both acids are in their basic form.

The third possibility was then eluded, but, as a side remark, the effects of acids alone at pH 4.3 or 4.8 were compared. The increase of the pH from 4.3 to 4.8 with AA at 1 or 0.5 g/L caused 25 % and 28 % less inhibition, respectively. As well, the LA at 1 or 0.5 g/L was 44 % and 31 % less efficient at pH 4.8 than at pH 4.0. The pH had therefore a strong impact on the reduction of the mycelial growth.

2.2.1.b Impact of chitosans in the medium on the mycelial growth

Five concentrations of chitosan and acid (Figure III.6) have been evaluated over time for each condition to see the evolution of the activity and to determine the concentration which inhibited 50 % of the mycelial growth (IC₅₀). Only the results with no pH adjustment and after pH adjustment to 5.6 are presented. The results of the kinetic study, mycelial diameter versus time, are presented in Figure III.7 and Figure III.8.

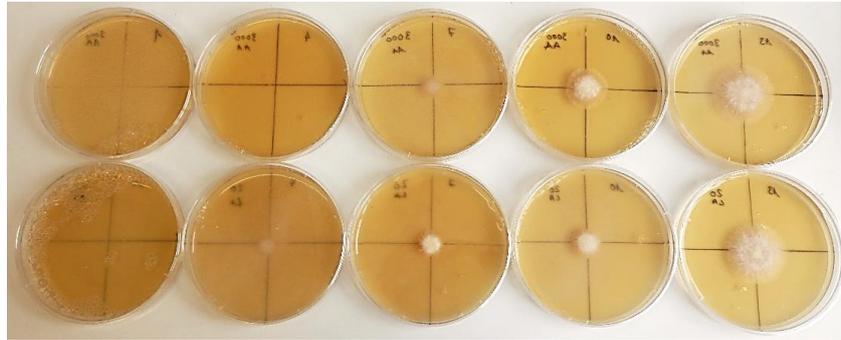


Figure III.6: Petri dishes of *F. graminearum* at day 3, grown on PDA medium, supplemented with Ch. 4.8 and AA (first line) and Ch. 7.8 and AA (second line) at concentrations 1.0, 0.8, 0.6, 0.4 and 0.2 g/L, without pH-adjustment.

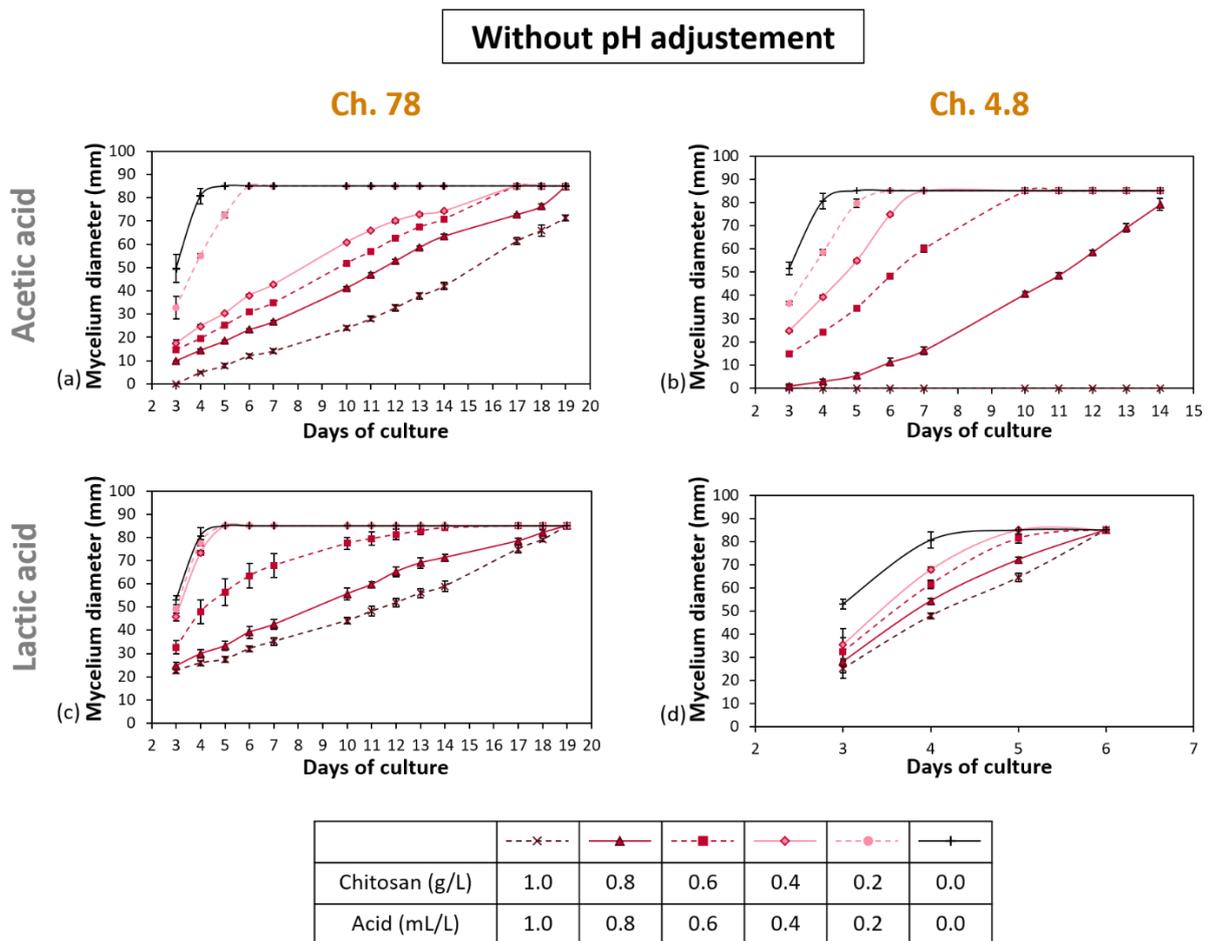


Figure III.7: Daily evolution of the mycelial growth of *F. graminearum* on PDA medium **without pH adjustment** supplemented with (a) Ch. 7.8-AA; (b) Ch. 4.8-AA; (c) Ch. 7.8-LA; (d) Ch. 4.8-LA at different concentrations at 25 °C and 70 % of R.H. Error bars correspond to the standard deviation of the measurements performed in triplicate.

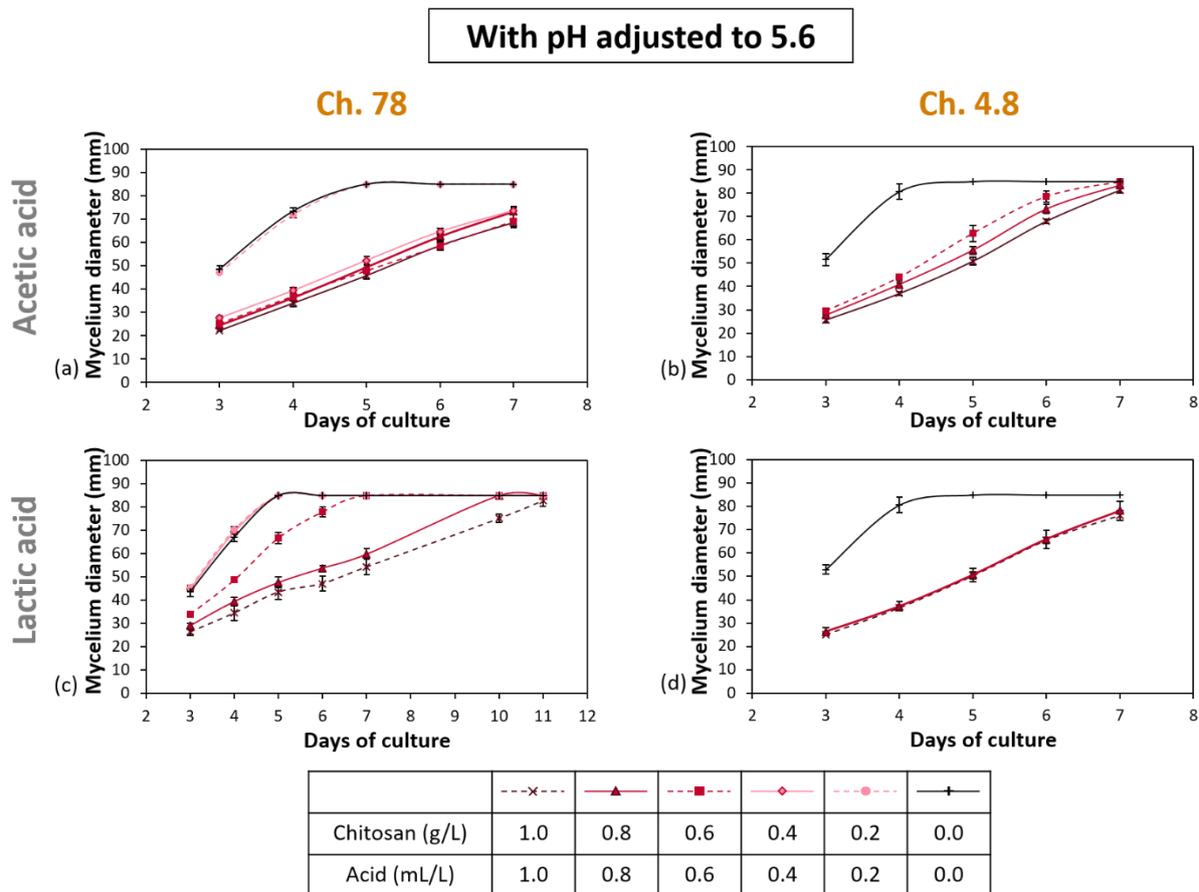


Figure III.8: Daily evolution of the mycelial growth of *F. graminearum* on PDA medium at pH = 5.6 supplemented with (a) Ch. 78-AA; (b) Ch. 4.8-AA ; (c) Ch. 78-LA; (d) Ch. 4.8-LA at different concentrations at 25 °C and 70 % of R.H. Error bars correspond to the standard deviation of the measurements performed in triplicate.

The first global observation is that chitosans and acids had a major impact on the fungal growth, whatever the tested concentrations.

Secondly, the higher the concentrations of chitosans or acids, the smaller the diameters of mycelia. Moreover, the higher the concentrations, the smaller the growth rate. For example, the mycelium was growing at approximately 20 mm/day for Ch. 78 at 0.2 g/L and 4.5 mm/day for Ch. 78 at 1.0 g/L, with AA without pH-adjustment (Figure III.7 (a)). It is common for traditional synthetic fungicide to slow the growth rate in a dose-dependent manner as for example on *F. graminearum* [343] or on *F. proliferatum* [344]. As chitosan is concerned, direct effects on the growth were also proven [345].

A slight effect on the germination can be suspected for Ch. 78-AA (1 g/L–1 mL/L) without pH-adjustment, as the mycelium was still not visible at day 3 -lag phase-, implying that the spore germination was surely delayed. Some authors found a more pronounced action of chitosan on spores than on growing hyphae for many fungi, including *F. oxysporum* [346]. As a result, the inhibition of the fungal growth strongly relies on the *total* inhibition of the spore germination and/or the inhibition of the growth.

The growth rates were maintained over time suggesting that the growth inhibition is constant, and that products are still efficient. Nevertheless, almost every condition was inefficient to totally inhibit the mycelial growth at the tested concentrations. Only the AA alone at 1 and 0.8 mL/L (graph not represented) and the Ch. 4.8-AA at 1 g/L completely stopped the growth (Figure III.7 (b)). The pH of the medium was certainly too low to enable the development of the fungi.

For a clarity purpose, IC₅₀ of chitosans and acids were calculated to compare the efficiency on the selected fungus.

2.2.1.c Calculation of the IC₅₀ after four days of incubation

The IC₅₀ was calculated from the percentages of inhibition compared to the control after four days of incubation, plotted *versus* the logarithm of the concentration. The Figure III.9 shows the linear regression obtained from the data plotted in Figure III.7 (a) with Ch. 78-AA, leading to an IC₅₀ of 0.3 g/L.

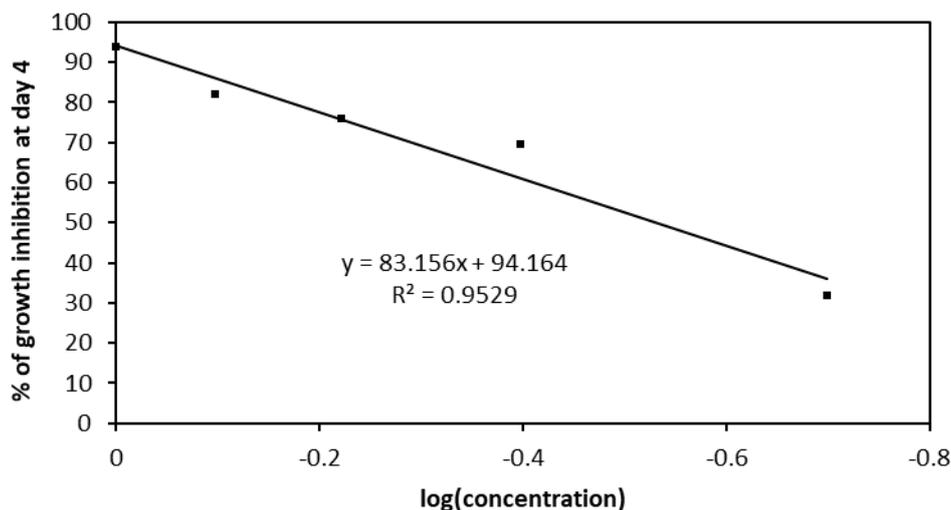


Figure III.9: Linear regression of the log(concentration) versus the percentage of growth inhibition with Ch. 78-AA at day 4, without pH-adjustment.

IC₅₀ results for the various conditions were presented in Table III.5. The smaller the IC₅₀, the more efficient the product.

Table III.5: IC₅₀ of chitosan acidic solutions (g/L and ml/L) and acidic solutions (mL/L) by *F. graminearum* in PDA medium, with and without pH adjustment after 4 days of incubation at 25 °C and 70 % R.H.

Chitosans and acids	IC ₅₀			
	Non-adjusted pH		pH adjusted to 5.6	
	Acetic acid	Lactic acid	Acetic acid	Lactic acid
Ch. 78	0.30	0.7	0.65	>1
Ch. 4.8	0.35	>1	1	>1
No chitosan	0.40	>1	>1	>1

III. CHITOSAN-BASED FORMULATIONS

The Ch. 78-AA possesses the lowest IC_{50} at 0.3 g/L, closely followed by the Ch. 4.8-AA and the AA alone at 0.4 mL/L, when the pH is not adjusted. As a comparison, a commercial formulation of chitosan chlorhydrate from France chitine® ($M_n = 33.3 \pm 0.3$ kDa) also gave an IC_{50} of 0.5 mL/L (data not shown).

Data obtained after the pH-adjustment were higher than those without pH-adjustment. It can be therefore assumed that a more acidic medium (from pH 4.3 or 4.8 to 5.6) plays a key role in antifungal properties of chitosan and acid solutions.

Regarding the results with LA without pH control, only the Ch. 78-LA showed an IC_{50} below 1 g/L, which confirms that the Ch. 78 is more efficient than the Ch. 4.8 and brings additional antifungal properties compared to LA.

When the pH is adjusted to 5.6, which corresponds to the pH of the PDA medium and thus, the pH of the control condition, IC_{50} are higher. The influence of AA is less pronounced at pH 5.6, as values with or without chitosans are further apart. All IC_{50} at adjusted pH with the LA as acid were higher than 1 g/L.

We will now discuss the influence of physicochemical parameters on the antifungal activity of chitosans.

2.2.1.d *Understanding relationships between structural & antifungal properties*

The influence of pH on chitosan bioactivity was predictable, as chitosans are assumed to possess an antifungal activity thanks to their $-NH_3^+$ groups. To study the impact of the pH on the charge density of the Ch.78, a titration with sodium hydroxide was carried out. The ratio of protonated on non-protonated amine groups at a given pH was calculated and plotted *versus* pH (Figure III.10).

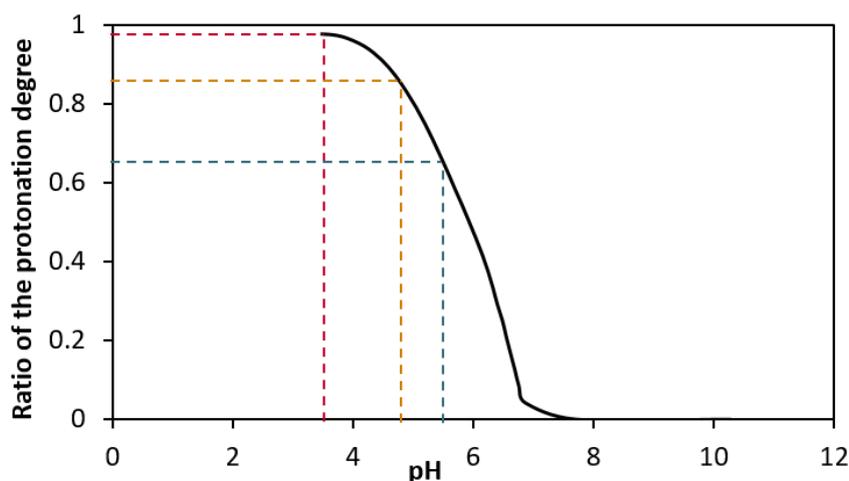


Figure III.10: Ratio NH_3^+/NH_2 given by the dosage of Ch. 78 with NaOH at 0.2 M.

At pH 3.5, chitosan should possess 97 % of the amino groups in $-\text{NH}_3^+$ form. In the PDA medium, at pH 4.8, the ratio was 85 % and at adjusted pH 5.6, the ratio fell to 63 %. The result highlighted again that the pH should have a large influence on the antifungal activity, by increasing the positive charges of chitosan. Many authors observed a better inhibition or a lower minimal inhibitory concentration for a given chitosan when the pH is lower, especially around 4.0-4.5 [149,341]. Avelelas et al. [342] found that water soluble chitosan (soluble at neutral pH, *i.e.* even when amines are not protonated) were less active than chitooligosaccharides, thus confirming that positive amine groups influence the antifungal activity of chitosans.

The influence of the counter-ion, here acetate or lactate depending on the acid used to solubilise chitosan, is more difficult to explain. In the work of El-Khadi *et al.* [347], acetic acid was the most efficient acid among eight organic acids for the inhibition of *Aspergillus flavus* and *Penicillium pururogenum* and one of the most efficient against *Rhizopus nigricans*. However, the growth of *F. oxysporum* was the most restricted by lactic acid. The commonly accepted mechanism for the action of acids on fungi is that they enter membranes, under osmotic pressure, then release H^+ in the fungus due to a higher pH inside the cell. The fungus must make additional efforts to keep its internal pH stable by eliminating H^+ , thus slowing the growth [347].

The DD, and therefore the density of free amine, was higher for Ch. 78 than for Ch. 4.8, which is another explanation for its stronger effect. We have already observed this trend between Ch. 130 and Ch. 127 in 2.1.

Regarding the molecular weight, Ch. 78 may possess chains short enough to enter inside fungal hyphae and spores and disturb some essential functions, while bigger chains could interfere with the fungal membrane. The SEC analyses (see 1.1.2) seemed to indicate different chain populations in the Ch. 78 sample indeed. We tried unsuccessfully to deconvolute signals.

To go further in understanding the mode of action of chitosan, SEM views were taken from the PDA solid medium (Figure III.11). Unfortunately, no obvious morphological changes can be seen, contrary to one study where huge modifications of the shape of the mycelium were observed [348]. When chitosan was put in the medium, the authors observed very small and swollen hyphae for all the species studied (*Alternaria alternata*, *Penicillium expansum* and *Rhizopus stolonifera*). The differences may be due to the medium of culture, which was liquid in their case and solid in ours, to the fungal species, and to chitosans, whose MWs were not mentioned.

III. CHITOSAN-BASED FORMULATIONS

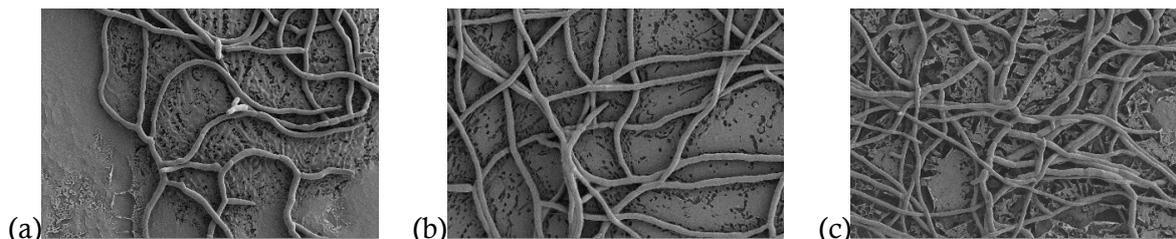


Figure III.11: SEM views of the mycelium of *F. graminearum* after 2 days of incubation on PDA medium, X400, high vacuum mode, 3 keV electron beam. PDA supplemented with (a) Ch. 78-AA 0.5-0.5*; (b) AA at 0.5 mL/L; (c) nothing (control).

* Ch.78 at 0.5 g/L and AA at 0.5 mL/L

Another attempt to examine the morphological changes was made by SEM in liquid culture. However, this task has proven to be arduous, for the aqueous medium has to be removed smoothly before the sample freezing in viscous liquid nitrogen. The sample was afterwards put under high vacuum. We assumed that one step went wrong because most of the mycelia were flat, marked with wrinkles like balloons inflated then deflated. The process was thus too damaging to distinguish characteristic morphological features due to one condition or another.

2.2.2 Antimycotoxigenic properties of selected chitosans

Because some antifungal molecules can exacerbate the production of toxic metabolites such as mycotoxins, the impact of chitosans on the production of toxins was then studied. The optimised medium for the selected strain was a minimal synthetic liquid medium, allowing a separation of the mycelium and the medium where toxins have been accumulated by a simple centrifugation. As a reminder, *F. graminearum* mainly produces trichothecenes B (TCTB) in Europe, including 15-acetyldeoxynivalenol (15-ADON) and deoxynivalenol (DON) as toxins. When toxins were extracted and analysed by HPLC a chromatogram close to Figure III.12 was obtained.

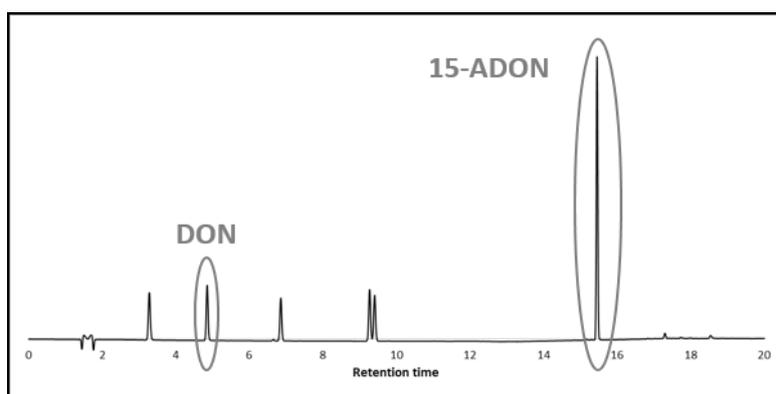


Figure III.12: TCTB analysis by HPLC (see experimental conditions p.69).

The Ch. 78 using AA or LA was first tested (Figure III.13). To study the impact of a product on TCTB production, an important point is that the active agent concentration must not change

the fungal growth. That is why concentrations were set at 100 mg/L with LA and 25 mg/L with AA, because fungi did not grow at 100 mg/L with AA.

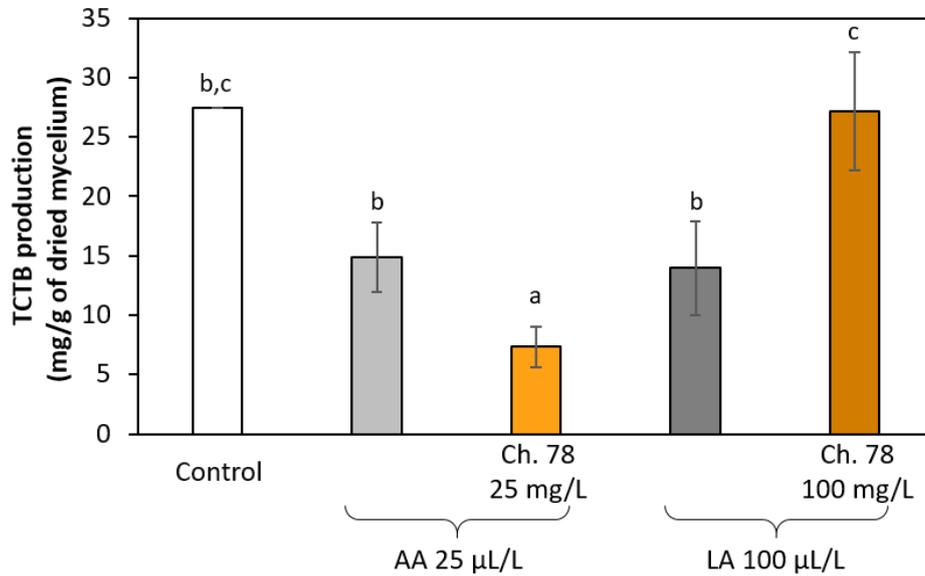


Figure III.13: TCTB production by *F. graminearum* in MS medium supplemented with Ch. 78 and AA or LA at different concentrations after 15 days of incubation at 25 °C and 70 % R.H. Error bars correspond to the standard deviation of the measurements performed on 5 Petri dishes. Different letters indicate significant differences.

Both acids reduced the production of toxins by approximately 50 %. Ch. 78–AA reduced the TCTB amount by 73 ± 6 %, which is the best and only significant inhibition. Ch. 78–LA did not show any effect on the TCTB accumulation, which is not what we would have expected, knowing that each product taken on its own reduced the TCTB quantity.

The same experiment was conducted with the Ch. 4.8 at 100 mg/L and acids at 100 µL/L (Figure III.14).

III. CHITOSAN-BASED FORMULATIONS

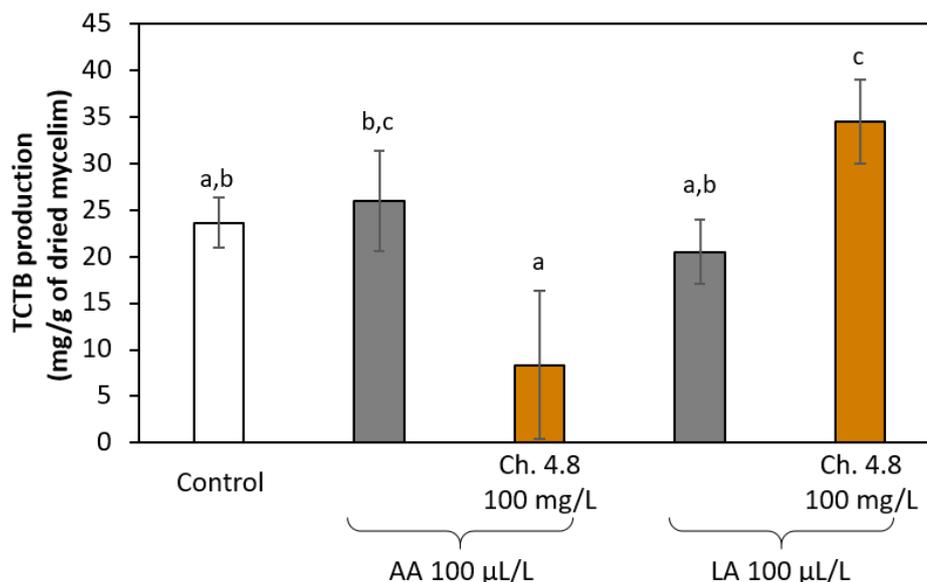


Figure III.14: TCTB production by *F. graminearum* in MS medium supplemented with Ch. 4.8 and AA or LA at different concentrations after 15 days of incubation at 25 °C and 70 % R.H. Error bars correspond to the standard deviation of the measurements performed on 5 Petri dishes. Different letters indicate significant differences.

The addition of Ch. 4.8–AA did not have a significant effect on the TCTB amount. On the contrary, the production of TCTBs with Ch. 4.8–LA significantly increased, which corresponds to the same trend observed with Ch. 78.

In a recent study, Zachetti *et al.* [349] showed the action of a chitosan (MW = 3.42 kDa, DD = 78 %) on the toxin production of *Fusarium* spp. For *F. proliferatum* and *F. verticillioides*, chitosan decreased the production of fumonisins (mycotoxins of these two species) up to 95 % in half of the studied conditions. No trend can be highlighted for the rest of the conditions. They also reported the action of this chitosan on two strains of *F. graminearum*. One strain was sensitive with a total reduction of the DON production at 2 mg/g of chitosan for each tested water activity (0.95, 0.98 or 0.99). The results for the other strain did not follow any trend.

Generally speaking, unpredictable influences of biocides on the TCTB production are often observed [216,230]. Ramirez *et al.* [343] emphasised the role of the environment on the accumulation of toxins and stated that the presence of sub-lethal doses of synthetic fungicide may have stressed the fungi. Knowing that the accumulation of mycotoxins is a stress-response [13,228,350], the presence of a biocide even in a small quantity may have led to an over-production of toxins.

Our results seemed to be in accordance with the literature, showing a reduction of the mycotoxigenesis, although, for some conditions, chitosan was not active.

As a complementary remark, chitosan was employed in some studies to absorb mycotoxins from a contaminated medium. Chitosan is known to remove various mycotoxins, heavy metal ions

and dyes [351]. Many toxins (aflatoxins, ochratoxins, zearalenone and fumonisins) were removed from palm kernel cake, except trichothecenes. The authors explained this phenomena by electrostatic interactions between the positively charged chitosan and the negatively charged toxins, except for DON whose pKa is 11.9 [352]. Ionic interactions also caused the removal of several toxins, especially ochratoxins and zearalenone, with the help of cross-linked chitosan-glutaraldehyde complexes [353]. Knowing that chitosan could trap mycotoxins, it could be hypothesised that some produced toxins are removed from the medium and not detected. The observed decrease in the toxins level could be due to the direct antimycotoxigenic properties of chitosan as well as on the trapping properties. An interesting experiment would be to check if chitosan and mycotoxins are present in the dried mycelia more than in the control.

2.2.3 Concluding remarks

Antifungal and antimycotoxigenic activities of different chitosans have been evaluated. Regarding the mycelial growth, the Ch. 78 was the most efficient in our study. A lower pH, linked with an increase of ammonium groups, had a positive impact on the reduction of the fungal growth. Contrary to LA, AA also helped the inhibition of the growth at both initial pH, probably due to intrinsic characteristics of the acid.

As previously found for the control of the fungal growth, the Ch. 78 solubilised with AA was also the best option to inhibit the accumulation of TCTBs. Although similar results have already been reported, no mechanisms of actions have been proposed yet. We suggest that, additionally to intrinsic effects against the mycotoxigenesis, chitosan could interact with already produced toxins and “purify” the medium, reducing the apparent concentration of TCTB.

3 Study of chitosan films as a model for the coating of leaves

Until this paragraph, all chitosans were studied in solutions, because we needed first to evaluate the bioactive properties of liquid formulations that should be pulverised on plants later. In addition, our results from chitosan solutions could be compared to those from literature, mainly made in solution. However, the solutions studied in this work are made to be sprayed on fields. This application implies that, when left out at the open air, the solution dries. And when a polymer with film forming properties such as chitosan is employed in a formulation, the evaporation of the solvent can lead to the formation of a thin film at the surface of the plant, depending on its concentration. That is why this part is dedicated to the study of chitosan films as a model of the resulting coating of the leaves of treated maize or wheat. One slightly yellowish chitosan film is pictured in Figure III.15.



Figure III.15: Picture of a chitosan film (Ch. 78 10 g/L and AA 10 mL/L).

As a result, this part is not devoted to the study of film mechanical properties, as films are not meant to be used for packaging or membrane applications. First, we checked if antifungal properties are maintained in a film form. The films are made from Ch. 78–AA solutions, since it was the most promising association in solution and because the Ch. 4.8 could not form films due to its very low molecular weight.

Secondly, a focus is made on the behaviour of the films towards water and UV radiations, as a future antifungal agent will be in contact with rain and sun. Barrier properties were also taken into account, as the chitosan coating should not prevent the gas exchanges at the surface of the leaves to ensure the proper development of the plant.

3.1 Antifungal properties

3.1.1 Effect of coatings on the development of the mycelium

To ensure that chitosan was still active as a coating, the antifungal properties of the Ch. 78–AA coating were studied against the growth of *F. graminearum*. Results were compared to coatings made by substituting chitosan with hydroxypropylmethyl cellulose (HPMC), knowing that HPMC has similar film-forming properties, similar oxygen barrier properties but without any direct antifungal action. Thus, it was possible to differentiate antifungal properties due to intrinsic properties of chitosan or due to film properties (gas barrier, nutrient barrier). The solvent controls (acid controls) did not, of course, form a film but permeated the medium and evaporated.

The daily evolution of the mycelial growth is plotted in Figure III.16.

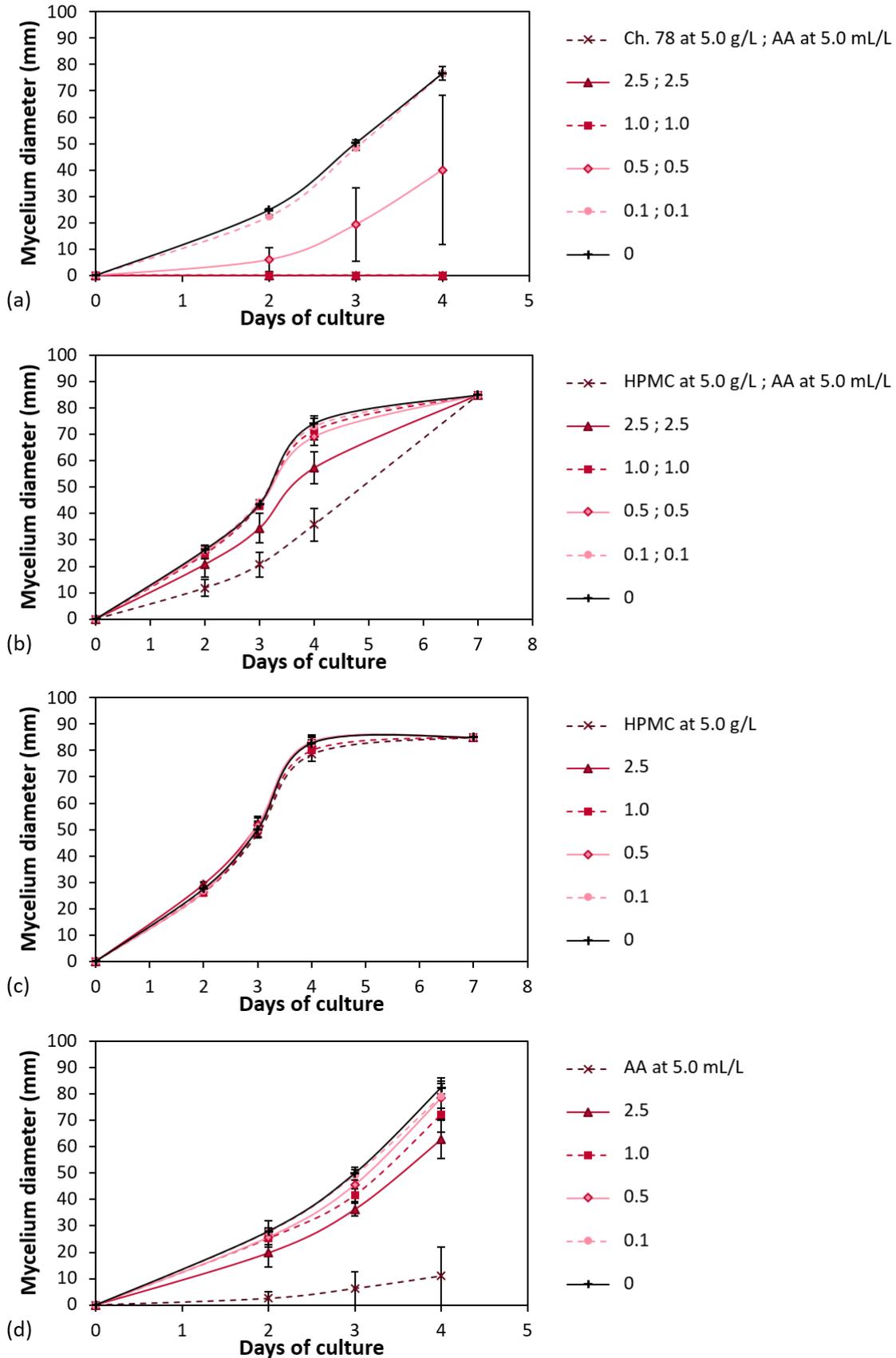


Figure III.16: Daily evolution of the mycelial growth of *F. graminearum* on PDA medium coated after inoculation with (a) Ch. 78-AA; (b) Ch. 4.8-AA ; (c) Ch. 78-LA; (d) Ch. 4.8-LA at different concentrations at 25 °C and 70 % R.H. Error bars correspond to the standard deviation of the measurements performed in triplicate.

III. CHITOSAN-BASED FORMULATIONS

A global overview showed that the Ch. 78-AA, the HPMC-AA and the AA coating had a significant impact on the mycelial growth, whereas HPMC films did not damage the fungi. Thus, the Ch. 78 and the AA did keep their antifungal properties in the form of a film. We observe a dose-dependent response, like we did with chitosan-acids in solution. The higher the concentration of the film forming solution, the more inhibition was obtained. Conditions with Ch. 78-AA with a concentration above 1.0 g/L totally inhibited the fungal development.

As the HPMC film only reduced the mycelial growth about 5 % (which was not significant compared to the control), the antifungal properties of the mentioned films are mostly due to their intrinsic antifungal activities than to the film form, assuming that chitosan and HPMC films have the same film properties (barrier properties).

The AA alone notably inhibited more the mycelial growth than the film of HPMC-AA, although the HPMC did not have any effect. We could assume that the AA in the polymer film was trapped and did not translocate in the medium, like it surely did when applied alone as an acidic solution, thus being more present in the medium and acidifying it.

As a remark, the concentrations required here to inhibit the fungi were higher than those used in solution. However, they were certainly much closer to the dose which could be applied in a field, as products would be cast on the surface of the leaves.

Then, we wanted to simulate the fact that the treatment would be applied at different moments and that the contamination of plants can therefore occur before or after the biocontrol treatment.

3.1.2 Impact of the treatment moment

The coating was performed at three different times of inoculation. These different moments simulated the delayed arrival of spores on the ear of the plant.

A coating before inoculation meant that the solution was cast on the medium and was dried for two hours, at the end of which the inoculation was carried out, thus simulating a preventive treatment.

The coating after inoculation corresponded to a coating two hours after the inoculation.

Finally, a coating 24 hours after the inoculation was also performed. These last two conditions simulated a curative treatment.

For more clarity, the percentages of inhibition at the fourth day after inoculation are given below. Only films with a concentration of the film forming solution more than 1 g/L did inhibit the growth of *F. graminearum*. That is why only these results are represented in Figure III.17, Figure III.18 and Figure III.19.

Films after inoculation (Figure III.18) were more efficient than the ones before inoculation (Figure III.17), probably because spores were directly in contact with the filmogenic solution while it was drying for 2 hours. Moreover, films before inoculation may actually act as a nutriment barrier, which can be pierced or may contain cracks through which hyphae can wriggle to reach the nutrient medium. In contrast, films after inoculation could form a gas barrier, which may be more detrimental to the hyphal development.

Moreover, films are also more damaging to spores than to a growing mycelium, because percentages for films before or after inoculation, involving spores (Figure III.17 and Figure III.18), are higher than those for films cast 24 hours after the inoculation, when spores had already germinated (Figure III.19). Minor changes in the time of application, 26 hours in total, caused up to five times less inhibition for the films. That is why an application of a potential biofungicide based on chitosan should coincide with the time when spores are most likely to infect the plant.

We now take a closer look to the results obtained at each application time. In Figure III.17, Ch. 78 and AA was the most efficient combination to reduce the fungal growth, with 62 % to 100 % of inhibition. It was more than twice than with the HPMC–AA film, proving once again the antifungal activity of Ch. 78.

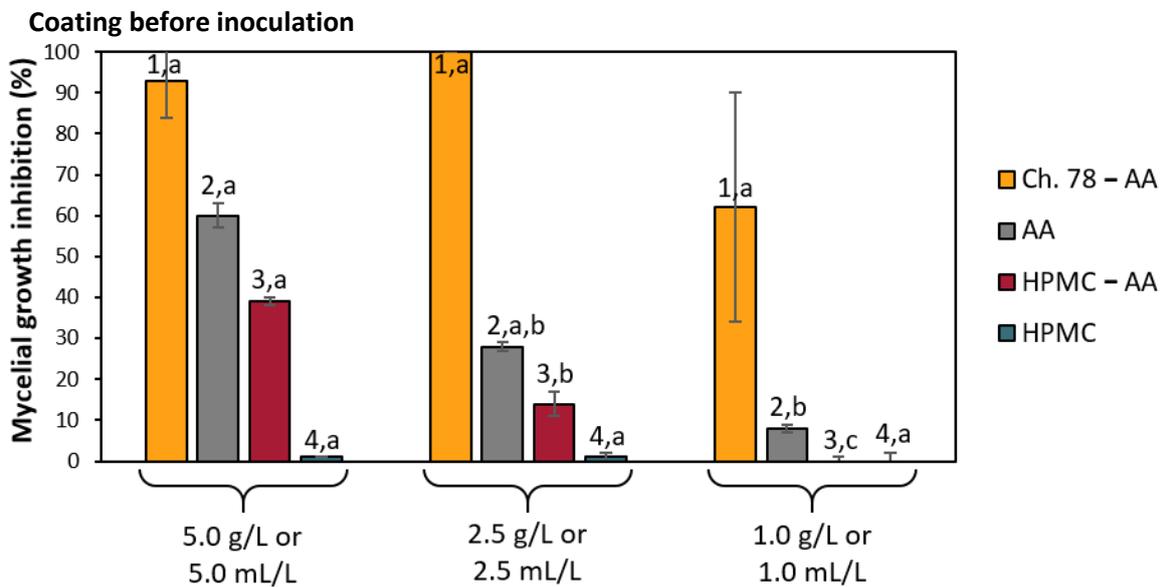


Figure III.17: Percentage of inhibition 4 days after incubation of the mycelial growth on solid medium coated by Ch. 78, AA or HPMC at different concentrations (AA in mL/L). Coating **before inoculation**. Figures indicate the same experiment and different letters indicate significant differences within the same experiment.

As a remark, the results in this Figure III.18 are the same than in 3.1.1. Results with Ch. 78–AA were particularly interesting after inoculation, as a total inhibition is kept whatever the

III. CHITOSAN-BASED FORMULATIONS

concentration while inhibition percentages for other products have dropped when concentrations decrease.

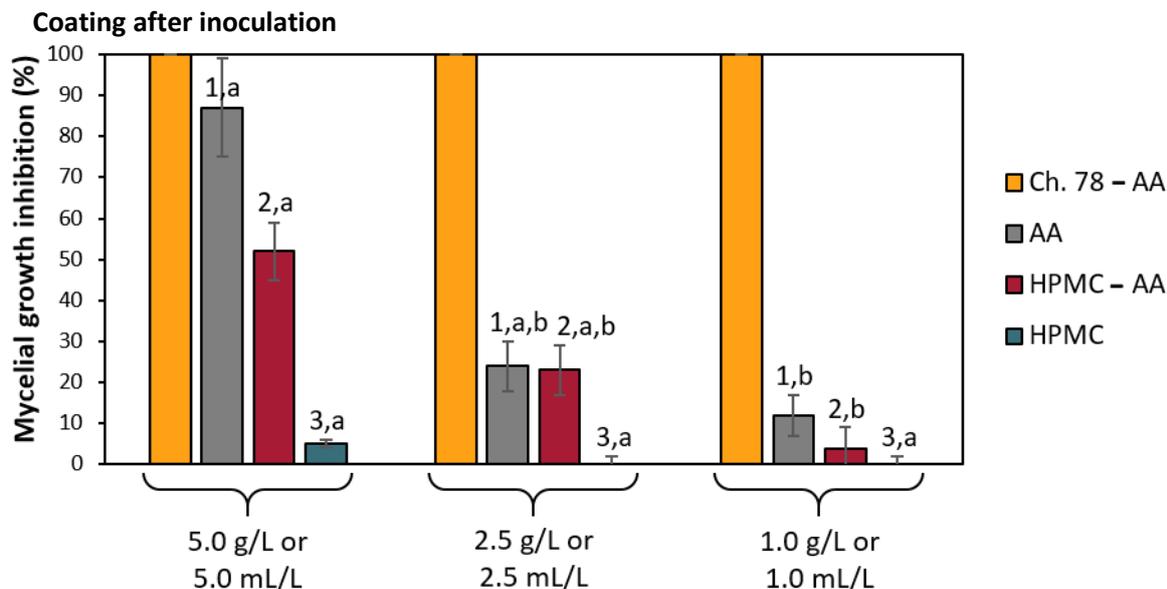


Figure III.18: Percentage of inhibition 4 days after incubation of the mycelial growth on solid medium coated by Ch. 78, AA or HPMC at different concentrations (AA in mL/L). Coating **after inoculation**. Figures indicate the same experiment and different letters indicate significant differences within the same experiment.

Results were different for the application 24 hours after the inoculation (Figure III.19). The effect of AA seemed to be predominant, with an inhibition of 89 % at 5 mL/L. Both chitosan and HPMC films with AA were less efficient, meaning that mycelia were more sensitive to a pH change brought by AA than to a thin film. Moreover, films on mycelia may not have coated all hyphae perfectly, which were already one centimetre long. Consequently, some of them were certainly not affected by the film and were therefore able to carry on with their normal operations.

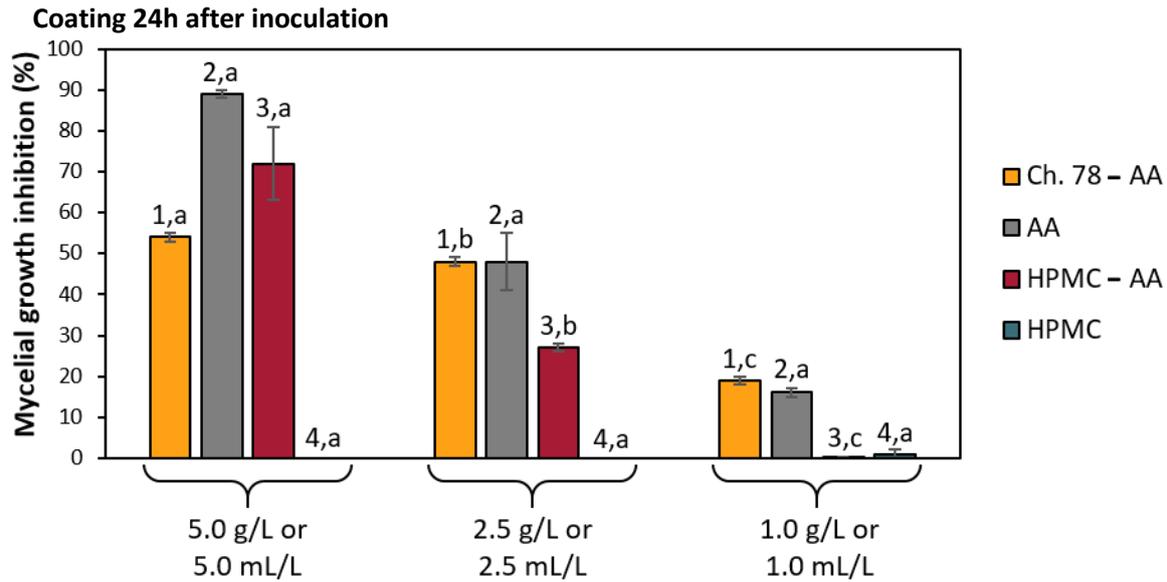


Figure III.19: Percentage of inhibition 4 days after incubation of the mycelial growth on solid medium coated by Ch. 78, AA or HPMC at different concentrations (AA in mL/L). Coating 24 hours after inoculation. Figures indicate the same experiment and different letters indicate significant differences within the same experiment.

To summarise these results, chitosan did maintain its antifungal properties in a film, which was mainly active due to intrinsic antimicrobial properties of chitosan rather than to the film form. The effect of AA, which is needed to form the film-forming solution, was less pronounced in films than in solution, thus clearly highlighting the specific antifungal activity of chitosan. Spores were more damaged by a chitosan coating than already growing hyphae.

Unfortunately, none of the tested conditions completely stopped the growth whatever the application time. Indeed, one might not forget that if the growth started, the mycelium would keep growing. The only way to totally inhibit the growth is to target the precise moment of the installation of the spores on the plant and to apply the chitosan-based formulation just after. Chitosan was really worth using, especially at 1 g/L just after the inoculation of spores.

3.2 Physicochemical characterisations

As chitosan films did maintain the antifungal properties as a coating, we wanted to see if this coating was suitable for an application directly on the leaves. Consequently, we simulated rain and sun on the films and analysed the gas permeability. The interaction with liquid water was studied from the determination of the contact angles and the surface free energy as well as the dynamic penetration analysis of water in the film. For the interaction with water vapour, moisture barrier properties were performed. Damaging sunlight was simulated with UV light.

3.2.1 Behaviour towards liquid water

3.2.1.a Determination of the contact angle and the free surface energy

The values of the air/solvent angles for three solvents are summarised in Table III.6.

Table III.6: Air/solvent angle of a sessile drop performed at 22 °C. Average and standard deviation of 6 measures, taken 5 seconds after the deposition of the drop.

Solvent	Air/solvent contact angle (°)
Water	103±10
Ethylene glycol	49±12
Diiodomethane	57±1

Films have an apparent hydrophobic surface with an air/water contact angle equal to $103\pm 10^\circ$. The value of the air/water contact angle was higher than the one found by other authors (87° [354] and 91° [355]). This value is also high compared to other biopolymers, like cellulose (26°), lignin (67°) or starch (43°) [356]. It could be explained by the presence of impurities, such as residual lipids, dyes or proteins from crustacean shells [354,355,357]. With the Owen, Wendt, Rabel and Kaelble method, the surface free energy was calculated at 32 ± 4 mN/m. This value is common for chitosans, although Cunha *et al.* [357] found a higher surface free energy for some “purified” chitosans, obtained through a Soxhlet extraction. They suggested that their purification removed the above-mentioned lipophilic impurities, thus increasing the surface free energy.

As just mentioned, the contact angle with water is high and the drop is well domed. However, this behaviour evolved over the time and particularly quickly during the first 30 seconds. The evolution of the shape of the drop is represented in Figure III.20. The drop flattened and the aspect of the film changed just around the drop. The water probably started to dissolve the film. Farris *et al.* [355] showed that it was not possible to apply the concept of water contact angle equilibrium and that the surface wetting was due to the spreading of the water at the surface. The values of the contact angles and the surface free energy are thus to be considered with caution and one must remember from this part that after a few second, the water seemed to wet the film.

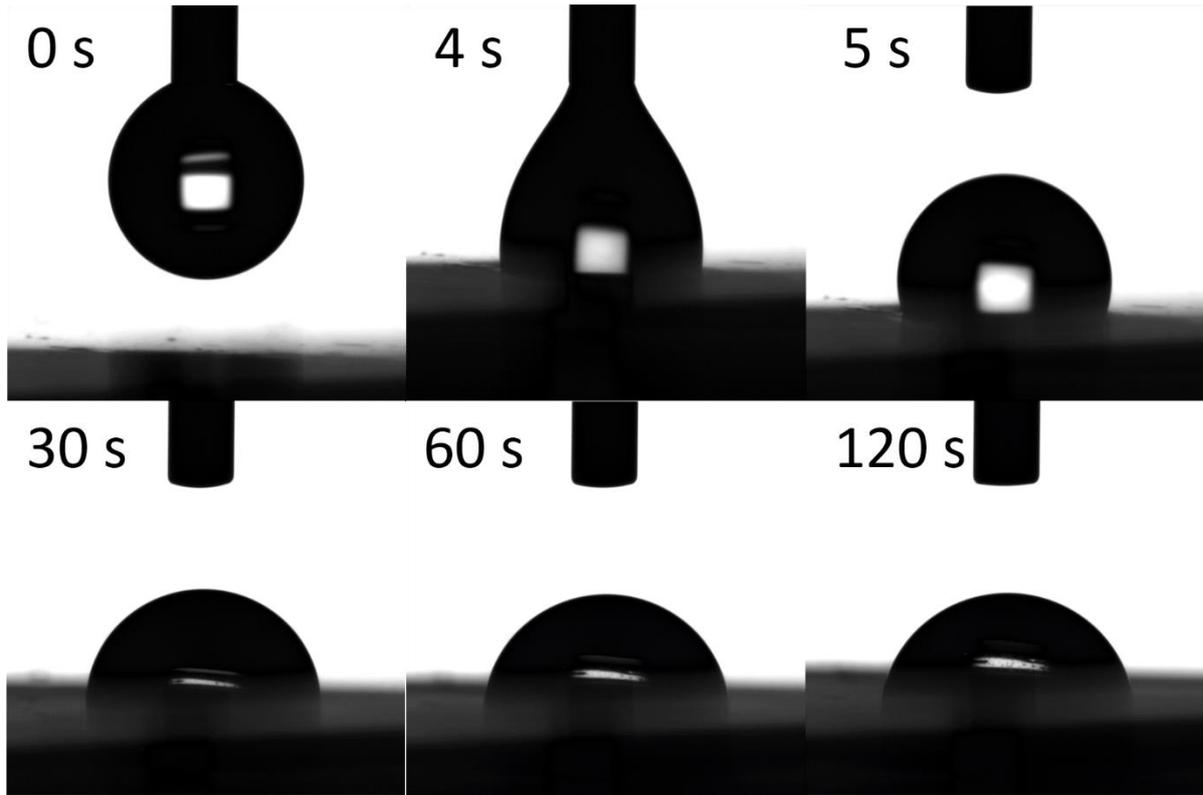


Figure III.20: Evolution of a water drop on a chitosan film.

3.2.1.b Dynamic penetration analysis (DPA)

The fact that water could go through the film was studied with DPA. Briefly, DPA is a two-part device that emits ultrasounds on one side and receives and records them on the other side. One part is fixed and has a water container and the other part, where the chitosan film is tapped, moved toward the first part to sandwich the film and water between the two parts. The signal crosses therefore the soaked chitosan film. Ultrasounds are transmitted without attenuation in water, but are attenuated in another medium or when crossing interfaces.

The experiment was very hard to set up because noise was visible at the beginning of the signal on most of the samples, probably due to an irregular surface of the films. The behaviour can be explained as illustrated in Figure III.21.

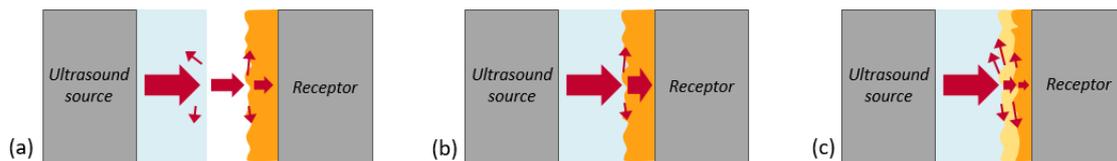


Figure III.21: Intensity of ultrasounds represented by red arrows when water (light blue) is wetting a chitosan film (orange). Red arrows symbolise the intensity of ultrasounds going from source to receptor and signal losses.

The resulting curve obtained from the chitosan film is shown in Figure III.22. When the water made contact, it first wiped out a layer of air (Figure III.21 (a)), causing an increase of the signal for 1 second (Figure III.22). Then, the water touched directly the film and wetted the film (Figure III.21 (b)) from 1 s to 5.5 ± 2.6 s (Figure III.22). Then, water entered the film, leading to a signal decrease due to the attenuation of ultrasounds (Figure III.21 (c) and Figure III.22) [35,36].

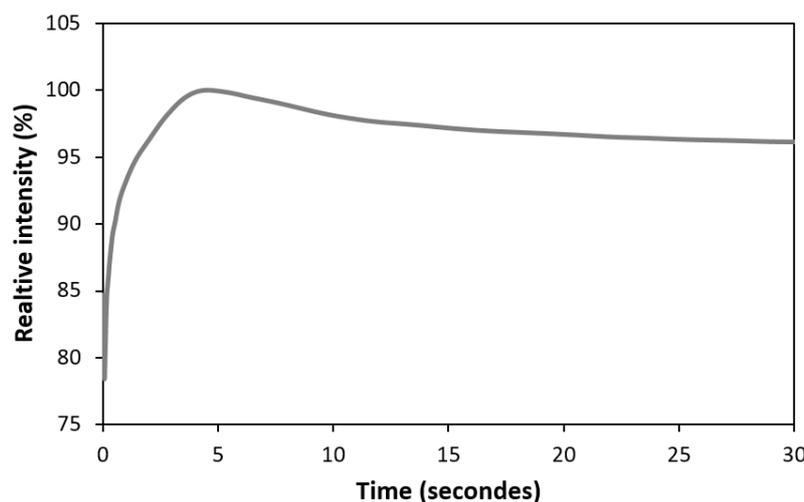


Figure III.22: Evolution of the penetration of water in a chitosan film. Measure performed in triplicate.

The main information is that chitosan films are permeable to water. Bordenave [35] also found that films were sensitive to water, but did not obtain the same curves. Water penetrated directly into the film and disintegrated it. As expected, this behaviour could be problematic for our application, because chitosan-based coatings would probably be washed away by rain.

3.2.2 Moisture barrier properties

The determination of the water vapour transmission rate (WVTR) deals with water as a gas. As seen in 3.2.1.b, water can penetrate the film and even go through it. The rate at which the vapour goes through the 30 μm -thick film is given in Table III.7.

Table III.7: Water vapour transmission rate of a chitosan film (thickness: 30 μm , diameter: 85 mm) stored at 23 °C and 50 % R.H.

Chitosan (\overline{Mn} , DD)	Thickness (μm)	WVTR rate ($\text{g.m}^{-2}.\text{day}^{-1}$)	Reference
78 kDa, 92 %	30 ± 10	225 ± 35	this work
400 kDa, 98 %	21 ± 1	63 ± 12	Bordenave [35]
380 kDa, 75 %	125 ± 3	650 ± 8	Le Goué [36]

We observe that the thicker the film, the higher the WVTR rate, which seems logical.

The chitosan solution was meant to be sprayed on whole plants. It was also interesting to know if the film was enough permeable to let the plant transpire. For this purpose, we needed to determine the liquid water flow through stomata. According to the literature, a grown maize plant transpires approximately as $60 \text{ g.plant}^{-1}.\text{day}^{-1}$ [358]. To convert and to compare this value with the one of the chitosan film, the area of stomata per plant needs to be calculated. This area was calculated from data obtained from the literature, such as leaf area, stomatal density and stomatal apertures. The detailed calculations were presented in the Supplementary Information. The result was $10^5 \text{ g.m}^{-2}.\text{day}^{-1}$. This value was 1000 times higher than the one found for the film and we could therefore predict that the chitosan coating would obstruct the stomata of a maize plant.

3.2.3 UV-radiations over the time

UV irradiation was performed to simulate damaging radiations from the sunlight. We chose to irradiate films at 375 nm, as a representative wavelength of UV solar light, comprised between 290-400 nm [359]. The UV-visible absorption of the films was measured over the time. Without UV, the absorption stayed the same during 10 days. In contrary, under UV, the film turned yellow. To better demonstrate the variations between an irradiated and a non-irradiated film, the difference of the absorbance between the two films was plotted in Figure III.23.

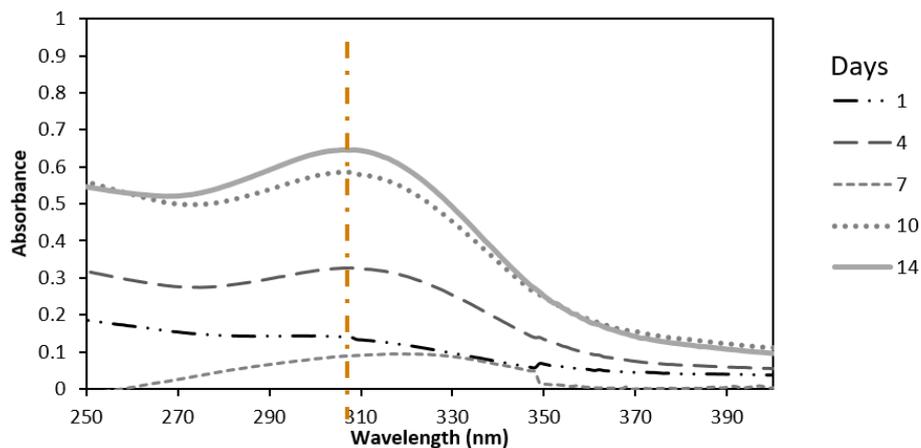


Figure III.23: Differences of absorption between the film put under UV light (375 nm) and the film kept in the dark.

One can clearly see the apparition of an absorbance band at approximately 308 nm, which corresponds to a UV-absorption. It was also already reported that, when chitosan was irradiated at 260 nm, a band at 310 nm appeared [360]. UV irradiations could have created scission reactions and the loss of carbonyl groups, thus increasing the number of $-\text{NH}_2$. When exposed at solar radiations, there was no change in the UV-visible spectra, but an absorbance appeared at approximately 300 nm when put under an artificial light (254 nm) [361]. This result suggests that UV irradiation was not the best option to simulate the solar spectra, but it gives an idea of what could happen in the most damaging case.

4 Concluding remarks

Chitosan has demonstrated promising antifungal and antimycotoxigenic properties. The best tested chitosan was a chitosan with a MW of 78 kDa and a DD of 92 %. It would have been interesting to see if chitosan chains could penetrate the cell. We could only hypothesise that the Ch. 78, the Ch. 130 and the Ch. 127 probably disturb the fungal membrane, and that the chitooligosaccharide could potentially enter the cell. Moreover, the counter-ion and the pH played an important role as far as the antifungal properties are concerned. The combination with acetic acid at pH 4.8 was the most deleterious one for the mycelium. Ch. 78 and AA can moreover reduce the production of toxins. Additional microscopic studies would have been welcomed as well as the analysis of the mode of action of chitosan on fungal genes.

Films of Ch. 78 and AA were cast to model the coating of the plant leaves. Films of chitosan were sensitive to water, meaning that they would probably be leached with rain. Further studies on the stability of chitosan coating onto the leaves need to be performed, like lessivability or the behaviour towards sunlight. The antimicrobial action of chitosan in a film form was maintained, but the influence of AA was less pronounced than for chitosan in solution, meaning that the activity of chitosan coating is mainly due to chitosan itself and not to the acid. Chitosan films had a strong antifungal action especially when cast just after the inoculation of spores.

CHAPTER IV – ADDITION OF TETRAHYDROCURCUMIN TO CHITOSAN-BASED FORMULATIONS

1	Structure and properties of THC	103
1.1	Chemical characterisation of THC	103
1.2	UV-visible absorption and stability	105
1.3	Antioxidant properties	106
1.4	Antifungal properties against <i>F. graminearum</i>	107
2	Association of THC to chitosan-based formulations	109
2.1	Study of chitosan-based solutions	109
2.2	Study of films as a model of plant coating	112
2.3	Concluding remarks	115

IV. ADDITION OF TETRAHYDROCUMIN

Tetrahydrocurcumin, also referred to as THC, is a bio-based phenolic compound, whose antioxidant and antifungal activity has already been proven [6,250]. Therefore, as mentioned previously, THC is a promising substance to add in biopesticide formulations, especially due to its positive action to control the production of mycotoxins *in vitro* [6].

In this chapter, after physicochemical characterisations of THC, its activity against the growth and the production of toxins of *F. graminearum* has been studied. Then, THC was added to the chitosan-based formulation, with the selected chitosan. As a reminder, the Ch. 78 solubilised in aqueous acetic acid was the one giving the best results regarding bioactive properties (see Chapter III).

The combination of both chitosan and THC has been studied as a solution but also, especially for antifungal properties, as a thin film to illustrate the plant coating after pulverisation of such pesticide formulation.

A global overview of the work presented in this chapter can be found in Figure IV.1.

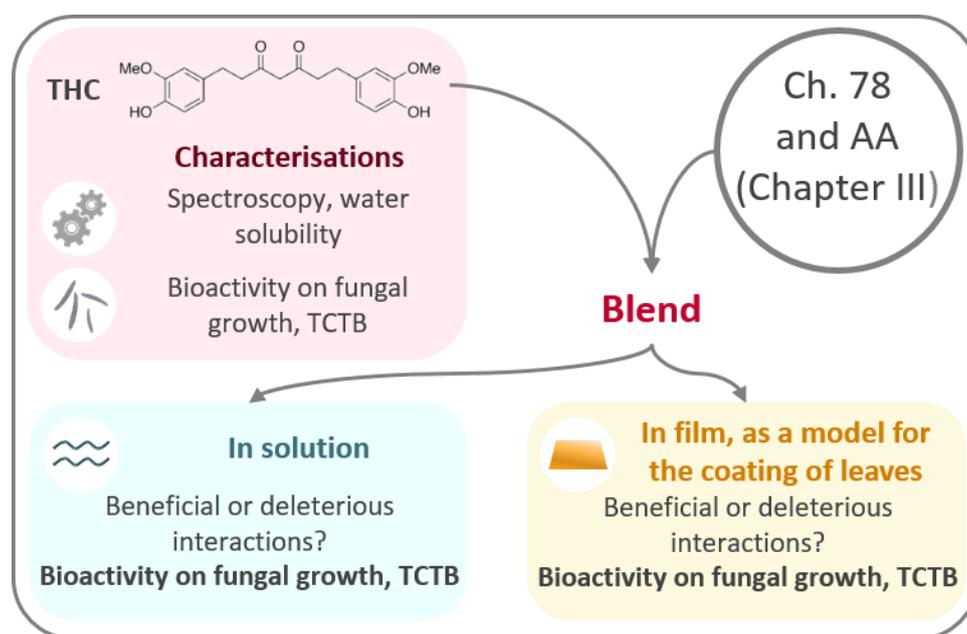


Figure IV.1: Overview of the study in this chapter.

1 Structure and properties of THC

1.1 Chemical characterisation of THC

Before its use, the commercial THC was characterised and its purity evaluated by FT-IR, ^1H -NMR and mass spectrometry.

1.1.1 Infra-red analyses

As expected, FT-IR spectrum of THC presented the characteristic bands of stretching of the aromatic carbons (1600 , 1511 and 1450 cm^{-1}) and the carbonyls at 1607 cm^{-1} (Figure IV.2 and Table IV.1). Specific bands of FT-IR were in agreement with results found in scientific literature (Trivedi *et al.* [362] and Mohan *et al.* [363]).

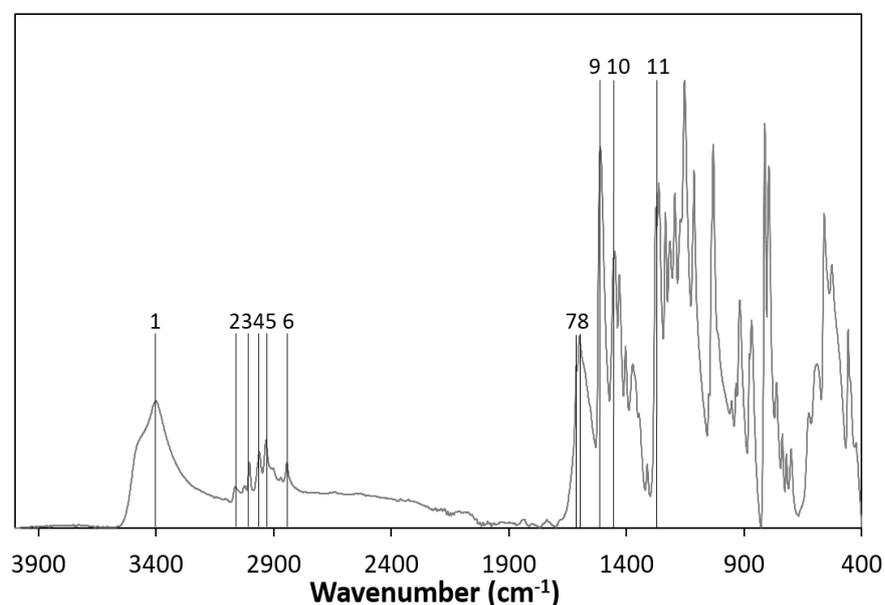


Figure IV.2: FT-IR spectrum of THC in KBr pellets.

Table IV.1: Attribution of principal bands in the FT-IR spectrum.

Number	Wavenumber (cm^{-1})	Type of vibrations
1	3399	νOH stretching
2	3061	$\nu\text{C}(\text{sp}^2)\text{-H}$
3	3005	$\nu\text{C}(\text{sp}^2)\text{-H}$
4	2962	$\nu\text{C}(\text{sp}^3)\text{-H}$ antisymmetric
5	2932	$\nu\text{C}(\text{sp}^3)\text{-H}$ symmetric
6	2843	$\nu\text{C}(\text{sp}^3)\text{-H}$
7	1607	$\nu\text{C}=\text{O}$
8	1600	
9	1511	$\nu\text{C}=\text{C}$ aromatic
10	1450	
11	1276	νCO enol

1.1.2 NMR characterisation

$^1\text{H-NMR}$ was first performed to verify if no undesirable signals were present. The signals that are presented in the spectrum (Figure IV.3) were those characteristic of THC [362]. Results were similar to those obtained by Trivedi *et al.* [362]. A detailed attribution and other spectra can be found in supplementary information.

Moreover, this technique was used to determine the ratio between the diketone and the keto-enol form, thanks to the signals of proton of the diketone and the signals of the enolic proton. It was equal to approximately 1:1 in DMSO, as the integration of the two protons of the diketone in-between the two carbonyl groups, “a diketone”, was equal to 1. The integrations of the enolic proton and proton “a enol” were respectively equal to 0.4 and 0.5.

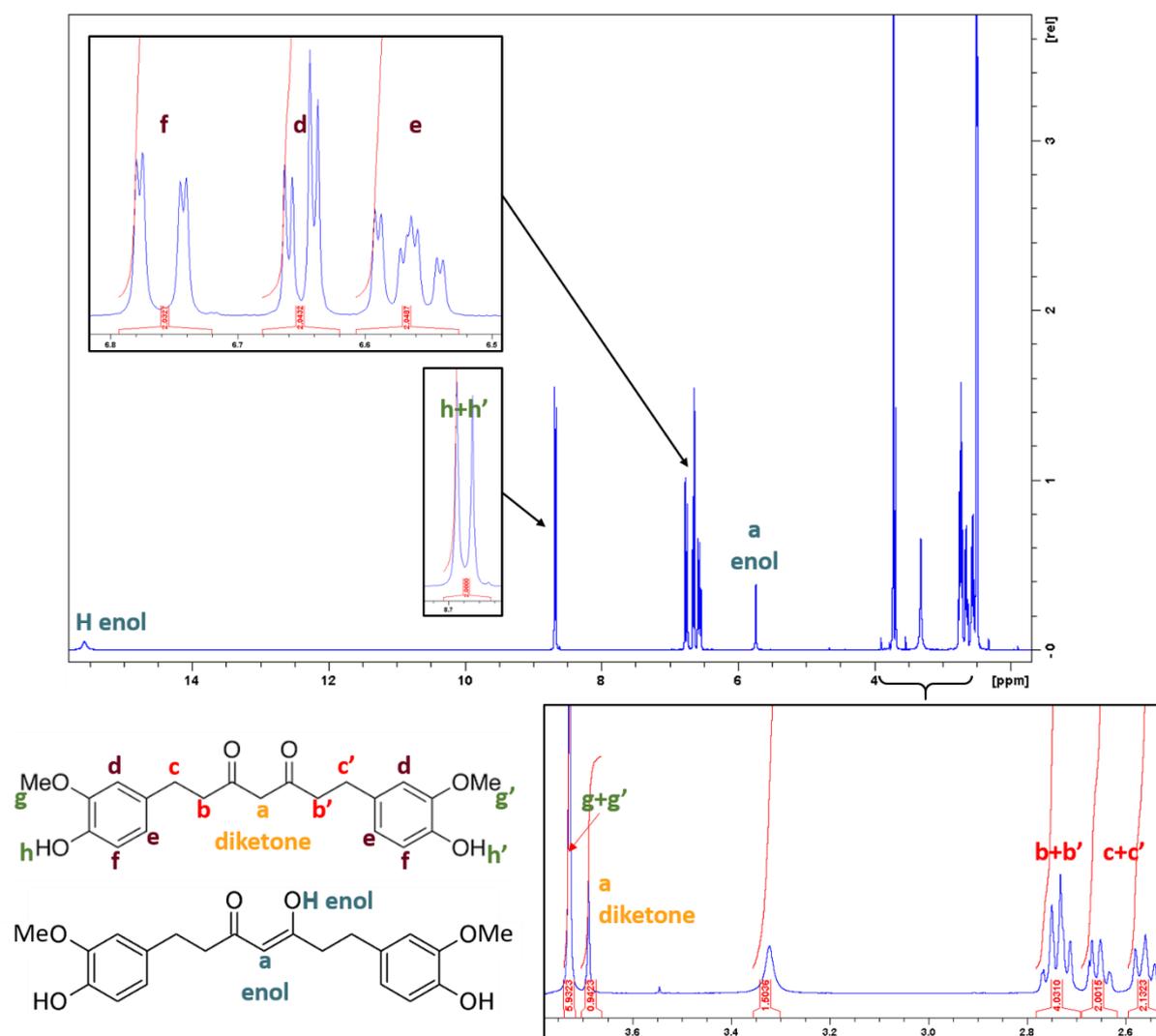


Figure IV.3: $^1\text{H-NMR}$ of THC in $\text{DMSO-}d_6$.

1.1.3 Mass spectrometry analyses

The commercial THC was analysed by MALDI-TOF mass spectrometry (Figure IV.4). The peak at m/z 395 corresponds to the cationised molecule of THC (372 g/mol) ($M + Na$)⁺. No impurity can be observed.

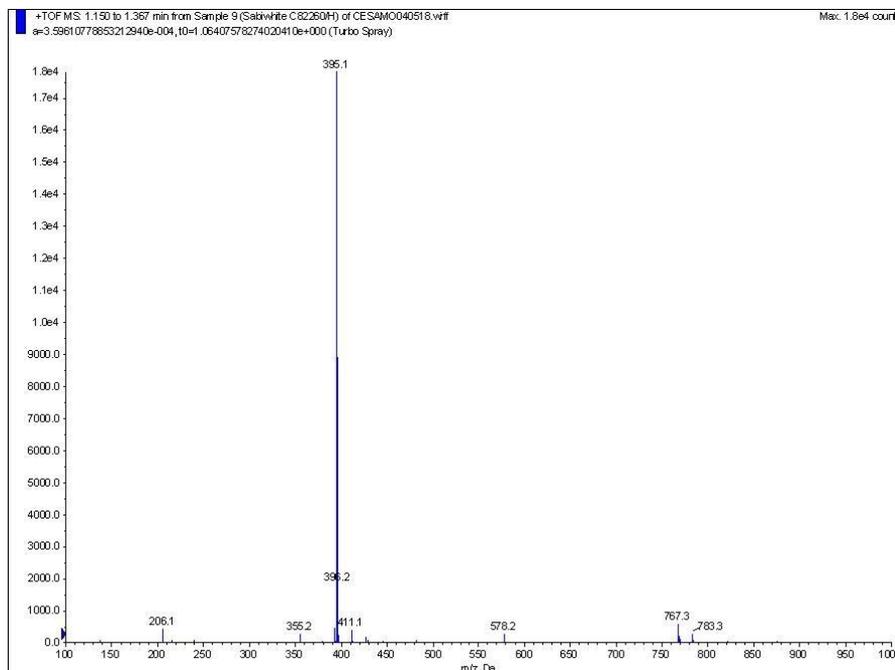


Figure IV.4 MALDI-TOF spectrum of THC (matrix: THAP).

1.2 UV-visible absorption and stability

The phenolic ring of THC is a chromophore that absorbs in the UV domain. This absorption is a powerful tool to track the presence of THC in solution. UV-visible spectrophotometry has been widely used in this work to dose the THC from various solutions. It was thus important to know the behaviour and the stability of THC in aqueous solution first, as we target an application in aqueous medium.

Briefly, THC was dissolved in distilled water and the pHs were adjusted to 4.8 or 5.6, which corresponded to the pHs that are used in the microbiological tests. These solutions were analysed by UV-visible spectrophotometry every day. Solutions were kept in the dark at ambient temperature between each analysis.

The spectra presented the commonly found band at 280 nm (Figure IV.5) [250]. The absorbance at this wavelength increased and was then stable after 3 days. A hypothesis is that, at 30 μ M, there was residual undissolved THC at the bottom of the bottle and that the two first samples taken from this bottle were less concentrated. This hypothesis also implies that it took three days for an aqueous THC solution to reach an equilibrium. The pH did not seem to have any effect on THC solubility between 4.8 and 5.6.

IV. ADDITION OF TETRAHYDROCUMIN

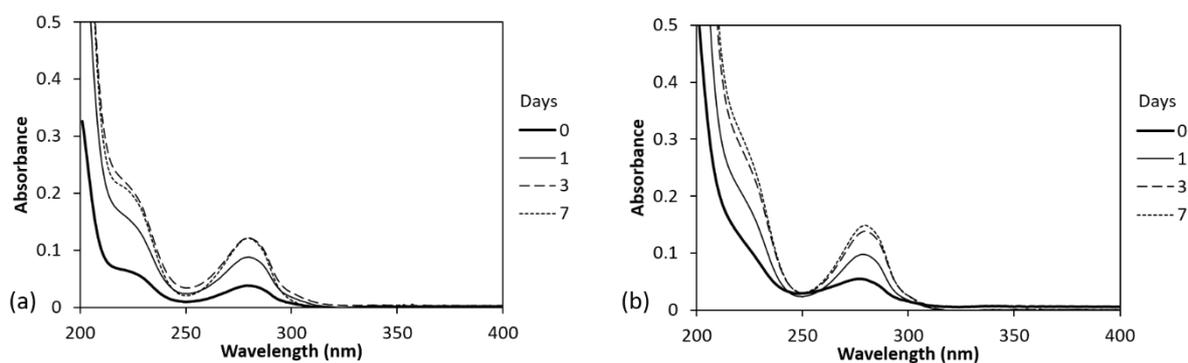


Figure IV.5: UV-visible spectra of THC solution in water at $30\ \mu\text{M}$ at pH (a) 5.6 and (b) 4.8 for different storage time in the dark conditions at ambient temperature.

1.3 Antioxidant properties

THC is a phenolic compound that possesses two phenolic rings conferring antioxidant properties. Let's reiterate that antioxidant activities may sometimes be linked to anti-TCTB properties. The ability to scavenge the DPPH^\cdot radical is a widespread test to measure the antioxidant activity of organic compounds [250,364].

The UV-visible absorption spectra of THC and BHT, a common antioxidant commonly used in foods, after 30 minutes of reaction with DPPH^\cdot is shown in Figure IV.6.

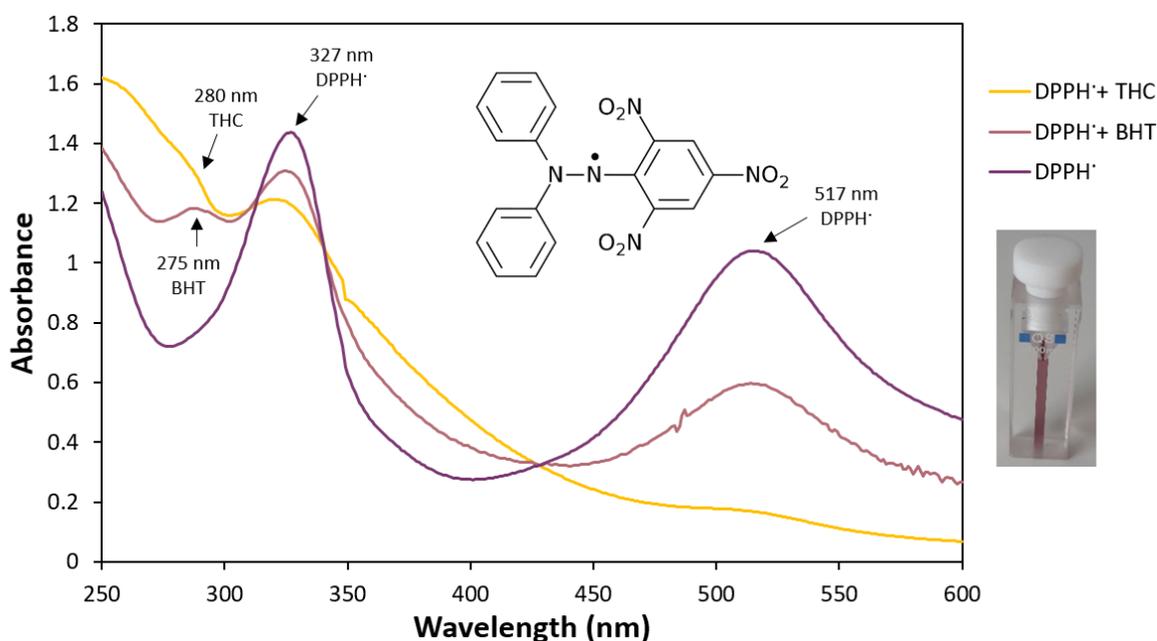


Figure IV.6: UV-visible absorption spectra of DPPH^\cdot (purple), BHT and DPPH^\cdot (pink) THC and DPPH^\cdot (yellow).

THC possessed a higher ability than BHT to scavenge the radical, with a DPPH^\cdot inhibition of 73 % (Table IV.2).

Table IV.2: Inhibition percentages of DPPH by THC and BHT.

Compound	Inhibition percentage (%)
THC	73
BHT	43

According to Portes et al. (2009), THC was more antioxidant than curcumin, eugenol or BHT [250]. This result demonstrated the potential of THC to act as antimycotoxigenic and antifungal agent.

1.4 Antifungal properties against *F. graminearum*

The addition of 10 μM of THC alone directly onto the solid medium did not show any inhibition of the fungal growth (data not shown).

The activity of THC against the production of toxins was then assessed with two concentrations of THC. As a reminder, *F. graminearum* produces trichothecenes B (TCTB), especially DON and 15-ADON. Moreover, to study the effect on the production of toxins, the concentration of the product must not affect the growth of the mycelium. This was the case, indeed, because the weights of dried mycelia were the same (data not shown).

As can be seen on Table IV.3, the effect of THC on the accumulation of TCTB not significant. The reduction of the mycotoxigenesis by phenolic compounds like chlorogenic or caffeic acid [214], resveratrol [365] or cinnamic acid derivatives [213] is reported in the literature, and can be related to their antioxidant properties. Indeed, many authors found a correlation between antioxidant and antimycotoxigenic properties [366,367]. Antioxidant compounds could scavenge radicals, thus reducing the stress. Therefore, as the production of toxins is probably linked with the fungal stress-response [350], the amount of TCTB could be reduced.

Table IV.3: Production of TCTB by *F. graminearum* after 14 days in liquid medium at 25 °C and 70 % R.H. Standard deviation of the measurement of 5 Petri dishes. Different letters indicate significant difference ($p < 0.05$) within an experiment (marked with figures).

	Production of TCTB (mg/g of dried mycelium)	Inhibition percentage (%)
Control	25 \pm 4 ^a	
THC at 5 μM	21 \pm 3 ^a	15
THC at 10 μM	20 \pm 2 ^a	20

Several studies have shown that the effect of active molecules is strain dependant [214,215,368–370]. Therefore, we have studied the effect on the accumulation of mycotoxins by

IV. ADDITION OF TETRAHYDROCUMIN

five other different strains of *F. graminearum* named Fg 164, Fg 156, Fg 215, Fg 605, Fg 812. These strains were selected because they mainly produced DON and 15-ADON, like the strains used for the whole study (CBS 185.32). It was interesting to know if the THC can decrease the TCTB production of different strains, knowing that numerous strains are present on fields.

As seen in Figure IV.7, the accumulation of toxins in controls varied from 0.14 to 5.4 mg/g of dried mycelium for Fg 156 and Fg 164, respectively. The production of toxins is thus strain-dependant. The effect of THC was also not the same for each strain. Indeed, the accumulation of TCTB by Fg 215 and Fg 812 was significantly reduced, up to 54 % for 10 μ M of THC on Fg 215. With regard to the three other strains, the results were ambiguous. No trend was observed for Fg 156 and Fg 605, even if changes were not significant. Finally, an increase in the mycotoxigenesis was noted for Fg 164.

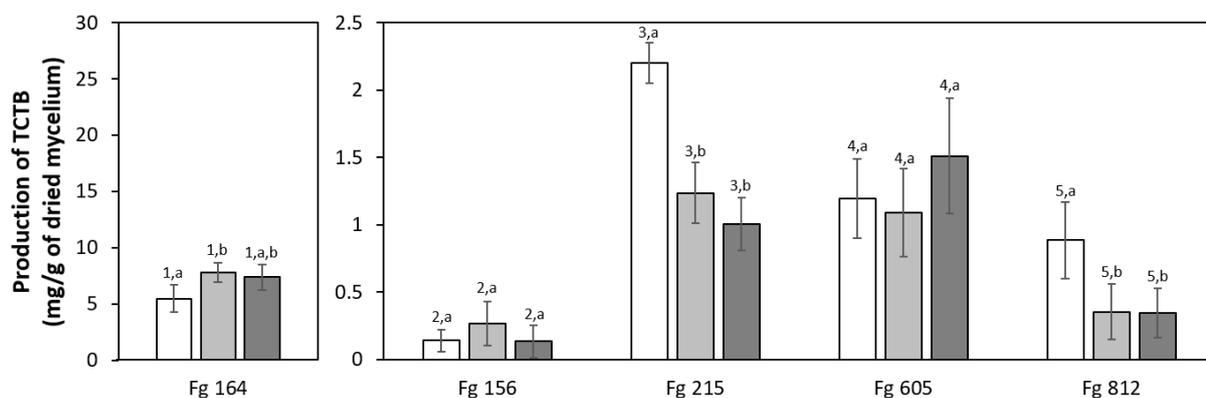


Figure IV.7: Production of TCTB for 5 different strains, Fg 164, Fg 156, Fg 215, Fg 605, Fg 812 of *F. graminearum* in liquid medium amended by (white) Control; (light grey) THC 5 μ M; (grey) THC 10 μ M at 25 $^{\circ}$ C and 70 % R.H. Error bars correspond to the standard deviation of the measurement of 5 Petri dishes. Different letters indicate significant difference ($p < 0.05$) within an experiment (marked with figures).

As already said, the reduction of the accumulation of toxins by different fungi with various phenolic compounds is common, but not constantly observed. The inhibition is component-dependent. For example, in the study of Ferruz *et al.* [229] all the tested phenolic compounds, excepted *p*-coumaric acid, reduced the amount of toxins by *F. langsethiae* or *F. sporotrichioides*. The impact also depends on fungal species, as phenolic compounds can reduce the production of mycotoxins by *F. graminearum* and *F. langsethiae*. With *F. poae*, quercetin or ferulic acid even had a positive or a negative impact depending on their concentration [230].

More interestingly for the present study, various strains seemed to react differently. For example, in a work of Ponts *et al.* [213], caffeic and syringic acids inhibited the production for one strain of *F. graminearum* and activated another. In another work on two *Fusaria*, the most sensitive strain for *F. culmorum* was the one producing the highest amount of toxins. However, for *F.*

graminearum, the two strains producing the lowest amount of toxins were the most sensitive [231]. In our case, no trend can be observed, but the action of THC is certainly strain-dependent.

This study was an opening concerning the effectiveness of THC on a panel of strains. As a result, THC was a promising component regarding the reduction of the toxins, which was probably linked to its antioxidant properties, although being not as effective as chitosan. Knowing that the latter cannot also totally inhibit the production of toxins, the idea was to combine both active agents in a global formulation and obtain a synergy.

2 Association of THC to chitosan-based formulations

Chitosan-based formulations using Ch. 78 were then loaded with THC to improve their antimicrobial properties. The bioactive properties of such blends were studied not only from the liquid formulations but also from the corresponding films, to get closer to the targeted application by simulating the plant coating after pulverisation.

To solubilise chitosan, acetic acid (AA) and lactic acid (LA) were compared, with a mass ratio chitosan/acid set at 1. A supplementation of 10 μM THC was chosen.

2.1 Study of chitosan-based solutions

2.1.1 Effect on the mycelial growth

Based on the results of the first chapter, we know that a concentration of Ch. 78 at 0.4 and 0.6 g/L have led to a medium mycelial inhibition in solution. We then chose a concentration of 0.5 g/L, to be able to track changes in the inhibition.

AA and LA were used to dissolve the chitosan, as it has always been the case in this study. The two pH options have been also tested in this part. For recall, one option consisted of no pH adjustment after the addition of THC, chitosan and acids and the other consisted of the adjustment of the pH at 5.6.

To begin with, all conditions with or without THC inhibit the fungal growth. To simplify the comparison of the results *with* and *without* THC in the formulation, we calculated the difference, Δ , between the inhibition percentages as presented in (IV.1). For example, 4 days after the inoculation, the Ch. 78–AA solution and the Ch. 78–AA solution + THC inhibited the mycelial growth by 63 % and 28 %, respectively.

$$\Delta = \%(condition\ with\ THC) - \%(same\ condition\ without\ THC) \quad (IV.1)$$

$$\Delta = 28 - 63 = -35 \%$$

IV. ADDITION OF TETRAHYDROCUMIN

Then, we observed that the addition of THC sometimes helped the action of chitosan on the fungi but sometimes hinder it (Table IV.4). The added THC led to a significantly lower inhibition than the chitosan-based solution alone, when the pH was not adjusted. Contrary to that, when the pH was adjusted THC and chitosan had a synergistic effect.

Table IV.4: Inhibition percentages of the mycelial growth on solid medium 4 days after inoculation at 25 °C and 70 % R.H. Medium supplemented or not with THC at 10 μ M, Ch. 78 at 5 g/L, AA at 5 ml/L or LA at 5 mL/L.

Components	No pH adjustment		pH adjusted at 5.6	
	Inhibition (%) ¹	Δ (%) ²	Inhibition (%) ¹	Δ (%) ²
Control	0 ^m			
THC	0.2 \pm 2 ^m	+0.2		
AA	53 \pm 1 ^{a,b,c}		4 \pm 2 ^{l,m}	
AA + THC	51 \pm 1 ^{b,c,d}	-2.2	3 \pm 1 ^{l,m}	-1.1
LA	13 \pm 1 ^{j,k}		-1 \pm 2 ^m	
LA + THC	16 \pm 1 ^{i,j,k}	+2.6	6 \pm 2 ^{k,l}	+6.5*
Ch. 78 + AA	63 \pm 1 ^a		19 \pm 2 ^{h,i,j}	
Ch. 78 + AA + THC	28 \pm 1 ^{f,g,h}	-35.0*	24 \pm 2 ^{g,h,i}	+4.8
Ch. 78 + LA	54 \pm 1 ^{a,b}		32 \pm 1 ^{e,f,g}	
Ch. 78 + LA + THC	44 \pm 1 ^{c,d,e}	-10.2*	40 \pm 1 ^{d,e,f}	+8.0

¹For the inhibition percentages, the different letters showed significant differences from three replicates ($p < 0.05$).

²For the differences between conditions with or without THC, the stars marked a significant difference between both conditions.

The global negative impact of THC on the bioactive properties of chitosan could be due to interactions between both molecules, probably involving the NH_3^+ of chitosan and reducing their availability to act on the fungi. The fact that the negative influence of THC on chitosan disappeared when the initial pH was 5.6 could be in favour of our assumption. Indeed, at this pH, the ratio $\text{NH}_2/\text{NH}_3^+$ was higher than at pH 4.8, so hypothetical interactions between charged amine group and THC would be less numerous.

2.1.2 Effect on the accumulation of TCTB

The effect of the blend THC and Ch. 78 was studied on the production of toxins and are presented in Figure IV.8.

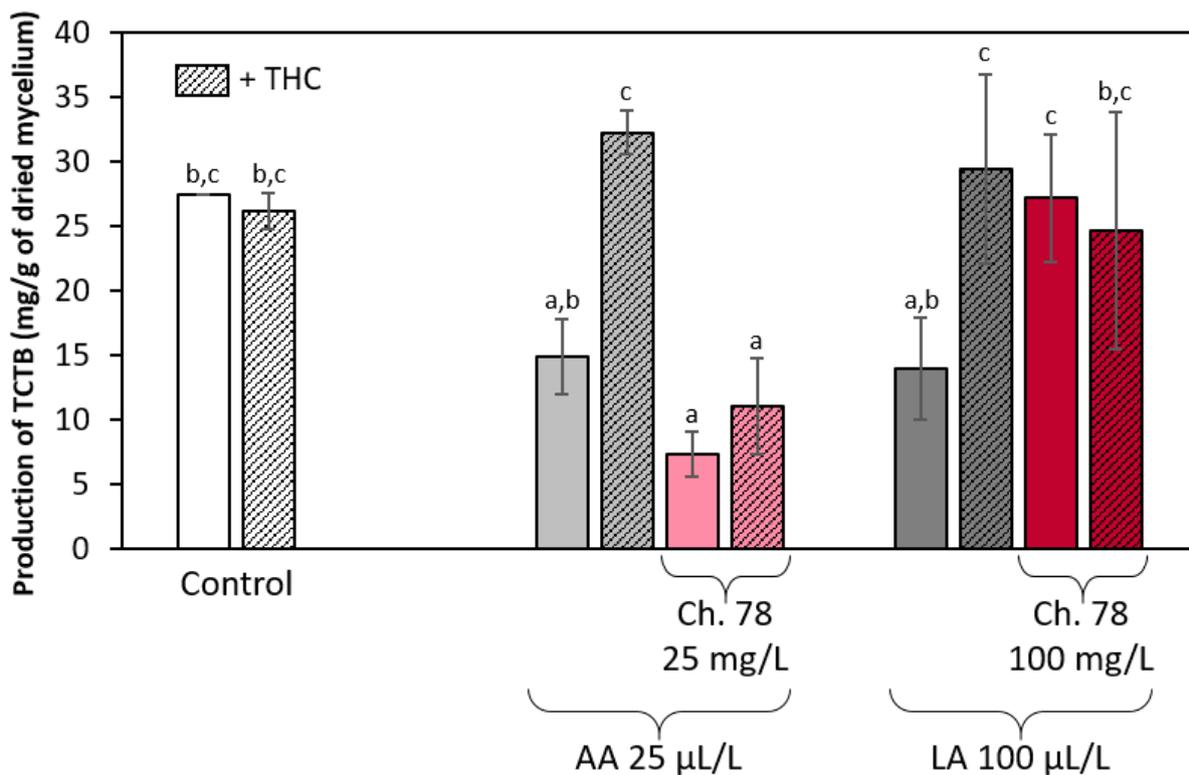


Figure IV.8: Production of TCTB in liquid medium amended by THC at 10 µM; Ch. 78 at 25 mg/L or 100 mg/L; AA at 25 µL/L or LA at 100 µL/L at 25 °C and 70 % R.H. Error bars correspond to the standard deviation of the measurement of 6 Petri dishes. Different letters indicate significant difference ($p < 0.05$).

Firstly, the effect of THC alone was surprising, because the decrease of the TCTB production was only about 5 %. We expected an inhibition about 20 %, as described in 1.4. This variability is commonly encountered in biological results on mycotoxin production because it depends on various parameters.

Globally, the addition of THC had no effect, because almost all changes were not significant. Only two conditions showed a significant impact on the accumulation of toxins, with a doubled production for acids + THC compared to acids alone. One can assume that the THC and these organic acids could interact with each other, so that acids are not efficient anymore.

The production of toxins with Ch. 78–AA + THC was higher than without THC, which may be due to interactions between THC and chitosan preventing both of these compounds to act properly against the production of toxins. However, as already said, the increase is not significant. Chitosans–AA, with or without THC, was still the most efficient conditions. The association of chitosans with LA, with or without THC, did not inhibit the accumulation of TCTB. So unfortunately, like on the fungal growth, the addition of THC into chitosan solutions did not have a positive impact.

2.2 Study of films as a model of plant coating

In parallel to chitosan solutions, it was also interesting to determine the influence of THC on the properties of chitosan films, as the targeted application is a coated formulation. Only the test on the mycelial growth was performed. Then, to study deeper the interactions between both active agents, films were analysed with UV-spectrophotometry.

2.2.1 Effect of chitosan and THC films on the mycelial growth

Briefly, the THC was added onto the solution of Ch. 78 and AA. The solution was cast at three different times. Films “before inoculation” were cast two hours before the inoculation, to see if the films can limit the installation of the spores, as a preventive treatment. Films “after inoculation” were cast two hours after the inoculation, to determine if coatings can restrain the fungal development, as a curative treatment.

The Figure IV.9 presents the evolution of the fungal growth with the different coatings. We observed a dose-dependent response for all application times, like when THC and chitosan were in solution. The effect was negligible for all coatings at 0.1 g/L. The onset of the growth was delayed for films before inoculation at 2.5 and 5.0 g/L (Figure IV.9 (a)). The growth was completely inhibited by coatings after inoculation at 1.0 g/L or more (Figure IV.9 (b)). It can be deduced that the formulation affected the spores, maybe by affecting the germination or by forming a nutrient barrier. The standard deviations of some conditions were high because one or two replicate(s) has/have been developing while the last one(s) has/have not grown. This may be due to some cracks in the coating, through which the mycelium can develop. These conditions were then susceptible to little changes and may not be reproducible.

The coating cast 24 hours after the inoculation was the less efficient to reduce the mycelial growth. The formulation was therefore not adapted to fight against the development of an already growing mycelium.

As already concluded in Chapter III, the best application time was directly after the inoculation, which is however complicated to implement.

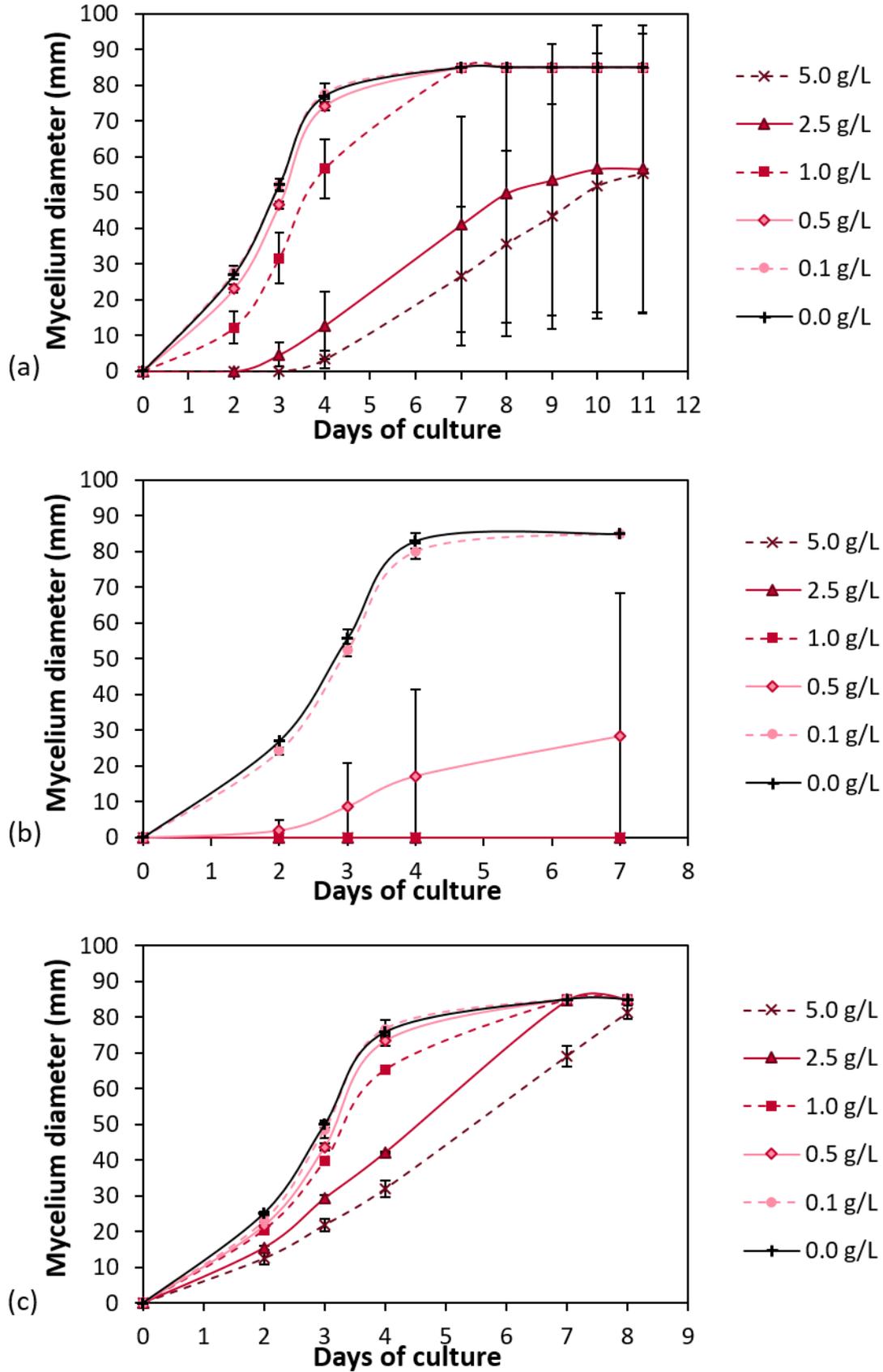


Figure IV.9: Daily evolution of the mycelial growth of *F. graminearum* on PDA medium, coated with 2 mL of chitosan-based solutions. Solutions composed of Ch. 78-AA at various concentrations and THC at 10 μ M. (a) before inoculation; (b) after inoculation; (c) after inoculation 24 hours. Error bars correspond to the standard deviation of the measurements performed in triplicate.

IV. ADDITION OF TETRAHYDROCUMIN

Results will now be compared with the results of films *without* THC. For clarity, these results were not repeated, as they were already reported in the previous chapter, but the parameter Δ introduced in 2.1.1 will be employed. Only films with the highest concentrations (5.0, 2.5 and 1.0 g/L) are compared. Calculated Δ at day 4 after the inoculation are represented in Figure IV.10.

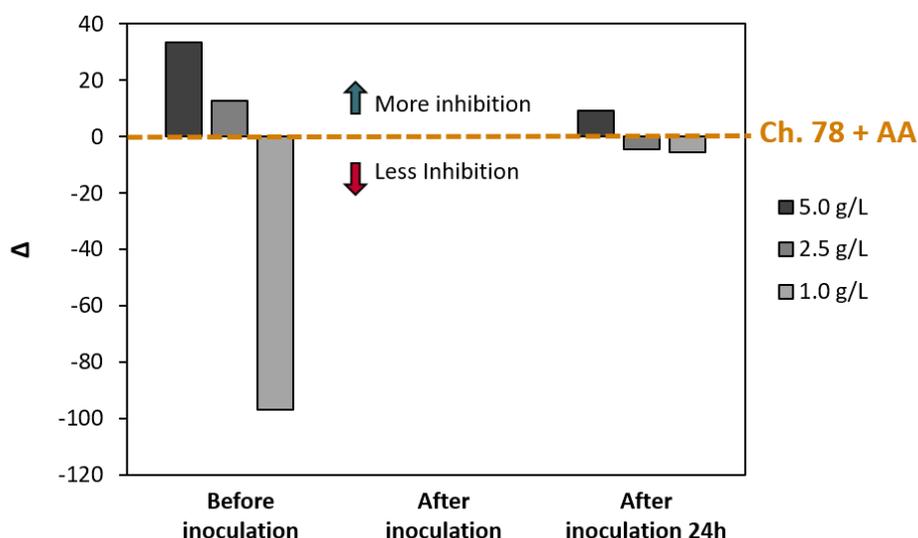


Figure IV.10: Differences between the mycelial inhibition percentages of THC-loaded chitosan films compared to chitosan films, after 4 days of inoculation at 25 °C and 70 % R.H. Films obtained from 2 mL of solutions of Ch. 78–AA and THC 10 μ M.

For the coatings at 5 g/L, THC seemed to help chitosan to control the growth. However, for lower concentrations of chitosan and AA, THC had a negative effect on the inhibition. It can be assumed that interactions that occurred in solutions are kept in coatings. For results after inoculation, chitosan/THC films showed the same efficiency than pure chitosan films, because the inhibition is total for both.

The effect on the accumulation of toxins would be an interesting study to complement the last result.

2.2.2 UV-absorption of films

Films were made out of film forming solutions at 1 % of Ch. 78. We wanted to compare films with or without THC.

Films with THC presented three absorption bands (Figure IV.11) compared to the chitosan film without THC. The one at 286 nm corresponded to the trapped THC, but shifted of 6 nm compared to the THC in solution. This finding highlights interactions between THC and chitosan also in films. A wide peak at 316 nm and a large absorption band at 454 nm can be detected. These

results are in accordance with the work of Portes *et al.* [34]. These newly formed bands were probably due to some interactions, which is in favour with what was already assumed.

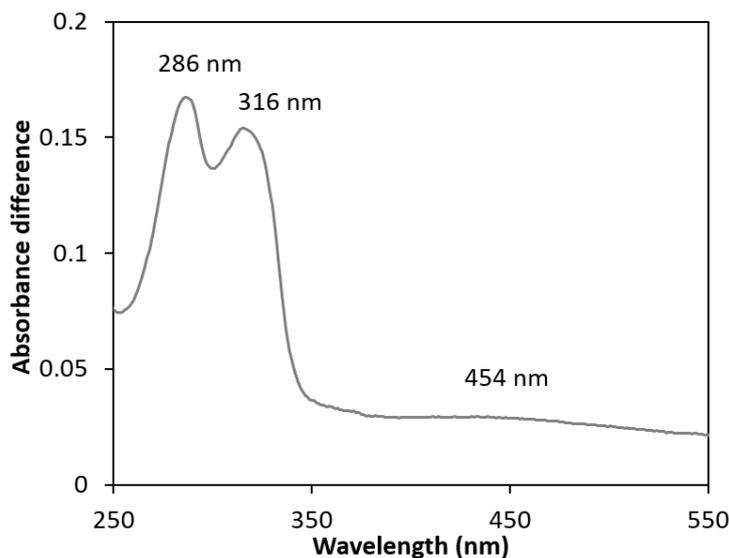


Figure IV.11: Difference between the absorbance of THC-loaded Ch. 78 film and Ch. 78 film.

2.3 Concluding remarks

THC was a promising antimycotoxigenic and antioxidant molecule. The addition of THC on chitosan-based formulations changed the efficiency of the solutions regarding both the mycelial growth and the accumulation of TCTB. We assumed that interactions occurred in solution between THC and chitosan, especially with the cationised ammonium groups of chitosan. As a coating, chitosan's action is also disturbed by THC, although no clear effect can be highlighted. Moreover, the impact of THC was slightly visible, due to its low solubility in aqueous solutions.

Interactions between chitosan and THC need to be deeper studied, for example by FT-IR, UV-spectrophotometry at various pH or concentrations. This understanding could help to target the right encapsulation or mode of association between the biopolymer and the natural compound. We could therefore fully benefit from inhibition properties of both components.

IV. ADDITION OF TETRAHYDROCURCUMIN

**CHAPTER V – ENCAPSULATION OF
TETRAHYDROCURCUMIN FOR FUTURE ADDITION TO
CHITOSAN-BASED FORMULATIONS**

1	Encapsulation <i>via</i> cyclodextrins	119
1.1	UV-absorption of THC and CDs to study interactions	119
1.2	Selection of a suitable CD	120
1.3	Proof of the inclusion	121
1.4	Study of the thermochemical equilibrium with Me β CD	124
1.5	Characterisation of the THC-loaded Me β CD complex powder	128
1.6	Activity against <i>F. graminearum</i>	130
1.7	Concluding remarks.....	132
2	Towards an encapsulation easy to implement industrially	133
2.1	Elaboration of emulsions with rapeseed oil	133
2.2	Drying processes: spray-drying and freeze-drying	136
2.3	Determination of the encapsulated amount of THC.....	136
2.4	Morphology of the powders by SEM	138
2.5	Differential scanning calorimetry analysis of the particles	139
2.6	Microbiological activity of THC-loaded particles against <i>F. graminearum</i>	140
3	Concluding remarks and prospects	144

The low water solubility of THC is a major constraint for the improvement of bioactive properties of our chitosan-based formulations. We decided to study the encapsulation processes to increase the water solubility of THC leading to a possible higher concentration in chitosan-based formulations. In addition, encapsulation can protect THC against environmental factors such as UV, resulting on a better stability. Finally, the formation of protecting particles could prevent undesirable interactions between THC and chitosan.

In this study, inclusion complexes with cyclodextrins have been synthesised and studied first. Afterwards, more industry-oriented approaches have been considered, such as the use of chitosan or starch matrixes.

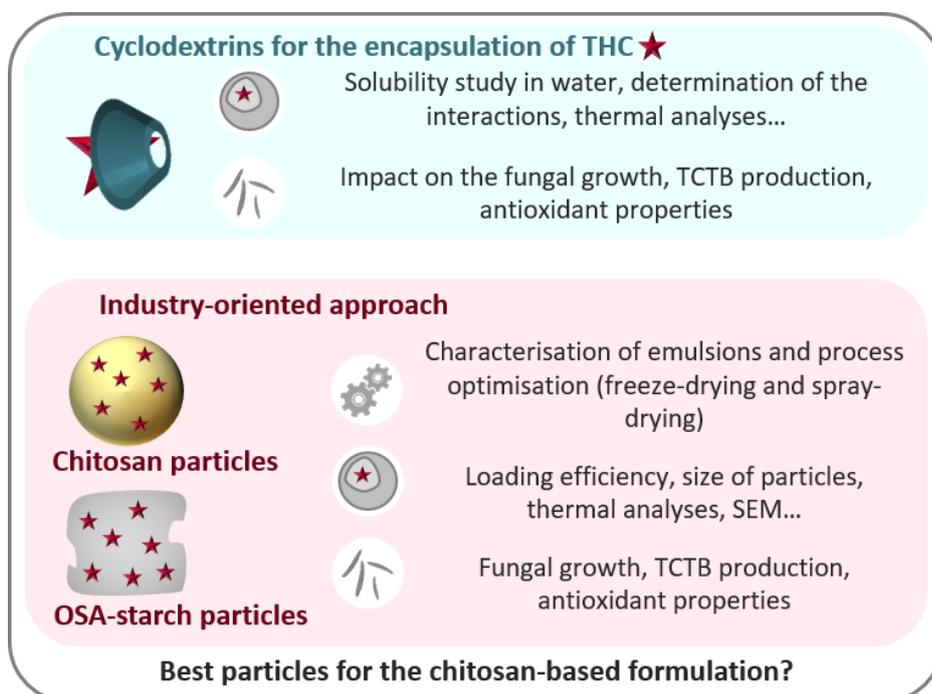


Figure V.1: Overview of the work of encapsulation.

1 Encapsulation *via* cyclodextrins

Cyclodextrins (CDs) are widely used to enhance the water solubility of lipophilic compounds. They are bio-based polymers of glucose units assembled to form a ring. The inner part is more lipophilic, and can theoretically trap lipophilic compounds, while the outer part is hydrophilic, guaranteeing the solubility of the host/guest complex in aqueous solutions.

As part of the collaboration with the UMET lab in Lille, THC has been encapsulated in various CDs in aqueous solutions (Table V.1). In order to select the best CD with regards to the application, the ability to trap the most of THC has been evaluated by UV-visible spectrophotometry. Then, specific interactions between Me β CD and THC were further detailed. Powders resulting from the drying of the CD solutions have been studied. The effects of the encapsulated THC have finally been assessed on fungi.

Table V.1: Abbreviations of cyclodextrins used in this work.

Cyclodextrins	Abbreviation
β -cyclodextrin	β CD
γ -cyclodextrin	γ CD
Randomly methylated β -cyclodextrin	Me β CD
Anionic polymer of β -cyclodextrin	Poly- β CD
Anionic polymer of Randomly methylated β -cyclodextrin	Poly-Me β CD

1.1 UV-absorption of THC and CDs to study interactions

The global absorbance, A , of a solution can be decomposed in several smaller absorbances, as many as the number of chromophores in the solution (V.1). The molar attenuation coefficient of a given component i , $\varepsilon_{j,\lambda}(i)$, depends on the surrounding solution (solvent and other components j) and the wavelength λ , as described in (V.2), where l is the length of the quartz cell and $[i]$ is the concentration of the molecule i .

In our case, the absorbance was due to CDs and THC, whose interactions might impact $\varepsilon_{CD,280}(THC)$. That is why, the concentration of THC, $[THC]$, in each sample was determined using six calibration curves, established with CD solutions at six concentrations in water (Figure V.2). It appeared for the case of Me β CD that $\varepsilon_{CD,280}(THC)$ did not depend on CD in the tested concentration range. The slope of each linear regression, corresponding to $\varepsilon_{CD,280}(THC)$, was indeed almost the same for each curve and variations did not seem to follow any trend. It was equal to $5.20 \pm 0.08 \times 10^3 \text{ L.cm}^{-1}.\text{mol}^{-1}$.

$$A = \sum_i A(i) \quad (\text{V.1})$$

$$A_{j,\lambda}(i) = \varepsilon_{j,\lambda}(i) * l * [i] \quad (V.2)$$

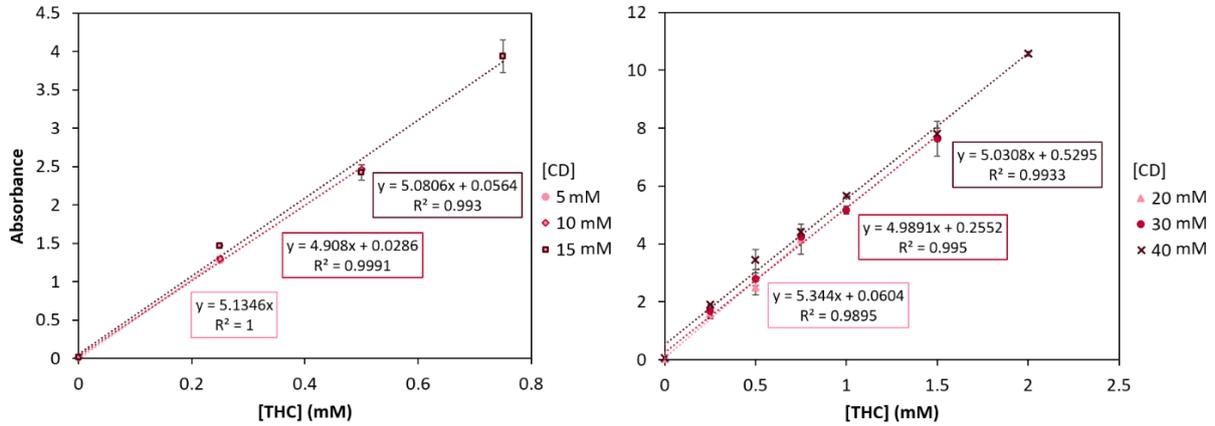


Figure V.2: Calibration curves of THC in solutions of Me β CD at different concentrations (5, 10, 15, 20, 30, 40 mM) in water. Measurements performed in duplicate.

This result was extrapolated for all CDs, meaning that we assumed that the contributions of THC and CD are independent and can therefore be added, whatever the ratio of the two products, in the range of the studied concentrations. The absorbance of solutions of CD at 15 mM, A_{CD} , was determined and reported in Table V.2. The contribution of CD to the global absorbance having been defined, it is now possible to calculate the absorbance of THC, A_{THC} .

Table V.2: Absorbance of CD solutions at 15 mM at 280 nm.

Cyclodextrins (15 mM)	Absorbance at 280 nm
β CD	0.10 ± 0.04
γ CD	0.38 ± 0.53
Me β CD	0.02 ± 0.01
Poly- β CD	0.09 ± 0.01
Poly-Me β CD	0.45 ± 0.01

1.2 Selection of a suitable CD

We wanted to select the CD that best solubilised the THC. For this purpose, THC was put in excess in aqueous solutions with each CD. The control condition consisted of THC in excess in freshly distilled water. Supernatants of resulted solutions were analysed with an UV-spectrophotometer. The higher the absorbance of THC, the higher its concentration and the better the CD for the application.

As explained in the last paragraph (1.1), A_{THC} was determined with the measurement of the absorbance at 280 nm minus A_{CD} (Table V.2), except for THC in water only. Since $\varepsilon_{CD,280}(THC)$ is known, the concentration of THC can be deduced with the equation (V.2).

As shown in Table V.3, excepted for β CD, all types of CDs improved the THC solubility in water with a proportional factor of 3 and 10 for γ CD and Me β CD, respectively. The different behaviour can be due to the diameter of the cavity (0.60–0.65 nm and 0.75–0.83 nm, respectively). The significant improvement of the solubility of THC by using Me β CDs can be explained by the presence of hydrophobic methyl groups leading to a higher hydrophobic character of such CDs compared to non-modified ones, which certainly played a role in the encapsulation process of THC within cavities, either by helping to “capture” the THC in the environment or by better retaining it in the cavity. Modified cyclodextrins, with methyl or hydroxypropyl groups, present a better ability to trap hydrophobic compounds [15,298].

Polymers of cyclodextrins were made of CDs reticulated with citric acid. The threefold increase in solubility could be therefore due to their network, which could trap more THC [371]. However, the solubility of THC did not seem to depend on the structure of CD, as the values were close to each other. One hypothesis might be that THC was mainly loaded into the network created by citric acid chains, which was the same for both polymers. Another hypothesis is that THC was encapsulated in the CD’s cavity, thus suggesting that the complexation process is highly influenced by reticulation chains, acting like substituents on CDs. Regarding the huge reduction of solubility with Poly-Me β CD compared to Me β CD, the high electronic density of phenolic rings in THC could eventually lead to unfavorable interactions with carboxylate anions of citric chains, thus preventing the molecule from entering the cavity. In addition, reticulation chains may hide methyl groups, which were thought to be on prime importance to enhance the solubilisation of THC in Me β CD.

Finally, Me β CD gave the best results with 1 mM of THC in solution and was then selected for the rest of the work.

Table V.3: Absorbance of THC at its maximal solubility, in water solutions of different CDs at 15 mM at 280 nm.

Compounds	Absorbance of THC at 280 nm	Solubility of THC (μ M)
THC (control)	0.61 \pm 0.1	119 \pm 20
CDs at 15 mM		
β CD	0.51 \pm 0.05	99 \pm 9
γ CD	0.90 \pm 0.60	177 \pm 121
Me β CD	5.10 \pm 0.40	1005 \pm 77
Poly- β CD	1.84 \pm 0.07	363 \pm 14
Poly-Me β CD	1.91 \pm 0.03	376 \pm 5

1.3 Proof of the inclusion

The Me β CD solution enabled the solubilisation of about 10 times more THC compared to a water solution.

The commonly admitted mechanism to explain this fact is an inclusion of one part of the guest molecule into the cavity of the CD. To prove that, $^1\text{H-NMR}$ and 2D-NMR were carried out. Protons of THC and Me β CD were annotated as the Figure V.3 shows:

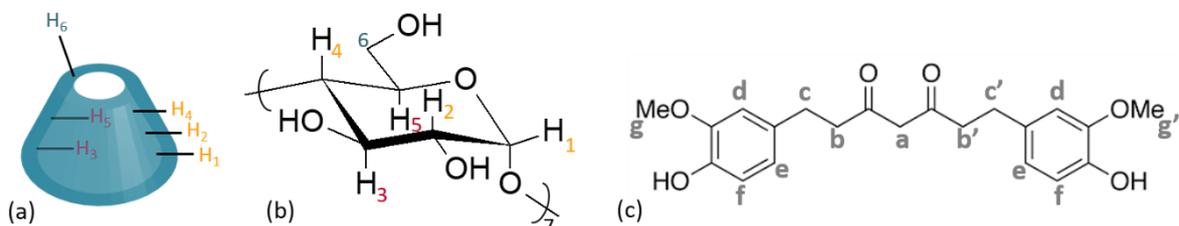


Figure V.3: Annotated protons of (a) CD in 3D-view, (b) glucose unit of a CD in chair conformation, and (c) THC.

1.3.1 $^1\text{H-NMR}$

A comparison of the $^1\text{H-NMR}$ spectra of Me β CD in the presence or absence of THC was carried out to highlight variations of chemical shift on H₃ and H₅ protons located inside the CD's cavity (Figure V.3). In case of an inclusion with a guest, protons located inside the truncated cone are subject to changes in their environment, due to the proximity of H atoms from the guest. H₁, H₂ and H₄ protons remained at the same chemical shifts, but changes could be observed for H₃ (0.01 ppm) and H₅ (no value given due to a large signal width). On the contrary to Jahed *et al.* [14] and Chao *et al.* [372] these chemical shift variations have been estimated too small to conclude to the formation of an inclusion complex between Me β CD and THC).

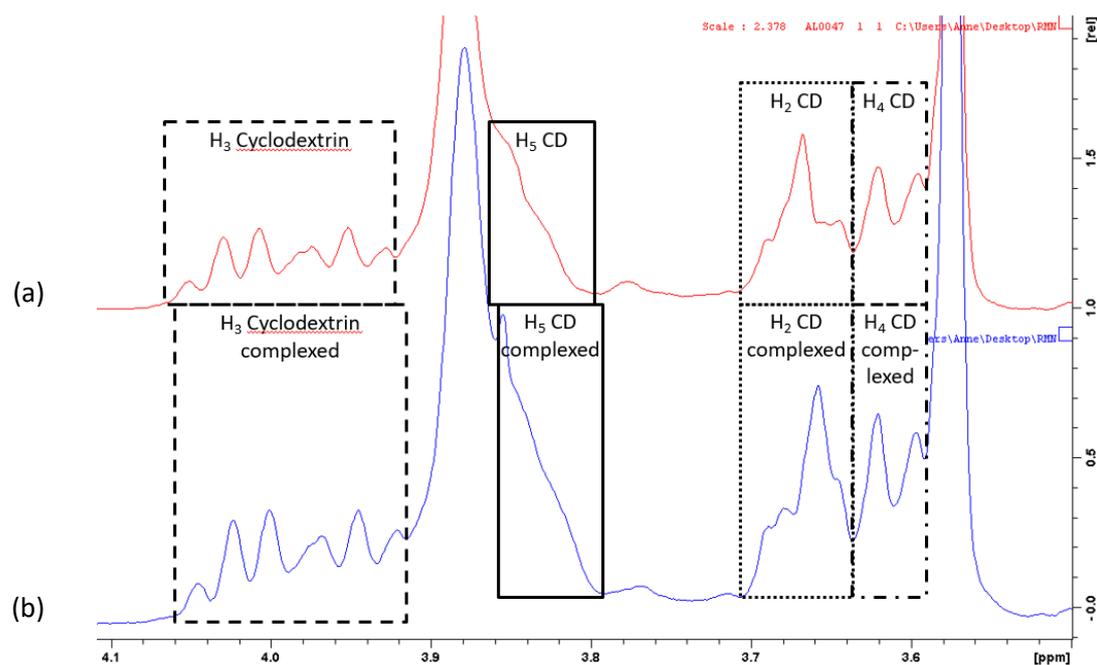


Figure V.4: $^1\text{H-NMR}$ of (a) Me β CD and (b) THC-loaded Me β CD in D_2O .

1.3.2 HSQC

To follow changes of chemical shifts from the THC point of view, we had to take a closer look at the aromatic protons of THC. However, the amount of encapsulated THC was too low to obtain defined signals detached from the noise. The NMR was performed in a mixture of ethanol- d_6 and D_2O at 1:4, to increase the concentration of the THC alone, which was used as a control. HSQC was needed to attribute the protons of THC. Aromatic protons d, e and f in the Figure V.5 (a) were attributed according to the 1H -spectrum of THC in DMSO- d_6 (Figure IV.3). Signals of carbon atoms were then deduced. Figure V.5 (b) shows the spectrum of THC-loaded Me β CDs where the signal of the proton f varied of -0.03 ppm while the proton “a” shifted of +0.04 ppm, which was easily linked with the formation of inclusion complexes. It can now be assumed that the phenolic parts of the THC were inside cavities of Me β CDs. As a remark, aromatic protons of THC undergo more significant changes of chemical shifts than H $_3$ and H $_5$ of the CD, probably indicating that the change in their environment is more drastic (going from free to trapped) than for inner protons of CDs (going from cavity to filled cavity).

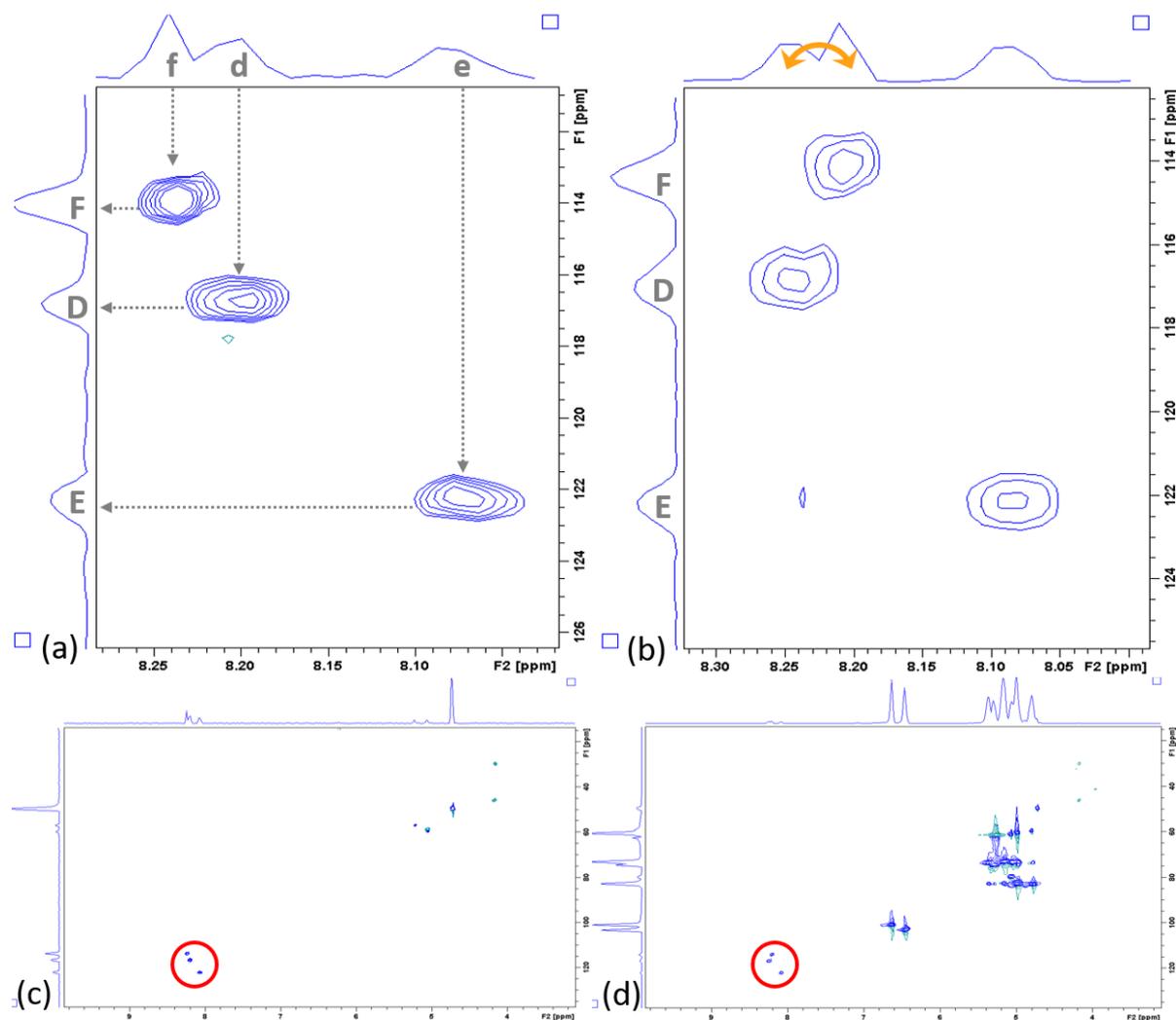


Figure V.5: HSQC of ((a) and (c)) THC and ((b) and (d)) THC-loaded Me β CD in D_2O .

1.3.3 ROESY

To totally confirm the inclusion of THC, 2D-NMR ROESY was employed, through which non-covalent interactions can be easily highlighted (Figure V.6). Aksamija *et al.* [297] observed interactions between protons of the guest molecule (rosmarinic acid) and both H₃ and H₅ of βCD, thus proving the inclusion of aromatic rings in CD's cavities. In this current study, the significant signal at 3.76 ppm corresponds to interactions between the aromatic protons and the protons of methyl groups, both from THC. Unfortunately, protons H₅ of CD are also located at 3.76 ppm. As a result, potential interactions with aromatic protons of THC could not be clearly evidenced. However, the second signal at 3.87 ppm proved unambiguously the presence of interactions between the protons H₃ of the inner cavity of CD and the aromatic protons of THC.

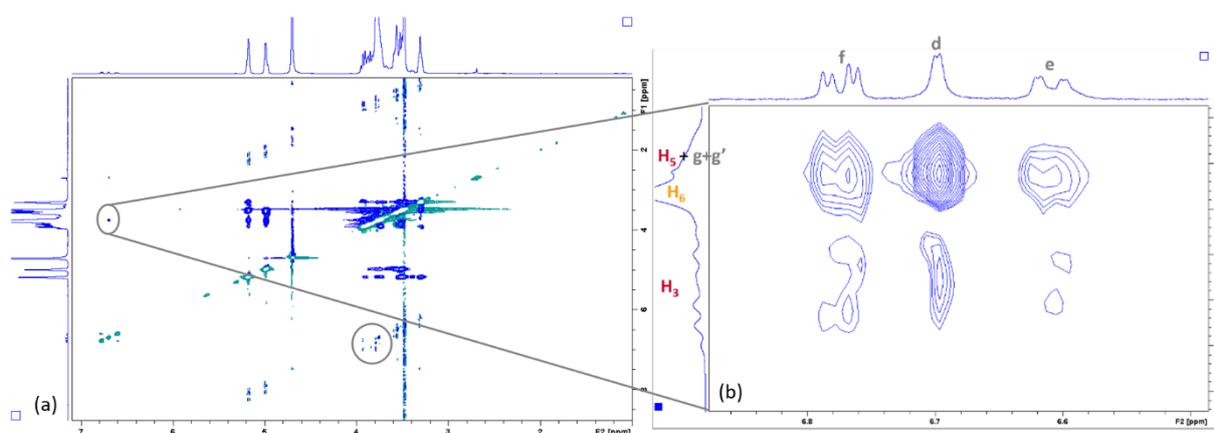


Figure V.6: (a) ROESY of THC-loaded MeβCD in D₂O and (b) zoom in on the interactions between the two components.

1.4 Study of the thermochemical equilibrium with MeβCD

1.4.1 Solubility study

The solubility study enabled the calculation of the solubilisation constant, according to Higuchi and Connors as described in the Material and Methods. First, THC was added in excess to solutions of CD at various concentrations. Afterwards, the concentration of THC, [THC], was determined *via* calibration curves, as described in 1.1, from the absorbance of the supernatants. The plot of [THC] *versus* [CD] can be modelised by a linear regression, meaning that a complex was made of one THC and one CD, for the studied ratios (Figure V.7). A linear plot of [THC] as a function of [CD]² would have proven a stoichiometry THC/CD of 1:2. As THC has two phenolic rings, it is possible that a second complexation occurs, maybe at a higher concentration of CD. The complexation constant is equal to 591±46 M⁻¹. Values commonly found in literature are ranging

from 10 to 10^5 M^{-1} , with most of the values ranging from 10^2 to 10^3 M^{-1} [373]. Curcumin-loaded βCD complexes possessed a constant ranging from 100 to 1500 M^{-1} [14,18]. Duarte *et al.* [299] found a solubility constant of 1483 M^{-1} with a resveratrol-loaded $\text{Me}\beta\text{CD}$ complex and Goto *et al.* [19] obtained a value of 1200 M^{-1} with THC and Hydroxy Propyl β -Cyclodextrin.

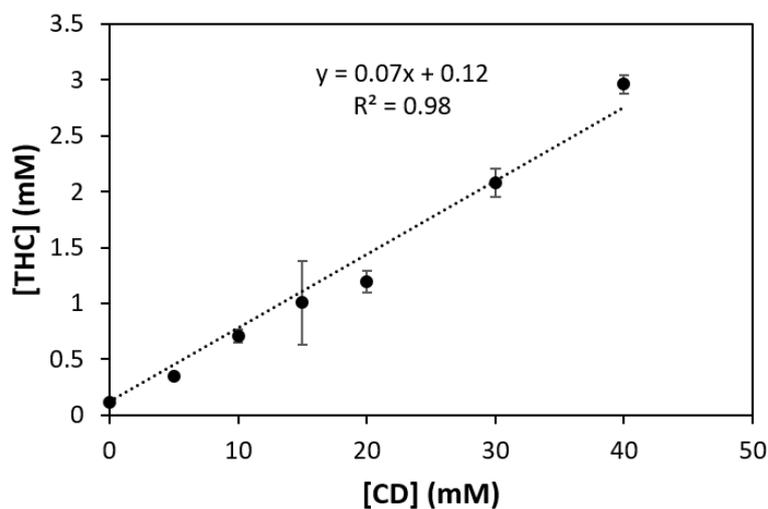


Figure V.7: Evolution of the maximal concentration of THC as a function of the concentration of $\text{Me}\beta\text{CD}$. Measurements performed in duplicate.

1.4.2 Isothermal Titration Calorimetry (ITC)

ITC is a powerful way to determine thermochemical parameters of a given system and was used to study the interactions between THC and CD. This technique is based on the measurement of small heat variations, usually in the μJ domain, corresponding to the formation of weak or strong chemical bonds in solution. This is especially relevant for non-covalent interactions, like the complexation of a compound by a ligand, particularly in biology [374] or for cyclodextrin complexes [375,376]. To explain the principle in more in detail, we will decompose the titration in three steps. The Figure V.8 is a theoretical figure, where the measured heat (negative value) is plotted as a function of the number of injections, which corresponds to the molar ratio.

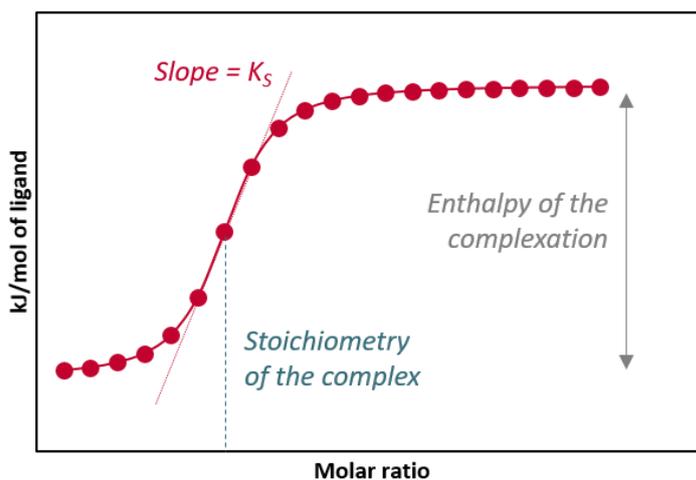


Figure V.8: Theoretical characteristic sigmoid curve of an ITC experiment.

The first step corresponds to the first injections of the CDs into the THC solution. All injected CDs react with THC, creating new bonds. The released heat is high, as many complexes are formed. Then, less and less THC remain free to react, as the number of CD injections increases. The absolute value of the measured heat is thus decreasing (the heat is being less negative). Finally, no more free THC is available to form a complex during the last injections. The released heat reaches a plateau.

In the experiment carried out in this current work, the titration was performed with multiple injections. Each injection had a different ratio of CD and THC and the curve was plotted manually with areas obtained from each experiment. However, even with this precaution to increase the signal/noise ratio, areas were inconsistent with ratios. The plot of the areas as a function of the molar ratio was an indecipherable scatter plot. The amount of reactants may still be too low to detect properly the signal, but this hypothesis seemed the less probable. The most likely source of error is probably the weighing of THC to prepare the solution at $10\ \mu\text{M}$. Moreover, we saw that aqueous solutions of THC needed 3 days to equilibrate. The freshly prepared THC solutions must have had a different concentration of what was expected. All these inaccuracies could have changed molar ratios CD/THC, leading to an incorrect plotting.

Some areas were obtained with a 3-day solution and are presented in Figure V.9. The curve cannot be fully interpreted. It seemed that several reactions occurred. The fact that areas are decreasing, increasing and decreasing again would mean that there was exo-, endo-, then exothermic reactions. The first two dots could correspond to the addition of one THC with one CD, studied in the solubility study. Complex formation with CDs leads to exothermic reactions, which are driven by a strong enthalpic gain because the lipophilic compound enters the cavity and

is dehydrated. Phenolic rings can even form H-bonds with OH from the CD, increasing the contribution of the enthalpy in the free energy. As a remark, the contribution of the entropy can be either positive or negative, but it is often negative, because both CD and THC are less free complexed than separated [373]. If the reaction of one CD with one THC occurred first, we can imagine that other heat variations, and especially the last one between the molar ratios 80 and 100, may be due to the fixation of a second CD. The middle step could eventually be linked to the move of water molecules in the environment of the complex (Figure V.9).

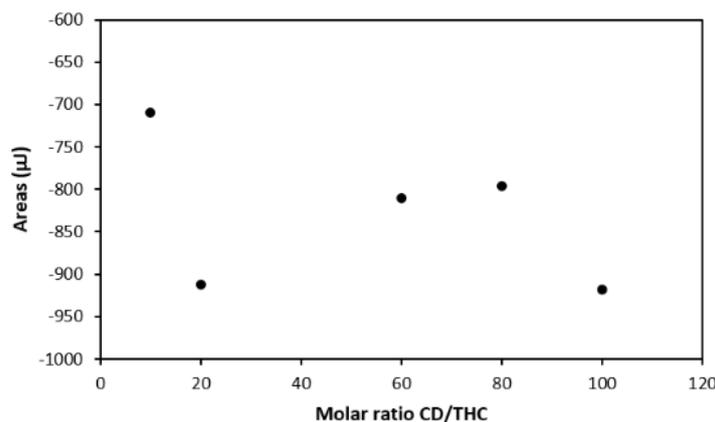


Figure V.9: Evolution of the dissipated energies of reaction as a function of the molar ratio of the two reactants.

Darby *et al.* [377] studied a specific guest-protein interaction and found that the guest has been linked in the first injections of the titration with one protein and that, meanwhile and later during the titration, a second protein was fixed. The first complexation was exothermic, due to bonds formation, and the second fixation was endothermic, because many water molecules had to be removed from the complex to let the second protein approach.

1.4.3 Concluding remarks

The study of the complexation process of the THC and Me β CD with NMR revealed that the phenolic ring of THC was located inside the cavity. Thanks to the solubility study, only one CD was found to be associated with one THC. If we make the approximation that a β CD is a cylinder of 0.79 nm length and 0.6 nm in diameter [378], its volume is 0.223 nm³. The volume of a phenolic ring is 0.162 nm³ [379]. As a consequence, only one phenolic ring should be inside and the second one outside, maybe fold onto the CD to create stabilising H-bonds between hydroxyl moieties of the CD and the phenol or the methoxy group [373]. The following Figure V.10 can be suggested:

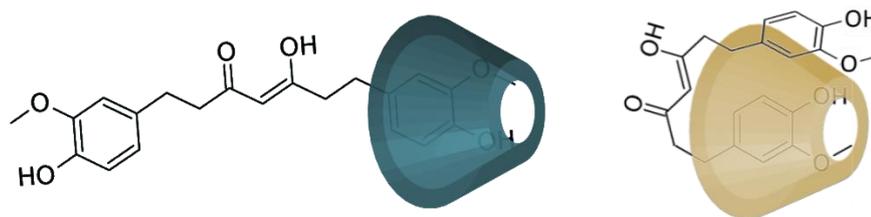


Figure V.10: Possible arrangements of THC inside the Me β CD.

1.5 Characterisation of the THC-loaded Me β CD complex powder

1.5.1 Morphology of the complex powder by scanning electron microscopy

Scanning Electron Microscopy (SEM) was used to know if complexation induced any changes in the morphology of powders. Powders from the starting material, raw or freeze-dried, and from the complex were analyzed. THC appears as crystals, rough in raw powder and spikier in freeze-dried powder (Figure V.11 (a) and (b)). Differences between both samples of Me β CD were very pronounced. In the raw powder, Me β CD was round shaped, like golf balls or empty capsules, with a diameter from 3 to 30 μm , whereas in freeze-dried Me β CD, thin sponge-like structures have been observed (Figure V.11 (c) and (d)). The morphology of the freeze-dried complex presented thin sheets that were often broken (Figure V.11 (e)). It could be concluded that THC was trapped within Me β CD, since THC spiked structures would have been visible among sheet-like Me β CD if THC would have not been in the cavity of the Me β CD.

As an additional remark, it can be assumed that the morphology seems mainly determined by the drying process. Both lyophilized Me β CD and complex presented a similar structure. However, the Me β CD furnished by the supplier was round-shaped. In parallel, some authors synthesized cyclodextrin complexes with a spray-drying technique and observed round shaped complexes [380]. It can be deduced that freeze-dried powders look like expanded sheets and spray-dried powder are ball-like. This is in contradiction with Duarte *et al.* [299] who suggested that the new structure was due to the formation of inclusion complexes.

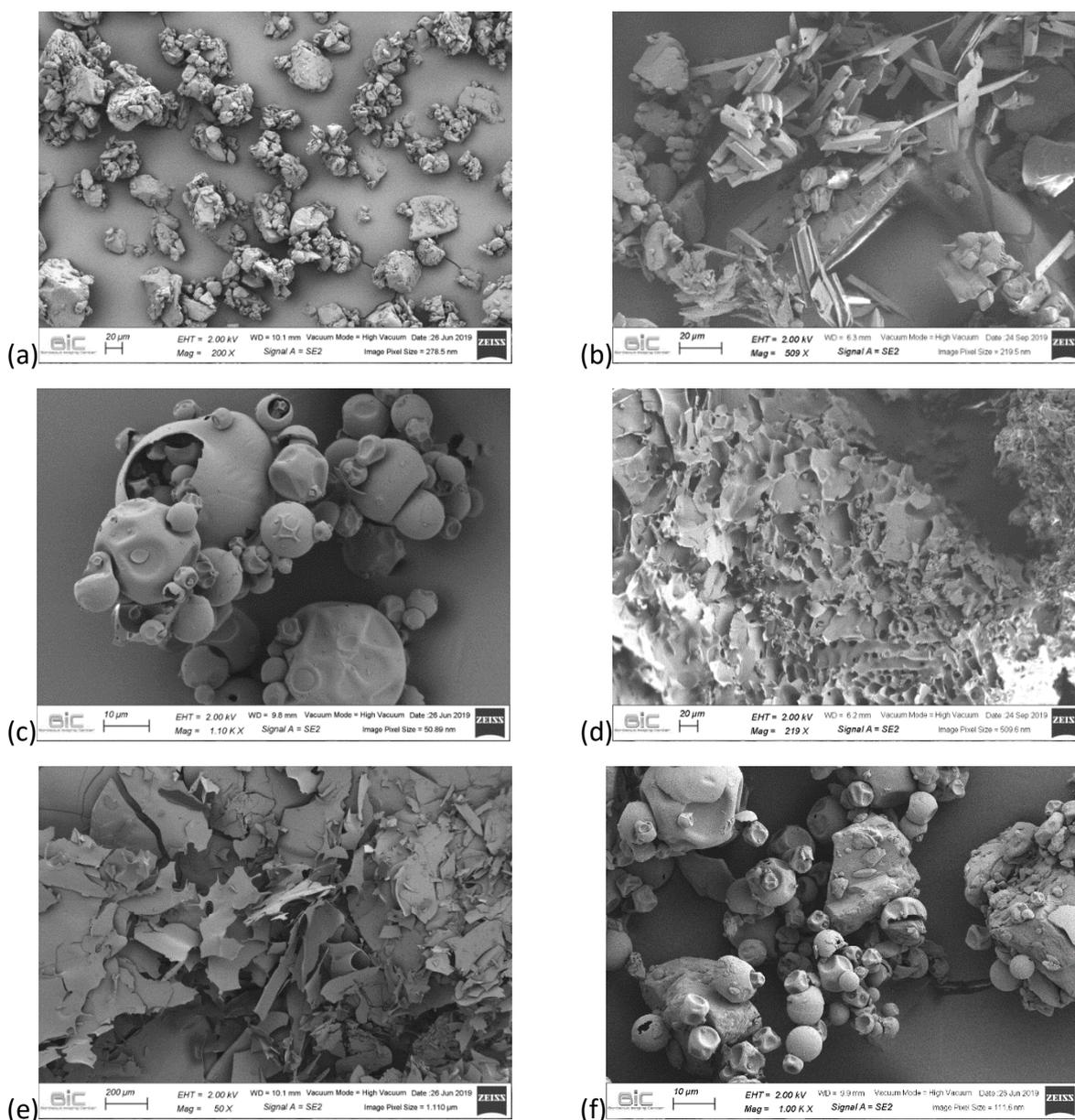


Figure V.11: SEM views of (a) THC; (b) freeze-dried THC; (c) MeβCD; (d) freeze-dried MeβCD; (e) freeze-dried THC-loaded MeβCD; (f) physical mixture of THC and MeβCD.

1.5.2 Differential Scanning Calorimetry (DSC) analysis

To supplement the study, a complex of MeβCD/THC and a simple mixture of THC and MeβCD in the same proportions were run on DSC. After a first cycle to eliminate water, the temperature was raised to a temperature above the melting point of THC (Figure V.12). In the physical mixture, a melting peak can be observed, corresponding to the fusion of THC but slightly shifted. The shift can be due to stabilising interactions with CD or maybe to the time the whole sample reached the temperature at which the THC should melt. However, no visible heat flow fluctuation could be seen in the curve corresponding to the complex. As a result, it could be

assumed that the THC was present in an amorphous form, as was also concluded by Maharjan *et al.* [381] or that the THC in the complex was protected from melting within a cavity of Me β CD.

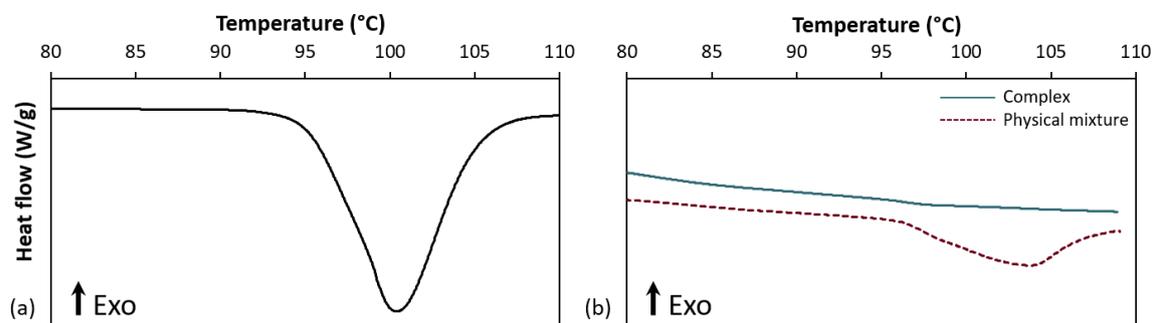


Figure V.12: DSC thermograms of (a) THC; (b) THC-loaded CD or THC and CD physical mixture.

1.6 Activity against *F. graminearum*

1.6.1 Impact on the fungal growth

The initial purpose of encapsulating THC was to enhance its antifungal action. The Me β CD solution containing 1 mM of THC was tested first, which is the maximal concentration achieved for the concentration of 15 mM. Unfortunately, the growth four days after inoculation was enhanced by 12 %, which putatively indicates the ability of *F. graminearum* to use CDs as carbon sources. However, after THC loading, the activation of the growth was reduced to only 2 %, indicating antifungal properties of THC, which may be related to the release of this active agent from CDs (Figure V.13). Unfortunately, inhibition percentages remained far below than expected.

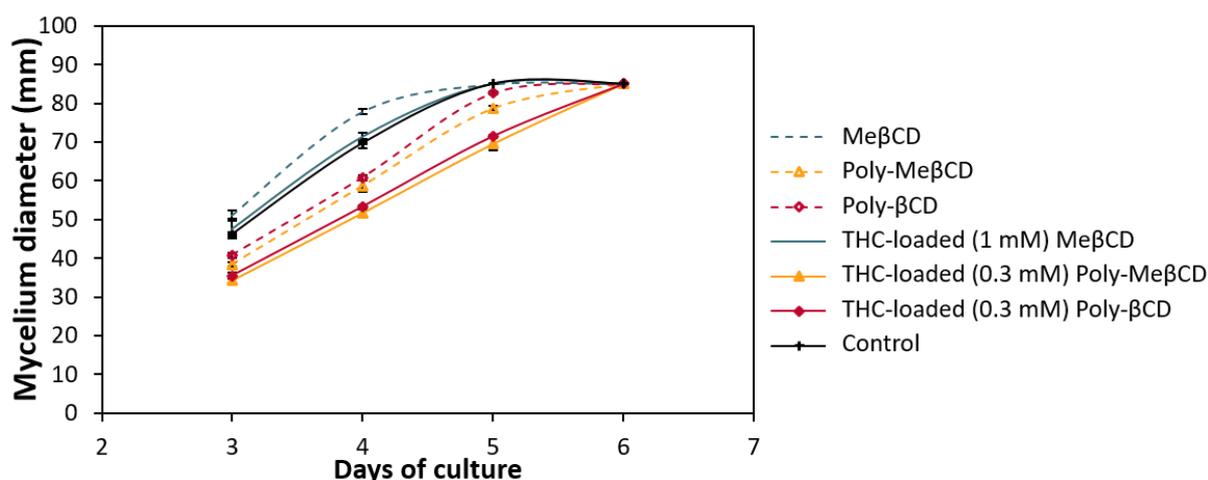


Figure V.13: Daily evolution of the mycelial growth of *F. graminearum* on PDA medium supplemented with Me β CD at 15 mM, Poly-Me β CD or Poly- β CD at 15 mM in CD cavities at 25 °C and 70 % R.H. Error bars correspond to the standard deviation of the measurements performed in triplicate.

Then, other CDs were tested, despite their lower impact on the solubility and the relatively lower THC amount. β CD at 6.6 mM and THC-loaded β CD (0.3 mM; 6.6 mM) also activated the growth by 29 % and 16 %, respectively.

The polymerisation of CD has helped to reverse the trend. Results showed that CD polymers, Poly-Me β CD and Poly- β CD, at 15 mM in cavities of CDs, even without the presence of THC loading, inhibited the mycelium growth of *F. graminearum* by 16 % and 13 % (Figure V.13). It could be due to the antifungal effect of free carboxylic acid functions of citric acid used for the synthesis of Poly-CDs [382]. After a THC loading at 0.3 mM, an improvement of 10 % was obtained, leading to an inhibition percentage of about 25 % and 23 % for Poly-Me β CD and Poly- β CD. The encapsulation of THC improved the inhibition percentage, thus showing once again THC ability to reduce the fungal development.

The inhibition percentage at day 4 have helped us to compare the efficiency of CDs between them. Regarding the long-term efficiency, all products, loaded as non-loaded ones, did unfortunately not stop the fungi from reaching the edges of the Petri dishes.

A previous study [6] has demonstrated that an inhibition of *F. proliferatum* of 67 % was achievable using, however, a higher THC concentration (13.6 mM), therefore requiring tetrahydrofuran to be fully soluble in water. In comparison, it was the first time that a significant inhibition was achieved without any organic solvent and with the use of a bio-based encapsulation system.

1.6.2 Impact on the production of toxins

The impact of the CD and the THC-loaded CD was evaluated against the accumulation of toxins (Table V.4).

Table V.4: Production of TCTB by *F. graminearum* after 14 days in liquid medium at 25 °C and 70 % R.H. Me β CD and complex at 125 mg/L. Standard deviation of the measurement from 5 repetitions. Different letters indicate significant difference ($p < 0.05$) within an experiment (marked with figures).

Production of TCTB (mg/g of dried mycelium)	
Control	20 \pm 2 ^a
MeβCD	19 \pm 3 ^a
MeβCD/THC	18 \pm 1 ^a

The CD and the complex were found to inhibit the production of toxins but not significantly, with 10 % of inhibition in the case of 125 mg/L of Me β CD. We assumed the THC activity can result either from its release or come directly from its complex form. With approximately 7 μ M of THC in the medium, which is the amount of encapsulated THC in 125 mg/L of Me β CD, the inhibition percentage was in the same order of magnitude as the results described in the Chapter IV.

In parallel, the antioxidant behaviour of THC-loaded CDs was determined. The antioxidant properties and the actions against toxins are related, so we checked if antioxidant properties of THC are kept in Me β CD.

Me β CD in methanol did not reduce the absorbance of DPPH \cdot radicals at all, as we can see from the purple solution in Figure V.14. The inclusion complexes inhibited radicals by 57 %, leading to an orange solution. The THC in methanol, at the apparent concentration in CD, completely inhibited radicals, forming a yellow solution.

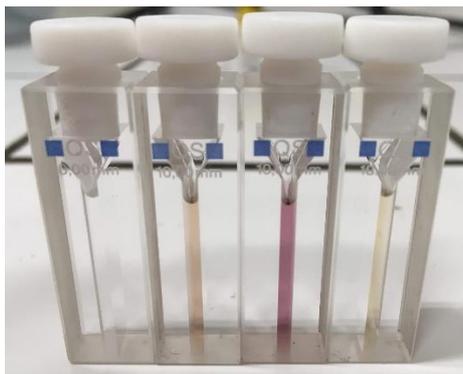


Figure V.14: Quartz cells after 30 minutes of reaction with DPPH \cdot . From left to right: methanol alone; THC-loaded Me β CD; Me β CD; THC.

In the complex, either the THC reacted with the radical while still being in the CD, or while being freed in solution. The dynamic equilibrium of the complex formation enables the presence of free THC at one time. Both hypotheses are possible, even if the second one seemed more likely to happen. In fact, one phenolic ring is probably free from CDs, but steric hindrances between CDs and voluminous DPPH \cdot radicals could happen.

Knowing that THC maintained some antioxidant properties and that antioxidant and antimycotoxigenic properties are linked, it is logical to observe an effect of THC complexes on the production of toxins, even if we could have expected a more pronounced action.

1.7 Concluding remarks

Encapsulation systems of CDs and polymers of CDs offered many advantages like their bio-based origin, the possibility to fully dissolve THC by using only water or a process at room temperature. Only Me β CDs gave an acceptable enhancement of the solubility of THC and however any application as a fungicide was ruined by their instability towards fungi. Although they trapped less THC, polymers of CDs did inhibit the growth of *F. graminearum*. The complicated elaboration of the complex in a multi-step encapsulation process and the formation of polymers, with all costs related, make such a process unthinkable to set up for industrial purposes. Therefore, some other options were evaluated like spray-dried and freeze-dried processes.

2 Towards an encapsulation easy to implement industrially

Because encapsulation by using CD is not really suitable for biopesticide applications notably due to the potential cost, another encapsulation process has been studied. It consisted of the preparation of an oil/water emulsion, which was then dried. This work was especially carried out at the Department of Polymer Chemistry and Technology at the Kaunas University of Technology in Lithuania. (Gilibert Project, Collaboration Ass. Prof. Ramunė Rutkaitė).

Emulsions of rapeseed oil with (1) octenylsuccinic acid (OSA) modified starch solution and (2) chitosan solution, both loaded with THC, were particularly studied. The two emulsions were dried by using two different techniques. The purity and properties of the resulting powders were then analysed.

2.1 Elaboration of emulsions with rapeseed oil

A way to incorporate lipophilic compounds into aqueous solutions is to create emulsions. In this current work, emulsions were made either with aqueous solutions of OSA-starch (Capsul®) and rapeseed oil or with chitosan (Ch. 78), Tween 80 and rapeseed oil. The study of the size of the droplets formed after homogenization and a visual observation helped us to trigger the parameters leading to the most stable emulsion. Once ratios between oil and water phases were chosen, THC was added into the oil and new emulsions were prepared.

2.1.1 OSA-starch based emulsions

OSA-starch is a commercial, easily available and food-approved modified starch [383]. The grafted OSA chains give the starch emulsifying properties. The lipophilic chains are pointing towards the oil phase, whereas the starch and the charges are in the water phase and are repelling each other (Figure V.15). It has been proved that OSA-starch was at the surface of oil droplets in emulsions [384].

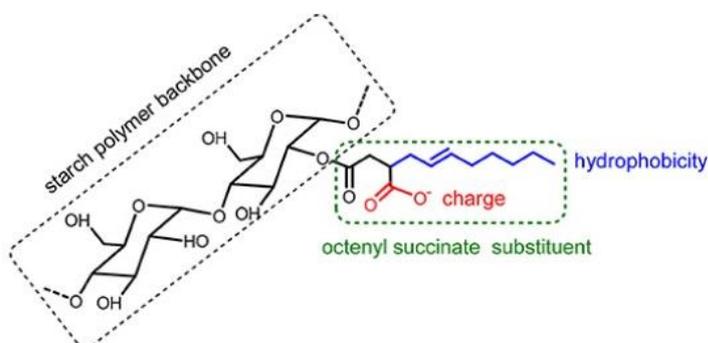


Figure V.15: Structure of OSA-starch from [385].

The impact on the size of the droplets of four parameters, homogenization time, ratio dried Capsul®/oil, ratio water/oil and speed of the rotation were studied. The objective was to determine the best conditions to obtain small droplets while limiting the experiment time, which is energy-consuming and can strongly impact the final cost of the materials.

As shown in Figure V.16, the longer the time of homogenisation, the smaller the size of particles. The size seemed to reach a plateau, so we chose to mix emulsions for 20 minutes before use.

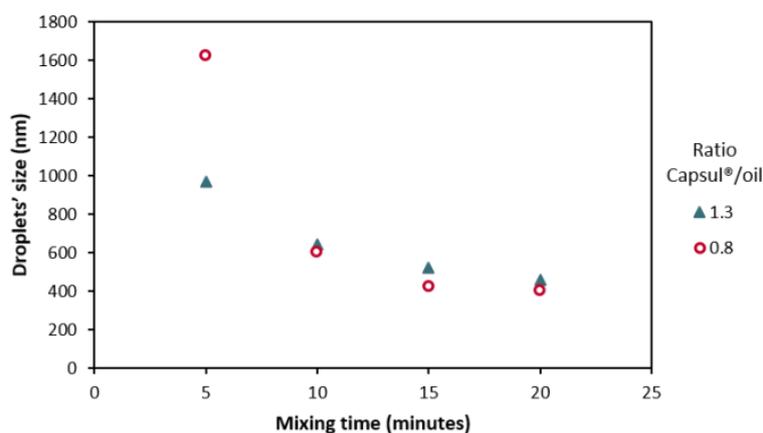


Figure V.16: Evolution of the droplet size as a function of the mixing time for a homogenization speed of 13,000 rpm.

Regarding the three other parameters to set, the ratio water phase/oil phase, the ratio Capsul®/oil and the mixing speed, their impacts on the droplet sizes are presented in Table V.5.

Table V.5: Evolution of the droplet size with the ratio water/oil, Capsul®/oil and with the homogenizer speed after a mixing time of 20 min. Measurements performed three times on duplicates.

Capsul®/oil	3	1.8	1.3	0.8	0.6	1.3	0.8
Water/oil	9	9	4	4	3	4	4
Speed (rpm)	10,000	10,000	10,000	10,000	10,000	13,000	13,000
Size of the droplets (nm)	797	1299±94	713	438±31	981±142	447±9	407±61

The best ratio water phase/oil phase was 4. The evolution of the droplet size with the ratio Capsul®/oil did not seem to follow any trend.

Enhancing the speed decreased the size of droplets, but the emulsion was getting hot during the homogenisation, thus potentially evaporating the water and changing ratios. Consequently, the maximum speed was set at 10,000 rpm, and in these conditions, the smallest droplets were obtained with the ratio Capsul®/oil 0.8.

The polydispersity indexes of the size of droplets was also studied (data not shown). We obtained the same conclusions regarding the setting of the process parameters.

As a result, we set a ratio water phase/oil phase of 4, a ratio Capsul®/oil of 0.8, with a speed of 10,000 rpm. These conditions led to droplets of 438 ± 31 nm, with a polydispersity index of 0.20.

Emulsions containing THC were obtained by the same way after adding 5 % (w/w) of THC in the rapeseed oil before mixing the two phases. Droplets were then 498 ± 42 nm large, with a polydispersity index of 0.22. Encapsulating THC in oil droplets did not change drastically their size.

A visual observation of the stability of emulsions was made at room temperature for 1 week, to confirm that the smallest droplets lead to the highest stability (without visual phase separation). As can be seen on Figure V.17, four behaviours were observed, although a lot of parameters have been changing. The selected conditions, gave the most stable emulsions (tube d).



(a) Yellowish phase (few mm) at the bottom
No clear phase separation

Most of the emulsions



(b) White upper phase
More transparent lower phase
Clear phase separation

ratio Capsul®/oil of 0.8, $t < 15$ min.



(c) Darker upper phase
White lower phase
No clear phase separation

Few tubes



(d) No changes in the emulsion

Selected conditions

Figure V.17: Visual observations of emulsions after one week at ambient temperature.

2.1.2 Chitosan-based emulsions

For the second option, we did chitosan-based emulsions. The aqueous phase was a solution of Ch. 78 at 10 g/L and AA at 10 mL/L prepared the day before the formation of the emulsion.

Chitosan emulsions needed the addition of Tween 80 as an emulsifier, because without it, large droplets reducing the stability of the emulsion are obtained.

As various conditions have been tested due to a more complex system, the preliminary results about the process were not presented here. The selected conditions were, Ch. 78/oil/Tween 80 = 1/0.2/0.1. Further characterisations could not have been performed¹.

2.2 Drying processes: spray-drying and freeze-drying

Emulsions were dried to obtain particles trapping THC, by two different techniques, freeze drying and spray drying. We compared the two drying processes, regarding the setup, the running time and the yield. Drying by freeze-drying was simpler than spray-drying. Indeed, the air flux in the spray-drying column was difficult to stabilise at the start of the equipment. The emulsion had to be diluted before not to block the nebulising head. We even had to change the apparatus to spray chitosan particles, due to a very bad yield (1-3 mg per day). This second spray-dryer was kindly lent by the Department of Food Science and Technology, Kaunas University of Technology, Lithuania.

The freeze-drying lasted 4 days (included 1 day for freezing) whereas the spray-drying was quicker.

However, the yield was far higher for the freeze-drying process and reached 87 % and 80 % for OSA-starch particles and THC-loaded OSA-starch particles and 68 % for both chitosan particles. The yield was not calculated for spray-dried OSA-starch particles, because the flow was too low to pass the 40 g of emulsion that was prepared for the day. The second spray-drier led to chitosan particles and THC-loaded chitosan particles with a yield of 21 % and 44 %, respectively. The gap might be due to handling problems, in particular because of the clogging of the dispersing head. Moreover, as the boiling point of acetic acid is 118 °C, the acid must have been removed during the process which was run at 170 °C. The resulting yield, without taking into account the AA, were 34 % and 71 % respectively.

2.3 Determination of the encapsulated amount of THC

Unfortunately, the amount of THC has only been quantified in OSA-starch particles.

To determine the amount of THC inside the OSA-starch particles, three techniques have been considered: UV-spectrophotometry, TGA and NMR. As THC absorbs at 280 nm, we wanted to dose its quantity by UV-spectrophotometry. Unfortunately, the starch, the THC and, to a lesser extent, the oil absorbed at the same wavelength in DMSO. In addition, the sum of the absorbance of each product measured separately was not equal to the absorbance of the mix of the products. This technique could not be applied to find the amount of THC without the creation of time-

¹ This work has been carried out in Lithuania at the beginning of March 2020. Due to the sanitary crisis and governmental measures that follows, the lab closed and my stay was shortened.

consuming calibration curves. We then changed our strategy for thermogravimetric analysis. If the THC was degraded before the starch, the mass of THC could be calculated. However, THC is very stable, as shown by the Figure V.18, where both particles and THC were degraded from 280°C. The discrimination of the degradation of THC, and thus its quantification was impossible by this way.

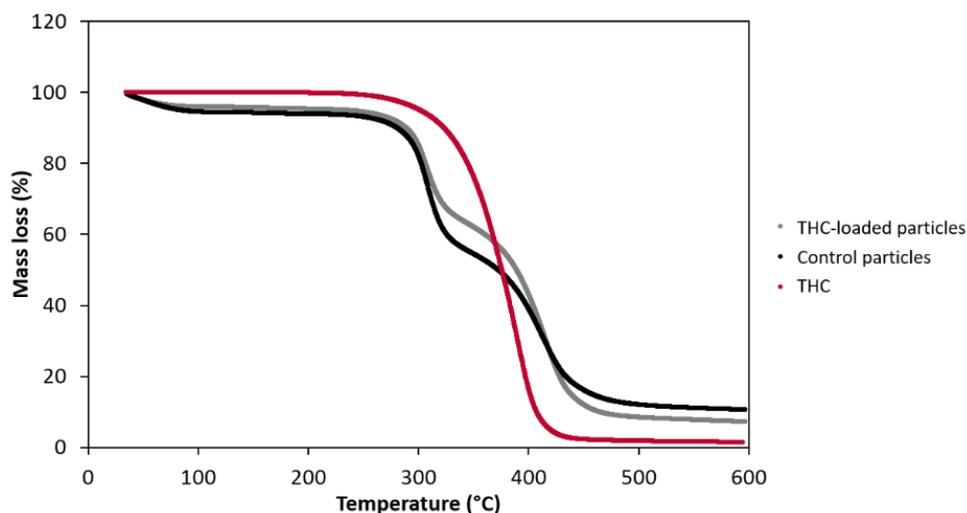


Figure V.18: Thermograms from thermogravimetric analysis of THC, spray-dried THC-loaded or non-loaded OSA starch particles.

Finally, NMR with pentafluorobenzaldehyde (PFBA) as an internal standard was chosen to determine the quantity of THC in OSA-starch particles. As this technique worked, it was employed for both freeze-dried and spray-dried starch particles. The spectrum for freeze-dried THC-loaded starch particles can be seen on Figure V.19 (knowing that its spray-dried analogue resembles it).

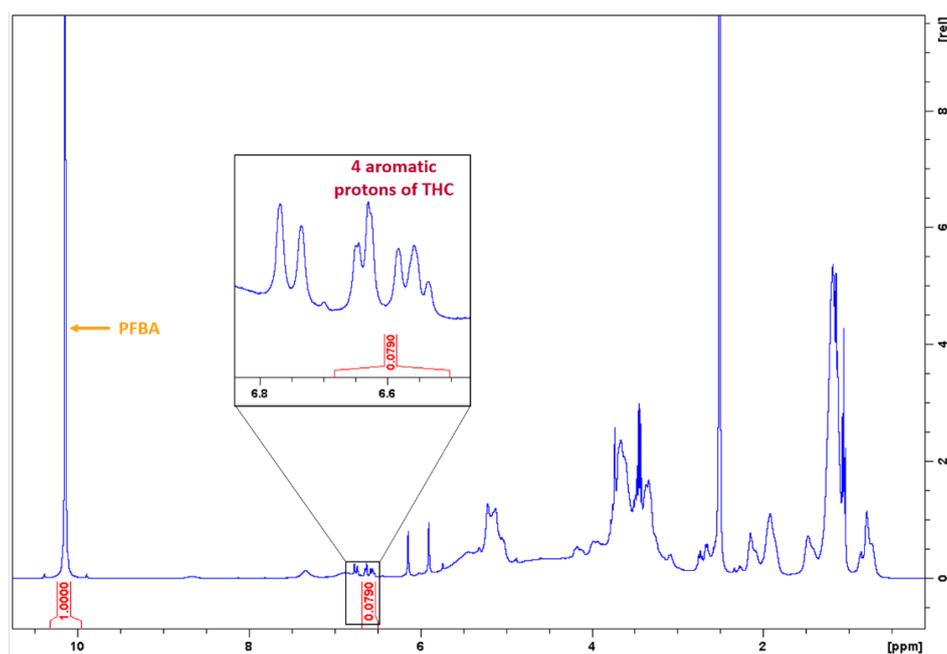


Figure V.19: $^1\text{H-NMR}$ spectrum of THC-loaded freeze-dried OSA-starch particles in DMSO-d_6 with PFBA.

The singlet at 10.14 ppm of the unique proton of PFBA was first integrated. The integration of the signal of four aromatic protons of THC enabled the determination of the mass fraction of THC inside the particles. In THC-loaded spray-dried and freeze-dried starch particles there were $1.9\pm 0.3\%$ and $5.1\pm 0.4\%$ of THC, respectively. It is common to get a better encapsulation efficiency for freeze-drying than for spray-drying [283].

2.4 Morphology of the powders by SEM

2.4.1 Freeze-drying

Particles from freeze-drying were observed by SEM (Figure V.20). No THC crystals were seen. As a reminder, freeze-dried objects are often in the form of spongy leaves (paragraph 1.5.1) but here, we can see spongy block of OSA-starch with air bubbles inside (a, b). These structures seemed more compact than the CD ones and, as a matter of facts, powders are also denser at the macroscopic level. Chitosan dried in this process was not a powder but rather flakes with the surface covered with round protuberances (c, d). This was rather uncommon, because freeze-dried chitosan generally presents a spongy network [386–388].

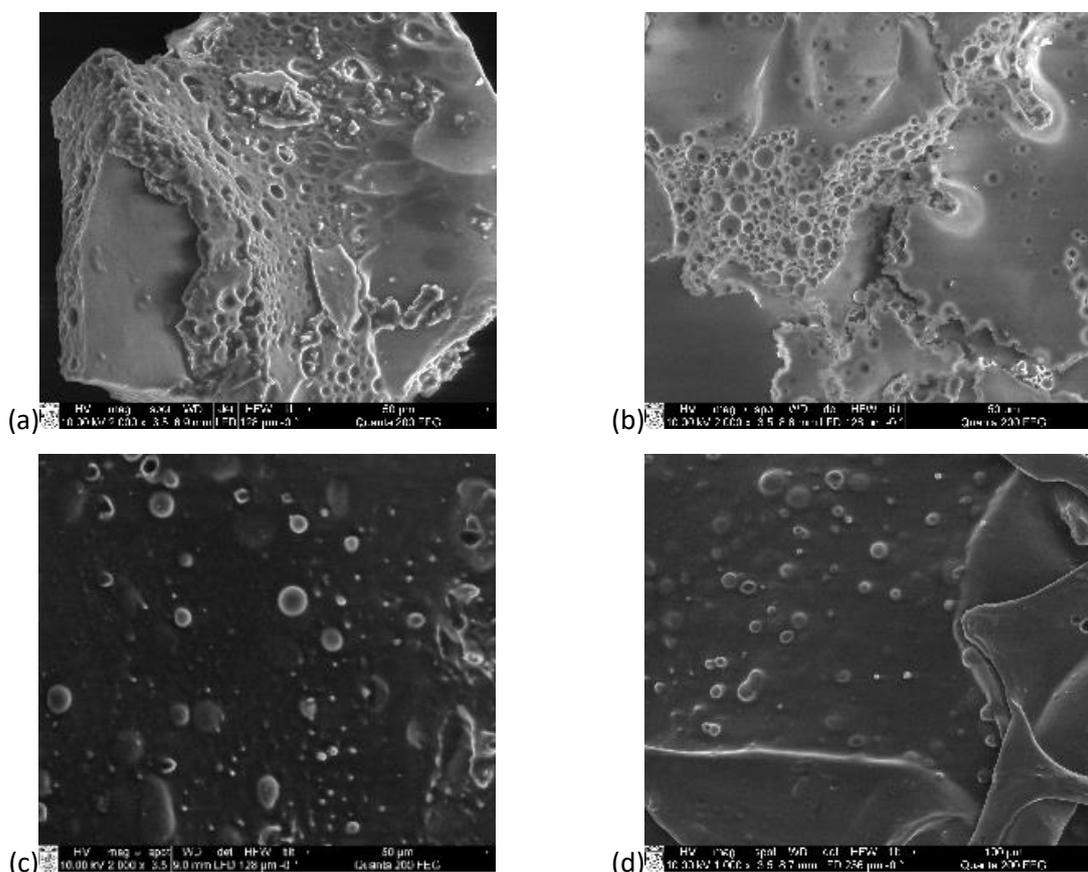


Figure V.20: SEM views of freeze-dried emulsions of (a) THC-loaded OSA-starch; (b) OSA-starch; (c) THC-loaded chitosan; (d) chitosan.

2.4.2 Spray-drying

This technique produced thin and compact powders. Their structures were balls with a diameter from some μm to $10\ \mu\text{m}$. We cannot see any crystal of THC throughout particles, meaning that THC should be inside them (Figure V.21). OSA-starch particles were spherical or irregular, like deflated balloons, with a smooth surface (a, b). Chitosan particles seemed to be less polydispersed than starch ones, since their size was between $1\ \mu\text{m}$ and $7\ \mu\text{m}$. They were spherical, some of them smooth and the others rough (c, d). Encapsulation with this technique seemed to produce exclusively spherical structures [389].

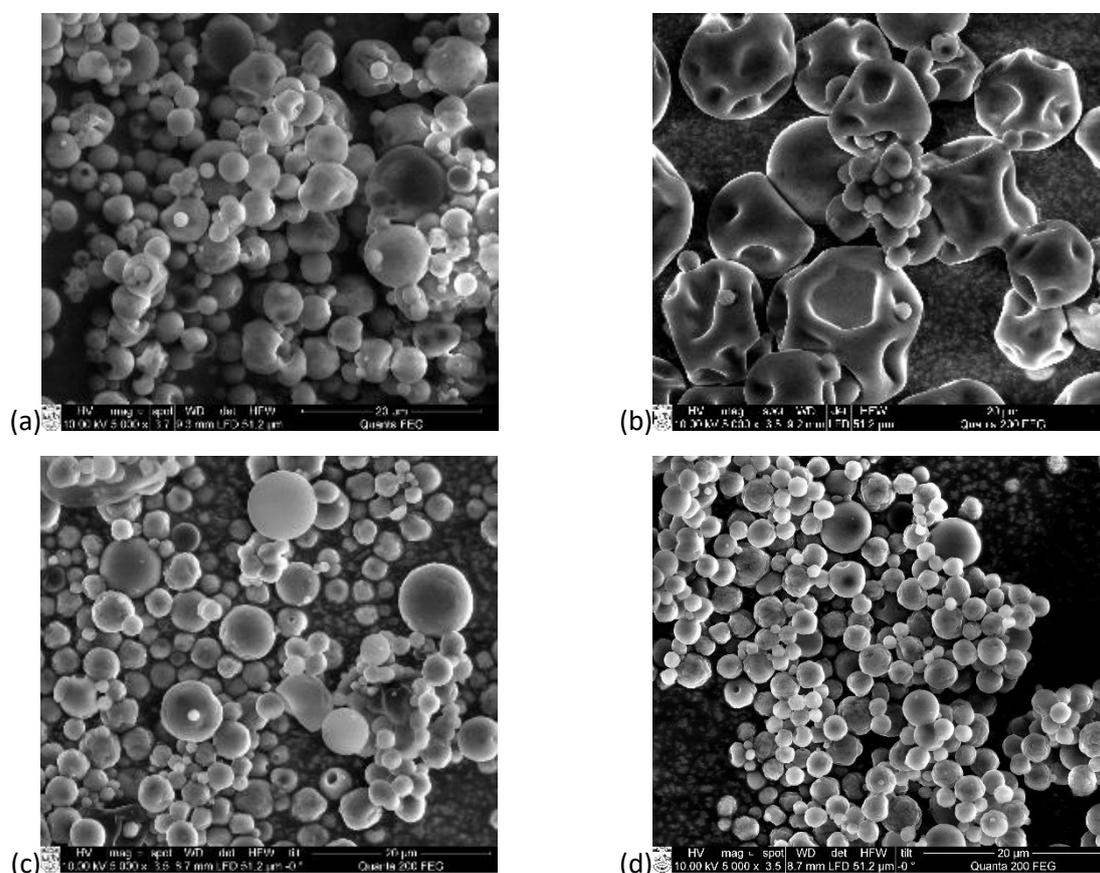


Figure V.21: SEM views (5,000x) of spray-dried emulsions of (a) THC-loaded OSA-starch; (b) OSA-starch; (c) THC-loaded chitosan; (d) chitosan.

2.5 Differential scanning calorimetry analysis of the particles

Like for CDs, the stability inside starch particles was evaluated by DSC analyses of particles loaded with THC and a simple mixture. The results are presented in Figure V.22.

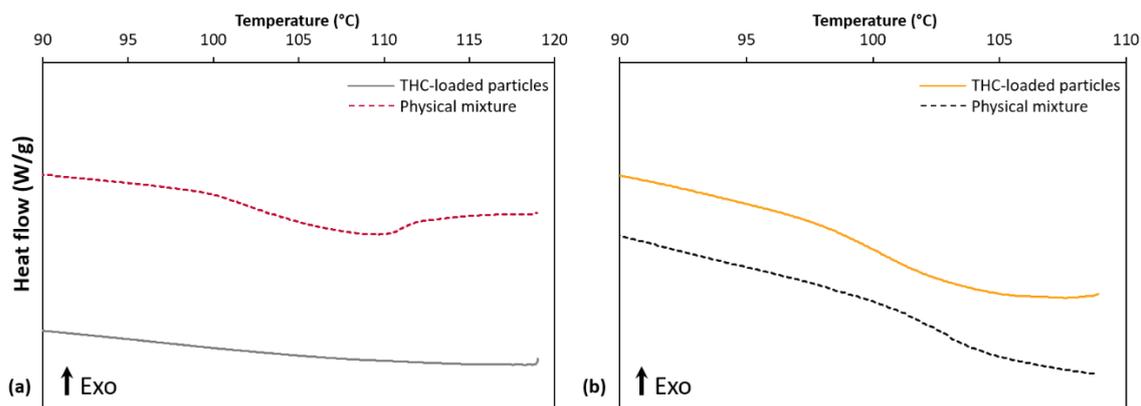


Figure V.22: DSC thermograms of (a) THC-loaded spray-dried OSA-starch particles and physical mixture of spray-dried OSA-starch particles and THC; (b) THC-loaded freeze-dried OSA-starch particles and physical mixture of freeze-dried OSA-starch particles and THC.

Spray-dried THC-loaded starch particles did not present any fusion peak, meaning that THC was in an amorphous phase or protected by the starch spherical particles, as previously observed with Me β CD. As expected, the physical mixture presented a shifted fusion peak for THC.

For freeze-dried particles, a new behaviour was observed. THC was melting in both cases, the one with THC-loaded particles and the one with a physical mixture of the two components. It means that THC was not protected by this kind of particles. This could be due to a loose matrix structure of the particles.

This experiment could not have been performed for chitosan particles as the THC amount inside these particles could not have been evaluated.

2.6 Microbiological activity of THC-loaded particles against *F. graminearum*

2.6.1 Antifungal activity of THC-loaded particles

As seen on Table V.6, the fungal growth was reduced by using these newly synthesized particles. Starch particles, free of THC, inhibited the fungal development, presumably because of the OSA-chain that could interfere with hyphae by either lipophilic or electrostatic interactions. Remember that polymers of CDs also had an effect due to their negative charges. Loading the THC improved the efficiency of freeze-dried and spray-dried particles by 14 % and only 5 %, respectively. As a comparison, THC alone in oil but twice as concentrated, gave an 11 % improvement compared to the rapeseed oil (Table V.6).

Table V.6: Inhibition percentage of the growth of *F. graminearum* on PDA medium supplemented with OSA-starch particles or rapeseed oil at 13.3 g/L, with and without THC, after 4 days of inoculation at 25 °C and 50 % R.H. Standard deviation of the measurements performed in triplicate.

	Mycelial growth inhibition (%)	
	Control compound	THC-loaded compound
Freeze-dried OSA-starch	7±1	21±2
Spray-dried OSA-starch	12±1	17±1
Oil	2±1	13±3

The THC in oil may be less efficient than THC in freeze-dried particles because the oil was rising to the surface of the Petri dish while the agar medium was cooling down as can be seen Figure V.23.



Figure V.23: Picture of the Petri dish from the test with rapeseed oil where oil droplets can be seen at the surface.

Ch. 78 particles demonstrated a higher antifungal activity than starch ones. For example, at four days of incubation, THC-loaded freeze-dried particles and spray-dried particles at only 0.4 g/L inhibited the mycelial growth by 15±2 % and 23±2 %, respectively, while the concentration of starch particles was 13.3 g/L for equivalent inhibition. It was then possible to estimate the antifungal activity by calculating the concentration at which 50 % of the growth is reduced (IC₅₀). Results are presented in Table V.7.

Table V.7: IC₅₀ of freeze-dried or spray-dried chitosan particles after 4 days of inoculation at 25 °C and 50 % R.H.

Particles	IC ₅₀ (g/L)	
	Control particles	THC-loaded particles
Freeze-dried Ch. 78	0.98	0.72
Spray-dried Ch. 78	0.46	0.61

IC₅₀ were in the range of what was observed for Ch. 78 solubilized in acetic acid, between 0.30 g/L and 0.65 g/L depending on the pH of the solution (see Chapter III). We observed undissolved chitosan particles in the medium. Although particles should actually contain AA, a significant fraction of acid must have been evaporated during spraying, leading to an insufficient amount to decrease the pH of the medium and totally dissolve the particles. Thus, the concentration might be incorrect and could explain why particles were less active than non-processed chitosan

(i.e. the chitosan from Chapter III). The result was the opposite to what was found by Ngan et al. [390] whose chitosan nanoparticles were more efficient against *Staphylococcus aureus*.

THC-loading improved the action of freeze-dried particles but worsen the one of spray-dried particles. No conclusive explanation has been found. So we assumed that THC may be released from freeze-dried particles and kept in spray-dried particles. In the first case, it could show antifungal properties whereas in the second case, it could hinder the antifungal activity of chitosan as seen in Chapter IV.

2.6.2 Antimycotoxigenic activity of THC-loaded particles

The production of TCTB in presence of particles, with or without THC, is available in Figure V.24. For the two conditions with spray-dried chitosan particles, the mycelium did not grow.

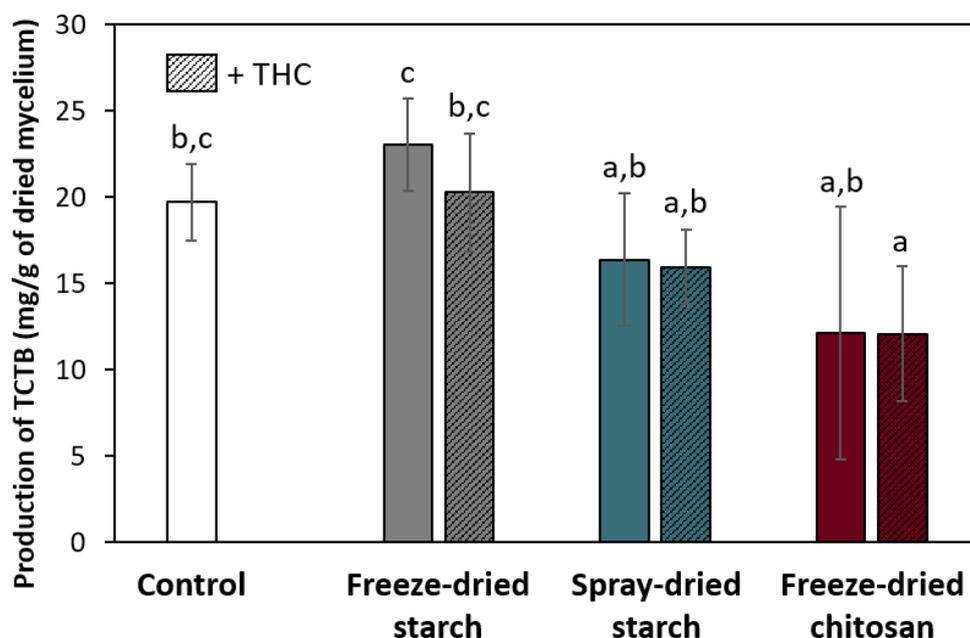


Figure V.24: Production of TCTB in liquid medium amended by non-loaded or THC-loaded particles at 125 mg/L after 14 days of incubation at 25 °C and 70 % R.H. Error bars correspond to the standard deviation of the measurement of 5 Petri dishes. Values with different letters presented significant differences.

To begin with, spray-dried chitosan particles totally inhibited the growth of the mycelium even at this concentration, which explains why they were not represented here.

The production of toxins was activated by freeze-dried OSA-starch particles, maybe because they act as a carbon source like CDs did. The addition of THC counterbalanced the undesirable trend. The difference between both conditions is about 10 %, which is what was usually obtained throughout this work thanks to THC.

Spray-dried starch particles were efficient although the addition of THC did not improve its action. We suggest that the THC is trapped inside particles without any release. Spray-dried

particles were already active against the fungal growth, probably due to residual anionic charges at the surface of those spherical particles.

Freeze-dried chitosan particles reduced the most the TCTB production, with 39 % of inhibition. As already discussed in Chapter III, chitosan reduced the amount of accumulated toxins. However, the THC-loading did not improve the effect of the particles. This can be attributed to the low amount of THC encapsulated in these chitosan particles or to the retention of THC inside them.

In addition, we assessed the antioxidant properties of particles. Interestingly, similar trends have been observed for antimycotoxigenic and antioxidant activities of particles. The results are presented in Table V.8.

Table V.8: Inhibition percentage of the DPPH[•] absorbance of particles.

Polymer matrix	DPPH [•] inhibition (%)	
	Control particles	THC-loaded particles
Freeze-dried OSA-starch	0	80±2
Spray-dried OSA-starch	2	0
Freeze-dried Ch. 78	4	13±2
Spray-dried Ch. 78	2	12±4

Freeze-dried starch particles did not have any antioxidant activity. Freeze-dried starch particles with THC quenched almost all radicals, whereas spray-dried ones were inefficient. The formulated hypothesis in the paragraph 2.5, that the THC was not protected in freeze-drying particles, was here supported. On the contrary, spray-dried starch particles must have protected the THC to prevent its release under these conditions, thus being in favour with what was assumed with TCTB.

Surprisingly, there was no difference between both THC-loaded chitosan particles, as it was the case with a starch matrix. The inhibition was lower, but the quantity of encapsulated THC was also lower. Ch. 78 possessed a small antioxidant capacity itself, although this antioxidant activity is controverted. It is often said to be linked with its chelating activity [391,392]. Intrinsic antioxidant activity of chitosan could eventually come from unshared pair of electrons on nitrogen atoms, on condition that they are not protonated. It could be the case, since, at the pH of the solution from which particles were formed, 3 % of amine groups are not protonated. The small antioxidant capacity of freeze-dried chitosan particles could explain its activity against mycotoxins. However, we should have expected for these THC-loaded particles to inhibit more the TCTB production than the non-loaded one.

Except the results with freeze-dried chitosan particles, results between antioxidant and antimycotoxigenic properties seemed to correlate.

3 Concluding remarks and prospects

For a clarity purpose, achieved goals of this chapter are reported in Figure V.25.

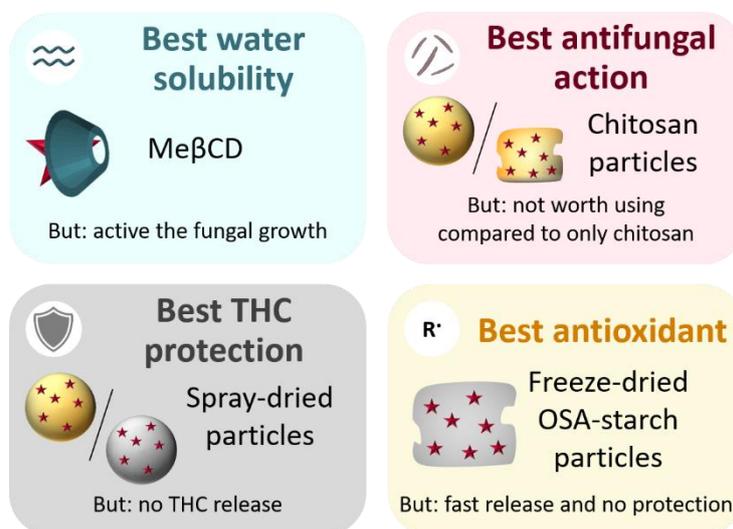


Figure V.25: Improvements obtained through the encapsulation of THC, with the main drawbacks for a given system.

First, encapsulating THC was an efficient way to improve its solubility in water, especially with Me β CD. With more suitable encapsulation processes for biocontrol formulations, it was found that THC was well protected by chitosan particles and spray-dried OSA-starch particles, but not by freeze-dried OSA-starch particles. The last one possessed a non-negligible antifungal activity, as much as high as the one of Poly-Me β CD. Chitosan particles showed logically the best global activity but they were unfortunately still less efficient than non-processed chitosan. The fast THC release from freeze-dried OSA-starch particles conferred them a high antioxidant capacity.

As a short term perspective, an interesting complementary study could be the improvement of one encapsulating system. Spray-dried OSA-starch particles deserve more attention. The parameters of drying could be optimised or the emulsion could be destabilised. Indeed, it appeared that these particles did not release enough THC, but were also promising, and reducing the amount of starch or adding a destabilising agent could change the protection and lead to a better and long-lasting efficiency against the fungi.

The association of encapsulated compound with chitosan should be, of course, the conclusive work of this part. The mix of Ch. 78, AA and THC-loaded OSA-starch particles would be interesting to create. Would the THC still interfere with chitosan, as it is almost not protected by this particle? Would this interaction be positive/negative, particularly against the mycelial growth or the accumulation of TCTB?

**CHAPTER VI – WOOD BY-PRODUCT EXTRACTS:
CHARACTERISATION AND ADDITION TO CHITOSAN-BASED
FORMULATIONS**

1	Extraction, characterisation and antifungal properties of extracts	146
1.1	Specific method of extraction	146
1.2	Extraction	147
1.3	Preliminary characterisation – total phenolic content.....	148
1.4	Antimicrobial activity	149
2	Addition of wood extracts to chitosan-based formulations for antifungal applications.....	153
2.1	Effect on the mycelial growth	153
2.2	Effect on the TCTB accumulation.....	154
3	First industrial assay on fields	155
3.1	Viscosity of the chitosan solution.....	155
3.2	Full-scale assay on field.....	155

Along with the curcumin derivative, other plant compounds have been selected in this study as potential biosourced antifungal molecules. We chose pine and vine resources as the silviculture and the wine production are two well-developed agricultures in *Nouvelle-Aquitaine*.

In this work, the wood-based extracts were obtained by using subcritical water, considered as an innovative and environmental-friendly process. Some of the results presented here were obtained from the MycSA laboratory team, and the companies Phenobio® and CRdistribution, as partner of project described in the introduction.

1 Extraction, characterisation and antifungal properties of extracts

1.1 Specific method of extraction

Wood extracts were obtained by our partner Phenobio® (Saucats, France) by using the subcritical water process. Generally speaking, water is an ideal inexpensive and environmentally friendly solvent for the extraction of plant extracts, but its use is limited due to a poor extraction efficiency for most organic compounds at ambient temperature. Nevertheless, its physicochemical properties can be modified by increasing its pressure and temperature. The so-called subcritical water remains liquid even above its atmospheric boiling point (100 °C) and until 374 °C and 221 bars [393–395] (Figure VI.1).

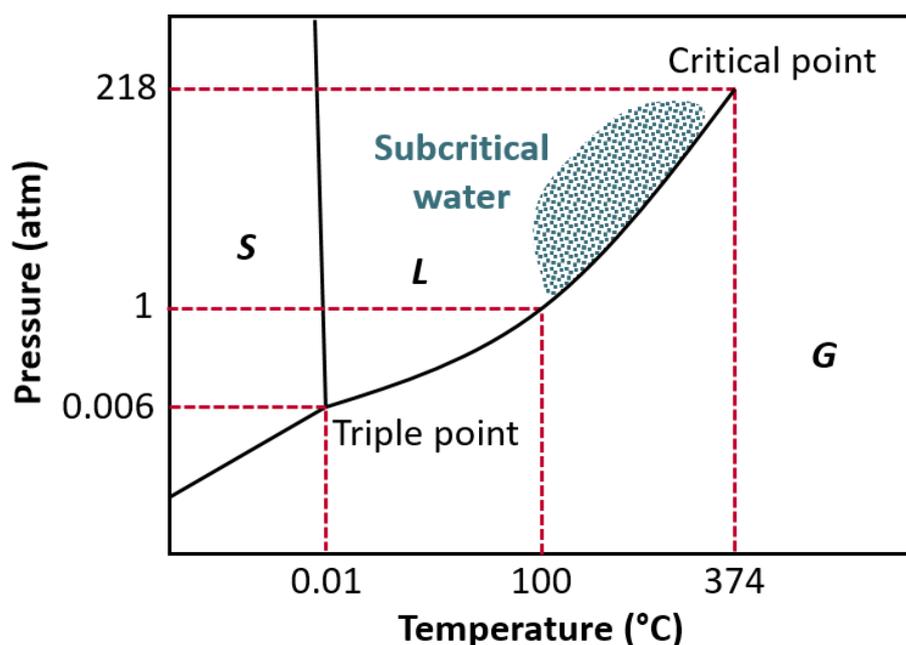
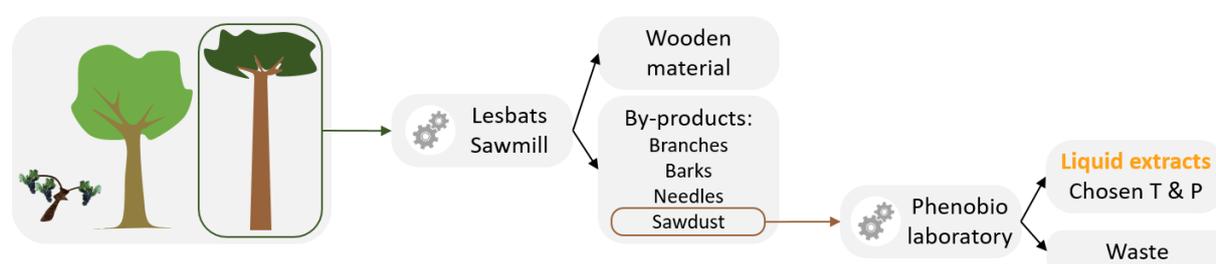


Figure VI.1: Temperature-Pressure diagram of water, highlighting the subcritical domain.

In these conditions, water presents significantly lowered values of dielectric constant, viscosity and surface tension in comparison to water at ambient temperature and pressure conditions. The higher the temperature, the smaller the dielectric constant of water under pressurised atmosphere. For example, the dielectric constant would drop from 79 (25 °C, 1 bar) to 44 (150 °C, 50 bars) [396]. Comparatively, DMSO possesses a dielectric constant of 47. Subcritical water extraction leads to efficient extractions due to high diffusion rates of the solvent through the raw material depending on the different chemical structures of organic compounds [397]. Solubility of less polar phenolic compounds increases when the temperature of subcritical water increases and short extraction time can be used [398,399].

Maritime pine bark, maritime pine sawdust and vine shoots were selected as plant sources. All extracts were obtained with a water temperature of 125 °C. For maritime pine sawdust, a second extract was prepared with subcritical water at 175 °C. This extract was called “maritime pine sawdust semi-polar (SP)”, for this water temperature enables the extraction of more apolar compounds. For example, the steps to obtain maritime pine sawdust polar and SP extracts are depicted in Figure VI.2.



	T (°C)	P (bars)	Organic solvent analogue*		Liquid extracts	Yield (% w/w)
Condition “polar”	125	15	DMSO	→	Polar extract	1.8
Condition “SP”	175	15	DMF/Acetonitrile	→	SP extract	2.3

*whose dielectric constant is close to the one of water under these conditions

Figure VI.2: Extraction of polar and semi-polar maritime pine sawdust extracts.

T = temperature

P = pressure

1.2 Extraction

The extracts tested in this work are summarised in Table VI.1. The extraction yield was the highest for vine canes with 5.7 % of total dry weight.

Table VI.1: Yield obtained with the subcritical water process for four extracts.

Natural extracts	Extraction yield (%)
Vine shoots	5.7
Maritime pine barks	4.7
Maritime pine sawdust	1.8
Maritime pine sawdust SP	2.3

Regarding the literature, hardwood, like vine, possess more extractives than conifers, such as maritime pine [400]. Barks contain a large proportion of extractive [401] with 17 % of the dry mass [402], most of which being polar compounds (91 % in *Pinus pinea* [403]). However sawdust contains more resin acids, terpenes, sterols, etc., which are more apolar components, and might therefore not be extracted with this technique [404]. Our extracts would be in adequacy with this because the yield for the semi-polar extract is higher than for the polar extract.

The extraction yields obtained with other techniques can be compared. Veggi *et al.* [405] reached an extraction yield of 24 % for Jatoba barks extracted with supercritical CO₂ and water (9:1). However, the extraction from eucalyptus barks with supercritical CO₂ and ethanol 8 %, lead to a yield of 1.5-2.0 % [406].

1.3 Preliminary characterisation – total phenolic content

Extracts are complex mixtures of products, which can be composed of phenolic compounds, terpenes or alkaloids. The quantity of phenolic compounds was approached by the Folin-Ciocalteu method for the four extracts, as shown by the Table VI.2 (MycSA team's results).

Table VI.2: Total phenolic content obtained from the four extracts by the Folin-Ciocalteu method.

Natural extracts	Total phenolic content (mg/g)
Vine shoots	92±4
Maritime pine barks	347±6
Maritime pine sawdust	121±18
Maritime pine sawdust SP	66±1

It is well known that barks contain a lot of phenolic compounds [407,408] especially tannins, which is the case here. Extracts of maritime pine sawdust contained more phenolic compounds than their semi-polar homologues. In fact, phenolic compounds are mainly polar. The maritime pine sawdust SP might contain more apolar compounds like resin acids, terpenes, sterols, as

already assumed, which are not phenolic compounds. The medium result of vine shoot can be explained because vine wood extractive are rich in stilbenes [409].

To go deeper into the characterisation, liquid chromatography coupled with mass spectrometry (LC-MS) was performed by the MycSA team (see p.56), knowing that we were involved in the interpretation of the results.

Only the most promising extract was analysed (see next sections about antimicrobial activity), which was the maritime pine sawdust SP. For this specific extract, some phenolic compounds were identified such as pinocembrin, pinobanksin, protocatechuic acid, pinoresinol, and nortrachelogenin (Figure VI.3).

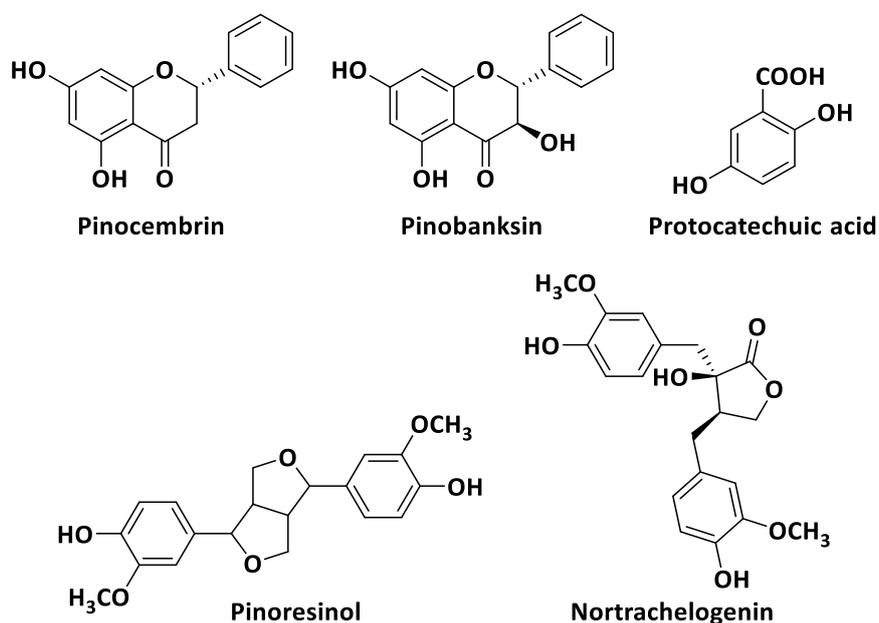


Figure VI.3: Structures of different molecules identified in extracts of maritime pine SP.

1.4 Antimicrobial activity

1.4.1 Impact on the fungal mycelial growth

F. graminearum, selected as the model pathogen fungus, was grown in presence of different concentrations of natural extracts. The daily evolution is presented in Figure VI.4.

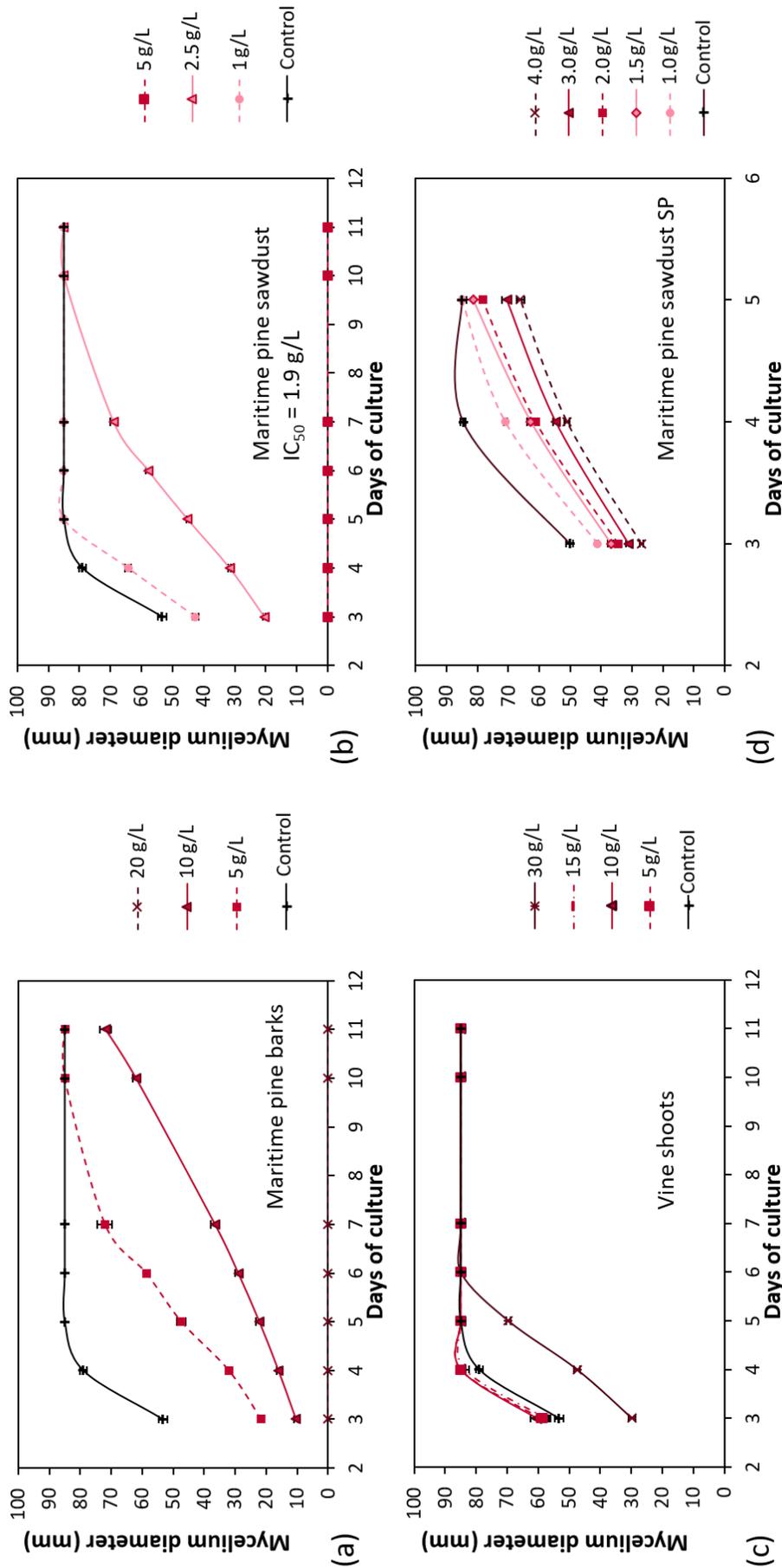


Figure VI.4: Daily evolution of the mycelial growth of *F. graminearum* on PDA supplemented with different concentrations of maritime pine barks; (b) extracts of maritime pine sawdust; (c) extracts of vine shoots; (d) extracts of maritime pine sawdust SP. Error bars correspond to the standard deviation of the measurement performed in triplicate.

Among these extracts, results are disparate, with an inhibition of 40 % and 60 % for vine shoots at 30 g/L and pine sawdust at 2.5 g/L, respectively. Vine shoot extracts exhibited the least antifungal activity. Extracts of maritime pine sawdust were more active with an IC_{50} of 1.9 g/L. Semi-polar extracts of pine sawdust had an intermediate activity, with 40 % of inhibition at 4 g/L.

When compared with the literature, Mezni *et al.* [8] showed that needle extracts of maritime pine decreased the growth of some *Aspergilli* and *F. solani* up to 43 %, for the extraction with acetone, which is more apolar than the subcritical water used in this work. Extracts of maritime pine and Aleppo pine exhibited antibacterial and antifungal properties in the work of Ghanmi *et al.* [9]. Alcohol-benzene extracts of maritime pine barks were shown to reduce the growth of *Trametes versicolor*, and *Coniophera puteana*, two xylophagous fungi. The growth was reduced up to 97 % with 0.13 mL/L of extract [10]. This is the first study to describe antifungal activities of wood extracts against *F. graminearum*.

About the possible modes of action, some authors showed that the cell membrane [184,185,191], plasma membrane [410], as well as the cytoplasmic membrane [187] were damaged by diverse essential oils or phenolic compounds. The loss of such membrane integrity causes leakage of intracellular contents, like soluble proteins [191], leading to cell death [411]. This could be due to direct hydrophobic interactions between lipophilic parts of natural compounds, *e.g.* alkyl chains, aromatic rings, etc., and fatty-acid-chain constituents of membrane lipid bilayers [411]. Phenolic compounds with one or more methoxy group(s) on their ring were indeed more antifungal than analogues bearing hydroxyl group(s) [412,413].

Moreover, mitochondria, proton pumps or ergosterol can be affected by natural extracts [181,188,189]. Peng *et al.* [191] studied the specific molecule pinocembrin, which was purified from propolis, and which is present in our extracts of maritime pine sawdust SP. They found that this molecule altered the fungal respiration and the mitochondrial respiration. This latter alteration caused an ATP deficiency, corresponding to a lack of energy.

In our case, there is no clear relationship between the quantity of phenolic compounds (see 1.3) and the inhibition of the fungal growth. The antifungal activity of maritime pine barks may be due to their high content in phenolic compounds.

The studies highlighted the link between antifungal and lipophilic properties. To summarise, the action of extracts against the growth of fungi may be due to several factors.

1.4.2 Impact on the TCTB production

The experiment to assess the production of TCTB by *F. graminearum* was carried out by project partner – MycSA laboratory. Their results are presented in Figure VI.5.

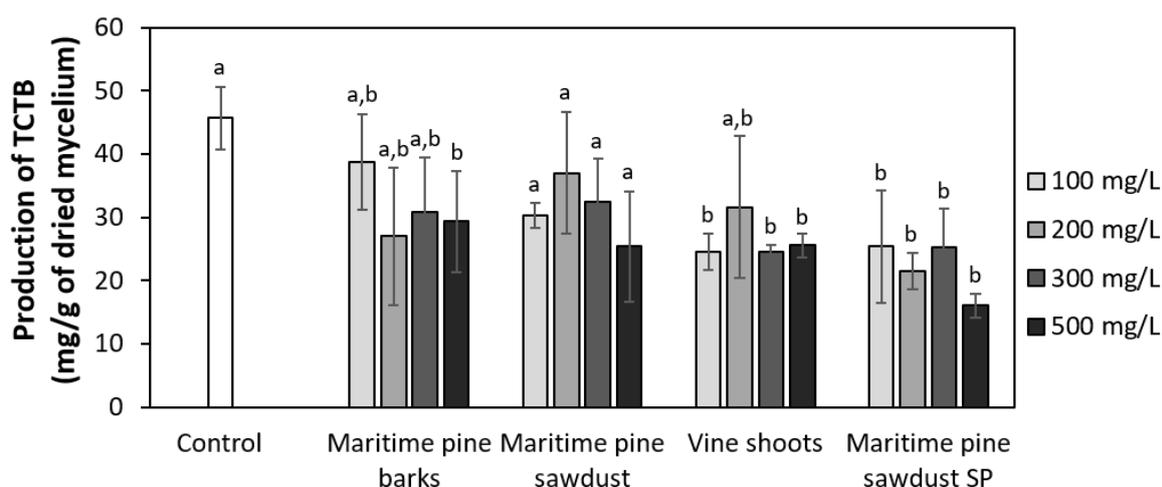


Figure VI.5: Production of TCTB of *F. graminearum* in liquid medium amended by extracts of maritime pine barks, maritime pine sawdust, vine shoots or maritime pine sawdust SP at 25 °C and 70 % R.H. Error bars correspond to the standard deviation of the measurement of 4 Petri dishes. Different letters indicate significant difference ($p < 0.05$).

All extracts inhibited the production of TCTB. Extracts of maritime pine barks and sawdust did not reduce the accumulation of toxins significantly, except for maritime pine barks at 500 mg/L with a reduction of 40 %. The inhibition caused by vine shoot extracts and maritime pine sawdust SP extracts was significant almost for all conditions, with a reduction from 31 % to 61 %.

Natural extracts have already been employed in other works to reduce the production of toxins of some fungi. However, no work with pine extracts has been reported. Perczak *et al.* [414] showed that eight essential oils were efficient for the reduction of the production of zearalenone and TCTB by *F. culmorum* and *F. graminearum* respectively. Methanolic extracts of *Agave asperrima* and *Agave striata*, obtained from simple soaking of the plant in the alcoholic solution, inhibited the production of aflatoxins by four strains of two species of *Aspergillus*. They obtained an inhibition of more than 95 % of the toxin production for almost all conditions, probably because they add a higher concentration of extracts than us [415]. Another example has demonstrated the efficiency of oregano and thyme aqueous or ethanol extracts to decrease the amount of aflatoxin, DON, zearalenone or T2 toxin in silage samples [416].

The maritime pine sawdust SP extract was selected to be associated with chitosan in the next part. It corresponded indeed to the best extract regarding the reduction of the TCTB production but also to the best extract in another experiment carried out at the MycSA laboratory. This latter was different, since extracts were directly put in contact with spores, and not mixed in the medium. It showed that semi-polar extracts of maritime pine were more active than the polar ones.

2 Addition of wood extracts to chitosan-based formulations for antifungal applications

As for the previous work with THC, the study has been finalised by developing chitosan-based formulations associating the extracts of maritime pine and by determining its effect on the mycotoxinogenic fungus. Comparatively to THC, used as a pure model phenolic compound, the industrial application of plant extracts-based formulations is easier and less costly, notably because no encapsulation is necessary. In an effort to scale-up such formulations, note that the production of extracts is a one-step, environmentally friendly and cheaper process than the synthesis of THC.

The combination of Ch. 78 and the extract of maritime pine SP was tested *in vitro* against fungal growth and toxin production. In the end, a full-scale test was carried out on wheat fields.

2.1 Effect on the mycelial growth

Natural extracts of maritime pine sawdust SP were added to an aqueous acetic acid solution of Ch. 78. The concentration of chitosan was set at 0.5 g/L because it leads to a medium inhibition, thanks to which potential changes in the reduction of the mycelial growth could be easily followed. The concentration of extracts was also chosen to give an intermediate fungal inhibition. The Figure VI.6 shows the fungal growth during one week.

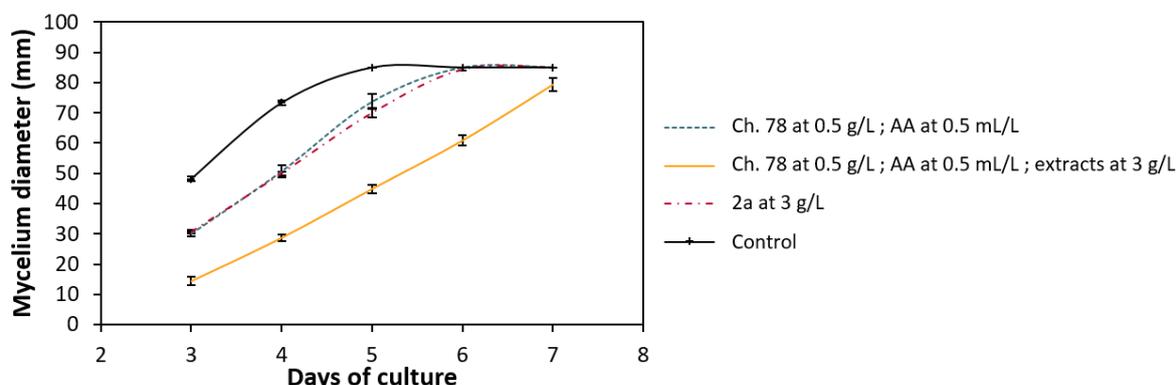


Figure VI.6: Daily evolution of the mycelial growth of *F. graminearum* on PDA medium supplemented with Ch. 78, AA or maritime pine extracts at 25 °C and 70 % R.H. Error bars correspond to the standard deviation of the measurements performed in triplicate.

The association of Ch. 78 and extracts was twice as active as a single compound. It could mean that both the Ch. 78 and the extract have kept their own antifungal activity. Contrary to what was observed with THC, the action of chitosan is maintained. The growth was not totally inhibited and the mycelium reached the edge of the Petri dish with a delay of two days anyway.

It is difficult to compare the activity of our compounds with the literature since plant extracts have only very rarely been associated to chitosan for crop protection application. In fact, Silva-

Castro *et al.* [417] employed chitosan (60–130 kDa) and chitoooligosaccharides with propolis ethanolic extracts obtained after soaking of grinded resin for 72 hours. Both chitosan and chitosan mixture improved the survival of *Pinus* spp. seedlings inoculated with *F. circinatum*. However, no significant difference between chitosan and chitosan/extract was found. In another study, chitosan (no MW nor DD mentioned) was associated with salicylic acid against *Penicillium digitatum*. Both compounds were active alone and their association was more efficient to reduce the disease incidence and the lesion diameter in grapefruit [418].

2.2 Effect on the TCTB accumulation

As a reminder, in an experiment to assess the effect on the mycotoxin production, the active compounds must not hinder the growth of the fungi. Concentrations of chitosan and acid were set to enable the normal growth of the fungi, 25 mg/L with AA and 100 mg/L with LA, and the concentration of extract was one of the ones used in the previous test, and did not hinder the growth either.

The production of toxins is given by the Figure VI.7.

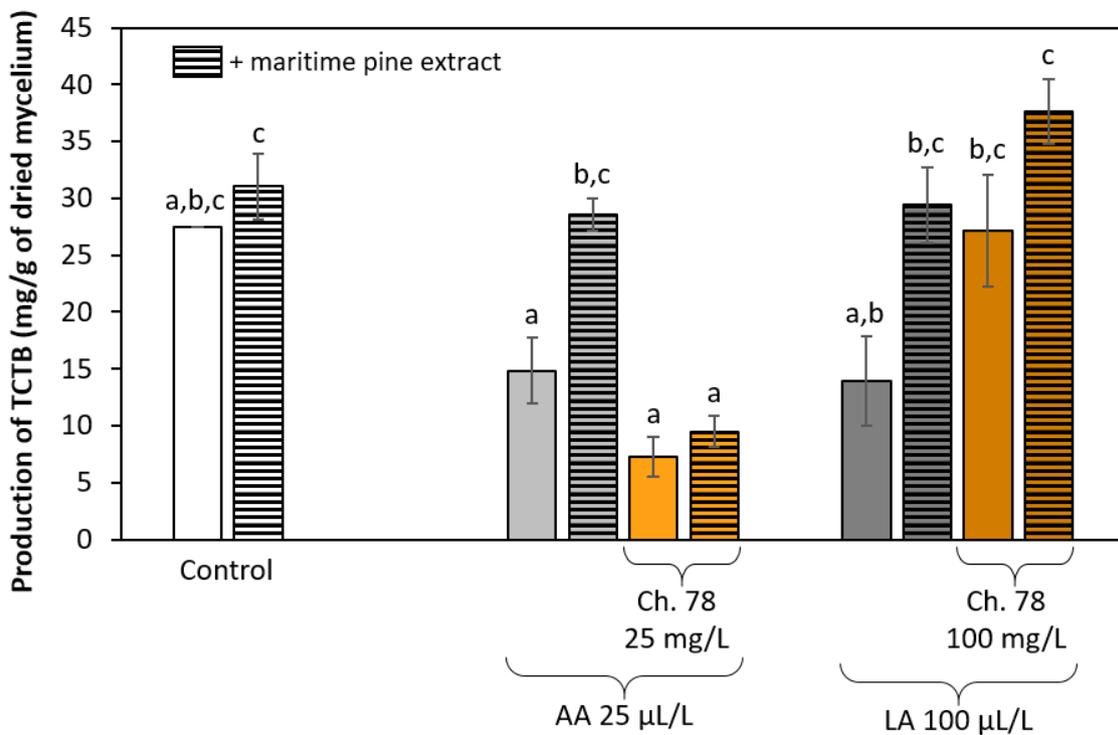


Figure VI.7: Production of TCTB of *F. graminearum* in liquid medium amended by AA at 25 µL/L; LA at 100 µL/L; Ch. 78 at 25 mg/L or 100 mg/L; maritime pine extract SP at 200 mg/L at 25 °C and 70 % R.H. Error bars correspond to the standard deviation of the measurement of 6 Petri dishes. Different letters indicate significant difference ($p < 0.05$).

First, unfortunately, the extract alone did not show any inhibition of the TCTB accumulation like we would have expected but an increase of 13 %. Accordingly, the blend of chitosan and extract is unlikely to work as well and the addition of extract did not help the action of chitosan and acids actually. We observed an activation of 92 % and 111 % with the extract added to AA and LA, respectively and of 30 % and 39 % with the extract added to Ch. 78–AA and Ch. 78–LA, respectively.

3 First industrial assay on fields

3.1 Viscosity of the chitosan solution

Chitosan solutions are known to be viscous, especially for chitosans with high molar masses. First, it was necessary to verify if the chitosan solutions were liquid enough to be sprayed on a device used in agriculture. The viscosity is 29 mPa/s or 11 mPa/s for film-forming solutions at 1 % or 0.5 % -w/v for chitosan and v/v for acid-, respectively, as can be seen in Figure VI.8.

CR Distribution, the partner in charge of the implementation on the fields, did not report any difficulties of spraying or manipulation with the products we sent to them.

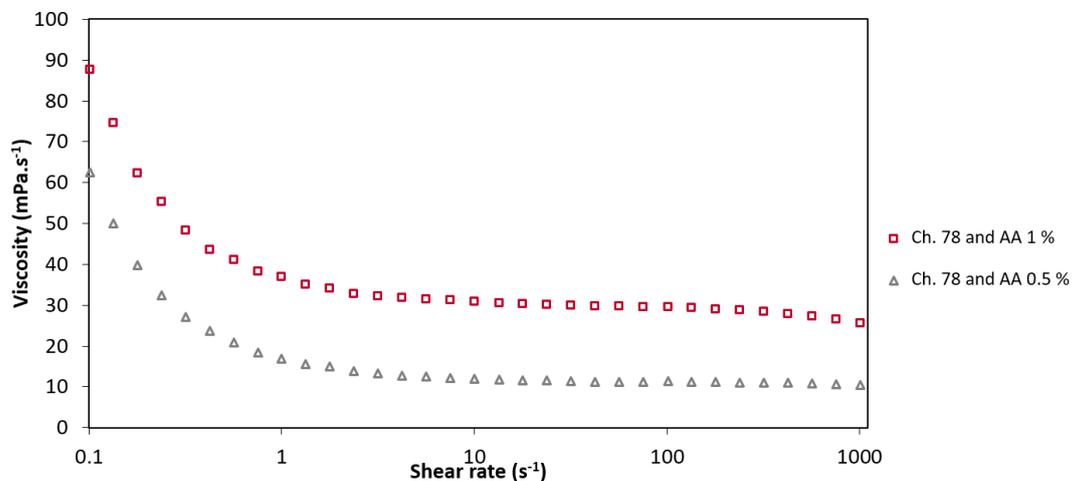


Figure VI.8: Evolution of the viscosity as a function of the shear rate.

3.2 Full-scale assay on field

The industrial test was carried out by CR Distribution, on durum wheat – variety ANVERGUR – in Benon (17170, France). The field has been ploughed on 15 January 2020 and sown on 25 January. The trial started in early May 2020 after the farmer had completed the fungicidal protection of the foliage. The concentration of the chitosan solution was 100 L/ha,

corresponding to 2,000 g/L, and the one of the extract was 800 g/ha. The durum wheat flowered after the rain at the beginning of May.

Characteristic data after the harvest are reported in the table below (Table VI.3).

Table VI.3: Yield of the harvest, humidity and protein content of the grains.

	Yield (q/ha)	Yield gap (%)	Humidity (%)	Protein content (%)
Control	87.7	0	11.4	13.1
Extract SP 800 g/ha	87.7	0	11.3	13.9
Ch. 78-AA 2,000 g/ha	85.5	-1.9	11.4	13.6

Unfortunately, the trial did not suffer any fungal contamination and therefore no scoring was possible. Although the statistical analysis of the yields was unfortunately not significant, a new trial has been planned.

GENERAL CONCLUSION

An innovative chitosan-based formulation against mycotoxigenic cereal pathogenic fungi has been developed. The study has been carried out on the strain CBS 185.32 of *F. graminearum* as a model strain producing deoxynivalenol and 15-acetyldeoxynivalenol. We exploited the interest of a commercially available plant-based curcumin derivative and employed local wood by-products, therefore valuing the resources of the region *Nouvelle-Aquitaine* to develop promising bio-solutions.

Chitosan was used in our formulation for two purposes: it is itself active by contact against fungi and can act as a matrix that traps active antifungal agents, in our case renewable compounds.

A graphical abstract of the main results is presented in Figure Co.1.

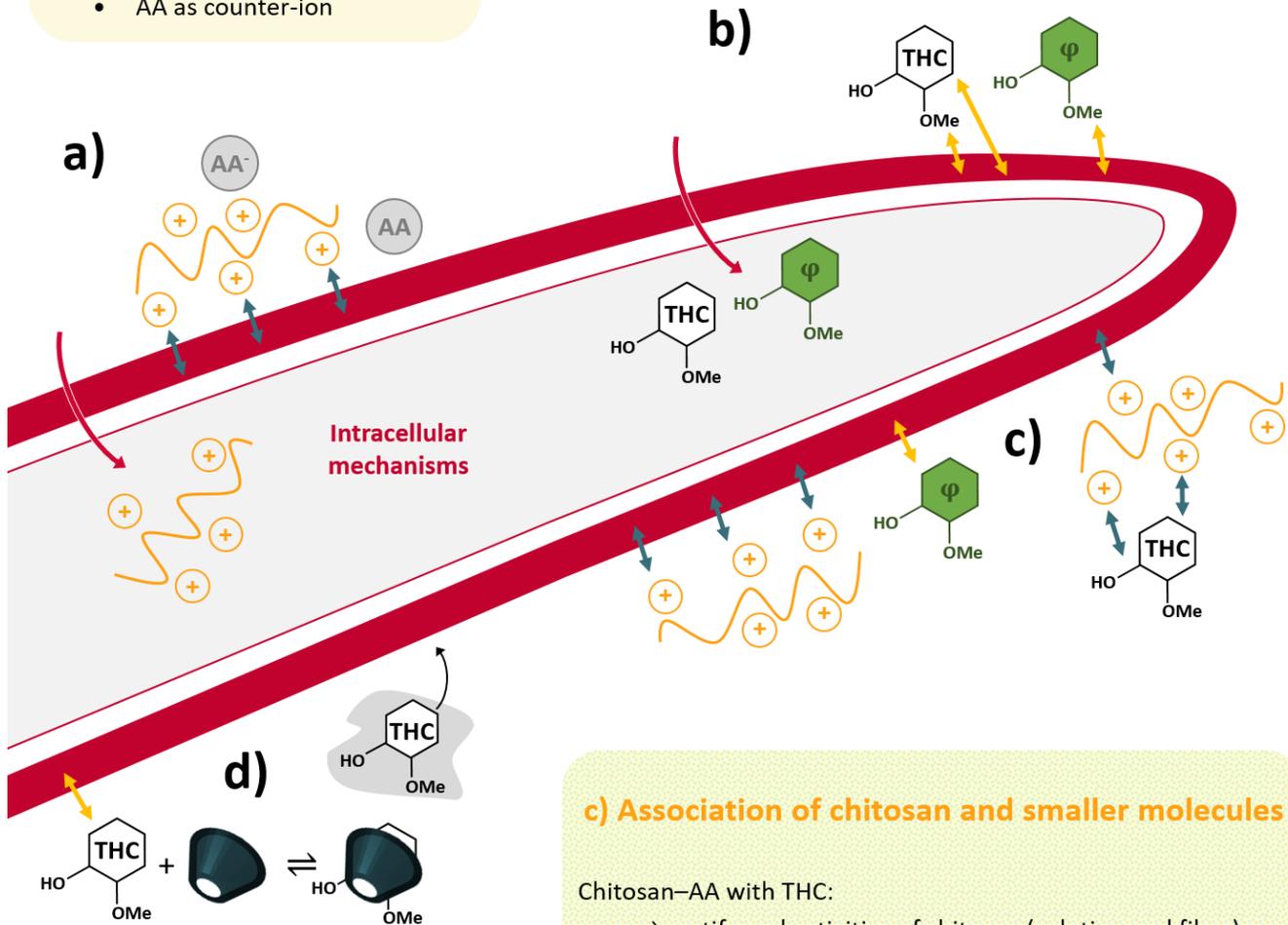
All tested chitosans were active against *F. graminearum* when dissolved in the culture medium with lactic or acetic acid used as solvents. The lower the pH value, the stronger the inhibition of the mycelial growth. Chitosan's ammonium groups are clearly involved in the antifungal properties, interfering notably with the cell membrane or ergosterol molecules located at the membrane surface [99]. The chitosan called **Ch. 78**, with an average molecular weight of 78 KDa, was found **to be the most active** among the four evaluated chitosans. This chitosan consisted, in fact, of chains of low and medium molecular weights. Therefore, the significant antifungal properties could be also due to shorter polymer chains that could penetrate through the fungal membranes, thus conferring to the Ch. 78 both intra- and extracellular actions (Figure Co.1 a). Further experiments could be envisaged to confirm this hypothesis, like tracking labelled chitosan inside/outside the cells as performed in similar works [419]. In addition to antifungal properties, the four selected chitosans were also efficient in reducing the production of toxins. The mechanism of action is not elucidated yet but it was shown that the acetate anions were better counter-ions. Moreover, the Ch. 78 kept its antifungal activity as a coating as a prevention treatment or as a curative treatment, reducing the mycelial development.

a) Chitosan

- ↗ antifungal activity when:
 - ↘ pH and ↗ the DD
⇒ Electrostatic interactions
 - Chitosan low \overline{M}_n
⇒ Intracellular penetration?
 - AA as counter-ion

b) Natural compounds

- THC is antioxidant
⇒ Could explain the antimycotoxigenic effect
- Extracts contain phenolic compounds in diverse proportion
- Both phenolic compounds cause antifungal effects



d) Encapsulation

- ↗ antifungal activity since the apparent solubility of THC is higher
- Conservation of antioxidant and antimycotoxigenic properties

c) Association of chitosan and smaller molecules

Chitosan–AA with THC:

- ↘ antifungal activities of chitosan (solution and films)
- ↘ antimycotoxigenic activity of chitosan

⇒ Interactions between both. Electrostatic? Hydrogen bonds?

Chitosan–AA with maritime pine sawdust extract:

- Additive effect of both compounds

↔ Electrostatic interactions φ Phenolic compound CD Starch particle Chitosan
↔ Lipophilic interactions

Figure Co.1: Antifungal activities of some products that have been explored in the present work.

To improve the efficiency of such bio-based formulation, plant based compounds, a curcumin derivative THC and wood extracts were added in chitosan solutions or films, leading to two distinct behaviours.

For **THC**, its **antioxidant and antimycotoxigenic properties** led to consider it as a very promising compound of the formulation (Figure Co.1 b)). However, by adding THC to chitosan, the antifungal and anti-TCTB actions of the biopolymer were hindered, although in insignificant proportions. This could be due to putative cation-induced dipole interactions between ammonium groups of chitosan and THC (Figure Co.1 c)). In addition, THC is unfortunately scarcely soluble in water, thus compromising its application in aqueous chitosan formulations. Due to this extremely low water solubility and to unwanted and deleterious interactions between THC and chitosan, encapsulation systems were considered.

As we evidenced by UV-spectrophotometry, among different **cyclodextrins** (CDs), encapsulation into randomly methylated β -cyclodextrins gave the most promising enhancement of the water solubility of the THC. Those CDs form an **inclusion complex** with one ring of THC, which has been highlighted by various NMR methods. However, the complex activated the fungal growth. Contrary to CDs, anionic polymers of cyclodextrins, obtained through the reticulation of cyclodextrins with citric acid, were found to reduce the fungal growth by 25 % (Figure Co.1 d). The antifungal activity of such complexes was conferred by both the THC and the anionic polymers. An interesting extension of this work would be to screen for other CDs, *e.g.* bearing short charged substituents, to enhance the solubility without being degraded by fungi.

Being easier to scale-up, an encapsulation of THC in **OSA-starch and chitosan particles** by **freeze-drying and spray-drying techniques** was the other studied option. After optimising the process, spherical or flat particles were obtained. Except for spray-dried chitosan particles, all possessed a significant antifungal activity, which increased after the THC-loading showing that THC can be efficiently released into the culture medium. Both THC-loaded particles also inhibited the TCTB production. Interestingly, the freeze-dried starch particles released almost all the THC instantaneously, as shown by an antioxidant capacity of these particles close to the one of free THC (Figure Co.1 d). In view of these results, a kinetic study on the release of THC by all particles would constitute an interesting and complementary study to perform. As a perspective, it would also be worth destabilising starch emulsions to obtain less stable spray-dried starch particles, which would better release THC. Another yet unexplored path could be to directly use starch emulsions in their liquid form without drying them. Finally, the next step would be to add these encapsulation systems into a chitosan formulation and to monitor changes in both antifungal and antimycotoxigenic properties.

For extracts from **wood by-products**, they also led to a growth inhibition of *F. graminearum*, especially the **maritime pine** ones. This is mainly explained by the content and the nature of their phenolic compounds, which can be attributed to lipophilic properties. But clearly, further studies are necessary to determine the chemical identity of the active molecules responsible for the

observed **antifungal and antimycotoxigenic effectiveness**. This essential step will allow the required basis to potentiate the properties of the maritime pine sawdust extract. Microscopic studies could help in the future to determine the targeted sites and to evidence membrane damages. The analysis of the fungal respiration or the ergosterol content could also provide some precious information on the mechanisms. In addition, we have shown that the extracts were reducing the production of toxins. Regarding this point, it would be interesting to explore more deeply the underlying modes of actions.

We observed a radically different behaviour from THC when adding natural wood extracts into chitosan solutions. When mixed into a chitosan solution, both the chitosan and the extracts kept their antifungal properties (Figure Co.1 c). This result is very promising since it could enable one to fully exploit the antifungal potential of both compounds at the same time. However, the assay on fields did not provide a satisfying demonstration, mostly due to the fact that the occurrence of the disease caused by *F. graminearum*, among others, on the fields was low during the test. Therefore, the efficiency of our products remains to be demonstrated under suitable conditions.

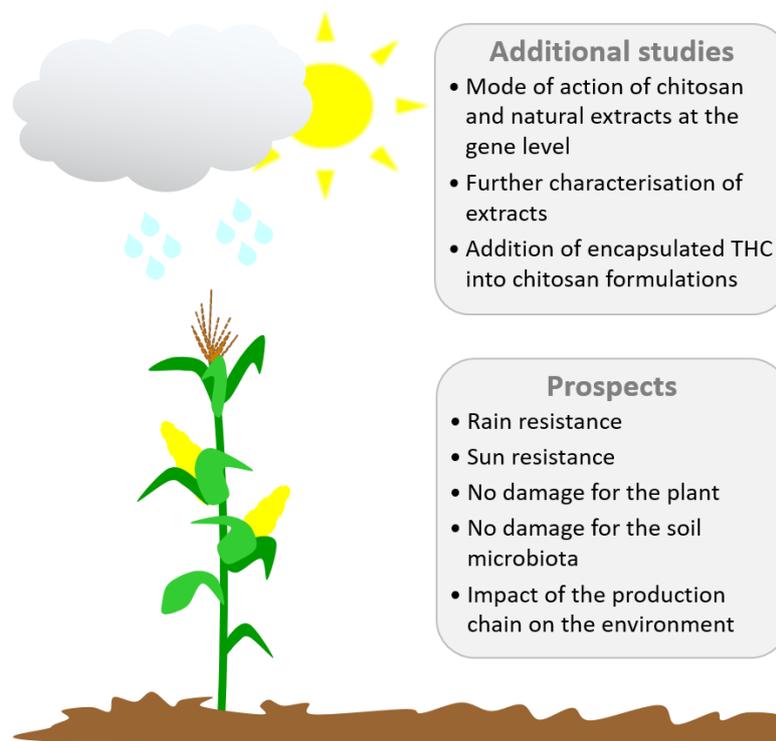
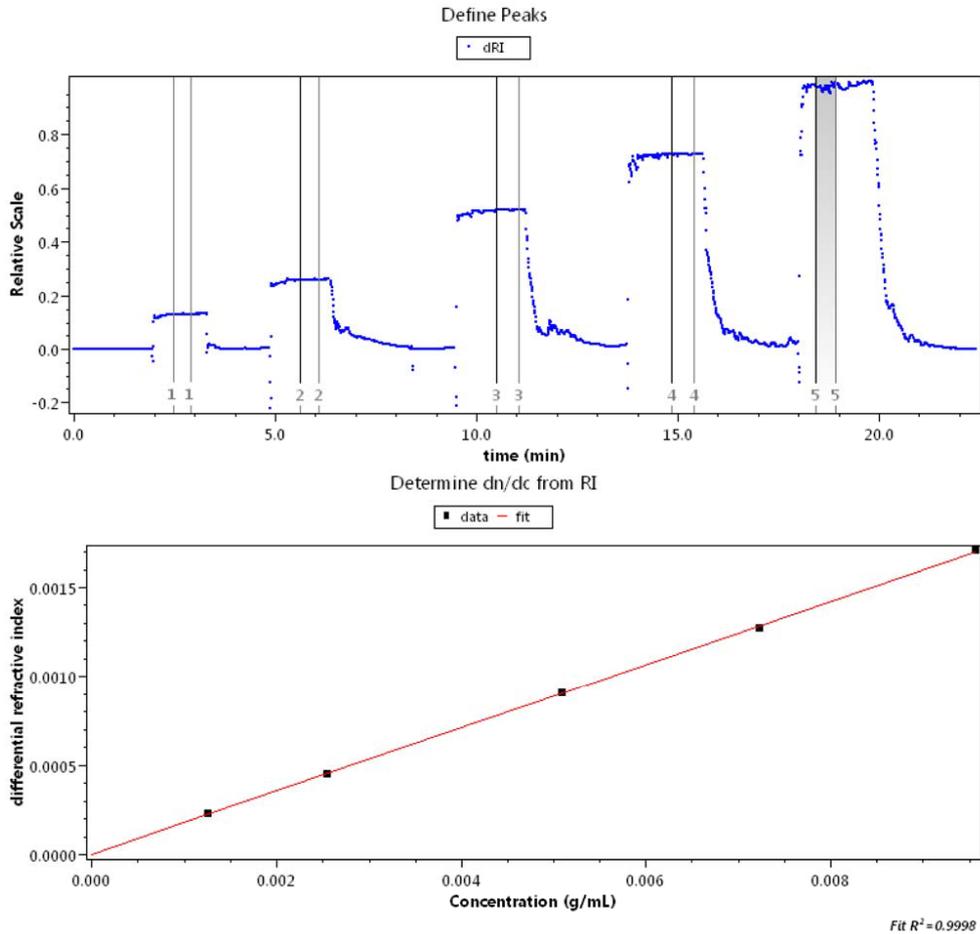


Figure Co.2: Some short-term and long-term prospects of this work.

Finally, regarding the application of chitosan-based formulations on fields, complementary projects could support and further continue our *in vitro* studies (Figure Co.2). For example, it would be interesting to see if the chitosan formulation adheres well enough to withstand rain. A comparison with other biocontrol solutions like COS-OGA would be of interest. A long-term prospect could be to analyse the life-cycle and the impact on the environment of all the components of such a formulation. In addition, it will be necessary to assess the dangerousness of the product on soils, underground waters, and living microorganisms in the fields. Deeper studies regarding the mode of action of THC on the fungi would also be welcomed, like the use of mutant strains to identify the gene getting involved.

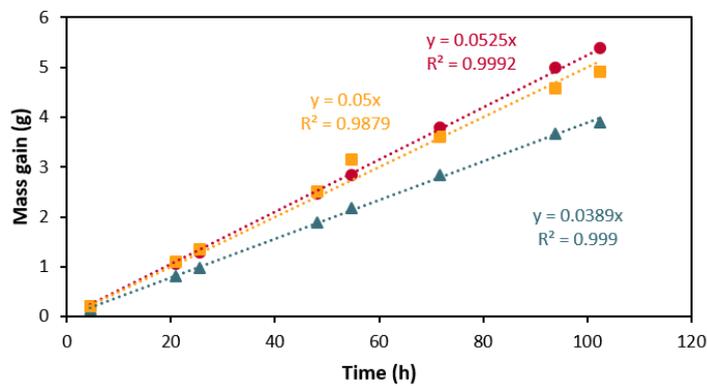
SUPPLEMENTARY INFORMATION

dn/dc:



Determination of the refractive index increment of Ch. 78.

Linear regressions for the determination of the WVTR:



Mass gain through water transmission without water absorption as a function of time for three replicates.

Detailed calculation of the transpiration rate of maize stomata:

Data		Values
Transpiration per plant	T_{pl}	60 g.plant ⁻¹ .day ⁻¹ [34]
Area of plant leaves	A_{pl}	350 cm ² - 500 cm ² [38]
Stomatal density	D_{st}	80 mm ⁻² [39]
Stomatal apertures area	A_{st}	100 μm ² [40]

Surface fraction occupied by stomata:

$$f_{st} = D_{st} * A_{st} = 80 * (100 * 10^{-3})^2 = 8 * 10^{-3}$$

Sum of all stomatal area per plant:

$$\sum_1 A_{st} = 8 * 10^{-3} * 350 = 2.8 \text{ cm}^2$$

$$\sum_2 A_{st} = 8 * 10^{-3} * 500 = 4 \text{ cm}^2$$

Transpiration per m² of stomata:

$$T_{st,1} = \frac{60}{2.8 * (10^{-2})^2} = 2.1 * 10^5 \text{ g.m}^{-2}.\text{day}^{-1}$$

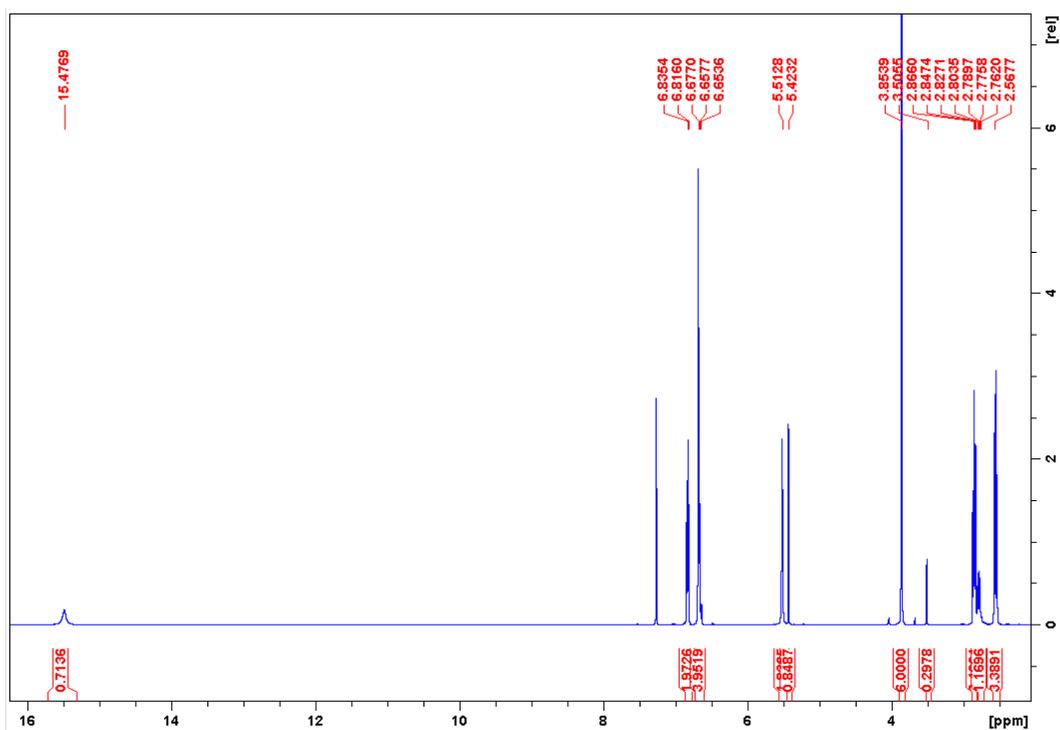
$$T_{st,2} = \frac{60}{4 * (10^{-2})^2} = 1.5 * 10^5 \text{ g.m}^{-2}.\text{day}^{-1}$$

$$T_{st} \approx 10^5 \text{ g.m}^{-2}.\text{day}^{-1}$$

NMR of THC:

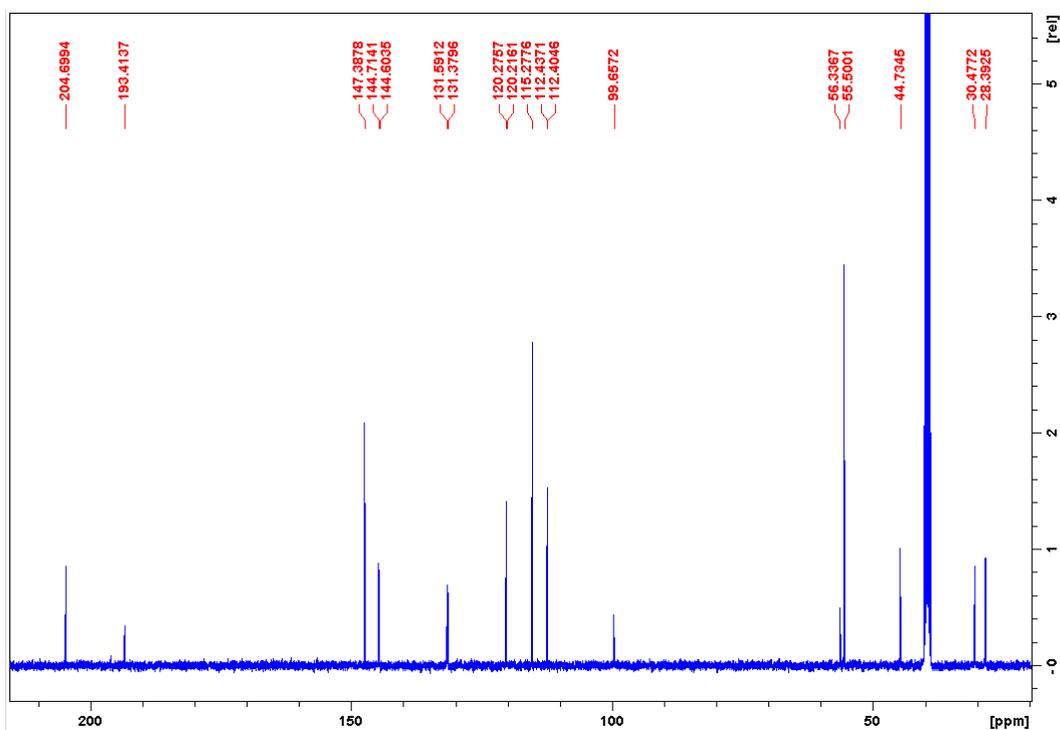
¹H-NMR (DMSO-d₆, 400 MHz) of 1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3,5-dione: δ 15.58 (s, 0.4H, H enol); 8.78 (d, 2H, 8.2 Hz, h+h'); 6.76 (dd, 2H, 1.8/13.7 Hz, f); 6.65 (dd, 2H, 2.5/8.0 Hz, d); 6.56 (m, 2H, 1.9/8.0 Hz, e); 5.74 (s, 0.5H, a enol); 3.73 (s, 6H, g+g'); 3.69 (s, 1H, a diketone); 2.74 (m, 4H, b+b'); 2.66 (m, 2H, c or c'); 2.56 (m, 2H, c or c').

¹H-NMR (CDCl₃, 400 MHz) of 1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3,5-dione: δ 15.48 (s, 0.7H, H enol); 6.83 (m, 2H, f); 6.66 (m, 4H, d and e); 5.51 (s, 2H, h+h'); 5.42 (s, 0.83H, a enol); 3.86 (s, 6H, g+g'); 3.51 (s, 0.3H, a diketone); 2.83–2.87 (m, 3.4H); 2.67–2.80 (m, 1.2H); 2.53–2.57 (m, 3.4H) (the last three attributions: 8H, b,b',c and c').



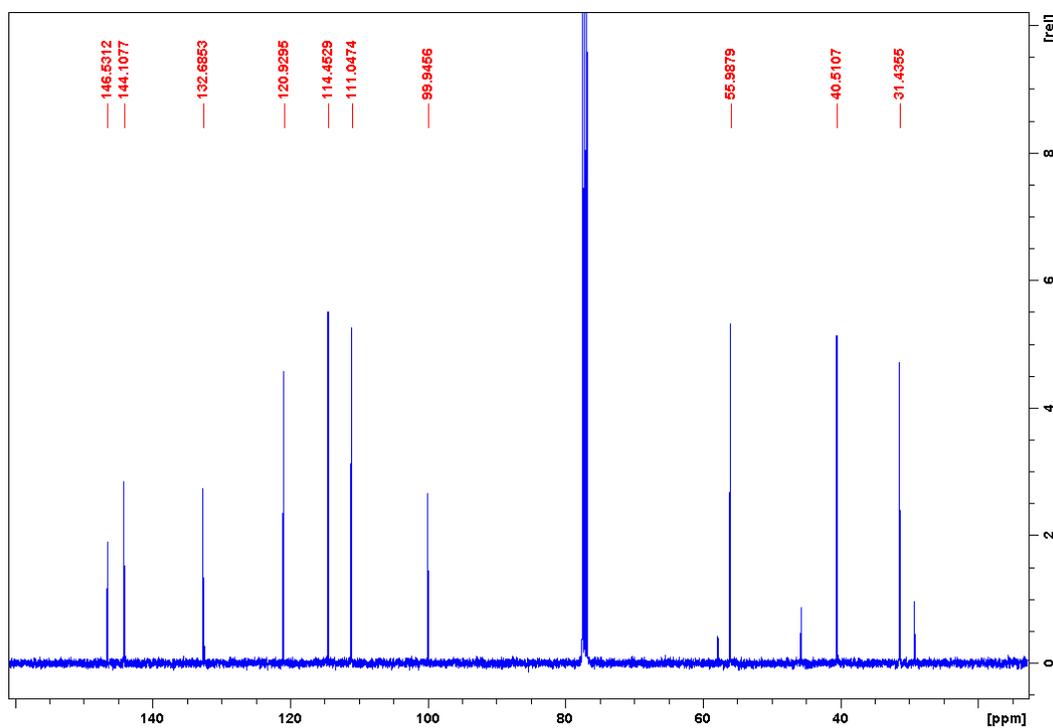
$^1\text{H-NMR}$ spectrum of THC in CDCl_3 .

$^{13}\text{C-NMR}$ (DMSO-d_6 , 400 MHz) of 1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3,5-dione: δ 204.7 (s); 193.4 (s, $\text{C}=\text{O}$); 147.4 (s, $\text{C}_{\text{ar-OMe}}$); 144.7 (d, $\text{C}_{\text{ar-OH}}$); 131.6 (d, $\text{C}_{\text{ar-Calk}}$); 120.3 (d, E); 115.3 (s, F); 112.4 (s, D); 99.7 (s, A); 56.3 (s); 55.5 (s, G); 44.7 (s, B and B'); 30.5 (s, C and C'); 28.4 (s).



$^{13}\text{C-NMR}$ spectrum of THC in DMSO-d_6 .

^{13}C -NMR (CDCl_3 , 400 MHz) of 1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3,5-dione: δ 193.3 (s, $\text{C}=\text{O}$); 146.5 (s, $\text{C}_{\text{ar}}\text{-OMe}$); 144.1 (s, $\text{C}_{\text{ar}}\text{-OH}$); 132.7 (s, $\text{C}_{\text{ar}}\text{-C}_{\text{alk}}$); 120.9 (s, E); 114.5 (s, F); 111.0 (s, D); 99.9 (v); 56.0 (s, G); 40.5 (s, B and B'); 31.4 (s, C and C').



^{13}C -NMR spectrum of THC in CDCl_3 .

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ABSTRACT

Cereals are subject to contamination by pathogenic fungi, which damage grains and threaten the public health with their mycotoxins. Recently, the raise of public and political awareness concerning environmental issues tend to limit the use of traditional fungicides against these pathogens in favour of eco-friendlier alternatives.

In this framework, this thesis work aims to create a formulation based on renewable products in order to limit the fungal development and control the production of mycotoxins from cereal fungi. Our work exploits the remarkable properties of three compounds: the chitosan, a chitin derived biopolymer, the tetrahydrocurcumin (THC), a curcumin derivative, and plant extracts.

In a first step, we studied and characterise the physicochemical properties of different chitosans. Chitosan solutions were shown to reduce the mycelial growth of a target model fungi *Fusarium graminearum*, and to divide by 2 the accumulation of mycotoxins. In addition, we showed that this biopolymer was able to maintain its antifungal properties as a form of a coating. In a second step, we focused on different plant extracts with antimicrobial activities. THC was able to inhibit the toxin production and a maritime pine by-product showed its potential to control the fungal growth. The combination of the THC or the wood extract with chitosan was then studied to increase the efficiency of the formulation. To this end, a significant work was made to increase the solubility of THC in water by forming an inclusion complex in cyclodextrins or by protecting it in starch or chitosan particles. In particular, we showed that the addition of pine extracts to a chitosan-based solution can double the effectiveness of the formulation.

Keywords: *Fusarium graminearum*, *chitosan*, *tetrahydrocurcumin*, *mycotoxins*, *biopolymer*

RÉSUMÉ

Les cultures céréalières sont sujettes aux invasions de champignons pathogènes, ce qui altère la qualité des grains et pose un problème de santé publique, en raison de mycotoxines potentiellement contenues dans ces grains. Face à la prise de conscience publique et politique de la nécessité d'inclure la composante environnementale dans nos modes de consommation et de production, les fongicides synthétiques traditionnels se voient petit à petit remplacés par des alternatives plus « vertes ».

Dans ce contexte, ce travail de thèse a pour but de créer une formulation à base de produits renouvelables, pour contrôler le développement et la production de toxines d'espèces fongiques pathogènes. Ce travail exploite les propriétés remarquables de trois composés : le biopolymère de chitosane, dérivé de la chitine, la tétrahydrocurcumine (THC), un dérivé de curcumine, et des extraits de pins et de vigne.

Les propriétés physico-chimiques des chitosanes ont tout d'abord été caractérisées. Ces solutions de chitosanes présentent des effets antifongiques prometteurs réduisant la croissance mycélienne du champignon modèle cible *Fusarium graminearum* et divisant sa production de mycotoxines de plus moitié. Un des principaux atouts de ce biopolymère réside dans le fait qu'il conserve ses propriétés antifongiques sous forme d'enduction. Des extraits végétaux possédant des activités antimicrobiennes ont ensuite été étudiés. Premièrement, la THC inhibe la production de toxines et est donc combinée avec du chitosane. Afin d'accroître la solubilité dans l'eau et l'efficacité de la THC, des complexes d'inclusion ont été formés avec des cyclodextrines. De même, cette THC a été encapsulée dans d'autres matrices de biopolymères variés, tels que l'amidon ou le chitosane. Deuxièmement, les extraits issus de ressources locales de pins maritimes et de vignes démontrent eux aussi des effets antifongiques et anti-mycotoxigéniques. En particulier, l'addition d'un de ces extraits, celui d'écorce de pin maritime, à une formulation de chitosane double l'efficacité de ce dernier contre le développement du mycélium.

Mots clés : *Fusarium graminearum*, *chitosane*, *tetrahydrocurcumin*, *mycotoxines*, *biopolymère*