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| | |
|---|---|
| A. s | *Armillaria sinapina* |
| AS | Amyrin synthase |
| AACT | Acetoacetyl-CoA transferase |
| AB | Acide bétulinique |
| BET | Bétuline |
| BLAST | Basic local alignement search tool |
| CYP | Cytochrome P450 |
| COG | Clusters of Orthologous Groups of proteins |
| DEG | Differentially expressed genes |
| DMAPP | Dimethylallyl diphosphate |
| DPS | Decaprenyl-diphosphate synthase |
| EBB | Écorce de bouleau blanc |
| FPP | Farnsyl diphosphate |
| FPS | FPP synthase |
| GPP | Geranyle diphosphate |
| GO | Gene ontology |
| HMG-CoA | 3-hydroxy-3-methylglutaryl-CoA |
| HMGS | HMG-CoA synthase |
| HMGR | HMG-CoA reductase |
| HMGL | Hydroxymethylglutaryl-CoA lyase |
| I. o | *Inonotus obliquus* |
| IPP | Isopentenyl diphosphate |

| | |
|---|---|
| IPI | IPP isomerase |
| LC-Qtof-MS/MS | Chromatographie liquide combinée à une spectrométrie de masse |
| LAS | Lanosterol synthase |
| LUS | Lupeol synthase |
| MVA | Acide Mévalonique |
| MVAP | Mévalonique acide phosphate |
| MVK | PhosphoMVA kinase |
| MUS | Muurolene synthase |
| PCR | Réaction en chaîne par polymérase |
| PRS | Protoilludene synthase |
| PMD | DiphosphoMVA decarboxylase |
| PMD | Phosphomevalonate kinase |
| TRI4 | Trichodiene oxygenase |
| TPM | Transcript per million |
| THIK | 3-ketoacyl-CoA thiolase |
| SQS | Squalene synthase |
| SQE | Squalene epoxidase |
| q-RT-PCR | PCR quantitative |
| RIN | RNA integrity number |
| YMA | Yeast malt agar |
| YMB | Yeast malt broth |
| βAO | 11-oxo-β-amyrin 30-oxidase |

# CHAPITRE I

# INTRODUCTION

## 1.1 Le règne fongique

Le terme « champignons » désigne généralement tout organisme étudié par les mycologues dans les deux principaux royaumes : les Protoctista et les Eumycota, au sens le plus large, soit les microorganismes. Le règne fongique est défini par sept caractères fondamentaux : les mycètes sont 1) hétérotrophes (non-photosynthétiques), 2) eucaryotes qui absorbent leurs nutriments (absorbotrophes ou osmotrophes), 3) leur organisation cellulaire basique est le thalle (avec une grande variété de structures), 4) ils ont développé un appareil végétatif diffus, ramifié et tubulaire, 5) ils utilisent les spores pour se reproduire, 6) ils sont non flagellés (à l'exception des Chytridiomycota), et 7) ils sont dotés d'une paroi cellulaire chitineuse et riche en stérols (Blandeau 2012; Kendrick 2017).

Fries fut le premier biologiste à s'être rendu compte de l'étendue de la diversité des champignons en 1825 (Fröhlich and Hyde 1999). Hawksworth estimait le monde fongique à plus de 1,5 million d'espèces en 1991 (Blackwell 2011; Hawksworth 2001; Fröhlich and Hyde 1999). Il a fallu plus de 200 ans et l'aide de la biologie moléculaire, notamment le séquençage de l'ADN, pour que les scientifiques décident de la classification des organismes qui devraient être considérés comme champignons. Au milieu des années 1970, les données nucléotidiques ont été utilisées pour la première fois pour les phylogénies moléculaires. Ces premières analyses ont été effectuées sur des ARN ribosomiques, abondants dans la cellule végétale et susceptibles d'être séparés par centrifugation (Sabbagh 2008). L'arrivée de l'amplification génique en utilisant les réactions par polymérisation en chaine (PCR) en 1988 et la publication d'un grand choix d'amorces ribosomiques universelles dans les années 1990 par des mycologues ont permis très rapidement la réalisation d'un large éventail d'études sur les champignons qui étaient

difficiles à distinguer sur la base des critères morphologiques (Blackwell 2011; Ochman, Gerber, and Hartl 1988).

En 2007, les banques de données génétiques, comme Genbank et EMBL, révèlent que plus de 60 000 séquences ribosomiques fongiques ont été répertoriées en utilisant la région de l'espaceur transcrit interne (ITS), qui est le principal marqueur génétique (Schoch et al. 2012), du type ITS-1-5.8S-ITS2 et 18S respectivement, et plus de 40 000 séquences en 28S (Ochman, Gerber, and Hartl 1988). Depuis, on utilise couramment les données moléculaires (séquence d'ADN) pour explorer les champignons et les communautés fongiques en utilisant des codes barres et des codes métaboliques pour une meilleure classification (Hawksworth and Luecking 2017). Jusqu'à présent, environ 100 000 champignons ont été décrits, mais cela ne représente que moins d'un dixième des mycètes de la Terre (Kendrick 2017; Chen and Liu 2017).

En 2011, grâce aux méthodes de séquençage à haut débit, l'estimation suggérait qu'il existe jusqu'à 51 millions d'espèces fongiques sur Terre dans les différents habitats comprenant les sols, l'eau et les organismes hôtes qui peuvent en abriter un grand nombre très peu étudié jusqu'ici (Blackwell 2011). Plus récemment, en 2017, le règne fongique a été revu à la baisse et estimé entre 2,2 à 3,8 millions d'espèces d'eucaryotes hétérotrophes (Hawksworth and Luecking 2017).

Dans l'arbre du vivant, le règne fongique (mycètes) constitue un phylum à part entière au sein des eucaryotes et est regroupé d'une manière classique dans le règne des eumycètes. En raison d'une séparation estimée à environ un milliard d'années (plus ou moins 500 millions d'années) d'un ancêtre partagé avec des animaux, le groupe monophylétique de l'arbre de la vie des mycètes qui comprend les moisissures, les levures, les champignons, les polypores, les rouilles et les smuts (parasites des plantes) constitue une lignée eucaryote majeure; en nombre égal à celui des animaux et supérieur à celui des plantes (Berbee and Taylor 2010; Blackwell 2011; Le Calvez 2009).

La configuration actuelle des mycètes s'articule autour de quatre principaux groupes qui sont :

- Les Myxomycétes
- Les champignons inférieurs (les Chytridiomycètes et les Oomycètes)
- Les Zygomycètes
- Les champignons supérieurs (les Ascomycètes et les Basidiomycètes)

En 2019, Genbank recense 36 projets de génomes fongiques, 29 relatifs à des *Ascomycota,* 6 à des *Basidiomycota* et 1 à des *Microsporidia* (https://www.ncbi.nlm.nih.gov/genomes/FUNGI/funtab.html).

## 1.2 Les champignons supérieurs

### 1.2.1 Les ascomycètes

Le phylum Ascomycota (champignons filamenteux) représente 60 % des espèces décrites. Ce groupe comprend de nombreux agents pathogènes graves pour les humains, les animaux et les végétaux (Schmidt-Dannert 2014). Malgré l'ignorance du nombre exact d'espèces fongiques au Québec, il a été considéré que la flore mycologique du Québec figurerait parmi les plus riches au monde (Pedneault 2007).

Les champignons étudiés dans cette recherche font partie de la famille des champignons dits supérieurs, les Basidiomycètes dont le nombre approximatif est de 23 000, distribués dans plus de 1 400 genres et 40 ordres (Blandeau 2012; Pedneault 2007). Les Basidiomycota représentent 30 % des espèces fongiques connues. D'un point de vue phylogénétique, le phylum Basidiomycota est le groupe frère du phylum Ascomycota, formant ensemble le sous-royaume Dicaryotes (Le Calvez 2009; Coelho et al. 2017). Ils peuvent être trouvés sous différentes formes de couleurs, de grosseurs et de goûts. Leurs thalles vivent généralement sur une terre riche en humus, de végétaux en décomposition, de vieux bois ou alors en présence d'agglomérations de feuilles mortes.

Ce sont des champignons qui produisent des basidiospores avec des basides (Figure 1.1). Trois sous-phylums composent les basidiomycètes : les *Agaricomycotina*, les *Ustilaginomycotina* et les *Pucciniomycotina*. Au Québec, on retrouve principalement les *Agaricaceae*, les *Amanitaceae*, les *Boletaceae*, les *Coprinaceae*, les *Lycoperdaceae*, les *Polyporaceae*, les *Russulaceae* et les *Tricholomataceae* (Pedneault 2007).

### 1.2.2 Les basidiomycètes

D'après la littérature, les champignons basidiomycètes représentent les principales lignées d'agents pathogènes et les principales espèces productrices de champignons comestibles (Coelho et al. 2017). Certains basidiomycètes sont porteurs d'arômes délicieux comme les chanterelles, les cèpes, les bolets ou bien la trompette de la mort, mais d'autres peuvent aussi être toxiques ou hallucinogènes comme les Psylocibe ou amanites (Blandeau 2012). Certains parasitent aussi des plantes vivantes.

Malgré leur rôle important dans la décomposition du matériel végétal ou même comme symbiotes des végétaux, très peu d'espèces de cet embranchement ont été caractérisées. À ce jour, très peu d'études approfondies ont été menées sur la caractérisation des voies de biosynthèse des produits naturels sur les basidiomycètes (Schmidt-Dannert 2014).

Le sous-phylum Agaricomycotina est celui qui contient la plupart des espèces décrites. On y retrouve environ 21 000 espèces, y compris de nombreux champignons tels que les saprophytes ou les symbiotes mycorhiziens des plantes et une grande diversité de levures, dont des espèces qui représentent des risques pathogènes pour l'homme.

Les Ustilaginomycotina constituent un sous-phylum qui comprend lui-même plus de 1 700 espèces fongiques. Il s'agit principalement de parasites des plantes. Les espèces bien connues des Ustilaginomycotina sont *Ustilago* et *Tilletia*, qui contiennent des espèces d'importance écologique comme la carie karnale du blé, et le charbon nu de l'orge et du maïs (Begerow et al. 2014; Coelho et al. 2017). Le sous-phylum Pucciniomycotina est un

groupe frère du clade contenant Ustilaginomycotina et Agaricomycotina, et il comprend plus de 8 400 espèces décrites à ce jour. La plupart des espèces Pucciniomycotina sont des parasites végétaux, tels que les champignons de rouille qui sont pathogènes (p. ex. *Puccinia spp.*) (Coelho et al. 2017; McLaughlin and Spatafora 2014).

## 1.3 Utilisation des champignons

Le règne fongique apporte une contribution majeure à notre vie tant dans le fonctionnement des écosystèmes que dans le nutritionnel, mais aussi dans les applications biotechnologiques. À mesure que de nouveaux champignons et leurs biomolécules associées sont identifiés, la biologie associée au monde fongique joue un rôle de plus en plus crucial dans le développement des secteurs biotechnologiques et aussi biomédicaux (Purchase 2016). En 2014, la Business Communication Company (BCC) a estimé le marché mondial total des microbes et des produits microbiens à près de 143,5 milliards de dollars US. Selon leurs estimations, cela devrait atteindre près de 306 milliards de dollars avec un taux de croissance annuel composé (TCAC) d'environ 14,6 % entre 2015 et 2020. En raison des avantages techniques et économiques liés à l'utilisation des microorganismes dans différents secteurs, ces derniers sont de plus en plus utilisés en remplacement des procédés synthétiques pour la production de suppléments nutritionnels tels que les vitamines et les acides aminés, les acides organiques, les métabolites jouant un rôle dans l'agriculture, les enzymes, les agents aromatisants, les colorants et les produits pharmaceutiques (Singh et al. 2017).

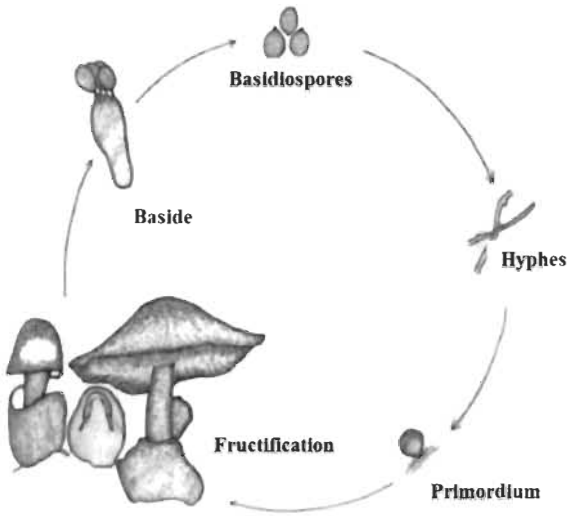


**Figure 1.1 Cycle de vie et fructification des champignons supérieurs.**
Les basidiospores matures germent dans des conditions favorables pour former du mycélium primaire. Le mycélium secondaire est formé grâce à la fusion des mycéliums primaires et donne naissance aux fructifications qui créent une autre nouvelle génération de basidiospores (Dapeng Bao et al. 2013).

Le rôle des microorganismes tels que certaines levures et certains champignons dans la fermentation était déjà connu à l'époque de l'Égypte antique. Un autre exemple serait le rôle des levures dans la fabrication du pain (*Saccharomyces cerevisiae*) et de l'alcool. À ce jour, les microorganismes sont souvent utilisés dans l'industrie agroalimentaire pour la fabrication des enzymes et arômes artificiels. Par exemple, les souches *Corynebacterium, Brevibacterium* et *Escherichia coli* sont utilisées pour produire des acides aminés alors que les vitamines sont obtenues par des cultures des souches *Propionibacterium* et *Pseudomonas*; les acides organiques d'*Aspergillus*, *Lactobacillus*, *Rhizopus* et des enzymes sont produites à l'aide d'*Aspergillus* et de *Bacillus* (Singh et al. 2017; Gurung et al. 2013).

### 1.3.1 Le compostage et les champignons

Les champignons peuvent aussi être utilisés dans le compostage et comme alternative aux produits agrochimiques pour lutter contre les maladies des plantes.

Le compostage est un mécanisme biologique par lequel des microorganismes dégradent ou décomposent, de manière physique ou chimique, de la matière organique. Les champignons jouent un rôle important dans la décomposition et la stabilisation des substrats organiques. La science du compostage a commencé par la culture du champignon de Paris (*Agaricus bisporus*), l'espèce de champignon la plus cultivée dans le monde, et reste aujourd'hui encore essentielle dans la culture du champignon de Paris (Purchase 2016). Dans de nombreux articles scientifiques portant sur la biologie du compostage, on énumère d'autres espèces bactériennes et fongiques isolées à partir de diverses matières et phases organiques de compostage (Ryckeboer et al. 2003; Purchase 2016). Ainsi, *Acremonium, Alternaria, Aspergillus, Chaetomium, Cladosporium, Emericella, Fusarium, Geotrichum, Mortierella, Mucor, Penicillium, Pseudallescheria, Scopulariopsis* et *Trichoderma* sont les champignons les plus couramment isolés des lieux de compostage (Purchase 2016).

### 1.3.2 Les champignons dans la valorisation des résidus ligneux

Depuis quelques années, les recherches s'intensifient sur la valorisation des résidus ligneux et le compostage s'est avéré être un procédé naturel et durable pour la valorisation de ces résidus. Le compostage des tiges de tabac génère une matière stable qui pourrait être utilisée comme conditionneur de sol ou comme engrais. Pour cela, Liang Yang et al., en 2019 ont étudié la dégradation des tiges de tabac en utilisant des champignons de pourriture blanche, *Trametes hirsuta* S13 et *Pleurotus ostreatus* S18. La lignine, retrouvée en grande quantité dans la tige du tabac, est connue pour être très résistante à la dégradation microbienne. Cette résistance limite l'efficacité du procédé de compostage. Le taux de dégradation dépend de la capacité lignocellulolytique des enzymes du champignon cultivé, qui comprend une large gamme d'enzymes telles que des laccases, peroxydases, xylanases et ligninases (Yang et al. 2019; Lo, Ho, and Buswell 2001).

Le bouleau blanc est un arbre très répandu dans les latitudes nordiques du monde. Il représente une source de bois naturelle abondante et peu utilisée (Zhao, Yan, and Cao 2007). Les écorces de bouleau blanc, produites en grandes quantités, sont généralement

considérées comme des résidus forestiers et sont souvent utilisées comme combustibles par les industries (Pedieu 2008). D'après la littérature, les écorces de bouleau blanc étaient utilisées par les Amérindiens en médecine traditionnelle (Zyryanova et al. 2010). Elles étaient rajoutées dans les infusions de thé et d'autres boissons pour traiter divers problèmes gastriques et intestinaux. On leur confère diverses propriétés pharmacologiques importantes telles que des activités antimicrobiennes, antidiabétiques, hépatoprotectrices, antiarthritiques et anticancéreuses (Géry et al. 2018a). En 1994, des scientifiques de l'université de Caroline du Nord ont montré que les molécules bioactives présentes dans l'écorce de bouleau blanc joueraient aussi un rôle dans le ralentissement de la croissance du virus de l'immunodéficience humaine (VIH) (Yogeeswari and Sriram 2005; Krasutsky 2006). La couche de liège des bouleaux à écorce blanche contient environ 20 à 22 % de bétuline et environ 3 à 5 % de triterpènes pentacycliques étroitement apparentés comme l'acide bétulinique, le lupéol, l'érythrodiol, la caféoyl-bétuline et l'acide oléanolique. Ce pourcentage peut varier d'un bouleau à un autre (Scheffler 2019). La bétuline peut être facilement extraite de l'écorce de bouleau blanc en utilisant des solvants organiques tels que le tétrahydrofurane (THF), et ce, à température ambiante. On attribue diverses propriétés pharmaceutiques à la bétuline et à ses analogues, notamment comme anti-VIH, antimicrobienne (apoptose cellulaire) et aussi on retrouve son utilité en cosmétique (Okada et al. 2019; Zhao et al. 2018; Ali-Seyed et al. 2016; Scheffler 2019). En raison de la cytotoxicité sélective et de l'index thérapeutique important qui est en contraste avec la majorité des agents antitumoraux utilisés en chimiothérapie, l'acide bétulinique est considéré également comme un agent anticancéreux très prometteur (Liu, Fu, and Chen 2011a; Ali-Seyed et al. 2016).

En raison de la faible concentration de cet acide dans les écorces (par exemple celles de bouleau), les chercheurs se sont intéressés au moyen de convertir la bétuline, présente en forte quantité dans les écorces, en acide bétulinique (acide 3β-hydroxy-lup-20(29)-en-28-oic) (Liu, Fu, and Chen 2011a; Chen et al. 2009). En 1997, Darrick S. H. L. Kim et al. ont démontré que grâce à une voie chimio-synthétique, il était possible d'obtenir l'acide bétulinique à partir de la bétuline (Kim et al. 1997). Cette voie de synthèse porte principalement sur l'oxydation et la réduction en position C-28 de la bétuline (Kumar and

Dubey 2017) (Figure 1.2). Parmi les techniques de conversion de la bétuline en acide bétulinique, une technique basée sur deux étapes est proposée ; une oxydation par Jones des hydroxyles en position des carbones C-3 et C-28 pour obtenir de l'acide bétulonique. Une réduction de l'acide bétulonique en position C-28 est réalisée en utilisant le borohydrure de sodium pour produire finalement l'acide bétulinique (Hordyjewska, 2019).

Bien que cette méthode soit possible, certains problèmes subsistent, notamment les conditions de réaction, la sécurité, les coûts de production et les problèmes liés à la pollution (Chen et al. 2009).

Compte tenu de l'intérêt croissant pour l'acide bétulinique et des difficultés citées précédemment, la production d'acide bétulinique par biotransformation est appelée à avoir un impact considérable dans un futur proche. De plus, les biocatalyseurs sont en voie de devenir des éléments-clés dans le processus chimique pour la production de nouveaux produits pharmaceutiques, en solution de remplacement des catalyseurs conventionnels. La biotransformation est un procédé alternatif préférable pour la synthèse chimique dans la mesure où elle ne nécessite pas une grande quantité de réactifs pouvant être dispendieux et permet la synthèse verte de produits chimiques à haute valeur ajoutée. De plus, grâce à la biotransformation, la plupart des réactions biocatalytiques peuvent être réalisées dans des conditions sûres, respectueuses de la nature et économiques (Kumar and Dubey 2017; Chen et al. 2009; Liu, Fu, and Chen 2011a).

À ce jour, plusieurs microorganismes ont déjà été utilisés pour convertir la bétuline en acide bétulinique tels que *Cunninghamella blakesleeana, Bacillus megaterium*, *Aspergillus oryzae* AS 3.498, *Armillaria luteo-virens* Sacc QH, et *Aspergillus foetidus* ZU-G1. (Bastos et al. 2007; Chen et al. 2009; Kumar and Dubey 2017).

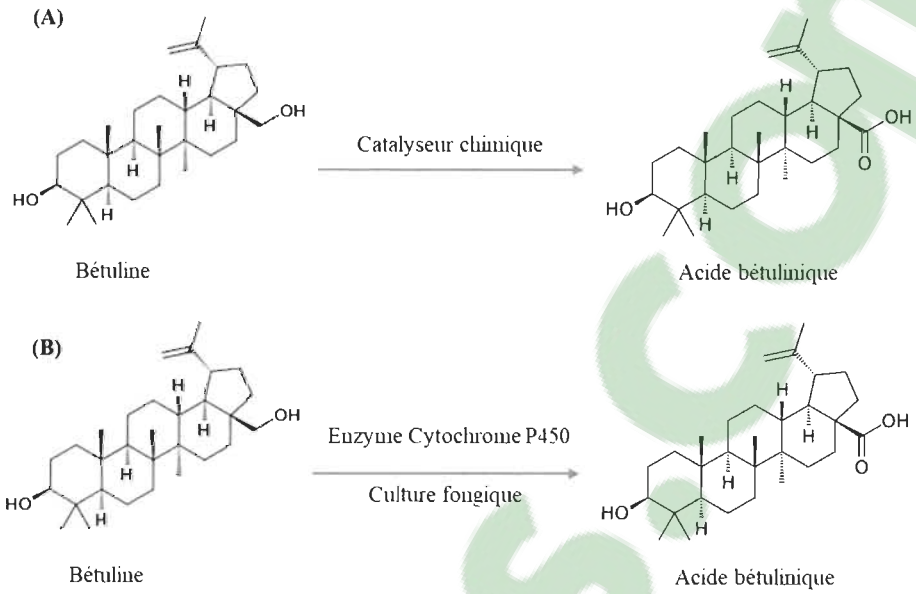


**Figure 1.2 Procédés de catalyse de la bétuline en acide bétulinique.**
(A) représente la conversion de la bétuline en acide bétulinique en utilisant un catalyseur chimique. (B) représente la biotransformation fongique de la bétuline en acide bétulinique en utilisant un catalyseur enzymatique.

### 1.3.3 Les applications médicales des champignons

Dans le domaine de la santé, à l'instar des plantes médicinales, les champignons trouvent aussi leur utilisation dans le procédé de fabrication des antibiotiques. En effet, avec la découverte de la pénicilline en 1928, qui est un antibiotique produit par la souche fongique *Penicillium notatum*, le potentiel de biosynthèse d'antibiotique dans le domaine pharmaceutique a commencé à être exploré dès 1940. Plus tard, un certain nombre d'antibiotiques ont été découverts spécifiquement à partir de champignons et d'actinomycètes. Ces découvertes font suite à des expérimentationsen quête de propriétés pharmacologiques de plus en plus efficaces, voire d'études portant sur la lutte antifongique contre de nouveaux agents pathogènes. Par ailleurs, les champignons sont largement utilisés dans le domaine biotechnologique en raison de leur capacité à produire des métabolites spécialisés tels que des molécules antibiotiques, anticancéreuses, antiinflammatoires et antidiabétiques (Le Calvez 2009; Kavanagh 2017; Chen et al. 2016; Singh and Kaur 2016; Lee et al. 2017; Dey et al. 2016; Gomes et al. 2019).

D'après une étude récente, le secteur de la santé représenterait le plus important marché d'utilisateurs des bioproduits produits par des microorganismes, soit environ 100,4 milliards de dollars US en 2014. Il devrait atteindre près de 111,5 milliards en 2015, et plus de 187,8 milliards en 2020 (Singh et al. 2017). Ces estimations reflètent l'importance et l'émergence de l'utilisation des métabolites spécialisés microbiens (Singh et al. 2017; Gurung et al. 2013).

Par exemple, le bêta-carotène, ou provitamine A, est nécessaire pour la vision, la croissance et la reproduction. Une carence ou une mauvaise absorption de cette vitamine aurait des effets néfastes pour l'individu. Les microorganismes *Blakeslea trispora*, *Phycomyces blakesleeanus*, *Mucor circinelloides*, *Rhodotorula spp.* et *Choanephora cucurbitarum* sont connus pour être utilisés dans la production du bêta-carotène.

Le cancer est la maladie du siècle. Selon l'Organisation mondiale de la Santé (OMS), environ 8,2 millions de personnes meurent chaque année du cancer, soit environ 13 % de tous les décès dans le monde. Une augmentation de près de 70 % de nouveaux cas de cancer est prévue au cours des deux prochaines décennies. En raison de ces chiffres, de nombreuses études se sont tournées vers la découverte d'agents anticancéreux. Le marché des produits chimiothérapeutiques est en pleine effervescence. Après la découverte du premier agent anticancéreux, l'actinomycine, par Wakesman et Woodruff en 1941, de nombreux métabolites produits par des microorganismes ont démontré des propriétés anticancéreuses.

Environ 60 % des composés ayant des propriétés anticancéreuses sont d'origine naturelle. En raison des limites associées à la productivité et à la vulnérabilité des espèces végétales en tant que sources de métabolites spécialisés, les microorganismes constituent une source ultime, reproductible, renouvelable et inépuisable de nouvelles structures avec un potentiel pharmaceutique. Les microorganismes, en particulier les champignons, ont longtemps été considérés comme une source importante de métabolites actifs ayant diverses activités biologiques prometteuses: antivirale, antibactérienne, antimycotique et antioxydante. De plus, un grand nombre d'agents chimiothérapeutiques utilisés pour le

traitement du cancer sont des métabolites spécialisés produits par des microorganismes, en particulier du genre Streptomyces (Singh et al. 2017; Chandra 2012). De plus, une étude réalisée par Lucas et ses collaborateurs a prouvé que les basidiomycètes avaient des propriétés anticancéreuses en réalisant des tests à partir d'extraits du champignon *Boletus edulis* pour le traitement du sarcome (Lemieszek et al. 2012).

Les Ascomycètes peuvent être facilement cultivés en laboratoire (exemple *Aspergillus, Penicillium, Fusarium*). Pour cette raison, de nombreuses études visant à mieux élucider le mécanisme (moléculaire et biochimique) des voies métaboliques spécialisées ont été axées exclusivement sur ce phylum (Gidijala et al. 2010; Atanasova-Penichon et al. 2018; Janevska and Tudzynski 2018; Andersen et al. 2013). En raison de la grande diversité fongique dans le monde, les champignons restent une source largement inexplorée/inexploitée pour la découverte de nouvelles molécules bioactives et leurs voies de biosynthèse, surtout les métabolites spécialisés dérivés des isoprénoïdes.

En raison de la difficulté de culture en laboratoire des basidiomycètes, à l'exception de quelques espèces, ce phylum reste généralement inexploité et peu d'informations sur les voies de biosynthèse des métabolites spécialisés ont été rapportées dans la littérature (Wasser 2011; Schmidt-Dannert 2014). Les champignons utilisent différentes voies métaboliques afin de produire des métabolites fongiques pour la synthèse de différents composés complexes. Les progrès récents de la science et de la technologie facilitent toutefois les manipulations génétiques où des espèces fongiques potentielles peuvent être génétiquement modifiées pour améliorer le rendement des métabolites bioactifs (Purchase 2016). Grâce à l'avancement technologique dans la science des omiques, il est possible d'avoir plus rapidement et de manière très précise des informations sur l'identification et la caractérisation des voies de biosynthèse des produits naturels fongiques. De plus, l'étude biologique des espèces fongiques a un rôle essentiel à jouer dans le développement des secteurs biotechnologiques et biomédicaux (Purchase 2016).

Cette recherche s'est particulièrement intéressée à deux espèces de basidiomycètes d'Amérique du Nord, le champignon médicinal, *Inonotus obliquus,* connus sous le nom du chaga et l'*Armillaria sinapina.*

### 1.3.4 *Inonotus obliquus* (chaga)

*Inonotus obliquus* (Figure 1.3), communément appelé chaga, est un polypore de pourriture blanche, appartenant à la famille des Hymenochaetaceae, du sous-phylum d'Agaricomycetes du phylum des Basidiomycètese. Il est considéré comme champignon parasitaire des espèces de feuillus, principalement ceux du genre *Betula* (Betulaceae) ainsi que dans quelques cas ceux des genres *Quercus* (chênes), *Populus* (peupliers), *Alnus* (aulnes), *Fagus* (cendres), et *Acer* (érables) (Ryvarden and Gilbertson 1993; Géry et al. 2018a).

Le chaga a été identifié et décrit pour la première fois par Persoon en 1801, qui l'a nommé en premier lieu « *Boletus obliquus* ». En 1883, Fries lui attribua le nom de « *Polyporus obliquus* » en raison de sa structure. En 1888, Quélet l'appela *Poria obliqua.* En 1927, Bourdot et Galzin le nommèrent *Xanthochrous obliquus*. Pilàt (1936, 1942) qui l'étudia plus en détail, lui donna son nom actuel, *Inonotus obliquus.*

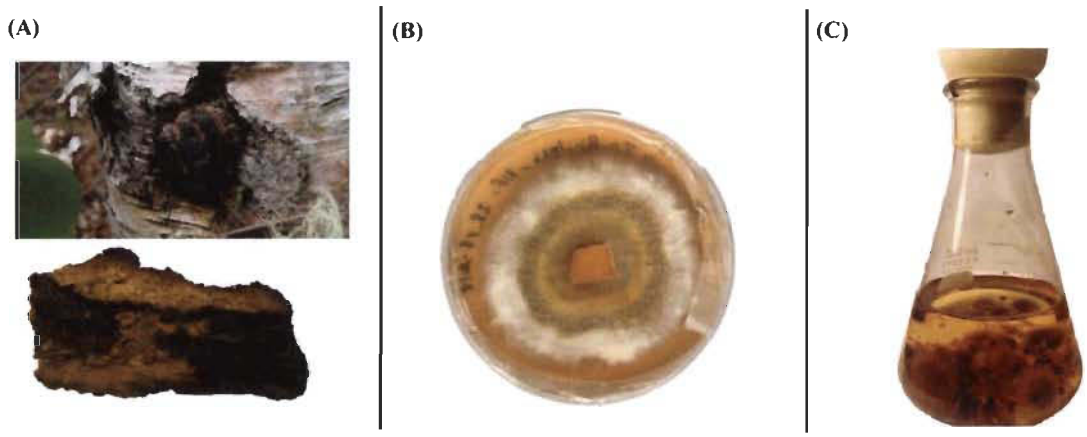


**Figure 1.3** **Représentation de *l'Inonotus obliquus* dans différents milieux.**
(A) *Inonotus obliquus* dans la nature, (B) *Inonotus obliquus* en culture solide sur une gélose YMA, (C) *Inonotus obliquus* en culture liquide dans un milieu YMB.

Le terme spécifique *obliquus* désigne le fait que les pores du corps reproducteur sont inclinés par rapport à l'horizon (Géry et al. 2018a; Haines ; Pilz 2004). Le chaga se trouve dans les forêts tempérées du subarctique d'Amérique du Nord et d'Eurasie. On le trouve notamment au Canada, au nord des États-Unis, au Kazakhstan, en Sibérie, en Ukraine, au Japon, en Corée du Sud, en Chine, ainsi qu'au nord et à l'est du continent européen (Géry et al. 2018a; Mishra et al. 2013; Lee, Hwang, and Yun 2009). Ce champignon se distingue des autres en raison de sa forme de conque. Celle-ci est constituée de bois et de mycélium qui se forme sur le bouleau après infection, due à une croissance invasive du champignon (Glamočlija et al. 2015a). La littérature rapporte que les extraits de chaga ont souvent été utilisés en médecine traditionnelle notamment en Chine, en Corée, en Russie et dans les pays baltes en raison de leurs activités présumées antibactériennes, hépatoprotectrices, anti-inflammatoires, antitumorales et antioxydantes, ainsi que comme agent gastroprotecteur et hépatoprotecteur. Ces propriétés ont été confirmées par plusieurs récentes qui ont démontré le potentiel thérapeutique des molécules bioactives présentes dans *I. obliquus* pour prévenir et guérir le cancer, les maladies cérébrales, vasculaires, etc. Il a été démontré que les extraits de chaga avaient un effet cytotoxique sur les cellules cancéreuses de la lignée PLP2 U251, ainsi que sur d'autres lignées cellulaires cancéreuses et qu'ils ont des effets inhibiteurs sur la prolifération des cellules cancéreuses du côlon. De plus, des études *in vitro* ont montré que les extraits de chaga étaient non-toxiques et surtout avaient des activités antivirales, antioxydantes, et anti-inflammatoires. Une mise en lumière de propriétés protectrices de type probiotiques dans la fraction mélanine de chaga *in vitro* a également été réalisée (Hu, Teng, et al. 2017; Glamočlija et al. 2015a; Parfenov et al. 2019; Mishra et al. 2013; Lee et al. 2009; Youn et al. 2009; Duru et al. 2019; Lee et al. 2019; Zhao et al. 2018; Rzymowska 1998).

Des études menées au cours de la dernière décennie ont révélé que l'extrait d'*I. obliquus* contient des molécules biologiquement actives, issues des métabolismes spécialisés. Ces métabolites spécialisés (*e.g.* polysaccharides, polyphénols et terpénoïdes) sont responsables des effets médicinaux qu'on confère aux extraits de *I. obliquus*, y compris les effets antioxydants (Mu et al. 2012a; Glamočlija et al. 2015a), antibactériens (Glamočlija et al. 2015a), antidiabétiques (Zhang, Bao, and Zhang 2018b; Géry et al.

2018a), anticancéreux (Géry et al. 2018a; Liu, Yu, Li, Liu, Zhang, Sun, Lin, Chen, Chen, and Wang 2019; Zhang, Bao, and Zhang 2018b) et anti-inflammatoires/immunomodulatoires (Lindequist, Niedermeyer, and Jülich 2005; Géry et al. 2018a).

D'après la littérature, plusieurs molécules terpénoïdes bioactives sont retrouvées dans le chaga comme des sesquiterpénoïdes (bergamote, sélinène, santalène, etc.) et des triterpénoïdes (bétuline, acide bétulinique, lanostérol, inotodiol, acide traméténol, etc.) (Zhao, Xia, et al. 2016b; Géry et al. 2018a; Ayoub, Lass, and Schultze 2009a).

### 1.3.5 *Armillaria sinapina*

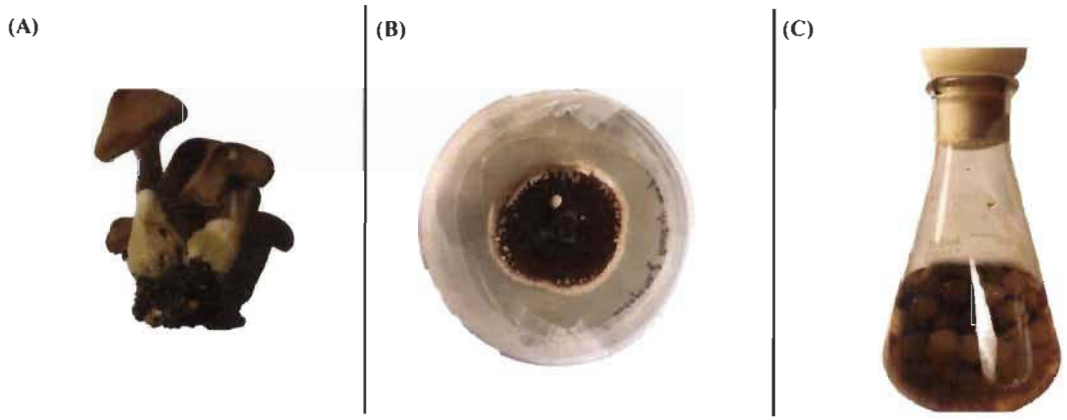


**Figure 1.4** **Représentation d'*Armillaria sinapina* dans différents milieux.**
(A) *Armillaria sinapina* dans la nature, (B) *Armillaria sinapina* en culture solide sur une gélose YMA, (C) *Armillaria sinapina* en culture liquide dans un milieu YMB.

Les *Armillaria* sont des champignons saprophytes et pathogènes communs appartenant au superclade d'Agaricales de la famille des basidiomycètes, de la grande famille des *Physalacriaceae*. D'après des études phylogénétiquesrécentes, les genres *Guyanagaster* et *Cylindrobasidium* seraient les plus proches du genre *Armillaria*.

On attribue aux *Armillaria* un rôle très important dans le cycle du carbone des forêts, mais aussi un rôle central dans la dynamique de nombreux écosystèmes ligneux. Le genre

*Armillaria* a une répartition mondiale et il est retrouvé aussi bien dans les forêts naturelles que dans les forêts plantées d'écosystèmes tempérés, boréaux et tropicaux. Ces champignons parasitent de nombreuses espèces d'arbres dans les forêts des hémisphères nord et sud. Le niveau de virulence des espèces *Armillaria* varie selon le type d'hôtes. À ce jour, plus de 70 espèces ont été répertoriées dans le monde et plus de 40 espèces du genre *Armillaria* ont déjà fait l'objet d'études complémentaires approfondies (Kolesnikova et al. 2019; Heinzelmann et al. 2019b; Sipos, Anderson, and Nagy 2018).

Différents projets de séquençage génomique ont été réalisés sur les *Armillaria*. Six génomes de la famille *Armillaria* ont été séquencés (*A. cepistipes, A. gallica, A. fuscipes, A. mellea, A. ostoyae* et *A. solidipes*) et une analyse transcriptomique d'une des espèces les plus virulentes qui affecte principalement les arbres, *A. solidipes* (*Armillaria ostoyae*) a été complétée (Ross-Davis et al. 2013; Sipos, Anderson, and Nagy 2018).

L'*Armillaria sinapina* (Figure 1.4) a été identifiée morphologiquement par J.A. Bérubé et D. M. Dessureault en 1988 en Amérique du Nord et a été très peu étudiée depuis (Bérubé and Dessureault 1988). L'*Armillaria sinapina* est considérée comme faiblement pathogène pour les feuillus et seulement saprotrophe pour les conifères. Elle serait présente dans toutes les forêts hormis en Europe centrale (Dettman and van der Kamp 2001; Zhelifonova et al. 2019).

Des études récentes montrent que les *A. borealis, A. cepistipes* et *A. sinapina* ont la capacité de synthétiser des métabolites spécialisés appartenant au groupe des esters de sesquiterpènes tricycliques (Zhelifonova et al. 2019). De plus, l'espèce *Armillaria* a démontré sa capacité à acquérir de nouvelles ressources alimentaires en utilisant différentes stratégies. Étant saprotrophes, ils sont en mesure d'exploiter tous les types de bois mort tels que les racines et les débris ligneux des différentes espèces présentes dans les écosystèmes naturels et artificiels. Le mycélium d'*Armillaria* renferme des enzymes qui ont la capacité de dégrader la cellulose et la lignine pour se nourrir, ce qui pourrait entraîner l'apparition d'une pourriture blanche (Heinzelmann et al. 2019).

Malgré l'impact considérable sur la sylviculture, l'horticulture, l'agriculture et la biotechnologie, peu de données sur les espèces d'*Armillaria* sont disponibles. À ce jour, les seules données omiques publiées sur l'*Armillaria mellea* et *solidipes* ont permis d'établir un important répertoire d'enzymes impliquées dans la dégradation de la paroi cellulaire des plantes (Sipos et al. 2017).

## 1.4 Les métabolites spécialisés chez les champignons

Les mycètes, comme les plantes, produisent un large éventail de molécules issues des métabolismes primaires et secondaires. Les métabolites spécialisés sont, par opposition aux métabolites primaires (protéines, glucides et lipides), des composés chimiques produits en faible quantité par les plantes et les microorganismes tels que les champignons. Ils diffèrent d'une espèce à une autre et sont responsables de multiples fonctions telles que la régulation des cycles catalytiques, l'interaction avec l'environnement et la réponse aux stress biotiques (microorganismes, nématodes ou insectes) et abiotiques (température, lumière, hydrométrie).

Contrairement aux métabolites primaires, ils ne sont pas directement impliqués dans la croissance des organismes vivants et leur absence n'est pas létale mais représente un handicap. De plus, ce sont ces métabolites qui sont utilisés par les humains depuis l'antiquité comme médicaments pour soigner plusieurs maladies ou comme arômes en cuisine.

Les champignons sont le groupe le plus diversifié et le plus abondant de la planète. Ils sont considérés comme d'excellents candidats pour la production de métabolites bioactifs en raison de leur ressemblance avec le système animal. Tout comme les végétaux, les fructifications de certains champignons peuvent accumuler un grand nombre de métabolites spécialisés, dont des acides organiques, des alcaloïdes, des terpénoïdes, des composés phénoliques et des flavonoïdesé. Leurs applications sont multiples, ainsi on peut les retrouver dans de nombreuses industries telles que la cosmétique, la pharmaceutique, les biotechnologies, ou encore les produits agroalimentaires [28].

Les champignons supérieurs, notamment les basidiomycètes, avec leurs impressionnantes diversités métaboliques et leurs molécules bioactives, offrent de nouvelles voies pour la production biotechnologique de nombreuses molécules autrefois considérées comme des molécules plantes-typiques (Dembitsky 2014). La figure 1.5 montre les voies de biosynthèse possibles en partant du glucose.

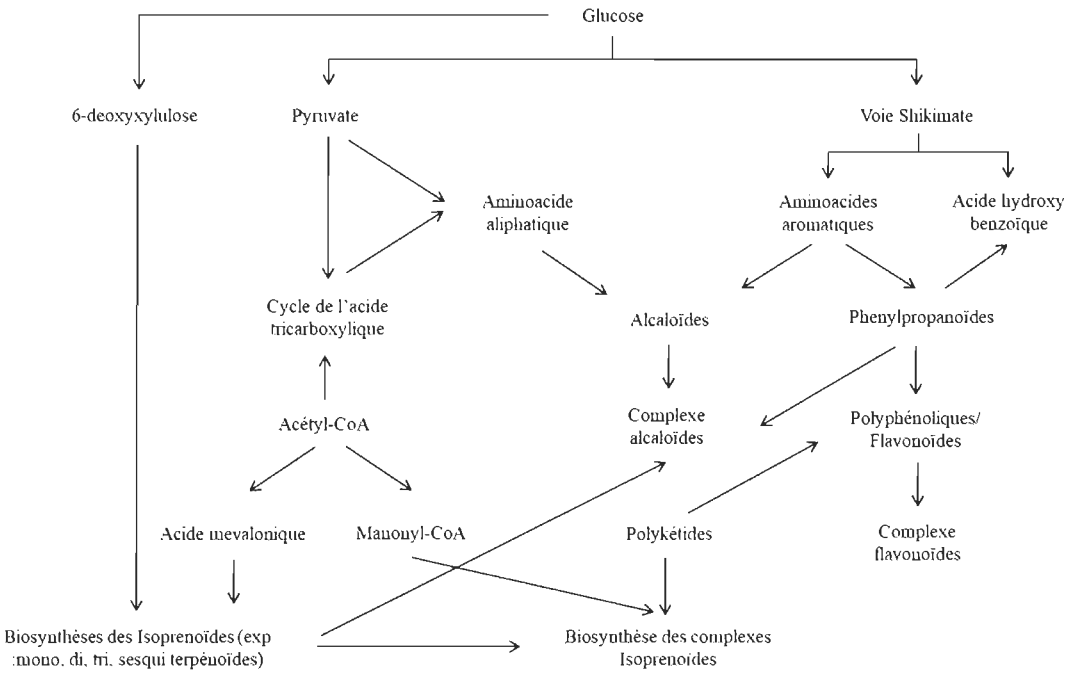


**Figure 1.5** Voies de biosynthèse de certaines classes de métabolites spécialisés à partir du glucose chez les mycètes.

### 1.4.1 Les polykétides

Parmi les métabolites spécialisés les plus répandus chez les champignons, on retrouve les polykétides (Figure 1.5). Les polykétides fongiques sont synthétisés grâce à des enzymes, appelés les polykétides synthases de type I (PKS), dont la structure est similaire à celle des protéines multi-domaines apparentées aux acides gras eucaryotes (Keller, Turner, and Bennett 2005). Ils sont considérés comme des composés à fort potentiel pharmaceutique. Ils trouvent leur utilité comme antibiotiques (rifamicine), anticancéreux (lankacidine), antifongiques ou encore comme antiparasitaires.

Plusieurs polykétides ont été caractérisés génétiquement comme la lovastatine, un composé commercial utilisé pour réduire le cholestérol. Les polykétides sont connus pour leurs grandes variétés structurales. Cette diversité moléculaire résulte du nombre de réactions d'itération, du nombre de réactions de réduction et de l'unité d'extension utilisée. Pour les polykétides aromatiques, il faut rajouter les cyclisations de la chaîne polykétide naissante (Keller, Turner, and Bennett 2005; Dorival 2016).

### 1.4.2 Les alcaloïdes

Les alcaloïdes sont un vaste groupe de métabolites spécialisés azotés qui sont largement répandus dans le règne des plantes et des champignons (Dembitsky 2014). Le nom « alcaloïde » vient du fait qu'un composé est « alcalin », c'est-à-dire de nature basique, et il contient au moins un atome d'azote. Dans les premières définitions d'un alcaloïde, ce dernier était uniquement d'origine végétale et avait comme caractéristique de contenir un moins un atome d'azote. La conine (ou coniine) est le premier alcaloïde dont la structure a été élucidée en 1870 et est aussi le premier à avoir été synthétisé avec succès. D'après un recensement en 2001, plus de 26 900 structures d'alcaloïdes, provenant de diverses sources comme les plantes, les champignons, les organismes marins et les mammifères ont été identifiés (Cordell, Quinn-Beattie, and Farnsworth 2001). Les champignons endophytes sont connus pour produire divers métabolites spécialisés tels que les alcaloïdes (Chujo et al. 2019). On leur confère différentes propriétés pharmaceutiques telles qu'anticancéreuses et antibactériennes (Li et al. 2018).

### 1.4.3 Les polyphénols

Les composés phénoliques sont des métabolites spécialisés très répandus dans les plantes et les champignons (Figure 1.5). Ils sont produits suivant la voie du shikimate à partir de l'acide shikimique des plantes et de pentose phosphate au cours de la métabolisation des phénylpropanoïdes (Lattanzio 2013). Les composés phénoliques sont des composés chimiques aromatiques caractérisés par la présence d'un ou plusieurs groupe(s) hydroxyle(s) (OHOn retrouve dans la nature des phénols simples et des

polyphénols, tels que les flavonoïdes et les tanins. Les phénols sont des molécules hydrophobes, ce qui complique leur extraction. En raison de leurs propriétés hydrofuges, les composés phénoliques chez les champignons se retrouvent de façon préférentielle dans les membranes lipidiques (Zabka and Pavela 2013). Par ailleurs, on reconnaît un fort potentiel antioxydant aux composés phénoliques et plusieurs études rapportent le pouvoir antioxydant de plusieurs champignons de la famille des basidiomycètes, tels que les *Agaricus blazei*, *Hypsizigus marmoreus* et *Boletus edulis* (Pedneault 2007; Martins et al. 2011).

### 1.4.4 Les polysaccharides

La formation des glucides se fait selon la formule empirique $(CH_2O)_n$. Ils comprennent les sucres simples, les polysaccharides et leurs dérivés. Ces composés sont les principaux nutriments dans la plupart des organismes, particulièrement sous la forme d'un sucre simple : le glucose. Les polysaccharides sont une classe de biomacromolécules importantes répandues dans les plantes, les animaux et les microorganismes. Les glucides sont principalement retrouvés dans les champignons sous forme de polysaccharides; les β-glucanes étant les plus abondants. Les polysaccharides isolés des champignons dits supérieurs ont attiré beaucoup d'attention ces dernières années en raison de leur large spectre d'activités. On leur attribue diverses propriétés telles que des activités antivirales, antibactériennes et anti-inflammatoires. Dans des études pharmacologiques, les polysaccharides extraits du champignon *Inonotus hispidus* ont révélé de nombreuses activités biologiques telles que la diminution de la glycémie, des activités antitumorales et l'amélioration du système immunitaire (Liu, Hou, et al. 2019; Angelini et al. 2019; Sánchez 2006).

### 1.4.5 Les terpénoïdes

Initialement, le terme "terpène" désignait les hydrocarbures cycliques en $C_{10}H_{16}$ dérivés des huiles essentielles végétales. Mais depuis, il regroupe tous les groupes de métabolites spécialisés naturels dérivés des unités isoprènes (isopentane) (Isah et al.

2019). Les terpénoïdes, appelés aussi terpènes ou isoprénoïdes sont des hydrocarbures naturels produits par les plantes, animaux et microorganismes comme métabolites spécialisés. Ils représentent l'une des familles de produits naturels les plus diversifiés sur les plans chimique et structurel. À ce jour, plus de 80 000 composés terpéniques ont été caractérisés incluant les stéroïdes et les caroténoïdes (Christianson 2017). Ils se composent de cinq unités isoprènes (unité diphosphate d'isopentényle (IPP ou IDP)) et peuvent être classés en différents sous-groupes selon le nombre de carbone et/ou le nombre d'unités isoprènes (UI) : monoterpènoïdes (C10), diterpènoïdes (C20), sesquiterpènoïdes (C25), triterpénoïdes (C30) et les terpénoïdes supérieurs tels que les caroténoïdes (Figure 1.6).

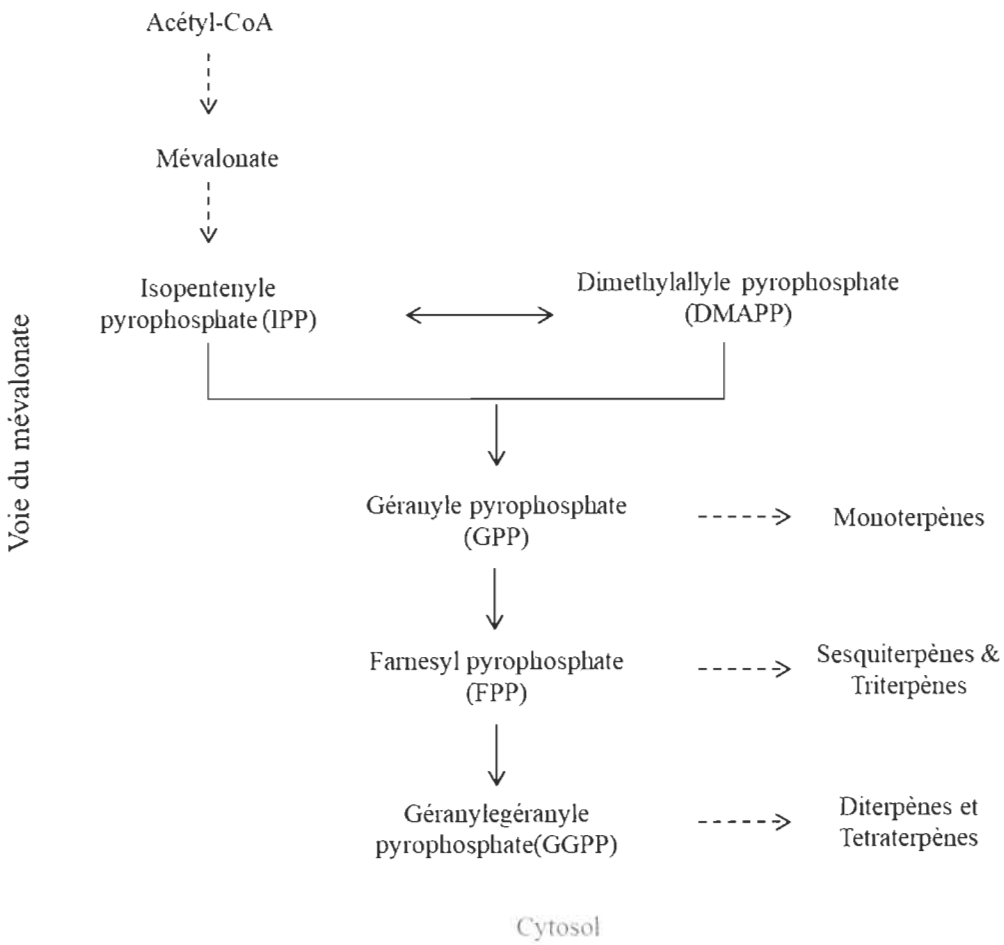


**Figure 1.6 Voie de biosynthèse simplifiée menant à la production de divers terpènes, suivant la voie du mévalonate.**
Une flèche cassée représente plus d'une réaction biochimique.

Les produits dérivés des isoprènes présentant moins d'atomes de carbone que ceux du groupe terpénoide avec lequel ils partagent une similitude structurelle sont classés comme dérivés « nor ». À l'inverse, ceux ayant plus d'atomes de carbone sont nommés dérivés « homo ». Cela génère des noms tels que nortriterpénoïdes. En ce qui concerne les homoterpénes, très peu ont pu être isolés naturellement. Hormis l'isoprène qui est un composé organique volatile (COV) libéré par plusieurs plantes, les monoterpénoïdes linéaires se retrouvent, le plus souvent, dans les huiles essentielles aromatiques comme les alcools, les aldéhydes, les hydrocarbures ainsi que les esters acétiques (Isah et al. 2019). Les terpénoïdes sont produits selon deux voies de biosynthèses : la voie du mévalonate (MVA) et la voie du 2-C-methyl-d-erythritol 4-phosphate, aussi appelée voie du non-mévalonate (MEP) ou 1-desoxy-D-xylulose-5-phosphate synthase (DXS) (Huang and Osbourn 2019).

L'isopentényl-pyrophosphate (IPP) et son isomère allylique, le diphosphate de diméthylale (ou le dimère diméthyl allyl diphosphate, DMAPP) sont générés chez les plantes suivant la voie Bloch-Lynen classique. L'IPP est formé de trois molécules d'acétyl-Coa via la voie de MVA dans le cytosol ou par la voie métabolique du MEP située dans le plaste, qui n'implique pas le mévalonate. L'IPP est formé de pyruvate et d'un glucide en C3, le glycéraldéhyde pour former le 1-désoxy-D-xylulose-5-phosphate (DXP) dans la voie du MEP (Volkman 2005).

Chez les champignons, en raison de l'absence du plaste, l'IPP et le DMAPP sont générés par la voie métabolique MVA dans le cytosol. Dans le cas de la voie de MVA, la condensation tête à queue 1'-4 successive d'une à trois unités d'extension IPP en DMAPP catalysée par toutes les trans-isoprényl-diphosphates synthases (IPS), donne lieu à des isoprényldiphosphates avec dix carbones (la géranyle pyrophosphate (GPP)), puis avec quinze carbones (le farnésyle pyrophosphate (FPP)), ou vingt carbones avec la production du géranylgeranyle pyrophosphate (GGPP). En ce qui concerne les chaînes plus longues (C-30 et C-40), elles sont formées par la condensation en 1'-1 de deux molécules FPP (squalène synthase) ou GGPP (géranylgéranyl pyrophosphate), catalysée par un autre groupe de synthases à chaîne prényle (Schmidt-Dannert 2014).

On attribue diverses fonctions aux terpénoïdes chez les plantes, que ça soit dans la croissance et le développement des plantes ou biendans les mécanismes de défense contre les insectes (Yadav 2019; Phillips et al. 2006). De plus, il a été rapporté que les terpènes peuvent agir comme un langage chimique important que les plantes utilisent pour communiquer avec d'autres organismes vivant dans le sol (Huang and Osbourn 2019). Les triterpènes, en particulier les pentacycliques, représentent des métabolites spécialisés largement répandus dans le règne végétal, comme le cholestérol et sont présents dans les feuilles, l'écorce des tiges, les fruits et les racines (Nazaruk and Borzym-Kluczyk 2015). Les terpénoïdes sont aussi considérés comme la plus grande classe des métabolites spécialisés produits par le règne fongique. On retrouve leurs applications dans plusieurs secteurs industriels comme agents aromatisants et comme additif dans les parfums (Yadav 2019). De nombreuses études empiriques issues de plusieurs essais biologiques soulignent le potentiel thérapeutique des terpénoïdes contre diverses maladies telles que les maladies parasitaires, les cancers et autres infections (Isah et al. 2019). Récemment, les études d'optimisation sur les voies de biosynthèse des terpénoïdes pour obtenir un meilleur rendement s'intensifient. Il est bien connu que les champignons sont capables de produire un large éventail de composés terpénoïdes, à qui on confère des propriétés pharmaceutiques diverses telles que des antibiotiques, des molécules antitumorales, des mycotoxines et des phytohormones (Chen et al., 2017).

Les sesquiterpènes et les diterpènes résultent de la condensation successive tête-à-queue d'une à trois unités d'extension IPP en DMAPP (Xiao et al., 2016). Les triterpènes (C30) sont des isoprénoïdes produits par fusion de tête-à-tête de deux molécules de farnésyl-pyrophosphate (FPP). Après la formation du squalène C30 par dimérisation de deux molécules de FPP, l'enzyme de la squalène monooxygénase ou époxydase forme le 2,3 oxydosqualène, qui est considéré comme étant le squelette des différents triterpénoïdes. La cyclisation du 2,3 oxydosqualène (dans les champignons, les animaux et les plantes) en triterpènes est l'une des réactions enzymatiques les plus complexes connues du métabolisme du terpène. La cyclisation du 2,3-oxidosqualène est catalysée par des enzymes appelées oxydosqualène cyclases (OSC), qui produisent des échafaudages de triterpènes dans un processus impliquant la liaison et la pré-organisation

du substrat (le pliage de la structure), suivies par le déclenchement de la réaction par protonation de l'époxyde, puis cyclisation et enfin réarrangement des espèces carbocations. Pour finir, une déprotonation ou la capture d'eau d'une molécule d'eau permet d'obtenir un produit final terpénique.

Pour former les différentes molécules triterpénoïdes comme le lanostérol, la bétuline, l'acide bétulinique, beta-amyrine ou l'ergostérol; le squelette initial des triterpènes subit, par la suite, des post-modifications induites par différentes enzymes, principalement des cytochromes P450 (CYP450s) (Thimmappa et al. 2014; Xiao and Zhong 2016).

À notre connaissance, les voies de biosynthèse des terpénoïdes par les champignons ont été peu étudiées, notamment sur des champignons d'Amérique du nord. En raison de l'importance pharmacologique grandissante des triterpènes, une meilleure compréhension de la synthèse des triterpénoïdes fongiques est nécessaire et repose sur la conjugaison de la découverte de nouveaux gènes et du génie métabolique pour contribuer à une meilleure productivité (Yadav 2019). De plus, la découverte de nouvelles enzymes provenant du règne fongique pourrait être une avancée majeure dans le secteur biotechnologique.

## 1.5 La science des omiques

La science des omiques regroupe essentiellement la science de la génomique, de la transcriptomique, de la protéomique et de la métabolomique (Figure 1.7). Grâce aux techniques bioinformatiques actuelles et au séquençage à haut débit avec un coût abordable des séquences d'ARN, d'ADN, des protéines et des métabolites, il est possible de mieux comprendre un phénomène biologique. De plus, la science des omiques constitue un moyen performant pour décrire précisément la complexité des vivants (Scheen 2015).

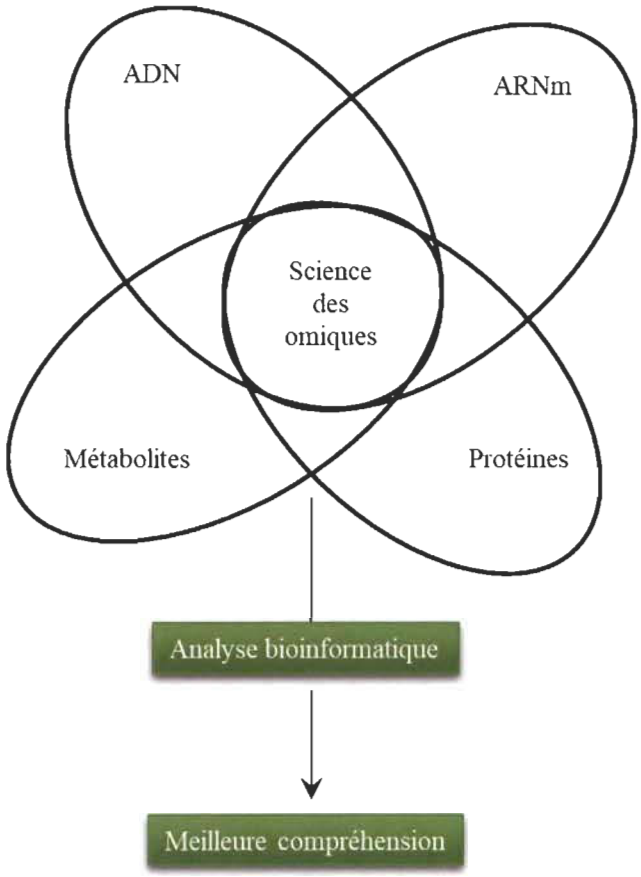


**Figure 1.7** Application de la science des omiques en utilisant les différentes bases de données (transcriptomique, protéomique et métabolomique) pour une meilleure compréhension d'un organisme.

Le séquençage de nouvelles générations à haut débit (NGS) est une technologie récente, qui regroupe l'ensemble des plateformes de séquençage mises au point depuis 2005. Les plateformes de séquençage à haut débit telles que les SOLiD, les plateformes Illumina avec MiSeq, Hiseq et Hiseq X ont été développées pour aider à l'étude transcriptomique des espèces modèles et non modèles, y compris les plantes, les animaux et les microorganismes.

Ces technologies de séquençage avancées se sont avérées être des outils rapides et puissants pour l'étude de nouveaux gènes fonctionnels et pour la détection de gènes exprimés de façon différentielle chez diverses espèces. Aussi, ces technologies ont permis l'ouverture du séquençage des organismes non-modèles (Yuan et al. 2017). Ces méthodes

permettent également d'analyser l'intégralité des exomes (c'est-à-dire l'intégralité des parties codantes des gènes) avec une très grande sensibilité de détection, en un temps réduit, et à un coût relativement abordable.

Le séquençage des fragments transcrits aide à caractériser de nouveaux transcrits à un moment donné ; par un organisme, un tissu, un organe ou une cellule donnée dans une condition choisie. Grâce au séquençage, il est possible d'étudier la diversité et d'identifier des sites d'initiation de la transcription des gènes à un temps déterminé. Le système de séquençage Hiseq 4000 permet un séquençage simultané de plusieurs millions de fragments d'ADN complémentaires générés par les ARN extraits.

La technique utilisée repose sur une amplification en forme de ponts des fragments d'ADN sur support solide suivi par un séquençage de synthèse chimique des ADN préalablement amplifiés. Chaque cellule génère 200 millions de lectures en mode simple lecture (Single read ou SR) ou 400 millions en mode double-lecture (paired-end ou PE) (Haas et al. 2013). L'étude transcriptomique est très importante car les ARN jouent un rôle clef au sein du transfert d'informations entre les molécules du vivant. De plus, il est possible d'observer lors de l'étude transcriptomique que certaines molécules d'ARN ont des fonctions catalytiques lorsqu'elles sont associées à des protéines (Loe-Mie 2012). Depuis quelques années, la disponibilité des séquences génomiques des champignons, qui a considérablement augmenté, a permis de faciliter la recherche sur la diversité génétique et l'identification des gènes impliqués dans la biosynthèse des métabolites spécialisés.

La métabolomique est une partie intégrante de la biologie systémique. Elle joue un rôle essentiel dans l'identification de plusieurs composés médicinaux extraits des plantes ou des microorganismes tels que les champignons. Cette science consiste à identifier, étudier la nature et quantifier tous les métabolites produits par un organisme. Le profil métabolique est réalisé grâce à diverses techniques spectroscopiques telles que la résonnance magnétique nucléaire (RMN), la spectrométrie de masse (SM) ou même la

spectrométrie de masse à haute résolution couplée à une chromatographie en phase liquide avec temps de vol (LCMS-QTOF) (Singh 2018; Bonvallot 2014).

L'analyse protéomique, quant à elle, a pour but de décrire, de façon exhaustive et quantitative, l'expression globale des protéines cellulaires exprimées dans un changement sous l'influence ou non de modifications biologiques comme les maladies ou le traitement. Cette analyse a pour but de fournir une compréhension systématique des événements au niveau moléculaire. Malgré l'importance des applications biotechnologiques des champignons, les études protéomiques des champignons n'ont commencé à faire leur apparition dans la littérature que récemment (Anderson et al. 1998; Kim et al. 2007).

## 1.6 Problématique

*Inonotus obliquus* et *Armillaria sinapina* sont disponibles en Amérique du Nord et parasitent divers types de feuillus et conifères. Le chaga (*I. obliquus*) est connu pour ses diverses propriétés pharmaceutiques telles que antitumorales, antibactériennes et aussi antimicrobiennes en raison de plusieurs métabolites présents dans le champignon (Lee et al. 2009; Youn et al. 2009; Hu, Teng, et al. 2017; Géry et al. 2018). Des molécules appartenant à la famille des terpènes comme la bétuline et l'inotodiol sont produites par *I. obliquus*. La bétuline est connue pour ses effets antimicrobiens et l'inotodiol est connu pour ses effets antitumoraux.

Des recherches ont montré qu'il était possible de convertir la bétuline en acide bétulinique en utilisant des catalyseurs chimiques (Chen et al. 2009). D'ailleurs, certains champignons ont démontré des capacités à biotransformer la bétuline en acide bétulinique (Chen et al. 2009). Une étude a démontré que l'utilisation de souches fongiques issues de la même source que celle du substrat pourrait aider à la production du dérivé du substrat (Bastos et al. 2007).

Le chaga et l'*Armillaria sinapina* sont deux espèces qui sont connues pour parasiter les bouleaux blancs. Des études ont démontré que les *Armillaria* pouvaient transformer la

bétuline en acide bétulinique (Liu et al. 2011). Pour sa part, le chaga, en présence de la bétuline ou des écorces de bouleau blanc qui renferment de la bétuline à forte concentration, pourrait être capable de biotransformer la bétuline en acide bétulinique. Cependant il n'existe aucune étude transcriptomique sur *Inonotus obliquus* et *Armillaria sinapina* en Amérique du Nord et sur l'identification de gènes prometteurs dans diverses voies de biosynthèses. De plus, aucune étude approfondie sur le pouvoir de biotransformation des triterpènes chez *I. obliquus* n'a été menée.

Ce projet de recherche s'inscrit dans le cadre de la valorisation de la mycoflore du Québec autour d'une perspective de valorisation des résidus forestiers et de biotransformation. L'objectif général de ce projet est l'étude transcriptomique de deux espèces fongiques, le chaga (*Inonotus obliquus*) et l'*Armillaria sinapina,* l'identification des gènes impliqués dans la voie de biosynthèse des terpènes et le rôle potentiel de l'*Inonotus obliquus* dans la biotransformation fongique.

Plus précisément, les objectifs de cette thèse sont :

### 1.6.1 Étude transcriptomique et identification des gènes impliqués dans la voie de biosynthèse des triterpènes chez *Inonotus obliquus* (chaga)

Ce premier objectif a permis l'optimisation des conditions de croissance d'*Inonotus obliquus* en milieu liquide et solide à différents pH et températures. Une analyse transcriptomique de la souche fongique dans les différentes conditions opératoires, en présence ou en l'absence de la bétuline ou d'écorces de bouleau blanc, a permis l'obtention d'informations importantes sur les organismes non-modèles étudiés. Le transcriptome a ensuite été assemblée puis les gènes ont été annotés. Cela a permis d'identifier des gènes impliqués dans les voies de biosynthèse des terpènes chez *I. obliquus.* Cet objectif est présenté dans le chapitre deux de cette thèse, sous forme d'article publié dans la revue *International Molecular Biology* en septembre 2019.

### 1.6.2 Évaluation de la production de triterpènes par *Inonotus obliquus*

Dans cet objectif, nous avons abordé la capacité du chaga à utiliser divers substrats comme la bétuline et les écorces de bouleau blanc pour produire différents triterpènes tels que la bétuline, l'acide bétulinique et l'inotodiol. Les cultures du chaga ont été incubées dans un milieu de fermentation durant 9 jours puis une extraction des métabolites spécialisés de la famille des triterpènes a été effectuée. La détection des métabolites spécialisés a été réalisée en suivant un protocole d'analyses pour les triterpènes en utilisant la chromatographie liquide combinée à une spectrométrie de masse (LC-qTOF-MS). Cet objectif est présenté dans le chapitre trois de cette thèse et une publication est en préparation, sous forme de courte communication, dans la revue scientifique *Natural Product Research*.

### 1.6.3 Étude transcriptomique et identification des gènes impliqués dans la voie de biosynthèse des triterpènes et des gènes susceptibles d'être impliqués dans la voie de dégradation de la lignine chez *Armillaria sinapina*

Dans ce troisième objectif, une analyse transcriptomique de la souche fongique *A. sinapina* en présence de la bétuline a permis l'obtention d'informations importantes sur un organisme dont le transcriptome n'a jamais été étudié. L'annotation et l'assemblage du transcriptome ont été réalisés. Des gènes impliqués dans les voies de biosynthèse des terpènes et dans la dégradation de la lignine ont été identifiés chez *A. sinapina*. Cet objectif est présenté dans le chapitre quatre de cette thèse et un article a été soumis pour publication dans la revue *PLoS One*.

# CHAPITRE II

## RNA-SEQ DE NOVO ASSEMBLY AND DIFFERENTIAL TRANSCRIPTOME ANALYSIS OF CHAGA (*INONOTUS OBLIQUUS*) CULTURED WITH DIFFERENT BETULIN SOURCES AND THE REGULATION OF GENES INVOLVED IN TERPENOID BIOSYNTHESIS

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### 2.1 Contribution des auteurs

Le projet a été conçu par Yacine BOUMGHAR, Hugo GERMAIN et Isabel DESGAGNÉ-PENIX. La méthodologie a été réalisée par Narimane FRADJ, Yacine BOUMGHAR, Hugo GERMAIN, Isabel DESGAGNÉ-PENIX. L'analyse des résultats a été faite par Narimane FRADJ, Nicolas DE MONTIGNY et Isabel DESGAGNÉ-PENIX. La rédaction et la correction de l'article ont été réalisées par Narimane FRADJ, Fatima AWWAD, Isabel DESGAGNÉ-PENIX, Hugo GERMAIN et Yacine BOUMGHAR. L'obtention des fonds a été réalisée par Yacine BOUMGHAR et Isabel DESGAGNÉ-PENIX. La supervision du projet de recherche a été menée par Yacine BOUMGHAR, Isabel DESGAGNÉ-PENIX et Hugo GERMAIN.

## 2.2 Article complet (anglais) : RNA-Seq de novo assembly and differential transcriptome analysis of Chaga (*Inonotus obliquus*) cultured with different betulin sources and the regulation of genes involved in terpenoid biosynthesis


### Abstract

Chaga (*Inonotus obliquus*) is a medicinal fungus used in traditional medicine of Native American and North Eurasian cultures. Several studies have demonstrated the medicinal properties of chaga's bioactive molecules. For example, several terpenoids (e.g., betulin, betulinic acid and inotodiol) isolated from *I. obliquus* cells have proven effectiveness in treating different types of tumor cells. However, the molecular mechanisms and regulation underlying the biosynthesis of chaga terpenoids remain unknown. In this study, we report on the optimization of growing conditions for cultured *I. obliquus* in presence of different betulin sources (e.g., betulin or white birch bark). It was found that better results were obtained for a liquid culture pH 6.2 at 28 °C. In addition, a de novo assembly and characterization of *I. obliquus* transcriptome in these growth conditions using Illumina technology was performed. A total of 219,288,500 clean reads were generated, allowing for the identification of 20,072 transcripts of *I. obliquus* including transcripts involved in terpenoid biosynthesis. The differential expression of these genes was confirmed by quantitativePCR. This study provides new insights on the molecular mechanisms and regulation of *I. obliquus* terpenoid production. It also contributes useful molecular resources for gene prediction or the development of biotechnologies for the alternative production of terpenoids.


**Keywords:** chaga; de novo transcriptome; *Inonotus obliquus*; biosynthesis; terpenoid; betulinic acid; specialized metabolism; RNA-Seq

### Introduction

*Inonotus obliquus*, a member of the Hymenochaetaceae family of Basidiomycetes, is a medicinal fungus used in the traditional medicine of Native American and North Eurasian cultures. *I. obliquus*, commonly known as chaga or black diamond, is used by

some Canadian First Nations to treat infections and tumors. Chaga is a black parasitic fungus that grows on the living trunks of the northern latitudes of America, Europe and Asia, and it is the primary pathogen of birch and other Betulaceae trees (Glamočlija et al. 2015b). In nature, instead of a fruiting body, it usually forms an irregular shape of a sclerotial conk with the appearance of burnt charcoal, and it has been used for centuries as a folk medicine to treat and prevent multiple diseases. Over the last decade, studies have revealed that the extract of *I. obliquus* contains biologically active molecules derived from the specialized metabolism, supporting the effectiveness of chaga in traditional medicine. These specialized metabolites (e.g., polysaccharides, polyphenols and terpenoids) are responsible for *I. obliquus*'s medicinal effects including those of the antioxidant (Hwang, Lee, and Yun 2016; Mu et al. 2012b; Anouar et al. 2014; Du et al. 2013; Glamočlija et al. 2015b; Lee et al. 2007; Lee et al. 2015; Liang, Zhang, and Wang 2009; Zheng, Zhang, et al. 2011; Zheng, Zhao, et al. 2011; Liu, Yu, Li, Liu, Zhang, Sun, Lin, Chen, Chen, Wang, et al. 2019), antibacterial (Lee et al. 2015; Geisler et al. 2013; Glamočlija et al. 2015b; Rastogi, Pandey, and Kumar Singh Rawat 2015), anti-diabetic (Wang, Chen, et al. 2017; Wang, Hu, et al. 2017; Lee and Yun 2011; Silva, Oliveira, and Duarte 2016; Xue et al. 2018; Zhang, Bao, and Zhang 2018a), and anticancer (Wold et al. 2018; Blagodatski et al. 2018; Géry et al. 2018b; Ali-Seyed et al. 2016; Chung et al. 2010; Drag et al. 2009; Gauthier 2006; Gheorgheosu et al. 2014; Lee and Yun 2011; Nomura et al. 2008; Pandey et al. 2015; Peron, Marzaro, and Dall Acqua 2018; Soljenitsyne 1970; Song et al. 2013; Sun et al. 2011; Zhang et al. 2015; Zhang, Bao, and Zhang 2018a; Zhao, Xia, et al. 2016a; Zhao et al. 2014; Zhao, He, et al. 2016) types.

Terpenoids consist of a large and diverse class of chemicals among the multitude of metabolites produced by fungi. Fungi employ terpenoids for an array of primary functions (growth and development) and for specialized activities, chemical interactions, and protection against abiotic and biotic factors. *I. obliquus* produces a diverse range of bioactive terpenoids exhibiting compelling therapeutic activity (e.g., anti-tumor, anti-inflammatory). Among the bioactive terpenoids found in chaga, sesquiterpenoids (e.g., bergamotene, selinene, and santalene) and triterpenoids (e.g., betulin, betulinic acid, lanosterol, inotodiol, and trametenolic acid) have been identified (Quin, Flynn,

and Schmidt-Dannert 2014; Yang et al. 2013; Yusoo, Yutaka, and Minoru 2002; Zhao et al. 2015; Zhao, Xia, et al. 2016a; Zheng et al. 2010; Ayoub, Lass, and Schultze 2009b; Glamočlija et al. 2015b).

In addition, *I. obliquus* is able to catabolize metabolites from its birch host to grow and develop. White birch bark contains large amount of triterpenoids including betulin and betulinic acid, which represent up to 75% and 2% of the extractives, respectively (Royer, Houde, and Stevanovic 2010; Blondeau et al. 2019). It is not clear if betulin and betulinic acid detected in *I. obliquus* sclerotia result only from fungal de novo synthesis, from the transformation of birch terpenoid metabolites, or from both. It has been shown that *I. obliquus* cells are able to produce these triterpenoids in a submerged culture, suggesting the presence of genes encoding biosynthetic enzymes involved in their de novo production (Xu, Zhang, and Chen 2016). However, the identification and regulation of these genes remain unknown.

In fungi, terpenoid biosynthesis occurs via the mevalonate (MVA) metabolic pathway which can be divided into three parts (Appendix A–Figure A1). The first part (precursor pathway) involves a series of enzymatic reactions converting acetyl-CoA to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are the precursors of all terpenoids. For the second part (the sesquiterpenoid pathway), the cytosolic farnesyl diphosphate synthase (FPS) condenses two molecules of IPP with one molecule DMAPP head-to-tail sequentially to produce farnesyl diphosphate (FPP). FFP serves as a precursor for sesquiterpenoids, which are synthesized by terpene synthases and can be processed by other assorted enzymes. Lastly, the third part of the pathway involves reactions that catalyze the formation of three groups of triterpenoids: The amyrin-type, the lanosterol-type and the lupeol-type.

In recent years, high throughput sequencing platforms such as the Illumina RNA-Seq have been developed to study the transcriptome of model and non-model species, including plants, animals and microorganisms. This advanced sequencing technology has proven to be a cost-effective, rapid and powerful tool for investigating

new functional genes and for detecting differentially expressed genes in diverse species. Currently, there is no genome information available for the Inonotus species; hence, the molecular basis of terpenoid production has not yet been elucidated. The aim of this study was to use de novo transcriptomic to generate a comprehensive profile of the genes related to terpenoid production in an *I. obliquus* cell culture supplemented or not with terpenoid substrates. Moreover, a better understanding of the metabolic pathway for the synthesis of *I. obliquus* pharmaceutically-relevant metabolites such as betulinic acid, betulin and inotodiol will enable tools to increase the production of these valued metabolites. Chaga's host, i.e., white birch (*Betula papyrifera*), also produces betulin in high concentrations (Zhao, Yan, and Cao 2007; Yu-hong, Tao, and Yang 2003). It has been reported that the use of microorganisms isolated from the same sources as the host substrate would increase the probability of obtaining derivatives of this substrate using the bioconversion approach (Bastos et al. 2007). Related to that, the co-culture of chaga with white birch bark residues could increase the conversion of betulin trapped in birch bark into betulinic acid to valorize forestry residues on one side, and, on the other side, it could elucidate the relationships between chaga and white birch bark.

In this study, the optimal growth conditions of *I. obliquus* cultured with or without betulin or white birch bark were investigated, and RNA was isolated. A cDNA library was then generated, and a whole transcriptomic analysis was performed using the Illumina HiSeq 4000. Whole-transcriptome was assembled de novo and analyzed, and differential expression studies confirmed by qRT-PCR unveiled the genes responsible for the terpenoid pathways in chaga.

## Results and Discussion

### *Optimal Growth Conditions*

The sclerotia of *I. obliquus* grow very slowly in nature, and artificial culture is difficult. To produce large amounts of biomass, *I. obliquus* was cultured using different growth conditions. Mycelial growth tests were carried out on a culture medium to

determine optimal conditions. The growth of the *I. obliquus* mycelium was conducted under nine conditions (three different pH (5, 6.2 and 7.5) and three different temperatures (22, 28, and 37 °C) over 16 days in a solid medium. Figure 1 shows the results for two temperatures because at 37 °C, no growth of *I. obliquus* mycelium was observed (data not shown). At 28 °C, *I. obliquus* growth was significantly higher for all tested pH; however, pHs 5 and 6.2 showed the best results (Figure 1). The effect of temperature on chaga's growth was only observed at pH 5. For example, on day 16 at pH 5 in the solid culture, fungal growth at 28 °C was twice the growth observed at 22 °C (Figure 1; from 12 mm at 22 °C to more than 24 mm at 28 °C). However, the effect of pHs 5 or 6.2 on fungal growth at 28 °C was not significantly different (Figure 1; circa 24 mm for both pH).

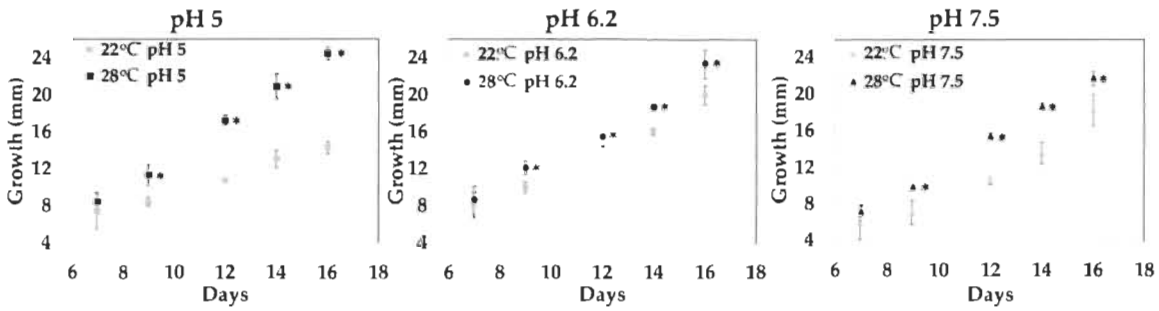


**Figure 1.** Effect of pH and temperature on *Inonotus obliquus* growth. *I. obliquus* was grown on a yeast malt agar medium (YMA) in Petri dishes for 16 days at 22 or 28 °C. YMA pH was adjusted with addition of an HCl or NaOH solution to obtain final pHs of 5, 6.2 and 7.5. Diameter (in mm) corresponding to fungal growth was measured every two days starting at day seven. Results represent the average (± error bars) growth of three biological repetitions per condition. Statistical significance is annotated with an asterix (*), according to ANOVA test results with $p < 0.05$.

In the wild, the growth and production of terpenoids by *I. obliquus* is likely influenced by substrates provided by its host. Thus, an investigation to determine the influence of white birch bark (WBB) on growth of *I. obliquus* under optimal pH and temperature conditions was carried out. For this purpose, *I. obliquus* cells were grown in presence or absence of WBB residues. Results showed that *I. obliquus* growth was significantly higher in presence of WBB (Figure 2). Specifically, after 16 days of co-culture, the *I. obliquus* control mycelia reached 23 mm diameter, whereas those that

were WBB-treated amounted to 27 mm (Figure 2). This indicates that *I. obliquus* growth was stimulated by the presence of bark residues from its host.

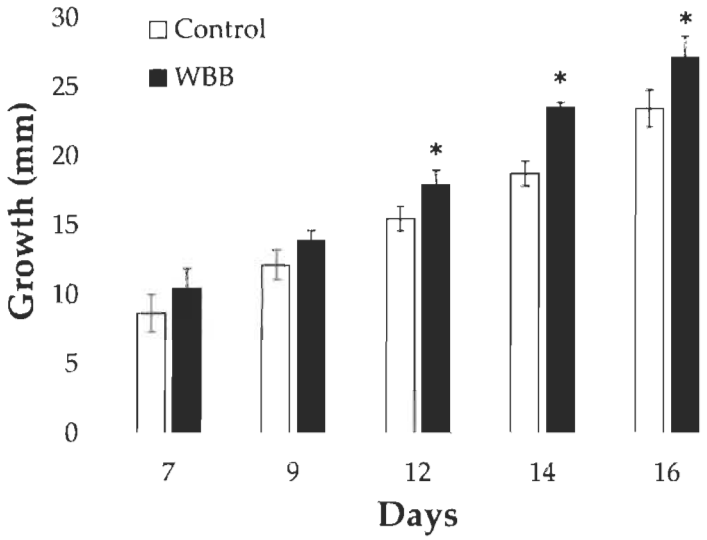


**Figure 2.** *Inonotus obliquus* cell culture growth in presence of white birch bark. *I. obliquus* was grown on a solid medium pH 6.2 at 28 °C for 16 days on agar plates containing a yeast malt agar (Control) medium (white bars) or YMA supplemented with white birch bark (WBB) fragments (black bars). The measurements of the diameter (mm) of growth were taken every two days starting at day seven. Results show the average (± error bars) growth of three biological repetitions per condition. Statistical significance is annotated with an asterix (*) according to ANOVA test results with $p < 0.05$.

The yield of fungal biomass is often higher in a liquid culture compared to a solid culture. Furthermore, a liquid culture is easier to manipulate in order to extract and obtain cell components such as metabolites, proteins or nucleic acids. Thus, the effect of pH on the kinetics of mycelial growth of *I. obliquus* in a liquid medium at 28 °C was investigated. A rapid increase in the biomass was detected within the first six days, concurrent with the cells being in the exponential growth phase with significant consumption of carbon. On day eight, the mycelial biomass reached the apex, and the optimal growth of *I. obliquus* was observed at pH 6.2 with 0.18 g/100 mL of mycelial dry weight compared to 0.10 g/100 mL at pH 5 and 0.14 g/100 mL at pH 7.5 (Figure 3).

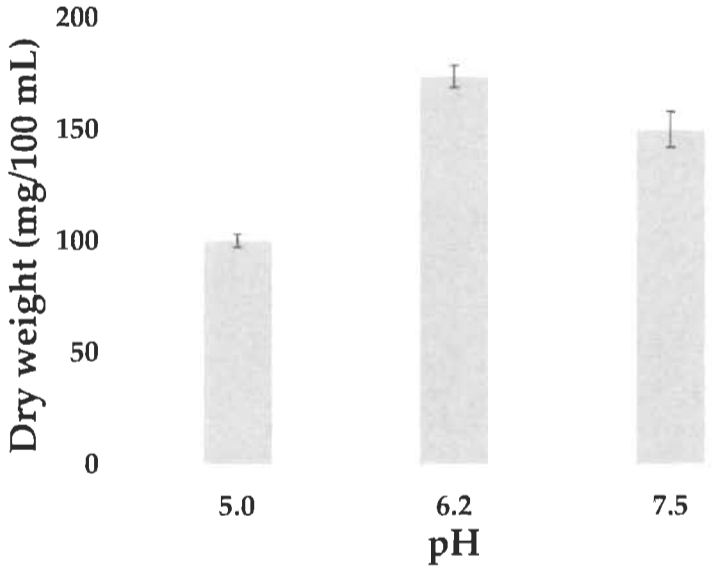


**Figure 3.** Effect of pH on *Inonotus obliquus* biomass. *I. obliquus* was grown in a liquid medium of a yeast malt broth medium (YMB) at 28 °C for eight days. The pH of the medium was adjusted by adding HCl or NaOH to obtain three final pH values of 5, 6.2 and 7.5. At day eight, *I. obliquus* cells were collected, filtered and dried at 70 °C overnight. Results represent the average (± error bars) dry weight (mg) of three biological repetitions per condition.

By-products of metabolism are shuttled throughout the cell and utilized to grow. Thus, optimal growth may influence the production of terpenoids [46]. In this study, different culturing conditions were used to establish the best possible parameters for *I. obliquus* growth. According to the results, the best condition for *I. obliquus* growth is at pH 6.2 and at 28 °C in a liquid culture that continuously shaken at 150 rpm. These results are supported by other studies on *I. obliquus* reporting similar optimal growth conditions [46,50,51]. The results of growth in the liquid and solid culture medium support that pH 6.2 would be the optimal pH for optimal mycelium growth. For subsequent analyses, an *I. obliquus* liquid culture grown at 28 °C and pH 6.2 were used, since they showed optimal growth and morphological properties. In addition, it provided an easier system for the collection of cells for RNA extraction.

*Illumina Sequencing and de Novo Assembly*

To better understand the molecular mechanisms underlying the differences in chaga's transcriptome caused by the presence of terpenoid substrates, RNA-Seq was performed. RNA was extracted from three biological replicates of cultured *I. obliquus* in three different conditions: In the absence (control, $n = 3$) or presence of the terpenoid substrate betulin (BET, $n = 3$) or white birch bark (WBB, $n = 4$). Ten corresponding cDNA libraries were generated and sequenced using Illumina HiSeq 4000 PE100. The raw reads for each library were deposited to the NCBI Sequence Read Archive under the accession PRJNA526077 (Appendix A–Table A1). A total of 229,970,255 raw paired reads were generated from all replicates of the three conditions of culturing. After filtering out low-quality sequences, 219,288,500 clean reads were obtained, corresponding to approximately 95% of the total raw reads (Appendix A–Table A1). Because of the lack of availability of information (i.e., genome, transcriptome) on *I. obliquus* or its related species, we combined all RNA-Seq libraries to build a deep transcriptome using de novo assembly. All clean reads obtained from the ten libraries were subsequently de novo assembled using the Trinity program (version 2.6.5), and a total of 196,273 transcripts with an average length of 2521 bp, and an N50 length of 4052 bp were obtained (Appendix A–Table A1). An evaluation of the size distribution showed that 96% of all transcripts of *I. obliquus* have lengths longer than 1 kb (Appendix A–Figure A2).

In a previous study, Zou et al. (2016) reported on the Illumina sequencing of *Inonotus baumii* (Zou et al. 2016a) and obtained a total of 27,259,264 reads. After the Trinity de novo assembly, 30,051 unigenes with an average length of 561 bp and an N50 length of 831 bp were generated, which is two-to-three times less than our results (Zou et al. 2016a). In another research work on the saprophytic fungus *Wolfiporia cocos*, the Illumina sequencing of the transcriptome yielded a total of 38,722,186 reads, which were assembled into 60,354 contigs with an N50 of 765 bp (Shu et al. 2013). In comparison to this, we obtained eight-to-ten times more reads, ensuring more coverage and allowing for a more contiguous assembly, as confirmed by the average transcript length of 2521 bp obtained here (Appendix A–Table A1).

The average transcript lengths of eukaryotic genes were greater than 1 kb. For example, the average transcript lengths of eukaryotic genes range from 1108 to 2667 bp in human and from 1135 to 1695 bp in yeast (Bertagnolli et al. 2013). Additionally, eukaryotic proteins have an average size of 472 amino acid residues (i.e., 1419 bp), although the size of proteins from plant genomes are smaller than those of fungi and animals (Ramírez-Sánchez et al. 2016). Based on our sequencing results, it could be concluded that the quality and the depth of this transcriptomic assembly is significantly improved compared to other fungal transcriptome studies.

*Functional Annotation of the RNA-Seq Data*

After the Basic Local Alignment Serach Tool (BLAST) annotation of the de novo assembly against the uniprot_sprot.trinotate_v2.0.pep protein database, a total of 20,072 transcripts with an average length of 4502 bp and an N50 length of 5512 bp were obtained (Appendix A–Table A1). A large number of transcripts had a similarity with known genes, suggesting a large amount of sequences specific to *I. obliquus*.

Gene function was annotated based on the following databases: Gene Ontology (GO), Clusters of Orthologous Groups of proteins (COG), and Protein family database (Pfam). A total of 86,246 transcripts (43.94% of assembled transcripts) had a match in the GO database with an E-value of 0 (Table 1). Additionally, 52,224 transcripts (20.05% of assembled transcripts) and 38,415 transcripts (19.57%) showed similarity to sequences in the COG and Pfam databases, respectively (Table 1).

**Table 1.** Summary of functional annotation for *Inonotus obliquus* transcripts in public databases.

| Description | Number of Transcripts | Percentage (%) |
|---|---|---|
| Blast-GO annotations | 86,246 | 43.94 |
| COG annotations | 52,224 | 20.05 |
| Pfam annotations | 38,415 | 19.57 |
| Total number of sequences not annotated | 105,655 | 53.83 |
| Total number of sequences annotated | 90,618 | 46.17 |
| Total of sequences transcripts | 196,273 | 100.00 |

The functional GO classification of genes is considered to be an effective tool, offering controlled vocabulary and strictly defined biological process for annotating and performing functional analysis of a large number of annotated genes and their products in a selected organism (Jiang et al. 2018). The GO classification was performed on the 196,273 transcripts, and a total of 86,246 transcripts were classified into 41 functional groups, categorized into three main GO ontologies: Biological process, cellular component and molecular function. The results indicated that the most highly annotated GO category was the cellular component (49.63%) with cell, organelle and membrane being the most abundant, while only a few transcripts were attributed to symplast, cell junction and nucleoid (Figure 4). Under the category of molecular function (38.42%), catalytic activity and binding activity were, respectively, the largest categories. Regarding biological process (11.95%), the dominant subcategories included genes involved metabolic process and cellular process (Figure 4). In addition, important categories with low numbers of transcripts were also represented in the GO, such as transcription factor activity, protein binding and nucleic acid binding.

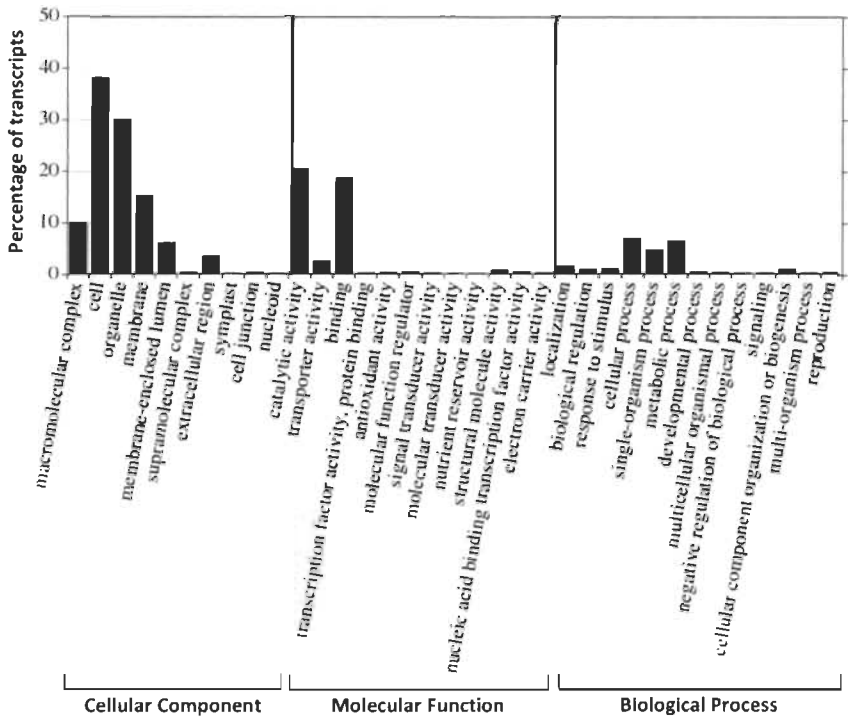


**Figure 4.** Gene Ontology (GO) terms of 41 functional groups of expressed transcripts from *Inonotus obliquus*.

COG is a database where gene products from a common family ancestor are classified. From the *I. obliquus* transcriptome, COG-annotated putative proteins were functionally classified into, at least, 25 protein families involved in basic function, such as transcription, translation, signal transduction, cellular structure, biochemistry, metabolism, and molecular processing. The COG analysis of *I. obliquus* transcriptome led to the classification of 52,224 transcripts to COG classifications. The top COG categories included basic function prediction (16.63%), followed by amino acid transport and metabolism (11.65%), carbohydrate transport and metabolism (10.06%), translation, ribosomal structure and biogenesis (7.94%), inorganic ion transport and metabolism (7.03%), signal transduction, mechanisms (6.84%) and posttranslational modification, chaperones (5.13%) (Figure 2.5a). It is interesting that 4.45% of the *I. obliquus* annotated transcripts were ranked in the specialized metabolites biosynthesis, transport and catabolism, which suggests that specialized metabolism occurs in cultivated *I. obliquus* cells.

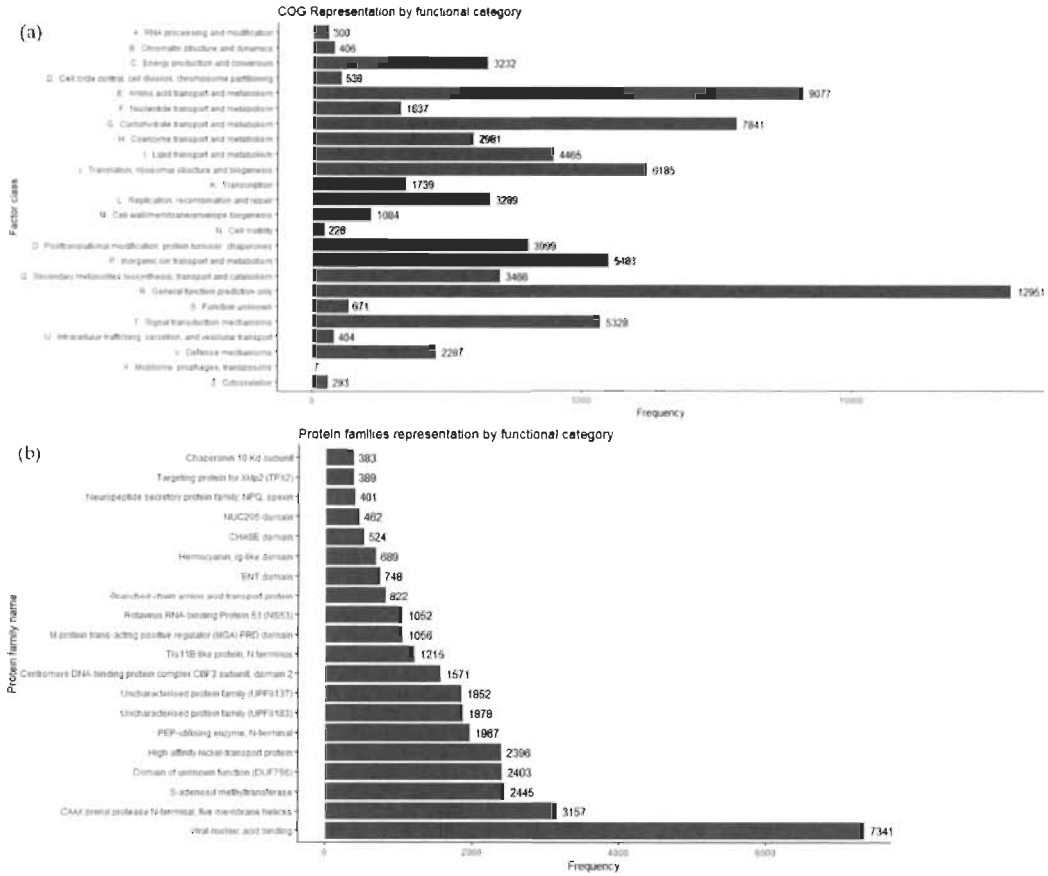


**Figure 5.** Annotation of the *Inonotus obliquus* transcriptome. (a) Cluster of orthologous groups (COG). (b) Protein family database (Pfam) classification of transcripts from *Inonotus obliquus*.

Protein domain analyses were performed using the Pfam database (Bateman et al. 2004). A total of 38,415 transcripts of *I. obliquus* were found to be associated with protein domains (Figure 2.5b). Compared to the *I. baumi* transcriptome, in which a total of 8276 transcripts contain at least one Pfam protein domain (Zou et al. 2016a), our *I. obliquus* Pfam analysis was six times superior. This result provides another indication that our transcriptome was of good quality and had more depth.

Among the abundant protein families expressed, several were linked to primary metabolism enzymatic activities such as prenylprotease, *S*-adenosyl methyltransferase and phosphotransferase (PEP-utilizing enzyme). In addition, an important number of the expressed genes encoded predicted proteins from families implicated in ion transport,

more specifically Nickel transport protein, a protein family proven to be essential for energy and nitrogen metabolism (Mulrooney and Hausinger 2003).

*Differential Expression Analysis*

Differential expression analyses were performed between databases of *I. obliquus*. Transcripts with adjusted *p*-values ≤0.05 and a fold change (log2FC) ≥1 were designated as significantly differentially expressed transcripts. For *I. obliquus* cells cultivated with betulin (control vs. BET), the results showed that 441 differentially expressed transcripts (139 up-regulated and 302 down-regulated) were identified. Twenty-two times more transcripts (9707) were differently expressed in cells cultivated with bark (control vs. WBB), with a total of 5070 annotated transcripts up-regulated and 4637 transcripts down-regulated (Figure 6). This suggest that the birch bark has very different molecular impacts and requires more transcriptome adjustment from *I. obliquus* than betulin.

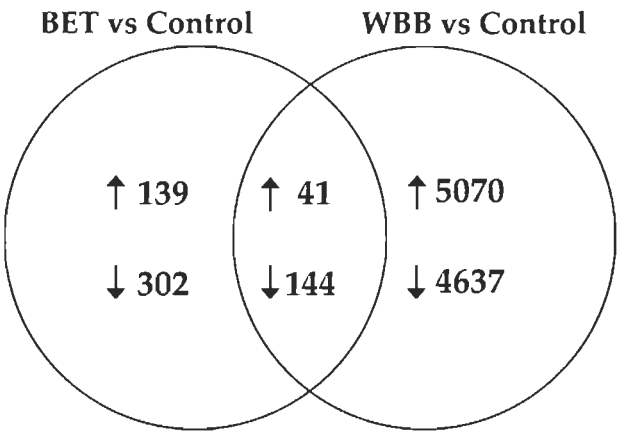


**Figure 6.** Venn diagram summarization of the differential expression comparisons. The number of differentially expressed genes (DEGs) in each circle represents the amount of DEGs between the different comparisons betulin (BET) versus control and white birch bark (WBB) versus control. Only the annotated genes were included. The overlapping number specifies the mutual DEGs between the distinctive comparisons and the non-overlapping numbers define the genes exclusive to each condition. Indicated in the diagram are the numbers of up-regulated (↑) and down-regulated DEGs (↓).

The top twenty-five up- and down-regulated expressed transcripts in *I. obliquus* cells cultivated with betulin (Appendix A–Tables A2 and A3) or white birch bark

(Appendix A–Tables A4 and A5) were identified. After blasting the top twenty-five up- and down-regulated transcripts against the NCBI database, 48% could not be annotated (no hit). Among top-regulated transcripts with predicted function, 16% have an E-value of $10^{-5}$ or less (Appendix A–Tables A2-A5). Interestingly, the transcript annotated as phosphatidylinositol 4-phosphate 3-kinase ranked first up-regulated for both treatments, suggesting the importance of the signaling pathways involved in cell proliferation, cell survival, and intracellular protein trafficking (Appendix A–Tables A2 and A4). Phosphatidylinositol 4-phosphate 3-kinase is a conserved enzyme involved in the regulation of phosphatidylinositol 4-phosphate, which is crucial for maintaining morphology, regulating lipid storage, Golgi function and actin cytoskeleton organization (Foti, Audhya, and Emr 2001). The signaling cascade activated downstream of this enzyme implicates the mitogen-activated protein kinase (MAPK) pathway, which regulates the filamentous growth of yeast (Adhikari and Cullen 2015). This suggests a putative role for phosphatidylinositol 4-phosphate 3-kinase in the regulation of the morphology of the mycelium and filamentous growth in *I. obliquus* cells in presence of WBB or betulin.

In addition, caffeic acid 3-O methyltransferase, which catalyzes the conversion of caffeic acid to ferulic acid, was up-regulated in both treatments (Appendix A–Tables A2 and SA4). Phenolic acids, such as caffeic acid and ferulic acid, possess antioxidant properties that play key roles in the synthesis of polyphenols and protection against UV and oxidative stress (Gowri et al. 1991; Jung et al. 2008; Lee et al. 2006; Lee et al. 2007). The up-regulated condition of caffeic acid 3-*O*-methyltransferase in our transcriptomic results suggests an important role for ferulic acid in cultivated *I. obliquus* cells for protection or polyphenol synthesis.

The most down-regulated transcripts found in the betulin-cultivated cells were annotated as transcripts involved in cellular processes such as DNA replication, transcription, cell division and proliferation, i.e., t-RNA ligase, cyclin-dependent kinase, ribonucleoprotein complex subunits, chromosome proteins, and condensin complex

subunits. This suggests betulin-induced cellular processes in *I. obliquus* cells (Appendix A–Table A3).

For WBB-cultivated cells (Appendix A–Table A5), most down-regulated transcripts found annotated for protein involved in metabolic processes such as several peroxidases, lipases, and racemases. This suggests that cultivation with WBB reduced the expression of genes encoding proteins involved in ligninolysis, i.e., to metabolize substrates/degrade bark molecules (Ali, Husain, and Ishqi 2019). Effective lignin and plant cell wall degradation is possible through the action of enzymes from filamentous fungi, and it has been reported that fungi of the basidiomycetes family express several enzymes of the peroxidase family in their transcriptomes, such as lignin- and manganese-peroxidases known for their ability to degrade plant cell walls (Hammel and Cullen 2008). The down-regulated peroxidase transcripts in *I. obliquus* cells cultivated with WBB suggests that WBB may leak repressors for peroxidases to prevent its degradation from the mycelium.

*Genes Involved in the Biosynthesis of Terpenoids in Cultured I. obliquus Cells*

The presence and identification of terpenoids in *I. obliquus* cells have been already studied and are well documented (Ayoub et al. 2009; Géry et al. 2018; Glamočlija et al. 2015; Liu et al. 2014; Nomura et al. 2008; Xu et al. 2014; Xu et al. 2016; Yusoo et al. 2002; Zhao et al. 2015; Zhao et al. 2016a). In this study, narrow BLAST searches were achieved to identify distinct transcripts encoding enzymes presumably taking part in terpenoid biosynthesis (Appendix A–Figure A1). Eighteen transcript sequences from the mevalonate pathway involved in terpenoid biosynthesis were found in the transcriptomic data of *I. obliquus* (Table 2). As expected, no genes involved in the non-mevalonate pathways were identified, suggesting the absence of this pathway in chaga. Similarly, the transcriptome from *I. baumii* reported no genes from the non-mevalonate pathway (Zou et al. 2016a).

From the precursor pathway leading to IPP/DMAPP (Appendix A–Figure A1), several transcript variants of orthologous genes were identified (Table 2). For example,

*AACT* genes have been cloned and characterized from various species including zebra fish, frog and human. Two human (*Homo sapiens* (Hs)) isoforms (HsAACT1 and HsAACT2) were identified to be ubiquitous and important enzymes found in different intracellular locations. The cytosolic HsAACT1 catalyzes the formation of the acetoacetyl-CoA required for sterol, including cholesterol biosynthesis. The exact function of HsAACT2 is not known, but patients with HsAACT2 deficiency have shown severe mental retardation and hypotonus (Fox et al. 2014). Similarly, the model plant *Arabidopsis thaliana* (At) has two isoforms of AACT, where *AtAACT1* is primarily expressed in the vascular system and *AtAACT2* is deeply present in root tips, top stems, young leaves, and anthers. The characterization of T-DNA insertion of mutated alleles for each *AtAACT* locus established that *AtAACT2* function is necessary for normal male gamete transmission and embryogenesis, whereas plants lacking *AtAACT1* are viable with no apparent growth phenotype (Jin, Song, and Nikolau 2012). In yeast, *AACT* (also called *erg10*) encodes a multimeric enzyme, but the exact subunit structure has not been defined (Fox et al. 2014).

Transcripts encoding each enzyme involved in the formation of terpenoid precursors IPP and DMAPP (Appendix A–Figure A1) were identified in the transcriptome of *I. obliquus* (Table 2). According to the number of reads, the *HMGS, HMGR,* and *PDM* transcripts were the most abundant with E values of 0, suggesting that these gene sequences are well conserved.

**Table 2.** Summary of the coding DNA sequence (CDS) of biosynthetic gene transcripts identified from the *I. obliquus* transcriptome known to be involved in terpenoid metabolism. All transcripts were deposited in the publicly available Genbank sequence database with the corresponding accession numbers listed in the material and methods section. * Accession number to UniprotKB/Swiss-Prot.

| Name | Number of Reads (Control) | Length (nt) | CDS (nt) | Top Annotation | Species | E Value | * Accession Number |
|---|---|---|---|---|---|---|---|
| *AACT1* | 8769 | 1655 | 816 | Acetyl-CoA acetyltransferase | *Danio rerio* | $1 \times 10^{-64}$ | Q6AZA0.1 |
| *AACT2* | 13 | 1247 | 1167 | Acetyl-CoA acetyltransferase | *Xenopus laevis* | $4 \times 10^{-149}$ | Q6GN02.1 |
| *HMGS* | 15,322 | 2277 | 1434 | 3-hydroxy-3-methylglutaryl CoA synthase | *Ustilago maydis* | 0 | Q4P3F1.1 |
| *HMGR* | 12,463 | 6041 | 2814 | 3-hydroxy-3-methylglutaryl CoA reductase | *Phycomyces blakesleeanus* | 0 | Q12649.2 |
| *MVK* | 323 | 1872 | 927 | Mevalonate kinase | *Homo sapiens* | $3 \times 10^{-39}$ | Q03426.1 |
| *PMK* | 4024 | 5489 | 1440 | Phosphomevalonate kinase | *Arabidopsis thaliana* | $2 \times 10^{-61}$ | Q9C6T1.1 |
| *PMD* | 10,182 | 4189 | 1209 | Diphosphomevalonate decarboxylase | *Ganoderma lucidum* | 0 | G9BIY1.1 |
| *IPI* | 1668 | 1830 | 969 | Isopentenyl diphosphate isomerase | *Xanthophyllomyces dendrorhous* | $2 \times 10^{-106}$ | O42641.1 |
| *FPS* | 5469 | 4639 | 771 | Farnesyl diphosphate synthase | *Schizosaccharomyces pombe* | $7 \times 10^{-113}$ | O14230.1 |
| *MUS1* | 5999 | 4901 | 1251 | Alpha-muurolene synthase | *Coprinopsis cinerea* | $1 \times 10^{-104}$ | A8NE23.1 |
| *MUS2* | 2651 | 5374 | 1281 | Alpha-muurolene synthase | *Coprinopsis cinerea* | $7 \times 10^{-169}$ | A8NE23.1 |
| *PRS* | 55 | 2054 | 258 | Delta (6)-protoilludene synthase | *Armillaria gallica* | $1 \times 10^{-20}$ | P0DL13.1 |
| *SQS1* | 2007 | 3770 | 1476 | Squalene synthase | *Ustilago maydis* | $2 \times 10^{-154}$ | Q92459.2 |
| *SQS2* | 4675 | 2525 | 1332 | Squalene synthase | *Ustilago maydis* | $4 \times 10^{-147}$ | Q92459.2 |
| *SQE* | 9978 | 6694 | 1164 | Squalene epoxidase | *Homo sapiens* | $3 \times 10^{-88}$ | Q14534.3 |
| *LAS1* | 3491 | 2500 | 1695 | Lanosterol synthase | *Pneumocystis carinii* | 0 | Q96WJ0.1 |
| *LAS2* | 5 | 2447 | 2020 | Lanosterol synthase | *Pneumocystis carinii* | 0 | Q96WJ0.1 |

| Name | Number of Reads (Control) | Length (nt) | CDS (nt) | Top Annotation | Species | E Value | * Accession Number |
|---|---|---|---|---|---|---|---|
| *AO* | 4214 | 6834 | 1596 | 11-oxo-beta-amyrin 30-oxidase | *Glycyrrhiza uralensis* | $2 \times 10^{-39}$ | H1A988.1 |
| *CEN3* | 1727 | 2261 | 1254 | Centromere protein 3 | *Schizosaccharomyces pombe* | $9 \times 10^{-22}$ | Q9USR9.2 |
| *GluRL* | 21 | 1656 | 831 | Glutamate tRNA ligase | *Pelodictyon luteolum* | $2 \times 10^{-35}$ | Q3B256.1 |

Sesquiterpene synthases play an essential role in diversifying the skeletal structure of sesquiterpenoids by catalyzing the very complex cyclisation of the common precursor, FPP (Agger, Lopez-Gallego, and Schmidt-Dannert 2009; Lopez-Gallego, Wawrzyn, and Schmidt-Dannert 2010; Quin, Flynn, and Schmidt-Dannert 2014; Ichinose and Kitaoka 2018). Sesquiterpenoids have been reported in Inonotus (Ayoub, Lass, and Schultze 2009b); however, no biosynthetic genes have been identified. BLASTx searches of *I. obliquus* transcriptome led to the identification of three sesquiterpene synthases: Two transcript variants of the muurolene synthase (*MUS1* and *MUS2*) and one of the protoilludene synthase (*PRS*) (Table 2). *MUS1* and *MUS2* are only 36% identical at the nucleotide level, suggesting different functions, i.e., substrates for enzymatic activities. Similarly, the E values of *MUS1*, *MUS2* and particularly *PRS* suggest that their function may be different than orthologous sequences, suggesting that *I. obliquus* possess the machinery to produce structurally different sesquiterpenoids. Muurolene, protoilludene, and derivatives, also considered volatile organic compounds (VOCs), are produced by several fungal species. The rich bouquet of several VOCs contribute to the aroma and flavor of mushrooms. Though the ecological function of fungal volatiles is unknown, studies showed their biological activities as antibiotics, antioxidants or inhibitor of germination (Dickschat, Wang, and Stadler 2018; Atiphasaworn et al. 2017; Ichinose and Kitaoka 2018).

Lee et al. (2016) performed a transcriptomic analysis of the white rot fungus *Polyporus brumalis* which led to the identification of two transcripts, germacrene A synthase and trichodiene synthase, involved in sesquiterpenoid biosynthesis (Lee et al. 2016). However, the final products of these enzymes were not detected in the cultivated fungus (Lee et al. 2016). To our knowledge, this study is the first to report the presence of transcripts encoding sesquiterpenoid-forming enzymes in Inonotus.

Triterpenoids from Inonotus species include high pharmaceutical valued metabolites. For example, the lanosterol-derived triterpenoid inotodiol and the lupeol derivative betulinic acid are well known bioactive triterpenoids from *I. obliquus* (Chung et al. 2010; Nomura et al. 2008; Sun et al. 2011; Zhang, Bao, and Zhang 2018a; Zhao et

al. 2014; Drag et al. 2009; Gheorgheosu et al. 2014; Rios and Manez 2018; Silva, Oliveira, and Duarte 2016; Zhao et al. 2013). Though several studies have shown the presence of triterpenoids in *I. obliquus*, only one gene, SQS, has been identified (Zhang et al. 2016). In order to increase knowledge on genes involved in triterpenoids production, a search for these genes was carried out in the transcriptome of *I. obliquus*. Two transcript variants of squalene synthase (*SQS1* and *SQS2*) were identified with a 95% sequence identity to each other (*SQS2* has an insertion at the 3' end) (Table 2). Our *SQS1* transcript sequence is identical to previously characterized *SQS* from *I. obliquus* (Zhang et al. 2016).

A single transcript for *AO* was identified in the transcriptome of *I. obliquus*, whereas two transcript variants, *LAS1* and *LAS2*, were found (Table 2). *LAS1* and *LAS2* possess a 98% sequence identity, but *LAS2* is longer and far less expressed than *LAS1*, suggesting the importance of *LAS1* in the production of lanosterol and its derivatives in *I. obliquus* cell cultures (Table 2).

According to the literature, only one terpenoid gene, *SQS*, has been characterized from *I. obliquus,* whereas nine genes related to terpenoids biosynthesis where identified in *I. baumii* (Zou et al. 2016a; Zhang et al. 2016). The transcriptome generated in the current study allowed for the identification of all (17) orthologous genes involved in terpenoid metabolism. Furthermore, variants of the *AACT*, *MUS*, *SQS*, and *LAS* genes were identified, indicating that these steps may be regulated differently in *I. obliquus*. For example, the higher expression of *AACT1*, *MUS1*, and *LAS1* suggests the importance of theses isoforms in terpenoid formation of *I. obliquus*-cultivated cells.

The digital expression of each terpenoid-associated transcript was studied for each growth condition (Figure 7). Upstream precursor genes, such as *AACT1* and *PMD*, had a higher expression in *I. obliquus* cells compared to specific downstream (sesqui and triterpenoid) genes such as *PRS* and *LAS2* (Figure 8). From the precursor pathway, the most strongly expressed transcript, *AACT1*, remained highly expressed across the different treatments, suggesting no transcriptional regulation under those conditions, whereas *PMD*, *SQS2* and *SQE* were up-regulated in presence of BET and WBB (Figure 7).

In contrast, *HMGR*, *MUS1*, and *AO* were down-regulated in presence of the terpenoid substrate, particularly in *I. obliquus* cells cultivated with WBB. HMGR is a rate-limiting enzyme in the terpenoid biosynthesis. It is possible that the addition of terpenoid substrates promotes negative feedback regulation mechanisms. For example, HMGR, which generates mevalonate, a critical intermediate in the biosynthesis of terpenoids, is exposed to large amount of feedback regulation through various mechanisms that are influenced by end-products, light, and hormones (DeBose-Boyd 2008; Hu, He, et al. 2017; Ma et al. 2018; Singh and Sharma 2015).

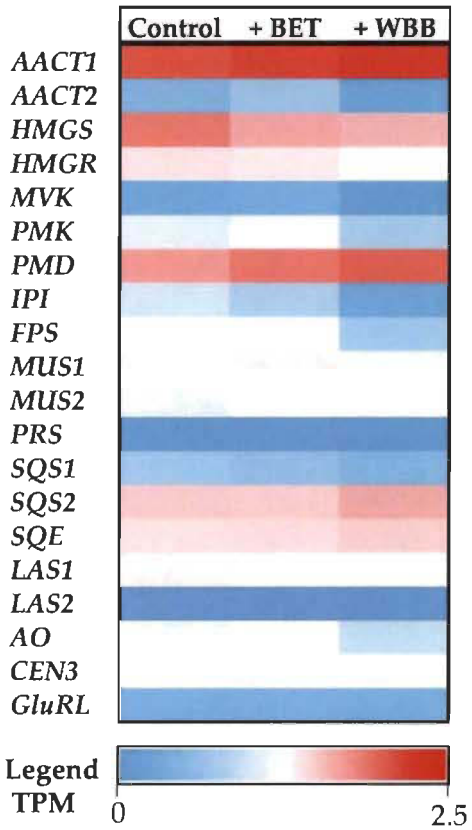


**Figure 7.** Heatmap of the digital expression levels of transcripts encoding terpenoid biosynthetic enzymes in the *Inonotus obliquus* transcriptome. Results are in transcripts per million (TPM) with the legend from low (blue) to high (red) expressed transcript.

*qRT-PCR Validation of RNA-Seq Gene Expression Data*

To validate the RNA-Seq digital expression data, eight transcripts encoding terpenoid biosynthetic enzymes were selected for the qRT-PCR analysis. For control

reference genes, transcripts with no/low variation were extracted from the database using a custom method developed by dos Santos et al. (2019), which has shown to outperform pre-defined references genes (Santos, Desgagne-Penix, and Germain 2019). Indeed, *centromere protein 3* (*CEN3*) and *glutamine RNA ligase* (*GluRL*) show no/low difference of expression among the conditions tested indicating that they are good reference genes for qRT-PCR study (Figure 7).

All eight transcripts qRT-PCR analyses are consistent with RNA-Seq results (Figure 8). This indicates that our transcriptome was reliable and that we could make reasonable inferences from the differentially expressed transcripts. For example, as observed for the digital expression, *SQS2* was up-regulated and *HMGR* was down-regulated in presence of terpenoid substrates (Figures 7 and 8).

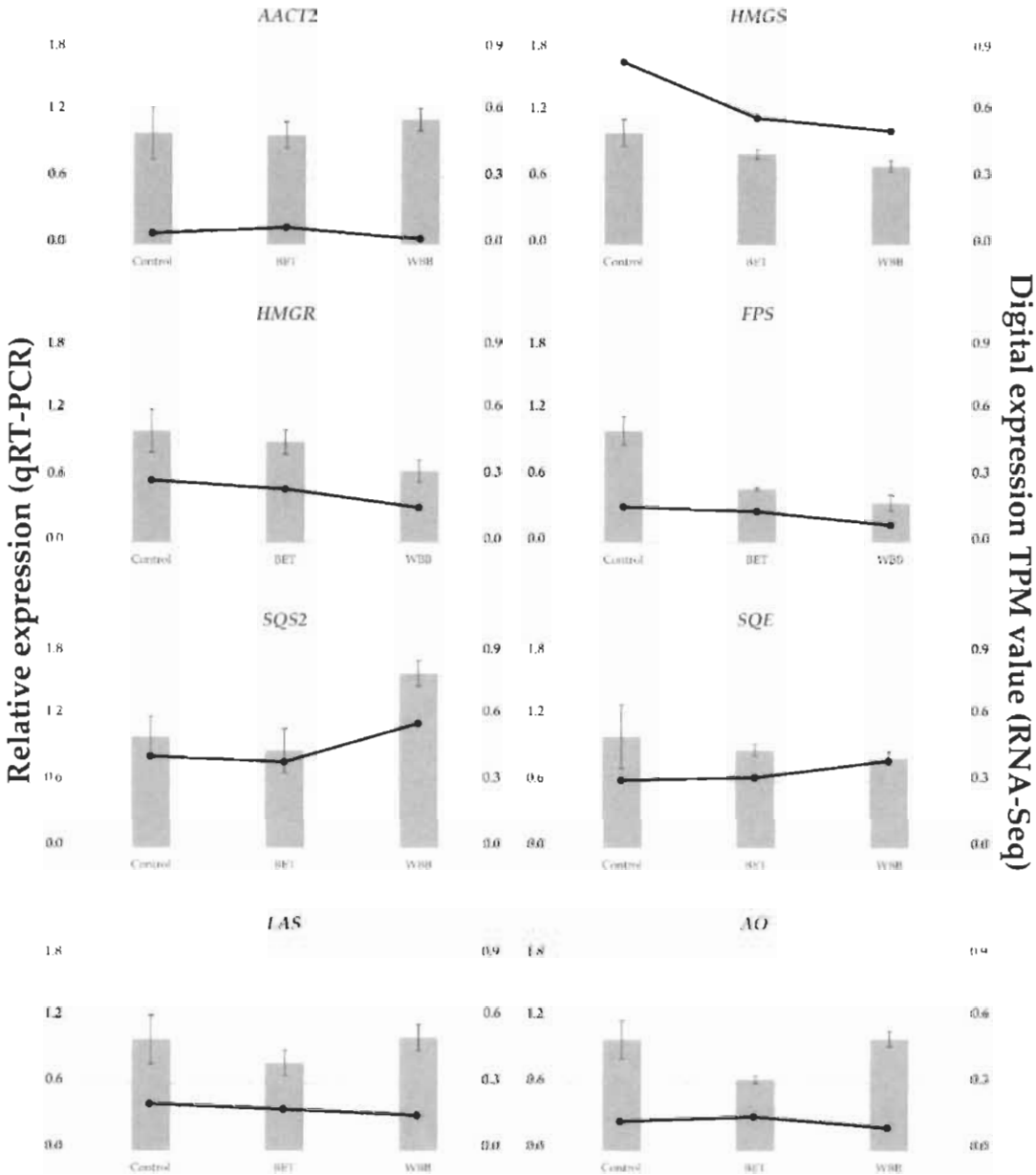


**Figure 8.** Comparison of expression profiles of eight representative transcripts from *I. obliquus* cells supplemented without (control) or with betulin (BET) or white birch bark (WBB), as measured by RNA-Seq and quantitative reverse transcription PCR (qRT-PCR). The eight transcripts are assigned to the terpenoid pathway in Appendix A–Figure A1. Columns represent expression determined by qRT-PCR (left *y*-axis), while lines represent digital expression by RNA-Seq in TPM values (right *y*-axis). The *x*-axis indicates different growth conditions (control, BET, and WBB). Graphs are plotted using normalized ddCt values scaled to control. *Centromere protein 3* (CEN3) was used for internal reference. Expression fold change and error bars were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Bars represent the mean standard deviation of three independent replicates. Abbreviations are defined in Appendix A–Figure A1.

Interestingly, qRT-PCR analysis showed a downregulation of *FPS* in the presence of terpenoid substrates, suggesting a negative feedback regulation on this important step in the sesqui and triterpenoid biosynthesis. In contrast, *SQS2* expression increased in the presence of WBB, suggesting a positive regulation. This opposite regulation, negative on *FPS* and positive on *SQS2*, may be due to the presence of terpenoids such as FPP in bark residues. Altogether, the results suggest that few key reactions in the terpenoid pathway are regulated at the transcriptional level in presence of terpenoid substrates in *I. obliquus* cell cultures.

The amounts of valuable terpenoids accumulated remained low under exogenous induction in a fungal culture. The metabolic engineering of microorganisms is an interesting alternate route for the production of these important compounds. To do this, a better understanding of the terpenoid biosynthetic pathway and the genes involved in this pathway is required. However, the lack of genomic information about *I. obliquus* hinders the development of alternative production methods. In this work, the best conditions for growth of *I. obliquus* cells cultivated in presence of terpenoid substrates, betulin or white birch bark were examined, and the corresponding transcriptomes obtained after next-generation sequencing and de novo assembly. Transcriptome analysis identified eighteen transcripts encoding enzymes implicated in the terpenoid metabolism. Comparative analyses of the transcriptomes yielded valuable information with respect to the wide variety of genes implicated in terpenoid metabolism in *I. obliquus* cells under the described conditions. It would be interesting to compare the laboratory-adapted isolate of *I. obliquus* to the wild type fungus to monitor gene expression implicated in the host-pathogen interaction between chaga and its host, birch trees.

## Materials and Methods

### *Chemicals and Reagents Standard*

Standard betulinic acid (97%) was purchased from Adipogen Corp. (San Diego, CA, USA). Standard betulin (98%) was purchased from Sigma-Aldrich (Saint-Louis, MO,

USA). Methanol, acetonitrile and dimethylsulphoxide (DMSO), ethyl acetate, and acetic acid were HPLC grade from Fisher Chemical. The other cited chemicals were of analytical grade. Media components (yeast extract, malt extract, agar, etc.) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

*Fungal Culture and Growth Conditions*

*I. obliquus* was provided and cultured by the Biopterre laboratory (La Pocatière, Qc, Canada). The stock of *I. obliquus* was maintained on a potato dextrose agar (PDA) medium, and the stock culture was stored at 4 °C. The identity of the isolate was confirmed by PCR amplification followed by Sanger sequencing of the ribosomal internal transcribed spacer (ITS) DNA region. The sequence data were deposited to the NCBI database under the GenBank accession number MN239482. To optimize the mycelial growth of *I. obliquus* in laboratory, yeast malt broth (YMB) and yeast malt agar (YMA) media were selected based on previous reported studies (Bai et al. 2012; Xu, Li, and Hu 2014; Zheng et al. 2007). The YMB and YMA contained 3 g. L-1 of yeast extract and 3 g. L-1 of malt extract. Different growth condition parameters of the liquid and solid culture of *I. obliquus*, were tested: Three pH (5, 6.2, and 7.5) and three temperatures (22 °C, 28 °C, and 37 °C) in presence or not of white birch bark (50 µg. mL) or betulin (15 µg. mL).

Optimization of Growth Conditions for Solid Medium

To optimize the mycelial culture on solid media, the effect of pH, temperature, and the presence of white birch bark (50 µg. mL) were investigated. *I. obliquus* was initially grown for 14 days on a YMA medium in petri dish in absence of light. A disc of 1 $cm^2$ was cut after 14 days of culture, and then it was transferred into other plate under different conditions for 14 days in absence of light. The growth diameter was measured, in triplicate, every two days on the Petri dish (Chen et al. 2009; Liu, Fu, and Chen 2011b). In order to sustain the culture of *I. obliquus,* mycelium was maintained on YMA Petri dishes for three months in an incubator at 28 °C and then stored at 4 °C. Based on

preliminary results obtained from the optimization of growth in a solid culture, and in order to reduce the parameters in liquid cultures, the optimization of the liquid cultures of *I. obliquus* was carried out at 28 °C, which yielded the best growth of the isolate.

Optimization of Growth Conditions for Liquid Medium

To initiate a liquid culture of *I. obliquus*, a one-centimeter$^2$ disc was collected from a fresh solid culture of mycelium in a YMA medium. The mycelium pre-culture was grown in flask of 250 mL with 30 mL of yeast malt broth (YMB) at 28 °C on a rotary shaker incubator at 150 rpm for 8 days. Then, to investigate the effect of pH (5, 6.2 and 7.5), an aliquot of 10 mL of the mycelium was transferred to 250 mL flask containing 100 mL of YMB at different pH, incubated at 28 °C on a rotary shaker (Thermofisher Scientific) at 150 rpm in absence of light for 8 days. The mycelium was sampled every 48 h by sequential filtration, washed several times with distilled water, and then was dried overnight at 70 °C.

*Isolation of RNA*

Ten mL aliquote of 8 days *I. obliquus* liquid pre-culture were aseptically added to 250 mL flask containing 100 mL of a YMB medium supplemented or not with 0.2% DMSO (Control), 15 µg. mL of betulin (BET), or 50 µg. mL of white birch bark (WBB), and grown for 8 days on a rotary incubator at 150 rpm and 28 °C in absence of light. The *I. obliquus* mycelium was collected after 8 days of growth. The mycelium was filtered, frozen with liquid $N_2$, and then placed in a 2 mL tube with 2 mL of TRIzol reagent (Fisher Scientific, Toronto, Canada). The mycelia were crushed using the TissueLyser II (QIAGEN, Qiagen Retsch GmbH, Hannover, Germany) using the stainless steel beads with 5 mm diameter (QIAGEN, Qiagen Retsch GmbH, Hannover, Germany) at speed of 30 strokes per second for 3 min until a homogeneous sample was obtained. The tubes were incubated on ice for 5 min, and then 400 µL of chloroform were added. The tubes were vigorously vortexed for 15 s and incubated in an ice bath for 3 min. After centrifugation at 13,000× *g* for 15 min at 4 °C, two phases were obtained, which were separated by

cellular debris – the clear phase of the supernatant was recovered and transferred into another clean 1.5 mL Eppendorf tube. RNA was precipitated with the addition of 700 µL isopropanol, followed by incubation at −20 °C for 3 h. Finally, precipitated RNA was collected by centrifugation at 13,000× *g* for 15 min at 4 °C, and the pellets were washed once with 1 mL of ice cold 75% ethanol and air dried briefly at room temperature. Then, they were centrifuged again at 13,000× *g* for 5 min at 4 °C. The RNA pellets were resuspended using 20 µL of nuclease free water. The quality and quantity of RNA extracted from the samples under different conditions were verified using the Nanodrop and the Bioanalyzer. Only samples with an RNA Integrity number higher than 8 were selected for Illumina sequencing.

*Transcriptome Sequencing de Novo Assembly and Functional Annotation*

Sequencing was performed using Illumina HiSeq 4000, PE 100 paired ends, at McGill University and Genome Québec Innovation Centre (Montreal, QC, Canada) on cDNA Libraries converted from isolated high-quality mRNA. Among the raw reads obtained from Illumina sequencing, low quality reads, reads with unknown nucleotides, and reads with adapters were found (Table 3). Trimmomatic was used to remove the adapters from sequencing and trimmed reads from the 3' end and further filtered all reads below 50 bp in order to obtain clean reads (Bolger, Lohse, and Usadel 2014). However, once the data from the surviving pairs were generated, Trinity normalization was performed to remove redundant reads in datasets without affecting its *k*-mer content (Brown et al. 2012). De novo assembly of cleaned and standardized reads was performed using Trinity (v2.6.5) assembler (Grabherr et al. 2011). Trinity method assembles the RNA-Seq reads into full-length transcripts, which are called contigs or unigenes. All the unigenes were aligned to the public protein database uniprot_sprot.trinotate_v2.0.pep protein database using the BLASTX program against the NCBI BLAST families using an E-value of $10^{-5}$. Top BLAST hits were used for annotation of component/gene for each transcript. To quantify the gene transcript abundance, the raw RNA-Seq reads were mapped to assembled transcripts with Bowtie (Langmead et al. 2009) using default

parameters. The gene transcript abundance was calculated as transcripts per kilobase million (TPM) using the RSEM package (Li and Dewey 2011).

**Table 3.** List of *Inonotus obliquus* RNA-Seq library sequenced using Illumina HiSeq4000 deposited to the NCBI Sequence Read Archive under the accession PRJNA526077.

| Library | Nb of Reads | Nb of Bases |
|---|---|---|
| RNA-Seq of *Inonotus obliquus* cell culture: Control 1 | 41,874,533 | 8,374,906,600 |
| RNA-Seq of *Inonotus obliquus* cell culture: Control 2 | 19,534,645 | 3,906,929,000 |
| RNA-Seq of *Inonotus obliquus* cell culture: Control 3 | 22,008,711 | 4,401,742,200 |
| RNA-Seq of *Inonotus obliquus* cell culture: Betulin1 | 25,264,465 | 5,052,893,000 |
| RNA-Seq of *Inonotus obliquus* cell culture: Betulin2 | 20,205,852 | 4,041,170,400 |
| RNA-Seq of *Inonotus obliquus* cell culture: Betulin3 | 22,305,852 | 4,461,046,200 |
| RNA-Seq of *Inonotus obliquus* cell culture: White birch bark 1 | 17,766,031 | 3,553,206,200 |
| RNA-Seq of *Inonotus obliquus* cell culture: White birch bark 2 | 16,318,641 | 3,263,728,200 |
| RNA-Seq of *Inonotus obliquus* cell culture: White birch bark 3 | 28,913,696 | 5,872,739,200 |
| RNA-Seq of *Inonotus obliquus* cell culture: White birch bark 4 | 15,777,829 | 3,155,565,800 |
| Total | 229,970,255 | 46,083,926,800 |

Functional annotations of unigenes were performed using Trinotate (http://trinotate.github.io/), by aligning our transcripts to Swiss Institute of Bioinformatics databases (Swiss-Prot) with BLASTX and by identifying protein domains (HMMER/PFAM), protein signal peptides, transmembrane domains prediction (signalP/tmHMM), and Clusters of Orthologous Groups (COG). Then, the functional annotation of unigenes was compared to currently curated annotation databases (EMBL Uniprot eggNOG/GO Pathways databases). The Trinity assembly and functional annotation of unigenes were integrated as an annotation report into an SQLite database.

Gene Ontology (GO) annotations for our unigenes were obtained using the Swiss-Prot. The WEGO 2.0 software was used to obtain the GO functional classification for all unigenes and to better understand the distribution of gene functions in *I. obliquus* at the macro level. All annotated sequences were linked to the GO terms in the database, and a quantification of the number of sequences for each of the different terms was

subsequently calculated. The raw reads under the accession number PRJNA526077 were deposited to the Sequence Read Archive (Leinonen et al. 2011).

*qRT-PCR Analysis*

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on *I. obliquus* in different culture conditions (control, with white birch bark (WBB) or with betulin (BET). The qRT-PCR assay was performed using a CFX connect Real-Time System by BioRad and analyzed using BioRad CFX Maestro 1.1. (BioRad laboratories) including statistics analysis using an ANOVA.

Two micrograms of total RNA extracted from different culture conditions were reverse transcribed into single-stranded complementary DNA using the High-Capacity RNA-to-cDNA™ Kit supplied by Thermo Fisher Scientific, following their protocol. qRT-PCR was performed in triplicates in a 10 µL total volume reaction containing 6 µL of SYBR Lo-ROX mix from Bioline SensiFAST SYBR Lo-ROX mix kit (FrogaBio, Toronto, ON, Canada), 2 µL cDNA and 2 µL of 200 µM of specific primers. The primers were designed using the conserved sequences of the variant of the transcripts selected using Clustal tool on Galaxy platform. The primers were aligned to the target genes sequences using the Integrated DNA Technology (www.idtdna.com), Primer3 and Tm Calculator (New England Biolabs, tmcalculator.neb.com) to select suitable annealing temperatures for each primer. The qRT-PCR was performed for the following gene transcripts: *Acetoacetyl-CoA synthetase (AACS), hydroxymethylglutaryl-CoA synthase A (HMGS); 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), farnesyl pyrophosphate synthase (FPS), squalene synthase (SQS), squalene monooxygenase (SQE), β-amyrin synthase (βAS)* and *lanosterol synthase (LAS).* The primers used for the qRT-PCR assay are listed in Table 2.4. qRT-PCR data were standardized using reference genes. From the transcriptomic data of *I. obliquus*, we selected as reference genes, the two genes with lowest variability in their expression under the different culture conditions. The Ct values for the seven genes of interests were normalized to the Ct value of the reference gene *centromere protein 3 (CEN3*) and *glutamine RNA ligase* (*GluRL*).

**Table 4.** Primers used for qRT-PCR validation of gene expression.

| Gene | Forward Primer (5′-3′) | Reverse primer (5′-3′) | Product Size (bp) |
|---|---|---|---|
| *AACT2* | CCGATCACTGTGAAGGGTAAG | TGGTGCGATAGGGAAATCTATG | 273 |
| *HMGS* | CAGTGTCGACTACCCCGTTT | GTGTACATGTTTCCGCAACG | 280 |
| *HMGR* | GTCGTTCCTGGTGTGAGGTT | GCGTCTTAGTGGCCAGAGTC | 243 |
| *FPS* | TCATGCACGAAACGACTCTC | CCGAAGCAGTCGAGGTAGTC | 276 |
| *SQS2* | CTTCGAGGGTTGGACACAAT | GACGTCGCGGTAAGAAGAAG | 282 |
| *SQE* | GTCCTGTCCTACTCTACCAAATC | GTCATTCCACCTCCAGTCAA | 289 |
| *LAS* | GCCTGAAGGCTGTGCTTTAC | TCTGCAAGGAAAGCATTGTG | 246 |
| *AO* | GCTTACTCCTGCGTTCAGTAA | TCCGAAACCTCCTCCATAGT | 375 |
| *CEN3* | AGGTCGACCGAGAAGTCG | CTTGAACTTTCTTACGTTTG | 300 |

The amplification was carried out under the following conditions: 2 min of polymerase activation at 95 °C, followed by 39 cycles of 5 sec of denaturation at 95 °C, 10 sec of annealing, and 30 s of extension and fluorescence data acquisition at 72 °C were measured. After verifications of unique and clear melt curve and unique band obtained on agarose gel electrophoresis, RT-qPCR efficiency and linearity were taken into account for next step of qRT-PCR. The percentage of efficiency qRT-PCR was chosen between 90% and 110% and a standard curve correlation coefficient (R2) ≥96 (Kennedy and Oswald 2011).

*Accession Numbers*

The sequences described in this paper have been deposited in the National Center for Biotechnology Information Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/) under the accession number PRJNA526077. Gene transcript sequences were deposited in Genbank with the following accession numbers for nucleotide sequences: Acetoacetyl-CoA transferase 1 (MK825552); acetoacetyl-CoA transferase 2 (MK825553); HMG-CoA synthase (MK825554); HMGR, HMG-CoA reductase; (MK825555); MVA kinase (MK825556); phosphoMVA kinase (MK825557); diphosphoMVA decarboxylase (MK82558); IPP isomerase (MK825559);

FPP synthase (MK825560); Muurolene synthase 1 (MK825561); Muurolene synthase 2 (MK825562); Protoilludene synthase (MK825563); Squalene synthase 1 (MK825564); Squalene synthase 2 (MK825565); Squalene epoxidase (MK825566); Lanosterol synthase 1 (MK825567); Lanosterol synthase 2 (MK825568); 11-oxo-β-amyrin 30-oxidase (MK825569); Centromere protein 3 (MK825570); Glutamine RNA ligase (MK825571) and Glyceraldehyde 3-phosphate deshydrogenase (MK825572).

## Conclusions

This research allowed us to define the best growth conditions to conduct qRT-PCR and RNASeq de novo assembly of Québec *Inonotus obliquus*. The best growing conditions in liquid and solid cultures were determined for optimal production yield of *Inonotus obliquus* mycelia. It was found that optimal conditions were obtained at pH 6.2, 28 °C, and using YMB as a culture medium. In this study, we provide the frst transcriptomic report generated by next-generation sequencing (Illumina HiSeq4000) of the medicinal fungus chaga, *I. obliquus*, grown under different conditions. The assembled transcriptomes generated 196,273 transcripts. High-quality transcriptomes were obtained, and based on genes annotations, we were allowed to identify of transcripts encoding 18 enzymes playing a key role in the terpenoid pathway. This led us to identify novel gene sequences encoding biosynthetic enzymes involved in the terpenoid pathway in chaga. The transcriptomic analysis of this medicinal fungus has led to the discovery of new genes that can be used as a reference for future genetic and genomic studies on chaga and other related medicinal species. Moreover, this transcriptome assembly may also be used as a reference platform for studies on fungus Hymenochaetaceae family. Beyond the biological impact, the data can be used as a reference to provide gene sequences for metabolic engineering and should pave the way for advanced fungal biology and biotechnology to produce valuable chaga terpenoids. pave the way for advanced fungal biology and biotechnology to produce valuable chaga terpenoids.

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**Conflicts of Interest:** The authors declare no conflict of interest.

**Abbreviations**

| | |
|---|---|
| YMA | Yeast malt agar |
| YMB | Yeast malt broth |
| WBB | White birch bark |
| HMG-CoA | 3-hydroxy-3-methylglutaryl-CoA |
| HMGR | 3-hydroxy-3-methylglutaryl-CoA reductase |
| MVA | mevalonic acid |
| MVAP | MVA phosphate |
| MVAPP | MVA diphosphate |
| IPP | isopentenyl diphosphate |
| DMAPP | dimethylallyl diphosphate |
| GPP | geranyl diphosphate |
| FPP | farnesyl diphosphate |
| AACT | Acetoacetyl-CoA transferase |
| HMGS | HMG-CoA synthase |
| HMGR | HMG-CoA reductase |
| MVK | MVA kinase |
| PMK | phosphoMVA kinase |
| PMD | diphosphoMVA decarboxylase |
| IPI | IPP isomerase |
| FPS | FPP synthase |
| MUS | Muurolene synthase |
| PRS | Protoilludene synthase |
| SQS | Squalene synthase |
| SQE | Squalene epoxidase |
| AS | Amyrin synthase |
| AO | 11-oxo-β-amyrin 30-oxidase |
| LAS | Lanosterol synthase |
| LUS | Lupeol synthase |

## Appendix A. Supplementary Information

In fungi, terpenoid biosynthesis occurs via the mevalonate (MVA) metabolic pathway (Miziorko 2011; Quin, Flynn, and Schmidt-Dannert 2014; Schmidt-Dannert 2015; Cordell 1976) which can be divided into three parts (Appendix A–Figure A1). The first part (precursor pathway) involves a series of enzymatic reactions converting acetyl-CoA to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) which are the precursors of all terpenoids. For the second part (sesquiterpenoid pathway), the cytosolic farnesyl diphosphate synthase (FPS) condenses two molecules of IPP with one molecule DMAPP head-to-tail sequentially to produce farnesyl diphosphate (FPP). FFP serves as a precursor for sesquiterpenoids, which are synthesized by terpene synthases and can be decorated by other various enzymes. For example, the cyclization of FPP by characterized fungal sesquiterpenoid synthases such as muurolene synthase (MUS) or protoilludene synthase (PRS) followed by subsequent reactions yield diverse sesquiterpenoids. MUS catalyzes the 1,10 cyclization of FPP to α-muurolene and germacrene A, and six corresponding genes (Cop1–6) were identified through mining of the genome of Coprinus cinerea (Agger, Lopez-Gallego, and Schmidt-Dannert 2009; Lopez-Gallego, Wawrzyn, and Schmidt-Dannert 2010; Engels et al. 2011). Lastly, the third part of the pathway involves reactions catalyzing the formation of three groups of triterpenoids: The amyrin-type, the lanosterol-type, and the lupeol-type. Briefly, at the endoplasmic reticulum, squalene synthase (SQS) catalyzes the condensation of two FPP molecules to produce squalene, which is the first committed precursor for triterpenoids and steroids biosynthesis. Squalene epoxidase (SQE) catalyzes the oxide squalene formation, which is modified by various tailoring enzymes. For example, lanosterol is formed from oxide squalene by a cyclization reaction catalyzed by lanosterol synthase (LAS) and is further metabolized into triterpenoids such as inotodiol (Lariviere and Desgagné-Penix 2017; Zou et al. 2016a). To date, only the SQS sequence has been identified, cloned and characterized from I. obliquus (Appendix A–Figure A1) (Zhang et al. 2016).

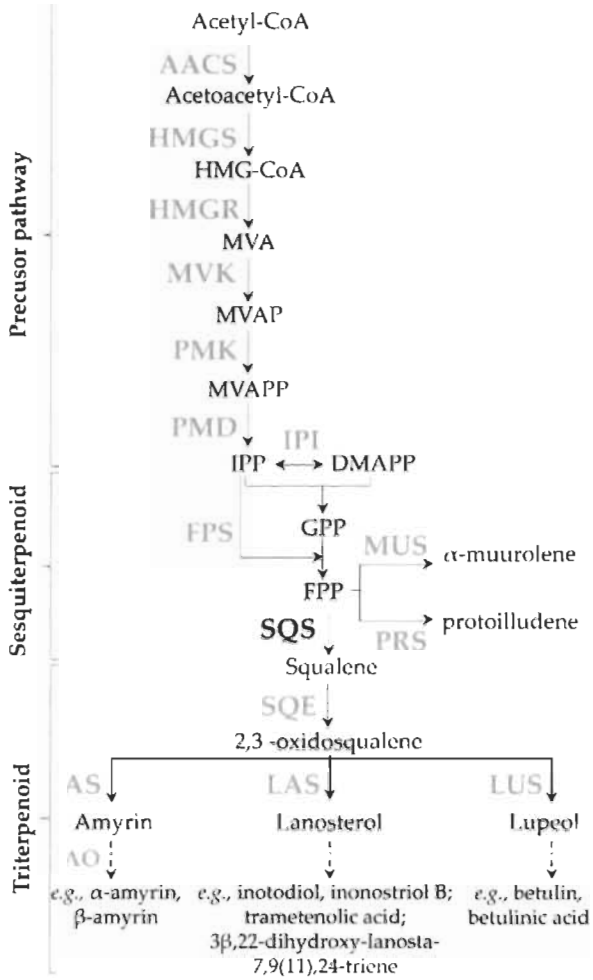


**Figure A1.** Proposed biosynthetic pathway leading to multiple terpenoids in *I. obliquus*. Enzymes for which corresponding genes have been isolated from *Inonotus* are shown in bold black whereas the ones from other species are shown in bold grey. Broken arrow represents more than one biochemical reaction. Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVA, mevalonic acid; MVAP, MVA phosphate; MVAPP, MVA diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; AACT, Acetoacetyl-CoA transferase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MVK, MVA kinase; PMK, phosphoMVA kinase; PMD, diphosphoMVA decarboxylase; IPI, IPP isomerase; FPS, FPP synthase; MUS, Muurolene synthase; PRS, Protoilludene synthase; SQS, Squalene synthase; SQE, Squalene epoxidase; AS, Amyrin synthase; βAO, 11-oxo-β-amyrin 30-oxidase; LAS, Lanosterol synthase; LUS, Lupeol synthase.

**Table A1.** Results of Illumina sequencing output and assembly for *Inonotus obliquus*.

| **Read Trimming and Clipping of Adapters** | |
|---|---|
| Raw paired reads [a] | 229,970,255 |
| Surviving paired reads [b] | 219,288,500 |
| Surviving paired reads (%) [c] | 95.36 |
| **Trinity de novo assembly** | |
| Nb. Transcripts [d] | 196,273 |
| Nb. Components [d] | 42,787 |
| Total Transcripts Length (bp) | 494,954,509 |
| Max. Transcript Length (bp) | 2338 |
| Min. Transcript Length (bp) | 201 |
| Median Transcript Length (bp) | 1953 |
| Mean Transcript Length (bp) | 2521 |
| N50 (bp) [e] | 4052 |
| GC percentage (%) | 50.07 |
| **BLAST annotation and filtered annotated components** | |
| Nb. Transcripts | 20,072 |
| Nb. Components | 2224 |
| Total Transcripts Length (bp) | 90,372,815 |
| Max. Transcript Length (bp) | 19,856 |
| Min. Transcript Length (bp) | 299 |
| Median Transcript Length (bp) | 4074 |
| Mean Transcript Length (bp), | 4502 |
| N50 (bp) [f] | 5512 |
| GC percentage (%) | 49.50 |

[a] Number of paired reads obtained from the sequencer. [b] Number of remaining paired reads after the trimming step. [c] Percentage of surviving paired reads/Raw Paired Reads. [d] Trinity has created a list of transcripts (contigs). The transcripts are grouped in components loosely representing genes. Transcript names are prefixed by the component/gene name, e.g., transcripts c115_g5_i1 and c115_g5_i2 are derived from the same isolated de Bruijn graph and therefore share the same component/gene number c115_g5. [e] Corresponding contig length distribution N50 = 4052 bp (Figure 4). [f] Corresponding contig length distribution N50 = 5512 bp.

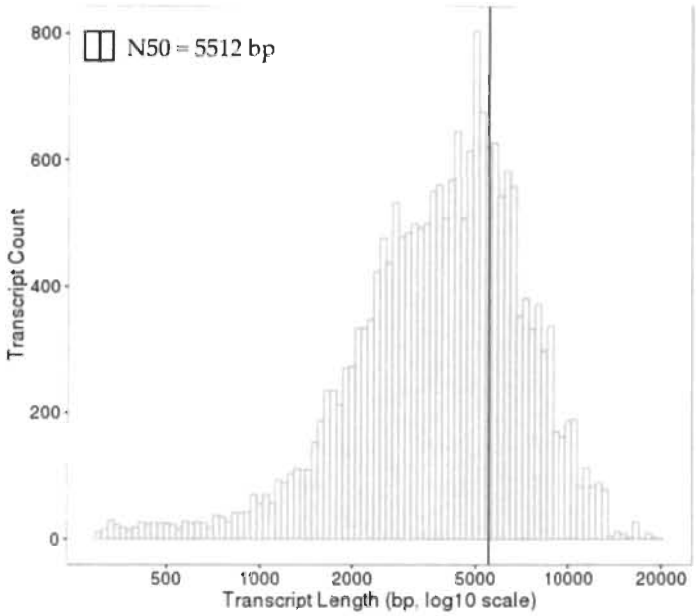


**Figure A2.** Sequence length distribution of transcripts of *I. obliquus* after Trinity de novo assembly.

**Table A2.** Top 25 up-regulated transcripts of *Inonotus obliquus* library in library of cells cultivated in presence of betulin.

| Rank | Log2 FC | Description | E-Value | Accession |
|---|---|---|---|---|
| 1 | 1.66 | Phosphatidylinositol 4-phosphate 3-kinase | 1.0 | O70173.1 |
| 2 | 1.65 | Alpha-ketoglutarate-dependent xanthine dioxygenase | $3 \times 10^{-28}$ | Q4QZZ9.1 |
| 3 | 1.59 | Probable lysosomal cobalamin transporter | 0.79 | Q8K0B2.1 |
| 4 | 1.57 | Major vault protein beta | 0.53 | P54659.1 |
| 5 | 1.48 | Diphthine methyl ester synthase | 9.8 | Q7S949.1 |
| 6 | 1.46 | ATP-dependent RNA helicase DEAH12 | $7 \times 10^{-38}$ | F4KGU4.1 |
| 7 | 1.44 | No hit | | |
| 8 | 1.44 | No hit | | |
| 9 | 1.43 | No hit | | |
| 10 | 1.42 | No hit | | |
| 11 | 1.40 | Superoxide dismutase [Mn] | 8.2 | Q9UQX0.1 |
| 12 | 1.40 | Orotate phosphoribosyltransferase | 6.1 | A6VLF2.1 |
| 13 | 1.33 | No hit | | |
| 14 | 1.32 | Caffeic acid 3-O-methyltransferase | 1.5 | Q43239.1 |
| 15 | 1.32 | No hit | | |

| Rank | Log2 FC | Description | E-Value | Accession |
|---|---|---|---|---|
| 16 | 1.31 | 30S ribosomal protein S7 | 0.23 | B3EUF4.1 |
| 17 | 1.29 | No hit | | |
| 18 | 1.29 | No hit | | |
| 19 | 1.28 | No hit | | |
| 20 | 1.28 | No hit | | |
| 21 | 1.27 | No hit | | |
| 22 | 1.27 | D-xylulose reductase | $2 \times 10^{-13}$ | Q07993.1 |
| 23 | 1.27 | No hit | | |
| 24 | 1.26 | No hit | | |
| 25 | 1.26 | No hit | | |

**Table A3.** Top 25 down-regulated transcripts of *Inonotus obliquus* library in library of cells cultivated in presence of betulin.

| Rank | Log2 FC | Description | E-Value | Accession |
|---|---|---|---|---|
| 1 | −1.61 | Isoleucine-tRNA ligase | $1 \times 10^{-42}$ | Q21926.1 |
| 2 | −1.55 | N-acetyltransferase eso1 | $2 \times 10^{-20}$ | O42917.1 |
| 3 | −1.52 | Probable inactive purple acid phosphatase 16 | 0.47 | Q9SR79.1 |
| 4 | −1.48 | No hit | | |
| 5 | −1.45 | ATPase family AAA domain-containing protein 1 | $2 \times 10^{-52}$ | F6QV99.2 |
| 6 | −1.44 | E3 SUMO-protein ligase NSE2 | $1 \times 10^{-8}$ | Q7ZXH2.1 |
| 7 | −1.42 | No hit | | |
| 8 | −1.42 | Cyclin-dependent kinase 1 | 0.21 | P04551.1 |
| 9 | −1.42 | H/ACA ribonucleoprotein complex subunit NHP2 | $3 \times 10^{-48}$ | P32495.2 |
| 10 | −1.40 | Structural maintenance of chromosomes protein 1 | $2 \times 10^{-31}$ | O94383.2 |
| 11 | −1.39 | No hit | | |
| 12 | −1.38 | No hit | | |
| 13 | −1.38 | Tyrosine-protein kinase receptor ver-4 | 1.8 | Q21041.2 |
| 14 | −1.37 | No hit | | |
| 15 | −1.37 | Proteasome-activating nucleotidase 2 | 2.4 | Q5UT56.1 |
| 16 | −1.37 | Structural maintenance of chromosomes protein 2 | 0.0 | P41003.2 |
| 17 | −1.36 | Structural maintenance of chromosomes protein 6 | $3 \times 10^{-159}$ | P53692.1 |

| Rank | Log2 FC | Description | E-Value | Accession |
|---|---|---|---|---|
| 18 | −1.36 | Condensin complex subunit 3 | $4 \times 10^{-110}$ | Q10429.1 |
| 19 | −1.36 | No hit | | |
| 20 | −1.36 | Deoxynucleoside triphosphate triphosphohydrolase | $1 \times 10^{-102}$ | Q0VCA5.1 |
| 21 | −1.35 | NFX1-type zinc finger-containing protein 1 | $5 \times 10^{-42}$ | Q8R151.3 |
| 22 | −1.35 | Cysteine-rich receptor-like protein kinase 41 | 0.001 | O23081.2 |
| 23 | −1.34 | U3 small nucleolar RNA-associated protein 3 | 0.40 | Q12136.1 |
| 24 | −1.34 | No hit | | |
| 25 | −1.33 | H/ACA ribonucleoprotein complex subunit cbf5 | $9 \times 10^{-102}$ | O43102.1 |

**Table A4.** Top 25 up-regulated transcripts of *Inonotus obliquus* library of cells cultivated in presence of white birch bark.

| Rank | Log2 FC | Description | E-Value | Accession |
|---|---|---|---|---|
| 1 | 6.09 | Phosphatidylinositol 4-phosphate 3-kinase | 1.0 | O70173.1 |
| 2 | 5.89 | Major vault protein beta | 0.53 | P54659.1 |
| 3 | 5.89 | No hit | | |
| 4 | 5.75 | DNA-directed RNA polymerase subunit beta | 1.1 | Q0ANP4.1 |
| 5 | 5.75 | No hit | | |
| 6 | 5.71 | Acetyl-CoA acetyltransferase IB | 2.0 | Q04677.3 |
| 7 | 5.70 | No hit | | |
| 8 | 5.70 | Orotate phosphoribosyltransferase | 6.1 | A6VLF2.1 |
| 9 | 5.67 | Methyl-CpG-binding domain-containing protein 11 | 2.5 | Q9LW00.1 |
| 10 | 5.64 | F-box DNA helicase 1 | 2.6 | F1ND48.2 |
| 11 | 5.62 | No hit | | |
| 12 | 5.59 | No hit | | |
| 13 | 5.58 | No hit | | |
| 14 | 5.56 | Glutamyl-tRNA reductase | 1.5 | A4SV44.1 |
| 15 | 5.54 | No hit | | |
| 16 | 5.53 | Segregation and condensation protein A | 4.5 | P47455.1 |
| 17 | 5.49 | Nascent polypeptide-associated complex subunit beta | 1.2 | A5DF06.2 |
| 18 | 5.48 | No hit | | |

| Rank | Log2 FC | Description | E-Value | Accession |
|---|---|---|---|---|
| 19 | 5.48 | Glucose-6-phosphate isomerase | 0.39 | Q83XM3.1 |
| 20 | 5.47 | Caffeic acid 3-O-methyltransferase | 1.5 | Q43239.1 |
| 21 | 5.44 | No hit | | |
| 22 | 5.43 | No hit | | |
| 23 | 5.43 | Protein AegA | 2.7 | P37127.2 |
| 24 | 5.43 | No hit | | |
| 25 | 5.43 | No hit | | |

**Table A5.** Top 25 down-regulated transcripts of *Inonotus obliquus* library of cells cultivated in presence of white birch bark.

| Rank | Log2 FC | Description | E-value | Accession |
|---|---|---|---|---|
| 1 | −5.03 | No hit | | |
| 2 | −4.91 | Tryptophan synthase alpha chain | 4.6 | P50382.2 |
| 3 | −4.30 | No hit | | |
| 4 | −4.15 | No hit | | |
| 5 | −3.98 | No hit | | |
| 6 | −3.76 | No hit | | |
| 7 | −3.75 | No hit | | |
| 8 | −3.74 | No hit | | |
| 9 | −3.71 | No hit | | |
| 10 | −3.69 | GTP-binding protein EngA | 7.9 | Q3AAU6.1 |
| 11 | −3.67 | No hit | | |
| 12 | −3.65 | Uncharacterized protein C12orf29 homolog | 6.0 | Q8BHN7.1 |
| 13 | −3.60 | Protein numb homolog | 0.33 | P49757.2 |
| 14 | −3.56 | No hit | | |
| 15 | −3.56 | No hit | | |
| 16 | −3.53 | No hit | | |
| 17 | −3.53 | Leucine-rich repeat transmembrane protein FLRT1 | 5.7 | Q6RKD8.2 |
| 18 | −3.51 | Manganese peroxidase H4 | $1 \times 10^{-132}$ | P19136.1 |
| 19 | −3.51 | No hit | | |
| 20 | −3.50 | L-ascorbate peroxidase, cytosolic | 3.0 | P48534.2 |
| 21 | −3.45 | No hit | | |
| 22 | −3.44 | No hit | | |
| 23 | −3.43 | DNA polymerase epsilon catalytic subunit A | 1.7 | Q9WVF7.3 |
| 24 | −3.41 | Putative lipase C4A8.10 | 0.001 | O14162.2 |
| 25 | −3.39 | Glutamate racemase | 3.4 | P63638.1 |

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# CHAPITRE III

# FIRST BIOTRANSFORMATION TESTS OF BETULIN TO BETULINIC ACID USING MYCELIUM FROM *INONOTUS OBLIQUUS*

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## 3.1 Contribution des auteurs

Le projet a été conçu par Yacine BOUMGHAR, Hugo GERMAIN et Isabel DESGAGNÉ-PENIX et réalisé au Centre d'études des procédés chimiques du Québec (CÉPROCQ). La méthodologie a été réalisée par Narimane FRADJ, Yacine BOUMGHAR, Hugo GERMAIN, Isabel DESGAGNÉ-PENIX. L'analyse des résultats a été complétée par Narimane FRADJ, Yacine BOUMGHAR et Isabel DESGAGNÉ-PENIX. La rédaction et la correction du court communiqué ont été faites par Narimane FRADJ, Isabel DESGAGNÉ-PENIX, Hugo GERMAIN et Yacine BOUMGHAR. L'obtention des fonds a été réalisée par Yacine BOUMGHAR. La supervision du projet de recherche a été assurée par Yacine BOUMGHAR, Isabel DESGAGNÉ-PENIX et Hugo GERMAIN.

## 3.2 Article complet (anglais) : First biotransformation tests of betulin to betulinic acid using mycelium from *Inonotus obliquus*


### Abstract

Triterpenoid like betulin, betulinic acid and lupeol found in many plant and microorganism species has attracted attention due to their important pharmacological properties, such as antimicrobial, anti-cancer and anti-HIV activities. In order to develop a bioconversion process instead of a chemical approach, the biotransformation process using fungi and white birch bark was studied in this research. Three different fermentation media without substrate, in presence of betulin or in the presence of white birch bark were investigated in the presence of *I. obliquus* (chaga) mycelium liquid culture. The extraction of metabolites from the mycelium of the chaga resulting from biotransformation was performed. The products a were quantified and identified using LCMS-QTOF. As a result, a small amount of betulin and negligible amount of betulinic acid were measured from the extraction of mycelium with no substrate available. For the fermentation medium with betulin, betulin was found to be at a higher concentration, and a slight increase in betulinic acid production has been observed. In the case of fermentation in the presence of white birch bark, we noted that *I. obliquus* was able to produce a greater amount of triterpenoids such as betulin, betulinic acid and lupeol.


### Introduction

Specialized metabolites are characterized by an impressive chemical and biological diversity of properties. They are organic molecules produced by specific species or tissues from plants, fungi, microbes, marine organisms or animals. For the past 15 years, interest in plant-derived drugs has increased and has risen steadily. Therefore, researchers have studied the potential of these natural products to treat diseases such as cancer or AIDS (Yogeeswari et al. 2005). Triterpenoids are specialized metabolites widely distributed in living organism, which display various biological activities and pharmaceutical proprieties (Zhao et al. 2018; Kumar et al. 2017). For exemple, betulin ((3β-lup-20(29)-en-3,28-diol) and betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) are naturally

occurring triterpenoids found in the bark of birch trees (genus *Betula*) in varying concentration (Scheffler 2019). Betulin and betulinic acid have many pharmacological properties including anti-cancer, anti-inflammatory and antioxidant activities (Zhao, Yan et al. 2007, Zhao, Li et al. 2018, Scheffler 2019). Betulinic acid and its derivatives shown various pharmaceutical proprieties including anti-cancer, anti-HIV, antiviral, anti-inflammatory, fungicidal, antimicrobial, anti-malarial, anti-leishmaniasis, anthelmintic and antiseptic activities (Kolobova et al. 2019). Indeed, betulinic acid is considered as a very promising anticancer agent due to its selective cytotoxicity and high therapeutic ratio, which is in contrast to the majority of antitumor agents used in chemotherapy (Liu, Fu et al. 2011, Ali-Seyed, Jantan et al. 2016). In birch bark, betulin can be found in large amount whereas betulinic acid is only detected at low concentration (Liu, Fu et al. 2011, Kolobova, Pakrieva et al. 2019). In 1997, Darrick S. H. L. L. Kim et al. demonstrated that by using a chemosynthetic reaction, betulinic acid was obtained from betulin (Kim, Chen et al. 1997). Although the transformation of betulin into betulinic acid is possible, various challenges remain to make it an efficient and cost-effective process. These challenges include conditions relatives to the process, safety, production costs and pollution problems. In addition, most of the proposed oxidation methods use product with high toxicities. Also, the separation of products containing toxic ions is very laborious and time-consuming (Liu, Fu et al. 2011, Kolobova, Pakrieva et al. 2019). Considering the growing interest for betulinic acid and the disadvantages of chemical transformation, the production of betulinic acid using alternative such as green chemistry or biotransformation is expected to be significant in the foreseeable future (Bastos, Pimentel et al. 2007, Liu, Fu et al. 2011, do Nascimento, Conceição et al. 2019). The biotransformation using microorganisms is a powerful tool to obtain derivatives of organic compounds and it is a cost-effective and sustainable process that does not require the use of toxic solvents. Microorganisms such as fungi have the potential to produce various enzymes capable of catalyzing a wide range of reactions, such as oxidation, hydroxylation reduction, dehydrogenation, epoxidation, demethylation, o-dealkylation, o-methylation and glycosylation (Arruda, Eugênio et al. 2018). Furthermore, biotransformation methods are more stereo and regioselective. In addition, few reactions that can be easily supplemented by in-depth chemical approaches are carried out by

microbial transformation in a simpler way without any toxic solvents (Bastos, Pimentel et al. 2007, Shao, Zhang et al. 2016, Kumar and Dubey 2017). To date, several microorganisms have already been used to transform betulin into betulinic acid such as *Cunninghamella blakesleeana*, *Bacillus megaterium*, *Aspergillus oryzae* AS 3.498, *Armillaria luteo-virens* Sacc QH, *Aspergillus foetidus* ZU-G1, etc. (Bastos, Pimentel et al. 2007, Chen, Liu et al. 2009, Kumar and Dubey 2017). On the other hand, there is not enough information on the biotransformation of betulin into its derivatives using fungi. Triterpenoids are produced from squalene or related cyclic and acyclic precursors of 30 carbon atoms.

*Inonotus obliquus* (chaga) is a rare polyporus medicinal fungus belonging to the family Hymenochaetaceae Donk. It is a parasitic fungus of hardwood species, mainly those of the genus Betula (*Betulaceae*) as well as in some cases those of the genera Quercus (oaks), Populus (poplars), Alnus (alders), Fagus (ash), and Acer (maples), which is found in the Russian Far East, northeast China, North America and other countries at latitudes ranging from 45 to 17 degrees latitude (Ryvarden and Gilbertson 1993, Xu, Zhang et al. 2016, Géry, Dubreule et al. 2018). *Inonotus obliquus* and other polyporous fungi have been used in folk medicine over many years due to the presence of a variety of biologically active compounds in their fruiting bodies. In the literature, Chaga's chemical analysis revealed that various compounds such as polysaccharides, triterpenes and polyphenols are present (Géry, Dubreule et al. 2018, TL, Raj et al. 2019). It has also been shown that *I. obliquus* cultures could produce biologically active metabolites such as sterols and derivatives compounds (TL, Raj et al. 2019). The purpose of this work is to study the possibility of fungal biotransformation of betulin to betulinic acid using *Inonotus obliquus* as a biocatalyst and to investigate the impact of its host, white birch bark, to synthesize compounds that are pharmaceutically relevant. Moreover, this bioprocess will be exploited to develop for the valorization of forest residues.

## Materials and Methods

*Chemicals and reagents Standard*

Standard of betulinic acid (97%) was purchased from Adipogen Corp. (San Diego, USA). Standard of betulin (98%) was purchased from Sigma-Aldrich (Saint-Louis, USA). Methanol, acetonitrile and dimethylsulphoxide (DMSO), ethyl acetate, acetic acid and milli-Q water (LC-MS and HPLC grade) were obtained form Fisher Chemical (Toronto, Ontario, Canada). The other cited chemicals were of analytical grade.

*Fungal culture and growth conditions*

*I.obliquus* was provided and cultured by the Biopterre laboratory (La Pocatière, Qc, Canada). The stock of *I. obliquus* was maintained on yeast malt agar (YMA) medium and stock culture was stored at 4 °C. Then, according to the work of Xu et al., mycelium of *I. obliquus* was cultured for 5 days in a rotary shaker at speed of 150 rpm at 28 °C, in the absence of light (Xu, Zhang, and Chen 2016). The media contained: glucose 20 g. L-1, peptone 3 g. L-1, yeast extract 2 g. L-1, $KH_2PO_4$ 1 g. $L^{-1}$, $MgSO_4$ 1.5 g. L-1, and $CaCl_2$ 0.1 g. L-1 with final pH of 6.2.

*Biotransformation process*

The biotransformation experiments were carried out at 28 °C, in flask of 250 mL, containing 90 mL of fermentation medium with 10 mL of the mycelia culture for 9 days on rotary shaker at 150 rpm and in the absence of light as done by other researchers (Xu et al. 2016; Liu et al. 2011a). In all experiments, substrate controls were composed of sterile medium with betulin and white birch bark and incubated without fungi. For the culture controls of fermentation blanks, fungus was grown under identical conditions without presence of substrate. The fermentation was carried o medium contained : glucose 30 g. L-1, peptone 4 g. L-1, $MgSO_4$ 0.5 g. L-1, $KH_2PO_4$ 1.5 g. L-1, and $CaCl_2$ 0.1 g. L-1 (Xu, Zhang, and Chen 2016).

Biotransformation with betulin

Flasks (250 mL) containing 90 mL of fermentation medium were inoculated with 10 mL of the 6 days precultured fungi of *I. obliquus*, and then the cultures were aseptically supplemented with 0.67 mL of the prepared substrate of betulin (98% betulin dissolved in DMSO 7.5 mg. $mL^{-1}$) and then, transformed on a rotary shaker under the identical conditions for 9 days.

Biotransformation of white birch bark

For fermentation in the presence of white birch bark (WBB), 10 g of WBB was added to a volume of 1L of fermentation medium and sterilized for 21min at 121 °C. Afterwards, aseptically, 90 mL of the fermentation medium with WBB was transferred to the 250 mL flacks. Then, 10 mL of mycelia preculture were added. At the end, the mycelia were transformed under the same conditions on a rotary shaker for 9 days.

*Preparation and isolation of crude triterpenoid*

According to the method described by Zhang et al., the triterpenoid concentration of *Inonotus obliquus* at different selected fermentation conditions was determined (Xu et al. 2016).

After 9 days of fermentation, the mycelium under the different culture conditions is recovered by filtration and rinsed with distilled water until the water is completely discoloured. Residues from white birch bark are also removed from the mycelium. The mycelium of *I. obliquus* was dried in an oven at 70 °C overnight. Once the mycelium was dried, the mycelium is ground with a pestle mortar until a fine powder was obtained. 100 mg of the mycelium powder under the various operating conditions is weighed and placed in 50 mL tubes and then 2 mL of pure isopropanol is added. Using a sonication prob (Branson Sonifier-S-150, Connecticut, USA), the mix (mycelium-isopropanol powder) is homogenized for 40 min with intervals of 4 seconds in an ice bath. Then, 8 mL isopropanol is added to all tubes and they are placed under rotary shake at 200 rpm and 50 °C for 24 h. Steps were repeated three times.

The supernatant is recovered after centrifugation at 10000xg for 6 minutes and then dried using the rotavap. The residues are resuspended using 2 mL of pure methanol in ultrasonic bath, then filtered through a 0.22 µm Millipore filter, before being injected into the LCMS-QTOF.

*LCMS-QTOF determination of triterpenoids from biotransformation*

The standards and samples obtained were analyzed by Agilent 6200 series LCMS-QTOF (Santa Clara, CA, USA) at CEPROCQ's installation. It is stated that the use of a positive mode APCI source is required for the ionization of triterpenes and their identification (Kosyakov et al. 2014). Moreover, the triterpene masses observed by APCI ionization in positive mode are losing the weight of a water molecule. The chromatographic conditions for triterpene analysis were as follows: mobile phase A: 50% Methanol; 49.9% Water and 0.1% formic acid, Mobile phase B: 99.99% Methanol and 0.1% formic acid. The column ZORBAX Eclipse plus C18, fast resolution 4.6X30 mm, 3.5-Micron (Agilent, Santa Clara, CA, USA) was used. Column temperature: 30 °C. Gradient: 0-1 min: 40% B; 1-9 min: 100% B; 9-13 min: 100% B, post-time: 5 min. Time of analysis: 13 min. Injection volume: 1 mL. Flow rate: 1 mL/min. The chromatographic peaks were identified and the content of the three triterpenoids were quantified by comparing the retention time against the known standards of betulin, lupeol and betulinic acid using Agilent MassHunter software. Extraction of the culture medium in the presence of white birch bark was done using the same analytical method to identify and quantify triterpenes.

**Results and discussion**

According to our previous transcriptomic analysis and research (Fradj et al., 2019), *Inonotus obliquus* mycelium was chosen as a biocatalyst to predict the potential betulin and betulinic acid production.

In a previous research, the mycelium of the following filamentous fungi, *Inonotus obliquus*, has been found capable of producing triterpenoids in fermentation media (Xu et al. 2016).

In biotransformation processes, composition of the fermentation medium plays a major role, as different nutrients could influence fungus functional and structural development (Carvalho et al. 2010). In order to investigate the influence of the fermentation medium on the ability of mycelium of *I. obliquus* to produce or biotransform triterpenoids compounds, three different fermentation conditions were considered. The first medium tested in this study was the fermentation medium, the second was the fermentation medium with a content of 10 g white birch bark and the third was with the concentration of 7.5 g. L-1 of betulin dissolved in DMSO. The different substrates were added to the fermentation media at the beginning. The three fermentations were carried out at 28 °C, in flask of 250 mL, containing 90 mL of fermentation medium with 10 mL of the preculture mycelia of *I. obliquus* cultured for 9 days on rotary shaker at 150 rpm, in the absence of light. Choosing this strategy is because mycelium of *Inonotus obliquus* parasites the trunks of white birches and its bark.

In order to identify and quantify the triterpenes produced by the mycelium of *Inonotus obliquus* under the three selected fermentations, an extraction using an organic solvent was carried out. The identification of the biotransformation products has been made based by liquid chromatography in combination with hybrid quadrupole time-of-flight mass spectrometry LCMS-QTOF with a positive mode APCI source for ionisation of triterpenoids compound.

The triterpenoids contents of *I. obliquus* with the different substrate are listed in table 1. The results obtained were carried out in triplicate. The estimated yield obtained for each triterpenoid compound was determined using a dilution curve of standards.

**Table 1.** Main peaks detected in the chromatogram of extraction of *Inonotus obliquus* in different biotransformation conditions.

| N° of compound | Retentions times (min) | m/z $[M+H-H_2O]^+$ | Identification |
|---|---|---|---|
| 1 | 3.958 | 425.3 | Betulin |
| 2 | 4.461 | 439.3 | Betulinic acid |
| 3 | 5.534 | 425.3 | Inotodiol |
| 4 | 9.726 | 409.3 | Lupéol |

According to the results from the extraction of mycelium of *I. obliquus* in the fermentation medium without substrate, peaks corresponding to the masses and retention times of betulin and betulinic acid were found at very low concentrations (Figure 1, table 1)). 35 mg. $L^{-1}$ of betulin and a slight amount of betulinic acid (0.16 mg. $L^{-1}$) were produced (Table 1). The identification of betulin and betulinic acid at low concentration in fungal extracts in the fermentation medium without substrates could be considered further as a signature of the relationship pathogen-fungi. These results could also be explained by the ability of *Inonotus obliquus* to produce triterpenes, because of the presence of all genes encoding for triterpene biosynthesis pathway in this fungus (Fradj et al. 2019).

Regarding the fermentation medium with betulin (Figure 2, table 1), a large peak of betulin and a small increased peak of betulinic acid were observed in the extraction process of triterpenes from *I. obliquus* mycelium. This could be explained through the storage capacity of *I. obliquus* mycelium of betulin contained in the medium and its possible ability to biotransform the betulin present into its derivative, betulinic acid.

It is interesting to note that, in the case of presence of white birch bark in the fermentation medium, mycelium from *Inonotus obliquus* was able to produce a very valuable amount of betulin and betulinic acid (Figure 3, table 1). Peaks corresponding to the retention time and molecular weight of betulin, betulinic acid and lupeol were found it. 678.57 ± 20.09 mg. $L^{-1}$ of betulin and 46.97 ± 1.39 mg. $L^{-1}$ of betulinic acid were measured. Furthermore, a peak corresponding to the retention time and mass of the lupeol load was found, and it was quantified at 20.55 mg. $L^{-1}$ (Figure 2). The presence of 10 g of

white birch bark in the fermentation media seems to increase the yield (678.57 mg. $mL^{-1}$ of betulin and 46.97 mg. $mL^{-1}$ for betulinic acid), compared to that obtained with betulin alone. This could be explained through the role of birch bark in inducing an enzymatic reaction within the mycelium of *I. obliquus* to produce an interesting amount of triterpenes and its derivatives. Also, this could be explained by the plant-fungal interaction with other white birch substrates such as cellulose or lignin that might have stimulated more important gene expression and thus enzyme activity to increase the observed yield.

Triterpenes are water insoluble or very poorly solubilized molecules. To confirm the possibility of presence of triterpenes in the biotransformation medium in presence of white birch bark, an extraction on the fermentation medium in the presence of white birch bark, shaken at 150 rpm for 9 days at 28 °C, was carried out in the initial mycelium culture medium. Very small amount of betulin (±2.36 mg. $mL^{-1}$) was observed after filtration (Table 2). Inotodiol, a triterpenoid compound found in chaga, was identified according to its molecular weight of 425.3g/mol, reported in the literature. Quantification of inotodiol wase not possible due to the unavailability of a standard.

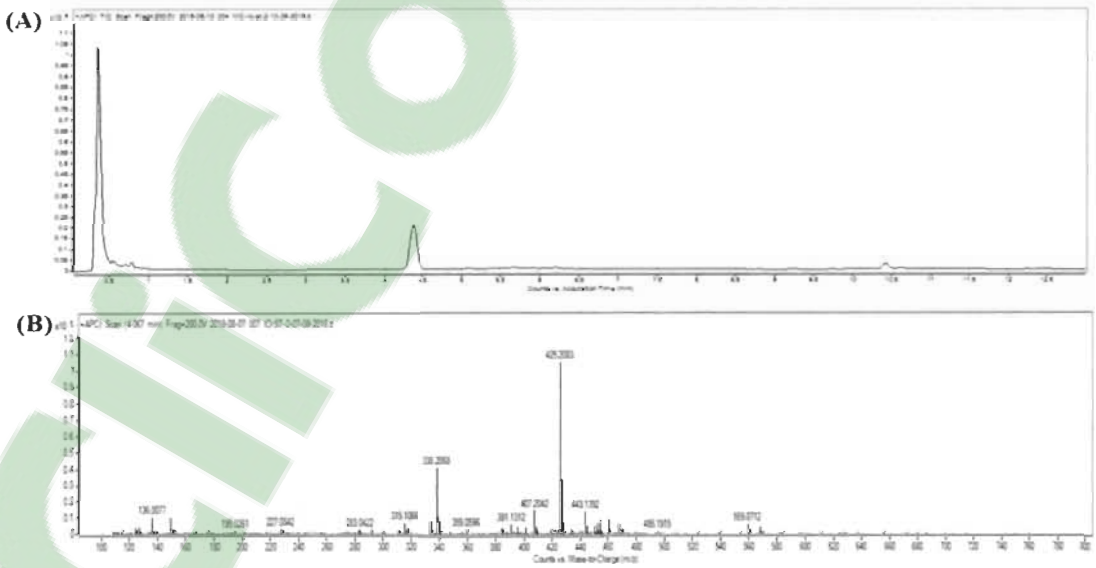


**Figure 1: (A)** Chromatograms of betulin in extraction of mycelium of *I. obliquus* in fermentation medium without substrate. **(B)** Mass spectra of betulin.

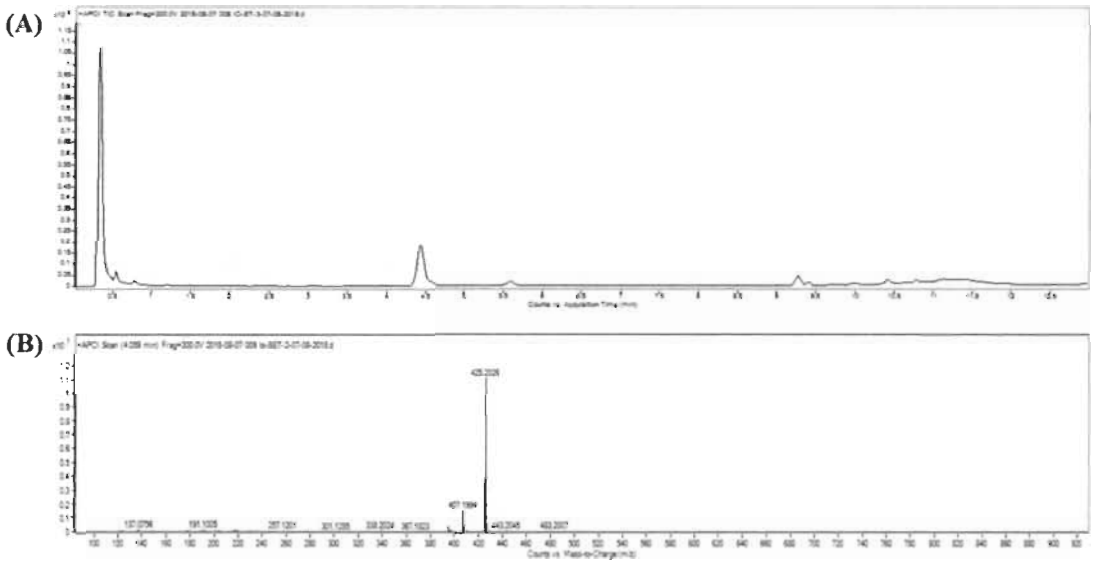


**Figure 2: (A)** Chromatograms of betulin in extraction of mycelium of *I. obliquus* in fermentation medium in presence of 7.5 mg. $mL^{-1}$ betulin. **(B)** Mass spectra of betulin.

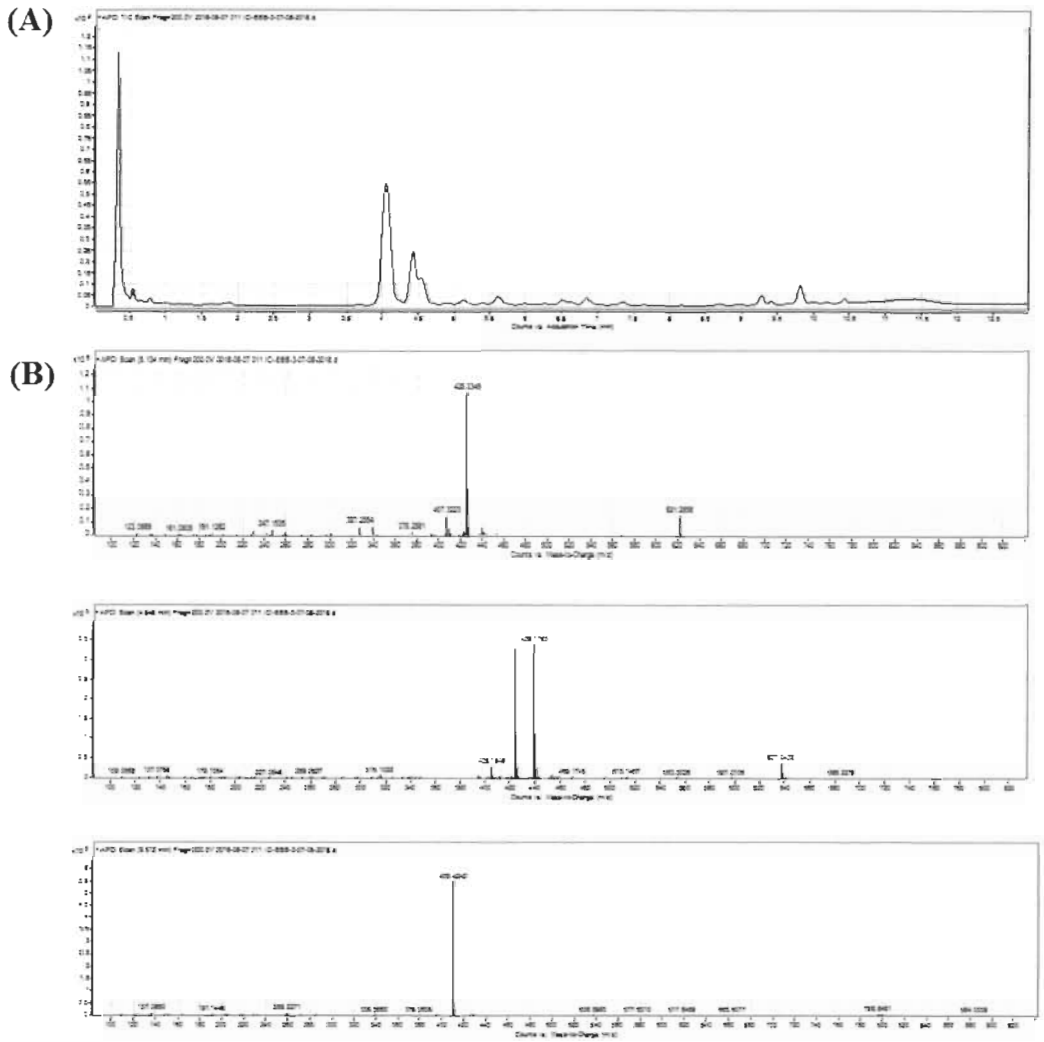


**Figure 3: (A)** Chromatograms of betulin, betulinic acid, lupeol in extraction of mycelium of *I. obliquus* in fermentation medium in presence of white birch bark. **(B)** Mass spectra of betulin, betulinic acid and lupeol.

**Table 2.** Quantification in mg. $mL^{-1}$ of betulin, betulinic acid and lupeol in fermentation media containing *I. obliquus* alone, in presence of white birch bark (WBB), in presence of betulin as well as fermentation media containing only WBB. Quantification was assessed by calculating the area under the curve corresponding to each peak compared to each compound's standard curve.

| **Compound** | ***I. obliquus*** | ***I. obliquus* +WBB** | ***I.obliquus* + betulin** | **WBB** |
|---|---|---|---|---|
| Betulin | 34.29 ± 1.95 | 678.57 ± 20.09 | 444.79 ± 10.36 | 2.36 ± 1.08 |
| Betulinic acid | 0.16 ± 0.03 | 46.97 ± 1.39 | 0.53 ± 0.04 | 0 |
| Lupeol | 0 | 20,55 | 0 | 0 |

These results confirm again the ability of chaga to biotransform white birch barks into valuable compounds such as betulinic acid and betulin.

Although the biotransformation was realized, more work should be carried to obtain optimal biotransformation yield, such as trying different substrate structures to insure higher plant-fungal interaction surface. Also, previous transcriptomic analysis revealed most of the enzymatic pathway responsible of triterpenoid pathway, an interesting way to improve the biotransformation yield would be enhancing the responsible enzymes' expression through targeted mutagenesis in bacteria, yeast or even chaga (Fradj et al., 2019). Moreover, it would be interesting to isolate the target enzymes and assess enzymatic activity through cell-free system. Such analysis would reveal more about the enzymes' reactional proprieties and advance the biotransformation process.

Finally, results shown in this short communication prove the importance of chaga in as green substitute to chemical catalysts in order to obtain pharmaceutically valuable compounds such as betulinic acid, inotodiol or lupeol.

## Conclusion

In this work, the results have shown the effect of the *I. obliquus* in the production of tritepernoids. The best biotransformation medium was in the presence of white birch bark. It was shown that, in the presence of white birch bark, the mycelium of *I. obliquus* is able to increase the production yield of betulin, betulinic acid, andlupeol. However, the yields of the different triterpenoids are still low, and furter researches are needed to optimize and enhance these yields.

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# CHAPITRE IV

# A FIRST INSIGHT INTO NORTHERN AMERICAN PLANT PATHOGENIC FUNGI *ARMILLARIA SINAPINA* TRANSCRIPTOME

Narimene FRADJ, Nicolas DE MONTIGNY, Fatima AWWAD, Yacine BOUMGHAR, Hugo GERMAIN and Isabel DESGAGNÉ-PENIX

Cet article scientifique est en préparation pour publication dans la revue PLOS One.

## 4.1 Contribution des auteurs

Le projet a été conçu par Yacine BOUMGHAR, Hugo GERMAIN et Isabel DESGAGNÉ-PENIX. La méthodologie a été réalisée par Narimane FRADJ, Yacine BOUMGHAR, Hugo GERMAIN, Isabel DESGAGNÉ-PENIX. L'analyse des résultats a été faite par Narimane FRADJ, Nicolas DE MONTIGNY et Isabel DESGAGNÉ-PENIX. La rédaction et la correction de l'article ont été faites par Narimane FRADJ, Fatima AWWAD, Isabel DESGAGNÉ-PENIX, Hugo GERMAIN et Yacine BOUMGHAR. L'obtention des fonds a été réalisée par Yacine BOUMGHAR et Isabel DESGAGNÉ-PENIX. La supervision du projet de recherche a été menée par Yacine BOUMGHAR, Isabel DESGAGNÉ-PENIX et Hugo GERMAIN.

## 4.2 Article complet (anglais) : A first insight into Northern American plant pathogenic fungi *Armillaria sinapina* transcriptome


### Abstract

*Armillaria sinapina* is a fungus pathogen of the main woody species across North American forests causing root diseases. RNA sequencing technologies have increased information at the transcriptome level of various pathogens and hosts for a better understanding, at the transcriptomic level of the interaction process. To this end, the transcriptome of *A. sinapina* was analyzed with or without the presence of betulin, triterpenoid found in high amount in birch bark in order to better understand the molecular mechanisms of Armillaria towards in white birch bark and fungal pathogens of woody plants. Furthermore, our results allowed us to identify specific genes with potential roles associated with pathogenesis, and to investigate the ability of *Armillaria sinapina* to be used as a biocatalyst in an innovative bioprocess. A de novo assembly and characterization of *Armillaria sinapina* transcriptome using Illumina technology were performed. A total of 170,592,464 reads were generated, then 273,561 transcripts were found. Several biosynthesis pathways have been listed using KEGG analysis such as the terpene biosynthesis pathways. 11 genes involved in the terpene biosynthesis pathway have been identifed. In addition, 25 genes that could play a significant part in lignin degradation have been listed. Several cytochrome P450s have been reported. However, number of transcripts are still unknown from the databases. To our knowledge, this research is the first transcriptomic study carried out on *Armillaria sinapina*.


***Keywords***: Forest plant pathogen, *Armillaria sinapina*, de novo assembly and analysis, transcriptomic, white root disease, fungi.

### Introduction

In forest ecosystems, fungi represent a complex group of microorganisms that vary from complex unicelluar to multicellular entities. Because of their wide range of

adaptation's, fungi are capable to adopt very different lifestyles going from saprophytes to plant, animal and human pathogens, parasites and symbionts. Through their adaptative capacities, these organisms play various roles, such as decomposition, beneficial symbiotic relationships or as biocatalyst agents. Some organisms have the ability to interact with each others and also with other ecosystem components in a multi-dimensional and complex way within the forest ecosystems (Stewart, Kim, and Klopfenstein 2018; Araujo and Sampaio-Maia 2018).

*Armillaria* species are globally distributed in natural and planted forests of temperate, boreal and tropical ecosystems. *Armillaria* species are regarded among the most famous and important phytopathogens of forest trees. Their benefits for horticulture and the growth of edible fungi have been recognized. More than 70 species have been listed worldwide, and more than 40 species of the genus *Armillaria* have been further studied (Sipos, Anderson, and Nagy 2018; Heinzelmann et al. 2019a; Ross-Davis et al. 2013).

Using partial sequencing of translation elongation factor gene – 1α (*tef1*), phylogenetic relationships between 15 known *Armillaria*_species and other unknown *Armillaria* species from several world regions were classified (Klopfenstein et al. 2017). The results of tef1 of *Armillaria sinapina* show that it belongs to a distinct phylogenetic clade. No phylogenetic separation between *A. sinapina* from North America and Japan has been found (Klopfenstein et al. 2017). The *Armillaria* species have shown their ability to acquire new food resources using different strategies. *Armillaria* species are known to be saprotrophic and can therefore exploit all types of dead wood, such as roots and wood debris of different woody species present in natural and artificial ecosystems. Mycelium of *Armillaria spp.* has the ability to break down cellulose and lignins to feed, leading to the appearance of white rot (Heinzelmann et al. 2018).

Some very interesting molecules (triterpenoids) are present in the birch barks. Among them, betulin is mainly highly concentrated in the birch's outer bark (genus Betula) (Dehelean et al. 2012). Betulinic acid, another triterpenoid found in birch bark, is present

at low concentrations and attracts more attention due its biological and pharmacological properties, such as anticancer activities (Joseph et al. 2019; Wang, Yuan, et al. 2019). Betulin may be transformed into betulinic acid through biotransformation using fungi. In order to improve betulinic acid production, a biotransformation study was conducted from betulin catalysed by the mushroom *Armillaria luteo-virens Sacc* ZJUQH100-6 (Liu, Fu, and Chen 2011a).

Further researches on fungi and their ecological roles in forests, such as symbiosis, disease, decomposition, demineralization and biological control, are crucial to a better understanding of their roles and how to be used in the industry such as biocatalysts (Stewart, Kim, and Klopfenstein 2018). The study of plant pathogenic fungi that are causing various problems in forests is constantly improving with increasing availability of genomic data banks. In addition, recent advances in omics science, such as genomics, transcriptomics, proteomics and secreotomics, have made possible the detection of peptides associated with plant-pathogen interactions and the various post-translational changes in these proteins during pathogenesis (Ross-Davis et al. 2013). Transcriptome sequencing (RNA-seq) is a highly efficient, high throughput method used to characterize gene expression profiles and provide large amounts of genetic information's, as well as for non-model organisms. The development of high throughput sequencing platforms, such as Illumina RNA-seq, has allowed genomic expression profiles to be studied in various species, including animals, plants and microorganisms. Due to its high sensitivity and specificity to microarrays, as well as its ability to detect new genes, rare transcripts and new SNPs can be used for associative investigations. Deep sequencing using RNA- seq technologie, such as NGS technology, are used to explain gene function, differential gene regulation and metabolic pathways in the presence of several inducers in a given organism (Mandlik et al. 2011; Cao et al. 2018).

Several genomic sequencing projects have been carried out on *Armillaria*. Up to now, six genomes of the *Armillaria* family have been sequenced (*A. cepistipes*, *A. gallica*, *A. fuscipes*, *A. mellea*, *A. ostoyae* and *A. solidipes*). To date, the transcriptomic analysis of one of the most virulent species that mainly affects wood *A. solidipes* (*Armillaria*

*ostoyae*) has been reviewed and published (Sipos, Anderson, and Nagy 2018; Ross-Davis et al. 2013).

*Armillaria sinapina* was morphologically identified by J.A. Berubé and D. M. Bessureault in 1988 in North America and has been very poorly studied since then (Bérubé and Dessureault 1988).

The aim of this research is to characterize the transcriptome of the fungal mycelium of the boreal forest species *Armillaria sinapina*. *Armillaria* is a pathogen of birch and can growth on it. Birch outter tissus are mainly composed of antimicrobial triterpenoids with betulin being the most predominant one. For example, in birch root, concentration of betulin is 5mg/g (Yin et al. 2013). In the wild, growth and production of metabolites by *A. sinapina* is likely influenced by substrates provided by its host. Thus, an investigation to determine the influence of highly abundant betulin from birch bark on *A. sinapina* transcriptome was carried out to better understand the genetic response of the betulin as well as to investigate its impact on the enzymes involved in degradation of cell walls of birch bark, and to discover possible new genes, including genes involved in the biotransfomation of betulin to betulinic acid.

## Materials and methods

### *Fungal culture and growth conditions*

*A. sinapina* was provided and cultured by the Biopterre laboratory (La Pocatière, Québec, Canada). *A. sinapina* mycelium was maintained on Potato Dextrose Agar (PDA) medium, and stock cultures were stored at 4 °C. Identity of the *A. sinapina* isolate was confirmed using PCR amplification, followed by Sanger sequencing of the ribosomal internal transcribed spacer (ITS) DNA region. Nucleotie BLAST (BLASTn) analysis showed that the sequenced PCR product displayed 100% sequence identity to *A. sinapina* isolate XY17_48 ITS NCBI sequence ID MH550358.1. To optimize mycelial growth of *A. sinapina* in laboratory, culture medium of Yeast Malt Agar (YMA) was selected,

based on literature (Engels et al. 2011; Liu, Fu, and Chen 2011b; Ming-liang et al. 2011; Zhang et al. 2008; Fradj et al. 2019). Different growth condition's parameters of liquid and solid culture of *A. sinapina*, were tested in presence or absence of betulin (15 µg. mL).

*Isolation of RNA*

RNA from *A. sinapina* was isolated from biological replicates following FRADJ et al. protocol (Fradj et al. 2019). Briefly, RNA was extracted from *A. sinapina* liquid culture of YMB medium supplemented or not with 0.2% DMSO (control) or 15 µg. mL of betulin (BET) and grown for 8 days on a rotary incubator at 150 rpm and 28 °C in absence of light. The mycelium was filtered, frozen with liquid $N_2$, and then placed in a 2 mL tube with 2 mL of TRIzol reagent (Fisher Scientific, Canada). The mycelia were crushed using 5 mm diameter stainless steel beads with the TissueLyser II (QIAGEN, Qiagen Retsch GmbH, Hannover, Germany) at speed of 30 strokes per second during 3 minutes until a homogeneous sample was obtained. Tubes were incubated on ice for 5 minutes, then 400 µL of chloroform were added. Tubes were vigorously vortexed for 15 seconds and incubated in an ice bath for 3 minutes. After centrifugation at 13,000 g for 15 minutes at 4 °C, two phases were obtained which were separated by cellular debris, and the clear phase of the supernatant was recovered and transferred into a clean 1.5 mL microtube. RNA was concentrated using the isopropanol method described in (Fradj et al. 2019) and RNA pellets were resuspended using 20 µL of nuclease free water. The quality and quantity of RNA were verified using the Nanodrop and the Bioanalyzer. Only samples with a RNA integrity number higher than 8 were selected for Illumina sequencing.

*Transcriptome sequencing, de novo assembly and functional annotation*

Sequencing was performed using Illumina HiSeq 4000, PE 100 paired ends, at McGill University (Montreal, Canada) and Genome Québec Innovation Centre (Montreal, Canada) on cDNA Libraries converted from isolated high-quality mRNA. Among the raw reads obtained from Illumina sequencing, low quality reads, reads with unknown nucleotides and adapters were found. Trimmomatic was used to remove the

adapters from sequencing and trimmed reads from the 3 'end and further filtered all reads below 50 bp in order to obtain clean reads (Bolger, Lohse, and Usadel 2014). However, once the data from the surviving pairs were generated, Trinity normalization was performed to remove redundant reads in datasets without affecting its *Kmer* content (Brown et al. 2012). De novo assembly of cleaned and standardized reads was performed using Trinity (v2.6.5) assembler (Grabherr et al. 2011). Trinity method assembles the RNA-seq reads into full-length transcripts, which are called contigs or unigenes. All the unigenes were aligned to the public protein database uniprot_sprot.trinotate_v2.0.pep protein database using BLASTX program against the NCBI BLAST families using E-value of $10^{-5}$. Top BLAST hits were used for annotation of component/gene for each transcript. To quantify the gene transcript abundance, the raw RNA-Seq reads were mapped to assembled transcripts with Bowtie using default parameters (Langmead et al. 2009). The gene transcript abundance was calculated as transcripts per kilobase million (TPM) (Li and Dewey 2011; Wang, Li, et al. 2019).

Functional annotations of unigenes were performed using Trinotate (http://trinotate.github.io/), by aligning our transcripts to Swiss Institute of Bioinformatics databases (Swiss-Prot) with BLASTX and identifying protein domains (HMMER/PFAM), protein signal peptides, transmembrane domains prediction (signalP/tmHMM), and Clusters of Orthologous Groups (COG). Then, the functional annotation of unigenes was compared to currently curated annotation databases (EMBL Uniprot eggNOG/GO Pathways databases). The Trinity assembly and the functional annotation of unigenes were integrated as an annotation report into a SQLite database.

Gene Ontology (GO) annotations for our unigenes were obtained using the Swissprot. The WEGO 2.0 software was used to obtain GO functional classification for all unigenes, and to better understand the distribution of gene functions in *A. sinapina* at the macro level. All annotated sequences were linked to the GO terms in the database, and a quantification of the number of sequences for each of the different terms was subsequently calculated. The raw reads under the accession number PRJNA565538 were deposited to the Sequence Read Archive (Leinonen et al. 2011).

Accession numbers

The sequences described in this paper have been deposited in the National Center for Biotechnology Information Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/) under the accession number PRJNA565538.

## Results

*Illumina sequencing and de novo assembly*

To better understand the molecular mechanisms underlying the differences in *A. sinapina*'s transcriptome caused by the presence of betulin, RNA-Seq and de novo assembly were performed. RNA was extracted from biological replicates of cultured *A. sinapina* in absence (control) or presence of the terpenoid substrate betulin (BET). Corresponding cDNA libraries were generated and sequenced using Illumina HiSeq 4000 PE100. The raw reads for each library were deposited to the NCBI Sequence Read Archive under the accession PRJNA565538. A total of 170,592,464 raw paired reads were generated from all replicates (Table 2). After filtering out low-quality sequences, 166,006,772 clean reads were obtained, corresponding to approximately 97.3% of the total raw reads (Table 1). Because of the lack of availability of information's (*i.e.* genome, transcriptome) on *A. sinapina*, we combined all RNA-Seq libraries to build a deep transcriptome using de novo assembly. All clean reads obtained from the libraries were subsequently de novo assembled using the Trinity program (version 2.6.5) and a total of 273,561 transcripts with an average length of 2,051 bp, and a N50 length of 3,523 bp were obtained (Table 1; Figure 1). An evaluation of the size distribution showed that 96% of all annotated transcripts of *A. sinapina* measured more than 1 kb (Figure 1).

**Table 1.** Results of Illumina sequencing output and assembly for *Armillaria sinapina*.

| **Read Trimming and Clipping of Adapters** | |
|---|---|
| Raw paired reads[a] | 170,592,464 |
| Surviving paired reads[b] | 166,006,772 |
| Surviving paired reads (%)[c] | 97.31 |
| **Trinity de novo assembly** | |
| Nb. Transcripts[d] | 273,561 |
| Nb. Components[d] | 99,358 |
| Total Transcripts Length (bp) | 561,145,023 |
| Max. Transcript Length (bp) | 16,405 |
| Min. Transcript Length (bp) | 201 |
| Median Transcript Length (bp) | 1,456 |
| Mean Transcript Length (bp) | 2,051 |
| N50 (bp)[e] | 3,523 |
| **BLAST annotation and filtered annotated components** | |
| Nb. Transcripts | 121,959 |
| Nb. Components | 13,544 |
| Total Transcripts Length (bp) | 288,576,284 |
| Max. Transcript Length (bp) | 16,405 |
| Min. Transcript Length (bp) | 298 |
| Median Transcript Length (bp) | 3,661 |
| Mean Transcript Length (bp), | 4,033 |
| N50 (bp)[f] | 5,081 |

[a] Number of Paired Reads obtained from the sequencer

[b] Number of Remaining Paired Reads after the trimming step

[c] Percentage of Surviving Paired Reads / Raw Paired Reads

[d] Trinity has created a list of transcripts (contigs) representing the transcriptome isoforms. The transcripts are grouped in components loosely representing genes. Transcript names are prefixed by the component/gene name e.g. transcripts c115_g5_i1 and c115_g5_i2 are derived from the same isolated de Bruijn graph and therefore share the same component/gene number c115_g5.

[e] Corresponding contig length distribution N50 = 3,523 bp

[f] Corresponding contig length distribution N50 = 5,081 bp

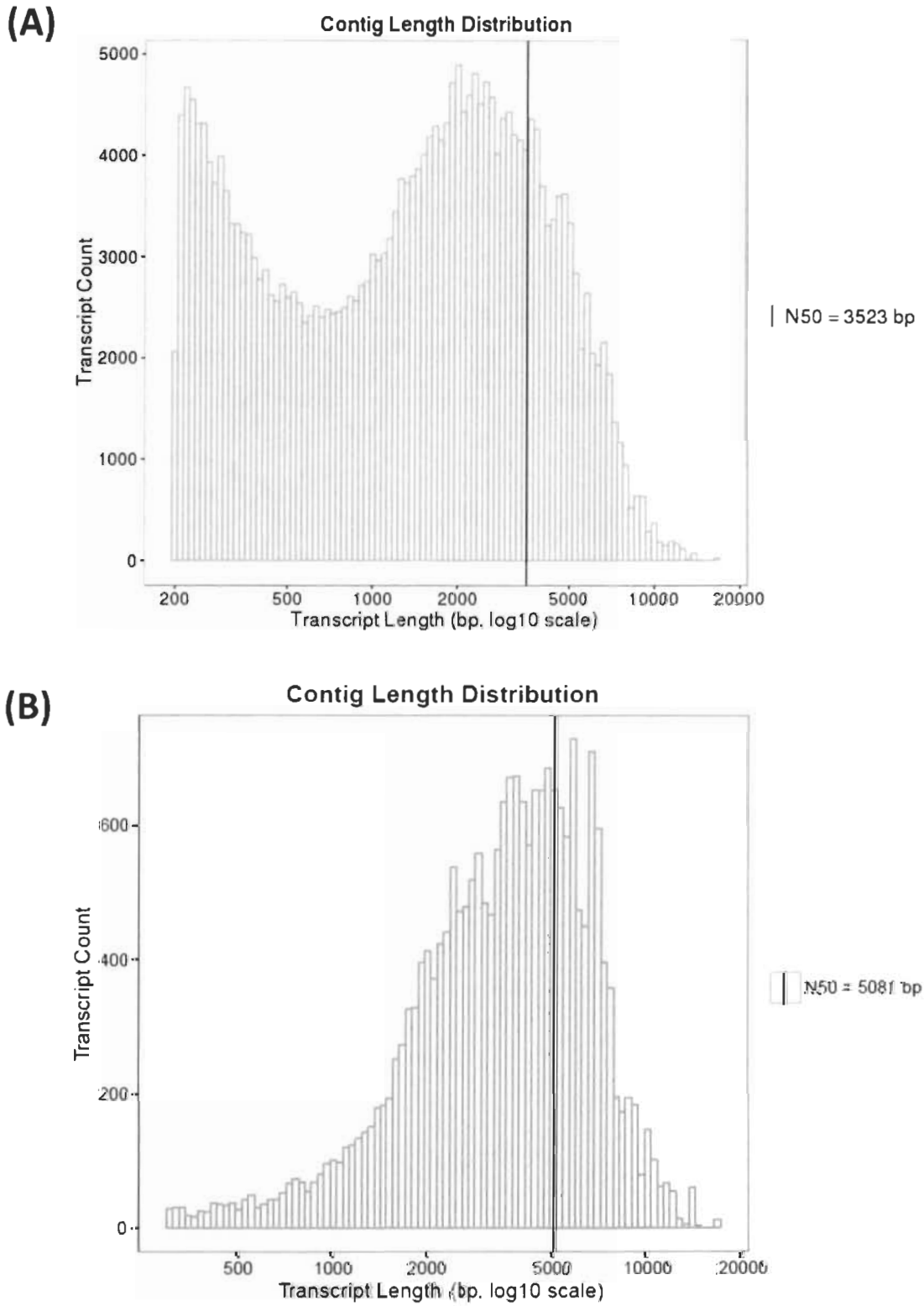


**Figure 1:** Sequence length distribution of transcripts of *A. sinapina* after Trinity de novo assembly (**A**; N50=3523bp) and after BLAST annotation and filtered annotated components (**B**; N50=5081bp).

*Functional annotation of A. sinapina transcriptome*

The potential protein-coding transcripts from *A. sinapina* transcriptome were identified using the BLASTx algorithm with an E-value cutoff threshold of $10^{-5}$ against the NCBI non-redundant (nr) protein database. A total of 121,959 transcripts (44.6%) similar to known proteins or conserved hypothetical proteins was obtained from this search.

Next, the functional classification of transcripts was performed using public databases such as the Swissprot protein databases, Gene Ontology (GO), Cluster of Orthologous Groups (COG), and the Kyoto Encyclopedia of Genes and Genomes (KEGG). Among the 273,561 assembled transcripts, 145,191 (53.07%) were annoted. 103,267 (37.75%), 61,272 (23.53%), 45,683 (16.70%), and 105,195 (40.39%) transcripts had corresponding sequences with the GO, COG, pFAM and KEGG databases, respectively (Table 2). Moreover, 128,370 (46.92%) transcripts showed no similarity with known sequences from the databases, which suggests that *A. sinapina* genes have been found and are worth investigating further.

Gene Ontology (GO) terms were annotated using Blast2GO, which assigned 103,267 (37.75%)

Transcripts were matched to known terms (Figure 2). Most abundant GO category is Cellular Component with 48.0% (95,002 transcripts), followed by Molecular Function with 39.1 % (77,267 transcripts), and last is the Biological Process category with 12.9% (25,492 transcripts). GO slim terms for cellular components consisted of 10 groups, in which the top three largest groups were cell, organelle and membranes (Figure 2). Most abundant GO slim terms for molecular function included catalytic, binding, and transporter activities reflecting the ability of *A. sinapina* to transport and metabolize diverse compounds.

The *A. sinapina* transcript sequences were blasted against the Cluster of Orthologous Groups (COG) of proteins database of the NCBI. A total of 81,735 unigenes were classified into 25 categories (Figure 3). A large number of transcripts (17,113) were classified in the functional category R (general function prediction only), followed by category E (Amino acid transport and metabolism; 10,164 transcripts), and category G (carbohydrates transport and metabolism; 9009 transcripts) (Figure 3A). Interestingly, the functional category secondary metabolites biosynthesis, transport and catabolism (Q) represented 8% (6,381 transcipts) of the COG annoted transcripts.

Also, the *A. sinapina* transcriptome was annotated by mapping the transcripts onto the pathways reported in the Kyoto Encyclopedia of Genes and Genomes (KEGG). A total of 105.195 (40.39%) annotated transcripts were assigned (Figure 3). Metabolism, genetic information processing, environmental information processing, cellular processes, and organismal systems were present. The most highly represented category of KEGG function was « the global and overview maps ». The KEGG metabolism contained such as biosynthesis of secondary metabolites, carbohydrate metabolism, energy metabolism, lipid metabolism and nucleotide metabolism, etc. These results indicate that active metabolic processes were ongoing in *A. sinapina*. Altogether, the result for GO, COG and KEGG annotations suggest that most of the transcripts expressed in *A. sinapina* cultured cells took part in basic biological processes, such as metabolism and biological regulation.

**Table 2.** Summary of the functional annotation of transcript sequences from *A. sinapina* obtained from public databases.

| "Public database | Number of transcripts | Percentage (%) |
|---|---|---|
| Blast-GO annotations | 103,267 | 37.75 |
| COG annotations | 61,272 | 23.53 |
| KEGG annotations | 105,195 | 40.39 |
| Pfam annotations | 45,683 | 16.70 |
| Total number of sequences not annotated | 128,370 | 46.92 |
| Total number of sequences annotated | 145,191 | 53.07 |
| Total of transcript sequences | 273,561 | 100.00 |

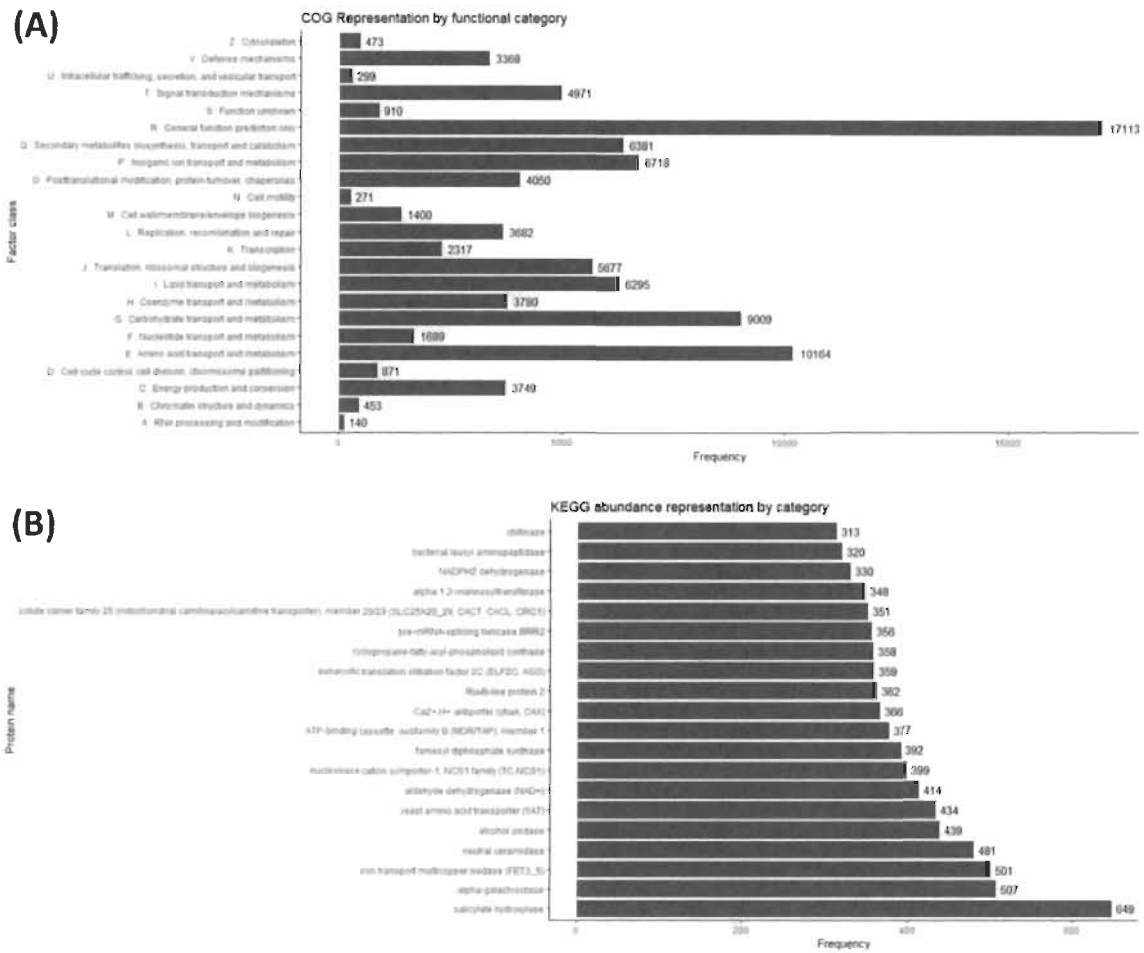


**Figure 3:** Functional classification of *Armillaria sinapina* transcriptome using (A) the Cluster of orthologous groups (CoG) and (B) the Kyoto Encyclopedia of Genes and Genomes (KEGG).

*Differential expression analysis*

Differential expression analyses were performed between databases of *A. sinapina*. Transcripts with adjusted P-values $\leq 0.05$ and a fold change (log2FC) $\geq 1$ were designated significantly differentially expressed genes (DEG). For *A. sinapina* cells cultivated with betulin (control vs BET), the results showed that 3,943 differentially expressed transcripts (3,863 up-regulated and 80 down-regulated) were identified. Fortyeight times more transcripts (3,863) were upregulated than down-regulated (80) in cells cultivated with betulin (control vs BET) (Table 3). This suggest that *A. sinapina* requires transcriptome adjustment in presence of betulin.

In the top 50 up-regulated transcripts, several transcripts encode proteins involved in lipid and carbohydrate metabolism such as ketoacyl-CoA thiolase, phosphoglycerate kinase, pyruvate decarboxylase, fumarate reductase, glycoside hydrolase, phosphopyruvate hydratase, and ceramide fatty acid hydroxylase (Table 4). This suggest that *A. sinapina* regulate cell process toward basic metabolism to produce energy (catabolism) and growth. Interestingly, a methylsterol oxidase (*erg25*), which catalyzes Fe-dependent first step in the removal of the two C-4 methyl groups of 4,4, dimetylzymsterol in zymosterol biosynthesis from lanosterol, ranked at position 5 (Table 4). This could indicate a feedback regulation step to process betulin. Also, a cytochrome P450 was up-regulated 9.91fold (Table 4), but its activity has not yet been elucidated. Only, experimental evidence at the transcript level has been reported. It should be noted that CYP450 often catalyze reaction at bottleneck rate-limiting step. In addition, the conversion of betulin to betulinic aldehyde or acid, involved redox reaction, often performed by enzymes from the CYP450 family, rendering it an interesting candidate, particularly for the biotransformation of betulin into something useful.

From the top 50 down-regulated genes from *A. sinapina* cultured cell, betulin treatment down-regulated transcripts involved in complex carbohydrate transport and degration such as pectate lyase, hexose transporter, glycosyltransferase, permease, and lactone oxidase (Table 4.3). This suggest that in presence of betulin, *A. sinapina* cells slow down processes involved in the transport and degradation of carbohydrates, most likely to focuses on lipid and triterpenoids degradation. This observation might indicate a shift in *A. sinapina* catabolism from sugars as carbon source to betulin as carbon source.

**Table 3.** The number of differentially expressed transcripts between control and betulin-treated *A. sinapina* cultured cells.

| Set | Differentially expressed transcripts | Up-Regulated | Down-Regulated |
|---|---|---|---|
| Control vs BET | 3943 | 3863 | 80 |

*Overview of gene expression with biotechnological relevance*

In the presence of betulin, the study of differential gene regulation revealed a significant number of genes and pathways responsible for triterpene biosynthesis, enzymes responsible for various redox reactions and survival approaches acquired by white rot fungi. Among several transcripts regulated upwards (3863) and downwards (80), a significant number of genes with various molecular functions were observed (Table4).

As betulin is a triterpenoid, and occurs in large amounts in white birch barks, special attention has been given to understand and identify enzymes involved in triterpenoid metabolism, in order to provide an insight into the genes involved in biotransformation of betulin to its derivatives. Furthermore, as *A. sinapina* is a white rot fungus that causes root diseases in trees, the enzymes that are involved in the degradation of these substrates have been developed and could be interesting candidates for further studies for the depolymerization of lignin, for example.

*Terpenoids*

Terpenoids have been mainly studied as specialized metabolites produced by plants. However, fungi have been shown to produce a wide range of terpenoids. It has been demonstrated that the fungi belonging to *Basidiomycota* family has the potential to produce a large amount of terpenoids that never before identified. Therefore, in addition, various studies demonstrate the ability of fungi to be used as biocatalysts for the production of secondary metabolites and their by-products. Through analysis of enzymatic pathways of *A. sinapina*, transcriptome using KEGG (Kyoto Encyclopedia of Genes and Genomes) shows that a highest number of genes are involved in biosynthesis of secondary metabolites, revealed 23 ORFs encoding components in terpenoid backbone biosynthesis pathway, 3 ORFs encoding for sesquiterpenoid and triterpenoid biosynthesis, 12 transcripts for fatty acid biosynthesis and other terpenoid quinones. With regard to terpenoid skeletal synthesis in *A. sinapina*, genes for thiolase, hydroxymethylglutaryl-CoA lyase, phosphomevalonate kinase and phosphomevalonate synthase were identified. As well, genes of Farnesyl pyrophosphate synthase, a precursor of the biosynthesis

pathways of triterpenoids and sesquiterpenoids, have also been identified (Figure 5). Muurolene synthase, Trichodiene oxygenase and protoilludane synthase have been identified for sesquiterpene biosynthesis. In addition, squalene epoxidase and monooxygenase were found (Table 4).

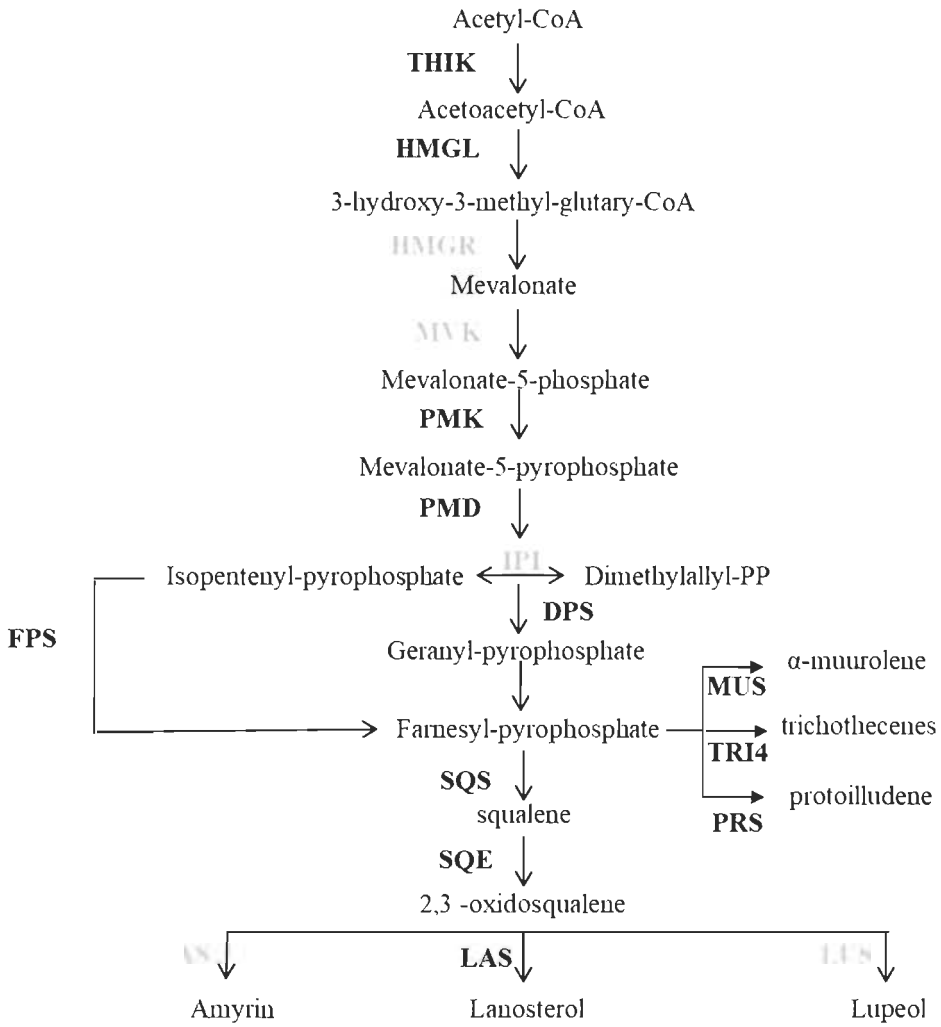


**Figure 5:** Identification of synthesis pathway enzymes and main synthases for terpenoid biosynthesis in *Armillaria sinapina*. Identified enzymes (in dark) included: MVK, Mevalonate kinase; PMK, phosphomevalonate kinase; PMD, phosphomevalonate kinase; THIK, 3-ketoacyl-CoA thiolase; FPS, Farnesyl pyrophosphate synthase; HMGL, Hydroxymethylglutaryl-CoA lyase; DPS, Decaprenyl-diphosphate synthase; MUS, Muurolene synthase; TRI4, Trichodiene oxygenase; PRS, protoilludene synthase; SQE, Squalene epoxidase; LAS, Lanosterol synthase. No Identified enzymes were in grey included: HMGR, hydroxymethylglutaryl (HMG)-CoA reductase; AS, Amyrin synthase; LUS, Lupeol synthase; SQS, Squalene synthase; IPI, isopentenyl diphosphate isomerase; MVK, Mevalonate kinase.

*Cytochrome P450s*

Proteins belonging to one of the largest protein families, Cytochrome P450, have been very well characterized for their role in cyclisation and terpenoids synthesis, such as lanosterol modification and secondary metabolite synthesis (Lin et al. 2014; Jain et al. 2019). Regarding the differently expressed genes in the transcriptome of *A. sinapina* following betulin treatment, a total of 108 transcripts of cytochrome P450 were present in *A. sinapina* transcriptome. Among them, 32 transcripts were found to be differentially regulated in mycelia of *A. sinapina* in presence of betulin and they were all up-regulated (Table 5). An interesting candidate, CYP450 annotated as Cytochrome P450 72A14, was found and which 8 different transcript variants were represented and up-regulated following BET treatment (Table 4).

*Enzymes involved in plant cell wall degradation*

In the pathogen-host relationship, it is important for the parasite to use the host as a carbon source (Ross-Davis et al. 2013). White rot fungi, such as *Armillaria* species, are well known to have genes encoding for enzymes that are implicated in degradation of all structural components of plant cell walls, particularly enzymes responsible for the degradation of lignin, cellulose, hemicelluloses and pectin (Heinzelmann et al. 2019b).

*A. sinapina* transcriptome revealed to contains majority of the key enzymes for the degradation of plant biomass. The decomposition of the plant cells requires groups of enzymes that act in tandem on cellulose, pectin and hemicelluloses (Table 4). Among genes involved in the degradation of plant walls and which are highly up-regulated in *A. sinapina* transcriptome such as Probable feruloyl esterase A, Laccase (Laccase-1 and Lcaccase-2), Glucan 1,3-beta-glucosidase D, Beta-mannosidase A, Probable glucan 1,3-beta-glucosidase D, Polysaccharide monooxygenase, SDO1-like protein C21C3.19, Mannosyl-oligosaccharide 1,2-alpha-mannosidase, Glucosidase 2 subunit beta, Invertase 2, Probable feruloyl esterase B-1, Probable glucan endo-1,3-beta-glucosidase, Glucosidase 2 subunit alpha, Glucan endo-1,3-alpha-glucosidase agn1, Glycogen debranching enzyme, Probable glucan 1,3-beta-glucosidase D, Glucan 1,3-beta-

glucosidase, Alpha-mannosidase, Probable alpha/beta-glucosidase, Mannosyl-oligosaccharide alpha-1,2-mannosidase, Mannosyl-oligosaccharide 1,2-alpha-mannosidase, Glucan 1,3-beta-glucosidase, Uncharacterized family 31 glucosidase, Probable endo-beta-1,4-glucanase D, Probable glucan 1,3-beta-glucosidase D, Versatile peroxidase VPL1, Probable glucan endo-1,3-beta-glucosidase, Probable endo-beta-1,4-glucanase D, Probable beta-glucosidase A, Xyloglucanase, Chanoclavine-I aldehyde reductase were found, while the probable pectate lysase A and pectate lyase A have been found to be down-regulated in presence of betulin.

**Table 4.** Differently expressed biotechnologically-relevant transcripts of *A. sinapina* in cells cultivated in presence of betulin.

| **Transcript name** | **Log2FC** | **Annotation** |
|---|---|---|
| ***Terpenoids*** | | |
| Armillaria_TRINITY_DN29150_c0_g1_i1 | 10,68 | Methylsterol monooxygenase |
| Armillaria_TRINITY_DN22917_c0_g1_i3 | 9,00 | Lanosterol synthase |
| Armillaria_TRINITY_DN3215_c0_g1_i1 | 8,85 | C-5 sterol desaturase |
| Armillaria_TRINITY_DN31064_c0_g1_i13 | 8,70 | Lanosterol 14-alpha demethylase |
| Armillaria_TRINITY_DN6566_c0_g1_i2 | 8,27 | Sterol 24-C-methyltransferase |
| Armillaria_TRINITY_DN34616_c0_g1_i1 | 7,28 | Alpha-muurolene synthase |
| Armillaria_TRINITY_DN17473_c0_g1_i1 | 7,22 | Lanosterol 14-alpha demethylase |
| Armillaria_TRINITY_DN45244_c0_g1_i1 | 7,17 | Squalene monooxygenase |
| Armillaria_TRINITY_DN18035_c0_g1_i1 | 7,13 | Delta(14)-sterol reductase |
| Armillaria_TRINITY_DN45179_c0_g1_i1 | 6,96 | Squalene synthase |
| Armillaria_TRINITY_DN54064_c0_g1_i1 | 6,94 | Sterol-4-alpha-carboxylate 3-dehydrogenase |
| Armillaria_TRINITY_DN13258_c0_g1_i1 | 6,93 | Sterol 14-demethylase |
| Armillaria_TRINITY_DN12757_c0_g1_i3 | 6,91 | Alpha-muurolene synthase |
| Armillaria_TRINITY_DN10601_c0_g1_i1 | 6,78 | Farnesyltransferase/geranylgeranyltransferase subunit alpha |
| Armillaria_TRINITY_DN10022_c0_g1_i1 | 6,78 | Isopentenyl-diphosphate Delta-isomerase |
| Armillaria_TRINITY_DN4217_c0_g1_i1 | 6,75 | Delta(24(24(1)))-sterol reductase |
| Armillaria_TRINITY_DN55954_c0_g1_i1 | 6,71 | C-8 sterol isomerase |
| Armillaria_TRINITY_DN9929_c0_g1_i1 | 6,66 | Diphosphomevalonate decarboxylase |

| **Transcript name**<br>***CYP450s*** | **Log2FC** | **Annotation** |
|---|---|---|
| Armillaria_TRINITY_DN20121_c0_g1_i1 | 9,91 | Cytochrome P450 72A14 |
| Armillaria_TRINITY_DN8574_c0_g1_i1 | 8,67 | Cytochrome P450 72A14 |
| Armillaria_TRINITY_DN61797_c0_g1_i1 | 8,50 | Cytochrome P450 4F5 |
| Armillaria_TRINITY_DN5903_c0_g1_i1 | 8,28 | Cytochrome P450 67 |
| Armillaria_TRINITY_DN4166_c0_g1_i1 | 7,74 | Cytochrome P450 61 |
| Armillaria_TRINITY_DN31064_c0_g1_i10 | 7,60 | Cytochrome P450 72A14 |
| Armillaria_TRINITY_DN23798_c1_g2_i3 | 7,44 | Docosahexaenoic acid omega-hydroxylase CYP4F3 |
| Armillaria_TRINITY_DN31064_c0_g1_i5 | 7,21 | Cytochrome P450 72A14 |
| Armillaria_TRINITY_DN23798_c1_g2_i1 | 7,16 | Docosahexaenoic acid omega-hydroxylase CYP4F3 |
| Armillaria_TRINITY_DN9123_c0_g1_i1 | 7,14 | Cytochrome P450 4F22 |
| Armillaria_TRINITY_DN23798_c1_g1_i1 | 7,00 | Cytochrome P450 3A9 |
| Armillaria_TRINITY_DN56547_c0_g1_i1 | 6,97 | Putative cytochrome P450 CYP13A10 |
| Armillaria_TRINITY_DN153_c0_g1_i1 | 6,95 | Cytochrome P450 3A24 |
| Armillaria_TRINITY_DN13346_c0_g1_i1 | 6,95 | Cytochrome P450 52E1 |
| Armillaria_TRINITY_DN7413_c0_g2_i1 | 6,90 | Cytochrome P450 3A24 |
| Armillaria_TRINITY_DN9340_c0_g1_i1 | 6,89 | Cytochrome P450 4A4 |
| Armillaria_TRINITY_DN31064_c0_g1_i27 | 6,89 | Cytochrome P450 72A14 |
| Armillaria_TRINITY_DN14362_c0_g1_i1 | 6,86 | Taurochenodeoxycholic 6 alpha-hydroxylase |
| Armillaria_TRINITY_DN21389_c0_g1_i1 | 6,85 | Docosahexaenoic acid omega-hydroxylase CYP4F3 |
| Armillaria_TRINITY_DN31064_c0_g1_i3 | 6,71 | Cytochrome P450 72A14 |
| Armillaria_TRINITY_DN6971_c0_g1_i1 | 6,65 | Putative cytochrome P450 CYP13A8 |
| Armillaria_TRINITY_DN3618_c0_g1_i1 | 6,58 | Cytochrome P450 72A15 |
| Armillaria_TRINITY_DN38009_c0_g1_i1 | 6,57 | Cytochrome P450 72A14 |
| Armillaria_TRINITY_DN31064_c0_g1_i21 | 6,50 | Cytochrome P450 72A14 |
| Armillaria_TRINITY_DN30372_c0_g1_i50 | 4,07 | Cytochrome P450 98A1 |
| Armillaria_TRINITY_DN32870_c0_g1_i19 | 3,84 | Fumitremorgin C synthase |
| Armillaria_TRINITY_DN32870_c0_g1_i5 | 3,63 | O-methylsterigmatocystin oxidoreductase |
| Armillaria_TRINITY_DN32011_c0_g1_i15 | 1,69 | Docosahexaenoic acid omega-hydroxylase CYP4F3 |
| Armillaria_TRINITY_DN33108_c1_g2_i4 | 1,55 | Leukotriene-B4 omega-hydroxylase 3 |
| Armillaria_TRINITY_DN31587_c1_g1_i22 | 1,02 | Cytochrome P450 4F1 |
| Armillaria_TRINITY_DN33118_c0_g1_i13 | 0,99 | Cytochrome P450 52A6 |
| Armillaria_TRINITY_DN33118_c0_g1_i20 | 0,93 | Cytochrome P450 52A6 |

| Transcript name | Log2FC | Annotation |
|---|---|---|
| ***Cell wall enzymes*** | | |
| Armillaria_TRINITY_DN19168_c0_g1_i1 | 9,19 | Probable feruloyl esterase A |
| Armillaria_TRINITY_DN31438_c0_g4_i1 | 8,93 | Laccase |
| Armillaria_TRINITY_DN22833_c0_g1_i1 | 8,87 | Glucan 1,3-beta-glucosidase D |
| Armillaria_TRINITY_DN20665_c0_g1_i1 | 8,76 | Beta-mannosidase A |
| Armillaria_TRINITY_DN8404_c0_g1_i1 | 8,75 | Probable glucan 1,3-beta-glucosidase D |
| Armillaria_TRINITY_DN21709_c0_g1_i2 | 8,68 | Polysaccharide monooxygenase |
| Armillaria_TRINITY_DN17910_c0_g1_i1 | 8,65 | SDO1-like protein C21C3.19 |
| Armillaria_TRINITY_DN44356_c0_g1_i1 | 7,99 | Mannosyl-oligosaccharide 1,2-alpha-mannosidase |
| Armillaria_TRINITY_DN15187_c0_g1_i1 | 7,52 | Glucosidase 2 subunit beta |
| Armillaria_TRINITY_DN2359_c0_g1_i1 | 7,42 | Invertase 2 |
| Armillaria_TRINITY_DN20388_c0_g1_i4 | 7,17 | Probable feruloyl esterase B-1 |
| Armillaria_TRINITY_DN31438_c0_g6_i2 | 7,09 | Laccase-1 |
| Armillaria_TRINITY_DN13563_c0_g1_i2 | 7,06 | Probable glucan endo-1,3-beta-glucosidase |
| Armillaria_TRINITY_DN16550_c0_g1_i1 | 7,05 | Glucosidase 2 subunit alpha |
| Armillaria_TRINITY_DN43871_c0_g1_i1 | 7,04 | Glucan endo-1,3-alpha-glucosidase agn1 |
| Armillaria_TRINITY_DN3408_c0_g1_i1 | 7,04 | Glycogen debranching enzyme |
| Armillaria_TRINITY_DN31438_c0_g3_i1 | 6,97 | Laccase-2 |
| Armillaria_TRINITY_DN71958_c0_g1_i1 | 6,94 | Probable glucan 1,3-beta-glucosidase D |
| Armillaria_TRINITY_DN11046_c0_g1_i2 | 6,91 | Glucan 1,3-beta-glucosidase |
| Armillaria_TRINITY_DN955_c0_g1_i1 | 6,83 | Alpha-mannosidase |
| Armillaria_TRINITY_DN55139_c0_g1_i1 | 6,82 | Probable alpha/beta-glucosidase |
| Armillaria_TRINITY_DN24913_c0_g1_i1 | 6,81 | Mannosyl-oligosaccharide alpha-1,2-mannosidase |
| Armillaria_TRINITY_DN33885_c0_g1_i1 | 6,78 | Mannosyl-oligosaccharide 1,2-alpha-mannosidase |
| Armillaria_TRINITY_DN11477_c0_g1_i1 | 6,68 | Glucan 1,3-beta-glucosidase |
| Armillaria_TRINITY_DN38622_c0_g1_i1 | 6,63 | Uncharacterized family 31 glucosidase |
| Armillaria_TRINITY_DN12447_c0_g1_i1 | 6,55 | Probable endo-beta-1,4-glucanase D |
| Armillaria_TRINITY_DN13227_c0_g2_i1 | 6,55 | Probable glucan 1,3-beta-glucosidase D |
| Armillaria_TRINITY_DN13426_c0_g1_i1 | 6,55 | Versatile peroxidase VPL1 |
| Armillaria_TRINITY_DN13563_c0_g1_i1 | 6,54 | Probable glucan endo-1,3-beta-glucosidase |
| Armillaria_TRINITY_DN12447_c0_g1_i2 | 6,53 | Probable endo-beta-1,4-glucanase D |
| Armillaria_TRINITY_DN15833_c0_g1_i1 | 6,49 | Probable beta-glucosidase A |
| Armillaria_TRINITY_DN32420_c0_g1_i1 | 2,03 | Xyloglucanase |
| Armillaria_TRINITY_DN30863_c0_g1_i6 | 1,48 | Chanoclavine-I aldehyde reductase |
| Armillaria_TRINITY_DN31740_c0_g1_i23 | -5,82 | Probable pectate lyase A |
| Armillaria_TRINITY_DN31740_c0_g1_i15 | -6,23 | Pectate lyase A |

**Discussion**

Owing to their diversity, and their ability to produce spcialized metabolites as well as their implication in different bioprocess due to their different biological niches, filamentous fungi and pathogenic fungi are of considerable importance. In order to survive and colonize their hosts and substrates, fungi require active systems that give them the capacity to breakdown complex external substrates, and then import nutrients into the cells. Additionnaly, they should have the possibility to modify or eliminate chemicaly toxic host defense that have been introduced into their cells. In this work, we are interested in *Armillaria sinapina*, a white rot fungus that affects conifers and leafy trees such as white birch, as well as its potential application in various bioprocesses such as betulin biotransformation, present in highly amounts in white birch bark and as well as in the degradation of cell walls.

In this study, we obtained a comprehensive transcriptome data from *A. sinapina* in presence of betulin by Illumina sequencing. A total of high-quality 166,006,772 of reads were obtained. A total of 343,289 transcripts with a minimum length of 201 bp and a N50 of 3523 bp and 99358 of contigs were genererated. Using Illumina sequencing and de novo assembly, the same method as A. L. Ross-Davis has used to assemble and analyze the transcriptome of *Armillaria solidipes* in 2013. Compared to their work (Ross-Davis et al. 2013), the average and N50 dimensions of the contigs, scaffolding and unigenes generated in this research were longer, with the exception of the N50 dimensions of unigenes. In another research work on the saprophytic fungus *Wolfiporia cocos*, Illumina sequencing of the transcriptome yielded a total of 38,722,186 reads which were assembled into 60,354 contigs with N50 of 765 bp (Shu et al. 2013). In comparison to this work, we obtained 4-7 times more reads, ensuring more coverage and allowing a more contiguous assembly, as confirmed by the average transcript length of 2,051 bp obtained in this study (Table 1). The average length of eukaryotic transcript genes is greater than 1 kb. For example, average transcript length of eukaryotic genes range from 1135-1695 bp in yeast to 1108-2667 bp in human (Bertagnolli et al. 2013). In addition, eukaryotic proteins have an average size of 472 amino acid residues (*i.e.* 1419 bp), although protein sizes in

plant genomes are smaller than those of animals and fungi. Based on our sequencing results, it could be concluded that the quality and the depth of this transcriptomic assembly is significantly improved, compared to other fungal transcriptome studies, and will serve as a basis for further transcriptomic research on North American *Armillaria* species. The unigenes were annotated using diferent functional databases. 103 267 (37.75%), 61 272 (23.53%), 105 195 (40.39%), 45 683 (16.70%) unigenes had similar sequences with the GO, COG, KEGG and Pfam databases respectively. 128 370 unigenes showed no similarity with known sequences based on these databases. Based on the wide distribution of GO, three GO categories, cell, binding activity and metabolic were the most abundant classes in cellular component, molecular function and biological process, respectively, which was consistent with the report from A. L. Ross-Davis about transcriptomic analysis of *A. solidipes*. These results confirmed that the transcriptome sequencing data from *Armillaria sinapina* have revealed new genes that had not been identified by A. L. Ross-Davis and other genomics researches about other *Armillaria* species. Unfortunately, in the transcriptomic report of *A. solidipes*, the KEGG, COG and Pfam databases were not realized in order to be compared with the results of *A. sinapina*. The KEGG annotation indicated that the three categories involving the most genes were the metabolic pathways, biosynthesis of secondary metabolites, biosynthesis of antibiotics and carbon metabolism. These results could indicate, from a transcriptomic view, that *A. sinapina* has probably more genes involved in the metabolism of secondary metabolites such as triterpenoids. The differential gene regulation in the presence of betulin has revealed a high number of genes, and biosynthetic pathways of metabolism and survival that have been developed by white rot fungi. It was interesting to note that 3863 transcripts were up-regulated, opposite of 80 transcripts down-regulated. Moreover, it is apparent from the results of differential gene expression that the presence of betulin has induced, increased or stopped the expression of genes involved in the biosynthesis pathway of secondary metabolism such as triterpenoids. Transcripts for enzyme, such as cytochrome P450, lanosterol synthase, glycoside hydrolase, 3-ketoacyl-CoA thiolase 5 and C4-methyl sterol oxidase involved in the secondary metabolite biosynthesis have been found through the top 50 of the upregulated genes. As for the largest family of cytochrome P450 in plant, human and fungal metabolism, Cythochrome P450 play an essential

biological pathway, including terpenoid biosynthesis as well as the production of other natural products. Because of their ability to introduce oxygen into non-activated C-H bonds, the P450s are very attractive for biotechnology applications such as for biotransformation methods that appear to be an interesting alternative to classical chemical synthesis, particularly in the field of triterpenoid oxidation (Janocha, Schmitz, and Bernhardt 2015). The CYP72A sub-family was found to be upregulated among the genes differentially expressed in the presence of betulin. In plants, a study has shown that the subfamily CYP72A is required for the metabolism of specialized triterpenoids that carry out species-specific functions, such as chemical defense response to specialized pathogens (Song et al. 2019; Prall, Hendy, and Thornton 2016). Several studies have been conducted on the sub-family function of the P450 CYP72A, sub-family found in plants, but no study has been yet published on the possible function of this sub-family in fungi. It has been reported that cytochrome P450 enzymes can degrade toxic substances into a more polar and water-soluble compounds, and therefore play an important role in detoxification, which may explain why several genes of the cytochrome P450 family have been upregulated in the transcripyome of *A. sinapina* in the presence of betulin (Cao, Wang, and Tan 2019). From differentially expressed genes in the presence of betulin, a number of genes of the cytochrome P450 family identified in the transcriptome of *Armillaria sinapina* have not yet been functionally defined or have been studied rarely in plants. The use of P450 enzymes in bioconversion processes allows the regional and stereoselective introduction of oxygen under less aggressive reaction conditions, which explains why P450 enzymes are very interesting for the production and modification of triterpenoids. However, despite their impressive synthetic properties and bioconversion capabilities, P450s remain under-researched and their application in industrial bioconversion processes is not widely known, especially the P450 from fungi (Janocha, Schmitz, and Bernhardt 2015).

In recent years, there has been a growing demand for fuels and chemicals based on renewable resources due to the sharp increase in greenhouse gas emissions (Liu, Le, et al. 2019). According to recent studies, the lignin polymer is to be one of the most abundant renewable sources of aromatic carbon and the second-largest polymer after cellulose.

Lignin-derived aromatic compounds have been found to be suitable for the production of biofuels as well as bulk and fine chemicals, which could be used to respond to energy and environment-related concerns (Rinaldi et al. 2016). In the lignin valorization process, lignin goes through two main steps, which are the depolymerization and degradation of aromatic compounds, these steps are followed by the biosynthesis of the target product (Liu, Le, et al. 2019). For pathogenesis, the ability to use the host polymers as a carbon source is essential (Ross-Davis et al. 2013). *Basidiomycota* white rot fungi have demonstrated their ability to degrade all components of lignocellulosic biomass, such as wood, and have therefore become promising candidates, in recent years, for biotechnological applications such as the pretreatment of plant feedstocks for conversion to bioethanol, other biofuels and the production of value-added bioproducts. Genomic and transcriptomic studies of certain fungi of the *Basidiomycota Agaricomycetes* family have revealed certain genes that cause wood degradation (Mäkinen et al. 2018). Oxidation and depolymerization of lignin are considered a significant barrier to the industrial use of lignocelluloses from multiple sources such as wood residues. The lignin may be modified, and may sometimes be mineralized by extracellular oxidoreductases produced by white rot fungi, including lignin and manganese peroxidases, in conjunction with some enzyme of hydrogen peroxide generating such as glyoxal oxidases or aryl alcohol oxidases (Mäkinen et al. 2018).

In the transcriptomic level of *A. sinapina*, enzymes such as laccase, MnP, VP, several $H_2O_2$-generating oxidases and polysaccharide monooxygenase were evident. The majority of these enzymes were also found in the transcriptome of *A. solidipes* and other white rot fungi (Jain et al. 2019).

The versatility of the North species, such as *A. sinapina* species to biodegrade all structural components of plant cell walls is clearly shown in its transcriptome which has a large number of genes associated to the degradation of plant cell walls, including enzymes that degrade lignin, cellulose, hemicellulose, and pectin. The BLASTx search of the de novo assembly of RNA-seq analysis of *A. sinapina* resulted into several transcripts encoding enzymes involved in the depolymerization and decomposition of cell walls.

Manganese peroxidase (MnP) that belongs to the glycoprotein family, is found in many different forms and in the fungal strain of *C. subvermisposa* with more than 11 isoforms (Bugg et al. 2011). In annotation of transcriptomic of *A. sinapina*, 2 diferent (MnP) were found.

Laccases are found in various organisms such as bacteria and fungi, plants as well as insects. They are a group of enzymes which have the ability to oxidize various phenolic substrates by reducing the oxygen in water (Bugg et al. 2011). Recently, a great interest has been shown in the enzyme family of mono-oxygenase polysaccharides, which has been found in large numbers in gene analyses of white rot fungi (Li et al. 2019). Polysaccharide monooxygenase is a class of copper-dependent enzyme that plays a role in boosting the degradation of polysaccharides in lignocellulosic biomasses. It is capable of weak hydrolysis of beta-glucan arley, carboxymethylcellulose, lichenan, wheat arabinoxylan and birch xylan. In addition, polysaccharide monooxygenase has the ability to stimulate the hydrolysis of lignocellulosic substrates, such as hydrothermally pretreated wheat straw, in conjunction with other cellulolytic enzymes (Dimarogona et al. 2012; Li et al. 2019). Genes encoding for polysaccharide monooxygenase Cel16 has been found in annotation of RNA-seq result of *A. sinapina* and were found to be upregulated genes in presence of betulin.

The deconstruction of plant biomass needs associations of enzymes that work in synergy on cellulose, hemicelluloses and pectin (Kuhad et al. 2016). The potential of *A. sinapina* in biomass deconstruction is evident due to the presence of various proteins belonging to the glycosylhydrolase family. Beta-glucosidase, alpha-xylosidase, probable exo-1.4 beta xylosidase, endo-1,4-beta-xylanase, bifunctional xylanase/deacetylase, xyloglucanase, polygalacturonase and exopolygalacturonase, endo-beta-1,4-glucanase D, alpha-xylosidase and alpha-glucosidase, Arabinan endo-1,5-alpha-L-arabinosidase A and beta-mannosidase were found in annotation transcriptome of *A. sinapina*.

Regarding differentially expressed transcripts encoding enzymes involved in cell wall modifications, it is noteworthy that there were more genes up-regulated than

down-regulated (Table 5). Laccase, Versatile peroxidase VPL1, beta-Glucosidase, Feruloyl esterase, Alpha-Mannosidase, Xyloglucanase, Endo-beta-1,4-glucanase D, Endoglucanase E-4, Beta-glucosidase 1A were up-regulated. On the other hand, pectate lyase was down-regulated. The cell wall is an important barrier for pathogens when they attack plants. As well known, pathogens produce a wide range of enzymes such as pectinases, polygalacturonases, pectins, pectate lyases and pectin esterases to digest against homogalacturonans and rhamnogalacturonans. This class of enzymes has been investigated in several studies because they are secreted at the very beginning of the pathogenesis case (Esquerré-Tugayé, Boudart, and Dumas 2000). The secretion of theses enzymes correlates to various factors. In 2013, Drori et al. indicate that secretion of pectate lyases could be influenced by nutritional factors, such as the presence of nitrogen in culturning medium. In addition, in 2006 Kramer-Haimovich et al. suggest that nutritional deficiency in primary nitrogen sources plays a critical role in PL secretion and fungal pathogenicity (Miyara et al. 2008; Drori et al. 2003). In addition, it has been reported that pectate lyases have an absolute requirement for $Ca^{2+}$ ions (Tardy et al. 1997). The negative regulation of pectate lyase could be explained by different factors, such as the culture medium poor in nitrogen or $Ca^{2+}$, pH of the medium, temperature, and the presence of triterpenoids (betulin) which could have been inhibitors to this enzyme (Tang et al. 2019). Most of the up reguler genes were referred to the genus Trametes versicolor while all the down regulated genes were referred to the genus *Armillara*. This could be related to the fact that these two white rot fungi are close families, and in addition, several transcriptomic studies have already been conducted on rametes versicolor fungi.

## Conclusion

In the study, we used *A. sinapina*, a root-disease pathogen of hardwood hosts as a model to explore the changes of transcriptional level after betulin treatment using Illumina paired-end sequencing method. About 343,289 unigenes were obtained from *A. sinapina* mycelium by de novo assembly. Various genes homologues, with important roles in the degradation of plant cell walls and involved in the production of metabolites, were identified and classified. 197,377 ungines were non-annotated. These results

demonstrate that Illumina paired-end sequencing is a fast and cost-effective approach for the discovery of new genes in non-model organism. This research may be used to better understanding of use of *Armillaria sinapina* and will facilitate the identification of candidate genes for uses in different processes, such as biotransformation, and also as an enzyme platform for the degradation and depolymerization of woody biomass to produce biofuels and a chemical products. Further work on the nature of the genes of the cytochrome P450 family ,not characterized to date, and the non-annotated genes could reveal a relevant element to understand the relationship between *A. sinapina* and its plant host, and thus its involvement in the life cycle of the forest ecosystem.

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**Competing Interests**

The authors have declared that no competing interests exist.

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Le chapitre V contient une discussion des principaux résultats présentés dans les chapitres II, III et IV avec de futures perspectives.

# CHAPITRE V

# CONCLUSION

Chaque année, on estime à plus de 20 millions de tonnes de matière sèche la quantité de résidus découlant de l'activité de l'industrie forestière canadienne (Meda, 2018). Les résidus forestiers ligneux tels que les écorces, et non-ligneux comme les champignons, sont généralement incinérés, enfouis ou utilisés comme combustibles à des fins énergétiques. De nombreuses études visant à valoriser ces résidus forestiers ont montré que les extraits de ces résidus forestiers renferment une grande diversité de molécules aux propriétés biologiques. Spécifiquement, l'écorce du bouleau blanc, une source d'énergie très importante pour les industriels, renferme de nombreuses molécules bioactives telles que les polyphénols et les terpènes (Zyryanova et al. 2010).

Avec l'arrivée et la popularisation de la science des omiques (le séquençage de nouvelles technologies, la protéomique et la métabolomique), il est plus facile de comprendre les phénomènes génétiques présents dans la nature et les exploiter dans des bioprocédés. Le séquençage à haut débit permet une exploration plus fine des interactions plantes-champignons et aussi une vue d'ensemble plus complète sur le potentiel biocatalytique des enzymes présentes dans l'organisme étudié. De plus, l'accumulation de ces données omiques permet l'émergence d'une nouvelle biologie « verte » dans le domaine des biotechnologies.

En raison de l'intérêt grandissant pour les molécules bioactives telles que les terpènes et pour les procédés de biotransformation, le travail entrepris dans le cadre de cette thèse visait à caractériser le transcriptome de deux champignons et leurs potentiels d'application dans les bioprocédés. *Inonotus obliquus* et *Armillaria sinapina* de la famille des basidiomycètes trouvés en Amérique du Nord parasitent les troncs des bouleaux blancs. Nous avons utilisé l'approche par analyse transcriptomique à large échelle

RNAseq de la plateforme Illumina Hiseq4000 suivie d'un assemblage de novo pour visualiser le profil complet de l'expression des gènes dans les différentes conditions de cultures.

Cette étude a permis dans le chapitre II i) de déterminer les conditions optimales de croissance à l'échelle du laboratoire en culture solide et liquide, ii) de caractériser des voies moléculaires conservées/uniques pour la biosynthèse des terpènes et iii) d'étudier les modulations de l'expression génique lors d'ajout de substrat (la bétuline) ou en présence d'écorces de bouleau blanc dans le milieu de culture du mycélium d'*Inonotus obliquus*.

Dans la nature, les sclérotes d'*I. obliquus* poussent très lentement et la culture artificielle est difficile. Afin de produire de grandes quantités de biomasse, *I. obliquus* a été cultivé dans des conditions de croissance différentes. Dans le deuxième chapitre, les résultats obtenus sont en corrélation avec les résultats des recherches publiées (Xu et al. 2019; Xu et al. 2017) : une température de 28 °C, avec une agitation à 150 rpm et un pH légèrement acide (pH 6.2) sont favorables à la croissance du champignon, et ce, en présence ou non d'écorces de bouleaux blancs. Ces résultats pourraient servir de référence pour des études ultérieures pour la production des mycéliums d'*I. obliquus* à échelle pilote/industrielle (Fradj et al. 2019).

Le chaga sauvage, présent dans les latitudes nord d'Amérique, de l'Europe et de l'Asie, a le potentiel de s'adapter aux changements de températures les plus extrêmes. Ainsi, il serait intéressant d'étudier la croissance de ce champignon médicinal à des températures proches de 0 °C.

À notre connaissance, aucune étude transcriptomique visant à une meilleure compréhension du mécanisme de production des métabolites secondaires n'a été publiée sur ce champignon à ce jour. Nos résultats transcriptomiques du chaga démontrent une très bonne qualité des données et une plus grande profondeur en comparaison avec d'autres études transcriptomiques sur des champignons de la même famille (Zou et al.

2016b). Des millions de lectures ont été générées et assemblées en utilisant de novo assembly à partir de l'ARN extrait des cultures liquides du mycélium d'*Inonotus obliquus* cultivé en laboratoire, en présence ou non de deux substrats : les écorces de bouleau blanc et la bétuline. Cette dernière est présente à forte concentration dans les écorces de bouleaux blancs. Ces résultats ont permis de générer deux à trois fois plus de lectures en comparaison avec l'étude menée sur l'*Inonotus baumii* par Zou et al. en 2016 (Zou et al. 2016b). Grâce aux outils d'annotations et aux bases de données, un grand nombre de transcrits ont pu être identifiés par similarité. De plus, de nouveaux transcrits non identifiés ont été découverts et dix-huit séquences de transcrits impliqués dans la voie du mévalonate pour la production des terpènes ont été répertoriés dans le transcriptome de *I. obliquus*. Huit gènes directement impliqués dans la voie de biosynthèse des triterpènes ont ensuite été confirmés par PCR quantitatives. En se basant sur les résultats obtenus, nous avons établi pour la première fois la voie de biosynthèse des terpènes chez le chaga. Un gène est considéré comme étant différentiellement exprimé s'il présente un *p* ajusté $\leq 0.05$ et un fold change (log2FC) $\geq 1$. En présence de la bétuline, 441 transcrits ont été exprimés différentiellement avec 139 gènes à régulation positive contre 302 gènes à régulation négative. En présence d'écorce de bouleau blanc, 9 707 gènes ont été exprimés différentiellement par rapport au contrôle, soit 22 fois plus en comparaison avec les gènes modulés en présence de la bétuline ; avec plus de 5 000 gènes régulés positivement, et plus de 4 000 régulés négativement. Ces résultats laissent supposer que la présence de l'écorce de bouleau a des impacts moléculaires bien différents et nécessite un changement transcriptomique plus important pour le mycélium de *I. obliquus* en comparaison avec la simple présence de la bétuline dans le milieu. Malgré ces résultats novateurs et intéressants, plusieurs de ses gènes différentiellement exprimés n'ont pas pu être identifiés en utilisant les différentes bases de données (Blast, Swiss-Prot)). L'approche couramment utilisée dans l'analyse des transcriptomes est l'annotation fonctionnelle par similarité de séquence avec les bases de données par blast. Toutefois, cela entraine souvent des erreurs d'interprétation des fonctions géniques en raison du manque d'homologie avec les gènes trouvés chez les organismes modèles. Pour contrer cette limite, une caractérisation fonctionnelle des gènes différentiellement exprimés est nécessaire. Ces informations

cruciales permettraient la découverte de nouveaux gènes qui pourraient être exploités dans les bioprocédés.

Dans le cadre d'une vision plus approfondie des gènes présents dans le chaga, une analyse transcriptomique du chaga type sauvage directement collecté sur le bouleau serait très intéressante. Une comparaison de ses résultats avec ceux du transcriptome du mycélium cultivé en laboratoire en présence d'écorce de bouleau blanc permettrait de récolter des données significatives. Une telle étude permettrait également l'analyse de l'expression génique différentielle dans les deux conditions. De plus, les résultats ainsi obtenus pourraient aboutir à une meilleure compréhension des mécanismes d'interaction entre l'hôte et ce champignon. Enfin, une caractérisation du mécanisme de production/stockage des terpènes par les cellules du chaga serait un axe de recherche intéressant.

Cependant, malgré la facilité et la disponibilité grandissante des outils de séquençage de nouvelle génération, le coût engagé pour l'analyse d'un échantillon reste un facteur limitant. De plus, en vue des millions de données récoltées par l'analyse, une infrastructure informatique appropriée est nécessaire pour le stockage et traitement des données engendrées par cette approche. En outre, en raison de l'évolution continue de ces techniques de séquençage, le développement d'outil bio-informatique est nécessaire pour l'amélioration des méthodes statistiques et de normalisations des données recueillies.

Dans le troisième chapitre, nous avons effectué une étude visant à caractériser le potentiel biocatalytique d'*Inonotus obliquus* dans le procédé de biotransformation de triterpènes selon diverses conditions de cultures. Les résultats préliminaires de l'analyse métabolique du mycélium d'*Inonotus obliquus* avec ou sans traitement ont montré sa capacité à bio-produire des métabolites spécialisés de type triterpène. L'analyse des métabolites produits a été réalisée en utilisant la chromatographie liquide à haute performance combinée à une spectrométrie de masse à temps de vol (LC-qTOF-MS/MS). Cette étude a démontré que l'ajout des écorces de bouleau blanc ou de la bétuline augmentait efficacement la production de métabolites bioactifs dans les cultures

d'*I. obliquus*. Ces résultats apportent une nouvelle méthode pour la production des triterpènes en utilisant une souche fongique.

L'élaboration d'un protocole d'extraction des métabolites spécialisés en utilisant des procédés moins polluants et peu coûteux tels que les procédés d'extraction supercritiques (utilisation d'un fluide supercritique tel que le $CO_2$) serait très intéressant et prometteur pour des applications futures. Étant donné que la production des métabolites spécialisés est corrélée à divers facteurs, tels que les cofacteurs, la période et les conditions de cultures, il serait judicieux d'investiguer plus en profondeur la possibilité d'améliorer les conditions de culture en ajoutant des cofacteurs enzymatiques pour un meilleur rendement de biotransformation. Une étude récente a démontré que l'ajout d'un composant organique, le jasmone de méthyle, aurait un effet positif sur la production des terpènes chez *Inonotus obliquus* (Zou et al. 2016b). Une étude transcriptomique et une étude sur la culture à échelle pilote du mycélium du chaga en présence de ce composé seraient également des axes de recherche intéressants à développer, et permettraient à la fois de mieux comprendre son rôle dans la production des terpènes et d'optimiser leur rendement.

Dans le dernier chapitre, nous avons présenté les résultats de l'analyse transcriptomique du champignon de la pourriture blanche *Armillaria sinapina.* Nous avons mis en évidence les gènes impliqués dans la voie de biosynthèse des terpènes, caractérisé l'expression génique différentielle en présence de bétuline et déterminé les gènes jouant un rôle majeur dans la dégradation des parois cellulaires des plantes pour la dépolymérisation de la lignine de ce champignon. Cette première étude transcriptomique *d'Armillaria sinapina* a généré 27 356 561 transcrits. Ces résultats obtenus pour *Armillaria sinapina* sont à mettre en comparaison avec ceux de l'étude transcriptomique de *Armillaria solidipes,* qui n'ont généré que 20 882 transcrits (Ross-Davis et al. 2013).

Plusieurs voies de biosynthèse ont été répertoriées dans l'analyse KEGG réalisée. Onze gènes impliqués dans la voie de biosynthèse des terpènes ont été identifiés. En outre, 25 gènes susceptibles de jouer un rôle significatif dans la dégradation de la lignine ont été

répertoriés. En présence de bétuline, 3943 transcrits exprimés de façon différentielle ont été identifiés. Quarante-huit fois plus de transcrits (3 863) ont été régulés à la hausse en présence de la bétuline avec seulement 80 gènes dont l'expression a été régulée négativement. Plusieurs cytochromes P450 ont été identifiés dans le transcriptome de *A. sinapina* et régulés positivement en présence de la bétuline. Les CYP450 sont connus pour être une superfamille capable de catalyser diverses réactions, souvent à des étapes limitantes. Par ailleurs, la conversion de la bétuline en aldéhyde bétulinique ou en acide bétulinique implique une réaction d'oxydoréduction souvent effectuée par des enzymes de la famille CYP450 ; ce qui fait que plusieurs candidats intéressants notamment pour la biotransformation de la bétuline sont présents dans le transcriptome caractérisé. Ainsi, une étude visant la caractérisation fonctionnelle des gènes des CYP régulés positivement en présence de bétuline serait intéressante. De plus, une étude transcriptomique du mycélium d'*Armillaria sinapina* en présence d'écorces de bouleau blanc ajouterait des informations cruciales sur son pouvoir de biotransformation et de dégradation de la lignine.

Finalement, l'utilisation des techniques de séquençage de nouvelle génération pour l'étude transcriptomique d'espèces n'ayant pas de génome de référence complet répertorié est une approche efficace pour la découverte de gènes et pour l'identification de transcrits impliqués dans des processus biologiques spécifiques. Les trois axes de recherche proposés ici ont tous pour but de valoriser les résidus forestiers et fournir un cadre pour de nouvelles orientations de recherche, afin de mieux comprendre les facteurs qui font des champignons des biocatalyseurs très intéressants dans les bioprocédés comme la bioconversion fongique. Les résultats obtenus lors de cette recherche devraient permettre d'orienter les futures recherches sur la bioconversion fongique pour la bio-production de métabolites spécialisés à valeur ajoutée, et sur les bioprocédés pour la dépolymérisation des matières végétales.

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