Biology, chemical characterization and antioxidant activities of the culinary medicinal mushroom Agaricus subrufescens

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Thèse présentée à

L'UNIVERSITE DE PAU ET DES PAYS DE L'ADOUR

Ecole Doctorale des Sciences Exactes et Leurs Applications ED211

Par

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Pour l'obtention du grade de

DOCTEUR

Spécialité : Sciences Agronomiques, Biotechnologies agro-alimentaires

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Biologie, caractérisation chimique et activités antioxydantes du champignon comestible et médicinal *Agaricus subrufescens*

Soutenue le 5 juillet 2013

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REMERCIEMENTS

Cette thèse n'appartient pas qu'à moi, puisque plusieurs personnes ont contribué directement ou indirectement à ce travail. Ces années passées en France furent parmi les meilleures de ma scolarité, en grande partie grâce à tous ceux qui m'ont accompagné au cours de ces trois dernières années.

C'est un plaisir de remercier les personnes qui généreusement ont partagé leur temps et leurs connaissances avec moi :

Je tiens en premier lieu à remercier vivement Madame Michèle Largeteau pour avoir accepté de co-diriger ce travail de recherche et s'être rendu disponible tout au long de ces trois années que ce soit pour un avis ou un conseil en matière de rédaction.

Je remercie également à Madame le Professeur Catherine Regnault-Roger, co-directrice de la thèse, pour ses conseils avisés sur le procès de ma formation comme docteur, et ses conseils en matière de rédaction.

Je tiens à remercier Monsieur le Professeur Marian Petre et Madame le Professeur Sylvie Rapior qui me font l'honneur d'accepter d'être mes rapporteurs.

Je souhaite remercier Madame Anne Marie Farnet qui a bien voulu juger ce travail.

Je remercie Monsieur Jean-Michel Savoie, pour m'avoir accueilli dans les locaux de MycSA, son support pour ses conseils et surtout pour le temps dépensé afin que cette thèse se réalise dans les meilleures conditions.

Un spécial remerciement à Philippe, pour nos conversations passionnantes sur les champignons, comme un bon ami et extrêmement aimable hôte durant mon séjour en France.

Je tiens à exprimer ma gratitude à Marie pour sa patience à m'écouter et ses précieux conseils sur la thèse et dans d'autres situations particulières comme dans « la cuisine ».

Je dois beaucoup à Nathalie Ferrer, pour ses précieuses collaborations dans les manips, et sa confiance depuis le début de ce projet. Heureusement que sa porte était toujours ouverte pour venir manger du chocolat, échanger, et discuter après une longue journée.

Je souhaite également remercier Magalie, Cathy, Christophe et Thierry. Merci pour vos coups de mains, votre gentillesse et votre bonne humeur.

Je n'ai oublierai pas Martine, Marie France, Corinne et Véronique qui depuis mon arrivé en France m'ont beaucoup aidé dans les démarches administratives, avec bonne humeur.

Merci encore à Anne-Marie Farnet du laboratoire de Marseille, où j'ai été merveilleusement accueilli (sur le plan scientifique et social) lors de mon stage pendant un mois. Merci à tous, et tout particulièrement à Fred, Anne Seline. Je me réjouis que la collaboration engagée ait pu aboutir à une publication.

Je remercie toute l'équipe de MycSA pour m'avoir accueilli pendant plus de 3 ans. J'ai beaucoup appris et partagé d'excellents moments dans les couloirs et la salle de café.

J'exprime toute ma reconnaissance à mes collègues du laboratoire qui sont déjà parti de MycSA, Aude, Laura, Laurie, Pilar, Sébastien, Adeline, Jawad, Aurélia, Guillaume et Yann, pour leur patience dans les leçons de français, pour les soirées, et surtout pour leur soutien comme amis.

Je souhaite également remercier tous les collègues avec qui j'ai pu interagir à MycSA, Ccori, Mathilde, Enric, Lea, Bettina, Marie Victoire, Manuella et Banafshe, pour leurs amitié, pour leurs aides, leurs sourires et pour l'agréable ambiance de travail.

A Linda, ma mycologue favorite, pour ton aide précieuse dans le domaine taxonomique, et les conversations rigolotes autour de la science. Et parce qu'avec toi le séjour en Thaïlande a été beaucoup plus agréable.

Finalement, la réalisation de ce travail a été possible grâce au financement d'une bourse par le Conseil National de Science et Technologie (CONACyT-Mexique) que je remercie.

PUBLICATIONS ET COMMUNICATIONS

Ce travail de thèse a donné lieu à :

- Quatre articles publiés dans des revues à comité de lecture,

Largeteau M. L., **Llarena-Hernandez R. C.,** Regnault-Roger C., Savoie J.-M. (2011) The medicinal *Agaricus* mushroom cultivated in Brazil: biology, cultivation and non-medicinal valorisation. **Review**. *Applied Microbiology and Biotechnology* **92** (5): 897-907.

Peter-Valence F., **Llarena R.C**., Largeteau M., Savoie J.-M., Ruaudel F., Ziarelli, F., Ferré E. Farnet A.-M. (2011) Chemical characterization of the biomass of an edible medicinal mushroom, *Agaricus subrufescens*, via solid-state ^{13}C NMR. *Journal of Agricultural and Food Chemistry* **59:** 8939-8943.

Llarena-Hernandez R. C., Largeteau M., Farnet A.-M., Foulongne-Oriol M., Ferrer N., Regnault-Roger C., Savoie J.-M. (2013) Potentiel of European wild strains of Agaricus subrufescens for productivity and quality on wheat straw based compost. *World Journal of Microbiology and Biotechnology* **29:** 1243-1253.

Llarena-Hernandez R. C., Largeteau M., Ferrer N., Regnault-Roger C., Savoie J.-M. (2013). Optimization of the cultivation conditions for mushroom production with European wild strains of *Agaricus subrufescens* and Brazilian cultivars. *Journal of the Science of Food and Agricultural* DOI 10.1002/jsfa.6200.

- Un article soumis à une revue à comité de lecture:

Llarena-Hernandez R. C., Largeteau M., Ferrer N., Regnault-Roger C., Savoie J.-M. (2013). Antioxidant activities in primordia, stipes and caps of cultivars and wild strains of the almond mushroom *Agaricus subrufescens*. *Journal of Agricultural and Food Chemistry*

- Un chapitre de livre ISBN:

Savoie J.-M., **Llarena-Hernandez R. C.,** Mata, G. and Largeteau M. (2012). Cultivation of Medicinal Almond Mushrooms, *Agaricus subrufescens*, in Europe. *In:* Mushroom biotechnology and bioengineering, 115-126. Petre and Berovic (Eds). Bucharest, Romania. ISBN 978-606-528-146-2.

- Une communication orale avec résumé dans un congrès international

Llarena-Hernández R.C., Largeteau M., Foulongne-Oriol M., Callac P., Savoie J.M (2012). Improvements in the production of the medicinal mushroom, *Agaricus subrufescens*, using biological resources and breeding. 18th Congress of the International Society for Mushroom Science, Beijing, China.

- Une communication par affiche et texte complet dans un congrès international,

Llarena-Hernández R.C., Largeteau M., Farnet A.-M., Minvielle N., Regnault-Roger C., Savoie J.M.(2011) Phenotypic variability in cultivars and wild strains of *Agaricus brasiliensis* and *Agaricus subrufescens*. *In*: Proc. of the 7th Int. Conf. on Mushroom Biology and Mushroom Products. Vol 2. pp 176-181. INRA, Fr.
http://www.wsmbmp.org/Previous_Conference_7.html

- Une communication par affiche dans un congrès national

Llarena-Hernandez R.C., Largeteau M., Farnet A.-M., Regnault-Roger C., Savoie J.M. (2011) Biology of *Agaricus subrufescens* and identification of bioactive molecules in plant protection. 9èmes rencontres Phytopathologie/Mycologie, 16-20 January, Aussois.

Biologie, caractérisation chimique et activités antioxydantes du champignon comestible et médicinal *Agaricus subrufescens*

RESUME

Récemment, *Agaricus subrufescens* est devenu un champignon d'intérêt de par ses propriétés médicinales. Sa production commerciale s'est développée dans des pays tropicaux à partir de matériaux locaux. Cependant, les données disponibles dans la littérature ne concernent que des cultivars qui sont génétiquement similaires. Notre étude sur un ensemble de cultivars et de souches sauvages a conduit à une meilleure connaissance de la biologie de l'espèce. Contrairement aux cultivars, les souches sauvages présentent un haut niveau de polymorphisme génétique. Une variabilité phénotypique élevée a été mise en évidence pour l'accroissement mycélien, la productivité et la morphologie. Des analyses chimiques par RMN du solide et l'estimation des activités anti-oxydantes ont montré que les souches sauvages comme les cultivars sont une source à exploiter comme aliment fonctionnel pour prévenir les maladies cardio-vasculaire, les cancers et le diabète. L'adaptation des conditions culturales à partir du substrat utilisé pour la culture d'*A. bisporus* a permis d'obtenir des fructifications d'*A. subrufescens*, puis d'augmenter le rendement et d'optimiser des caractères agronomiques d'intérêt. Du matériel sauvage avec une activité antioxydante et un rendement élevés a été identifié. L'évaluation d'hybrides intercontinentaux a montré la possibilité de transférer des caractères d'intérêt à une descendance. *Agaricus subrufescens* pourrait être proposé aux champignonnistes français comme alternative à *Agaricus bisporus* pendant la saison estivale.

Mots clés : Antioxydant, biodiversité, qualité, rendement.

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SUMMARY

Agaricus subrufescens is becoming a mushroom of interest because of its medicinal properties. Commercial production had developed in tropical countries using local materials. However, data available in the literature referred to cultivars that are genetically similar. Our study of a set of cultivars and wild strains led to a better understanding of the biology of the species. Contrary to cultivars, wild strains exhibited a high level of genetic polymorphism. High phenotypic variability was identified in mycelial growth, productivity and morphology. Chemical analyses by solid-state ^{13}C NMR and antioxidant activities showed that the wild strains as well as the cultivars proved a valuable source of functional food to prevent cardiovascular diseases, cancers and diabetes. Using cultivation substrate produced for *A. bisporus* and modifying cultivation conditions allowed fruiting of *A. subrufescens*, then increase in strain yield and optimization of agronomic traits of interest. Wild material with good antioxidant activity and high productivity was identified. The evaluation of intercontinental hybrids showed the possibility to transfer traits of interest to offspring. *A. subrufescens* could be proposed to French mushroom growers as an alternative to *Agaricus bisporus* during the summer season.

Keywords: Antioxidant, biodiversity, cultivation, quality.

GLOSSAIRE

ABTS: 2, 2'-azinobis (3-ethylenebenzothiazoline-6-sulphonic acid).

ADN : Acide désoxyribonucléique.

Anse d'anastomose : structure propre aux champignons basidiomycètes, qui permet à la structure dicaryotique de se transmettre à toutes les cellules du mycélium secondaire.

Blanc : semence commerciale sur un matériau support (grains de céréales) qui est habituellement utilisée pour inoculer le substrat de culture.

CGAB : Collection des Germoplams des Agarics à Bordeaux.

DAS: *Shared Allele Distance* (distance d'allèles partagés).

DPPH : Diphényl-2,2-pycryl-1-hydrazyle.

FDA: Diacétate de fluorescéine.

Gobetage : Opération consistant à recouvrir le substrat de culture avec une couche d'un matériau (appelé terre de gobetage) constitué, notamment en France, par un mélange de calcaire et de tourbe.

H_2O_2 : peroxyde d'hydrogène.

Lardage : ensemencement du substrat de culture avec du "blanc".

ppm: parties par million.

PSUMCC: Pennsylvania State University Mushroom Culture Collection.

Polymorphisme génétique. Coexistence de plusieurs allèles pour un locus donné dans une population.

RMN ^{13}C: Résonance Magnétique Nucléaire du carbone 13.

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Introduction

Le nombre d'espèces de champignons estimé actuellement sur la planète est de 150 000, dont, seulement 10% environ sont connus au niveau scientifique (Hawksworth, 2001). Plus de 3000 champignons sont définis comme des espèces comestibles majeures (*prime edible species*), parmi lesquels une centaine seulement sont, ou ont été, cultivés commercialement, et une dizaine parmi les 100 le sont à l'échelle industrielle. La production mondiale de champignons comestibles cultivés était de 24 000 milliers de tonnes en 2009 (Chang et Wasser 2012) et elle est en constante augmentation depuis une vingtaine d'années. La première raison de l'accroissement de la consommation est une combinaison de leur valeur comme aliment et de leurs propriétés médicinales ainsi que leur utilisation comme neutraceutiques (suppléments alimentaires). Le marché mondial était d'environ 36 000 milliards de dollars en 2006, dont 16 000 milliards pour les champignons médicinaux (Chang et Wasser, 2012).

Les projets d'amélioration des champignons s'adressent d'abord aux demandes des producteurs, pour lesquels des critères comme le rendement, la conservation après récolte et la résistance aux maladies sont parmi les plus importants. Cependant, les consommateurs et l'industrie alimentaire ont des intérêts différents tels que la qualité des traitements en cours de culture et post récolte, le goût, la valeur nutritionnelle, et la teneur en composés bioactifs. Ce dernier critère prend de l'importance ces dernières années.

Selon la littérature, les champignons médicinaux possèdent des propriétés anti-oxydantes, de piégeage de radicaux libres et de détoxification, et de nombreux effets thérapeutiques tels que des activités anti-tumorales, antivirales, antibactériennes, antiparasitaires, antifongiques, antidiabétiques, anti-hypercholestérolémie, hépato-protectrices, stimulatrices du système immunitaire, protectrices du système cardiovasculaire (Didukh et al., 2003; Gao et Zhou, 2003; Sullivan et al., 2006; Wasser, 1999 et 2010; Zhang et al., 2007).

Plusieurs espèces de champignons sont connues pour leur intérêt thérapeutique. La majorité des champignons médicinaux appartiennent à des espèces non comestibles, et un grand nombre d'entre eux montrent un gout amer et piquant, spécialement ceux qui appartiennent aux Polyporaceae, qui sont utilisés comme médicament en Chine depuis les

temps ancestraux. Des espèces non comestibles à potentiel médical, tel que *Ganoderma lucidum*, *Trametes versicolor* et *Inonotus obliquus*, sont très piquantes et/ou peu digestes. Ces champignons sont utilisés sous forme d'extrait, d'infusion ou de poudre. Ceux qui sont de bons comestibles portent le nom de 'champignons culinaires médicinaux' (*culinary-medicinal mushrooms*) (Reis et al., 2011).

Le genre *Agaricus* est composé d'espèces très connues, récoltées ou cultivées, dont l'intérêt n'est pas seulement alimentaire. *Agaricus sylvaticus* (Percario et al. 2009), *Agaricus bisporus*, et surtout *Agaricus subrufescens* (Kozarski et al., 2011 ; Wasser et Weis, 1999 ; Chang et Wasser, 2012) font partie des champignons culinaires médicinaux. Cependant, la biodiversité à l'intérieur du genre est peu connue, que ce soit au niveau inter spécifique ou intra spécifique.

Agaricus subrufescens est un nouveau champignon cultivé, développé surtout en conditions tropicales, ce qui peut représenter une opportunité pour les producteurs d'*Agaricus bisporus* dans les régions tempérées pendant la saison chaude. Toutefois, sa culture est souvent artisanale et demande encore à être améliorée. La connaissance de la biodiversité à l'intérieur de l'espèce est nécessaire pour une amélioration des caractères agronomiques et de la qualité comme aliment fonctionnel.

Dans ce contexte notre objectif est d'une part cognitif, contribuer à la connaissance de la biologie d'*A. subrufescens* avec un atout, une collection renfermant des souches d'origines sauvages, et d'autre part à but appliqué, l'adaptation de la culture en régions tempérées.

Après une revue de l'état de l'art, nous indiquerons les axes de notre étude, les résultats acquis en discutant leurs limites avant de poser les perspectives pour mieux valoriser les souches identifiées comme ayant le meilleur potentiel agronomique et neutraceutique.

Chapitre 1. Revue bibliographique

Agaricus subrufescens a fait l'objet de nombreuses publications dans les 10 dernières années à cause de ses qualités thérapeutiques et médicinales. Ces publications sont ciblées sur l'identification de molécules d'intérêt, sur ses propriétés comme aliment fonctionnel et sur les procédés de production soit du mycélium, soit des sporophores (culture). Un état de l'art concernant les cultivars est donné dans l'article 1 (page 31). Les données bibliographiques se rapportant à des souches sauvages ainsi que les apports plus récents sur les cultivars et des renseignements complémentaires sont présentés dans ce chapitre.

1.1. *Agaricus subrufescens*

1.1.1 Positionnement taxonomique, description et cycle de vie

Le champignon a été décrit pour la première fois par C.H. Peck en 1982 aux Etats Unis où il était cultivé aux XIX^{ème} et XX^{ème} siècles (Kerrigan, 2005). Il est connu dans la littérature tout d'abord sous le nom d'*Agaricus blazei* Murrill (*sensus* Heinemann). Ces dernières années, plusieurs articles ont montré que ce nom était incorrect et deux noms ont été proposés, *Agaricus subrufescens* Peck (Kerrigan, 2005) et *Agaricus brasiliensis* Wasser et al. (Wasser et al., 2002). Récemment, Wisitrassameewong et al. (2012b) ont établi que le nom correct est *A. subrufescens* Peck mais n'excluent pas l'existence de taxons infraspécifiques, ni le fait qu'il puisse s'agir d'un complexe d'espèces. Le nom *A. subrufescens* sera utilisé tout au long de ce document pour en faciliter la lecture, quel que soit le nom utilisé dans les articles cités.

La classification taxonomique d'*A. subrufescens* est la suivante :

Règne	Fungi
Phylum	Basidiomycota
Classe	Hymenomycetes
Ordre	Agaricales
Famille	Agaricacea
Genre	*Agaricus*
Espèce	*subrufescens*

Le champignon pousse de manière naturelle au Brésil (Iwade et Mizuno, 1997) comme saprophyte. D'après plusieurs articles, il a été découvert dans la ville brésilienne de Piedade, dans l'Etat brésilien de São Paulo, en 1960 par un producteur et chercheur nommé Furumoto, qui l'a envoyé au Japon en 1965 pour la recherche et l'identification de substances possédant une activité anti tumorale (Mizuno, 1995). Ce champignon a été identifié comme *Agaricus blazei* Murrill par un scientifique belge, le Dr Heinemann, en 1967 (Mizuno, 1995) et a reçu le nom commun de Himematsutake au Japon, tandis qu'au Brésil il a été nommé le « champignon Piedade » du nom de la ville où il a été découvert. Plus tard, il est connu sous le nom de champignon médicinal, ou champignon du soleil® (Cogumelo do Sol® en portugais) au Brésil, et Royal Sun Agaricus® dans d'autres pays. Après la mort de M. Furumoto, la culture d'*A. blazei* a été abandonnée et c'est seulement en 1990 qu'elle a été relancée à la suite de l'intérêt d'industriels japonais qui ont renvoyé au Brésil des souches d'*A. blazei* du Japon où elles avaient été utilisées dans des études sur l'activité antitumorale (Fujimiya et al., 1998 ; Fujimiya et al., 2000 ; Itoh et al., 2008 ; Kawagishi et al., 1989a ; Kawagishi et al., 1988 ; Osaki et al., 1994).

Plusieurs descriptions qui font référence au champignon cultivé mentionnent un chapeau de couleur brun doré (Figure 1.1), de 7 à 9 cm de diamètre, un pied blanc « comme la neige », souvent élargi à la base, des lamelles (matures) brun chocolat foncé et des basidiospores brun chocolat (5x4 µm). La longueur du pied et le diamètre du chapeau sont à peu près égaux (type campestroïde) (Wasser et al 2002 ; Firenzuoli et al., 2008 ; Pokhrel et Ohga, 2007). D'après d'autres auteurs, la morphologie du champignon est assez variable. La forme du chapeau varie de cubique à largement convexe. Le voile est élastique et reste généralement attaché à la marge du chapeau lors de l'expansion, tandis que la couche inférieure se décompose en un grand nombre de petits flocons cotonneux, ce qui constitue une caractéristique assez constante de l'espèce (Figure 1.2).

Malgré l'importance économique du champignon, son cycle de vie n'est pas bien connu. Le mycélium fertile de quatre cultivars brésiliens montre de rares anses d'anastomose, et une grande variabilité a été observée dans le diamètre et la longueur des hyphes (Dias et al., 2008). La taille hétérogène des spores pourrait s'expliquer par la présence d'un nombre variable de basides bi-, tri- et tetra-sporées chez des spécimens sauvages et cultivés d'*A. subrufescens*, qui est considéré comme une espèce amphithallique (Heinemann, 1993 ; Kerrigan, 2005). Un hybride a été obtenu entre une souche développée à partir du matériel

génétique du Brésil et un isolat de Californie (Kerrigan, 2005). L'hybride est plus précoce, a un rendement élevé, produit des sporophores de plus grand calibre et d'apparence attrayante. La ségrégation des allèles chez les descendants démontre que la méiose, la recombinaison et la répartition des noyaux recombinants dans les spores se produisent chez ces isolats d'*A. subrufescens*. Des spores hétérocaryotiques et homocaryotiques ont été observées, alors que d'autres spores étaient homoalléliques pour les marqueurs utilisés. Les auteurs (Kerrigan et Wach, 2008), ont conclu que ces observations sont compatibles avec un cycle de vie amphithallique, c'est-à-dire en partie hétérothallique et en partie pseudohomothallique. Une telle coexistence de cycles de vie différents a été largement étudiée chez *A. bisporus*, et diverses stratégies utilisant les propriétés des cycles de vie ont été développées pour les programmes de sélection (Callac, 2007 ; Callac et al., 2006).

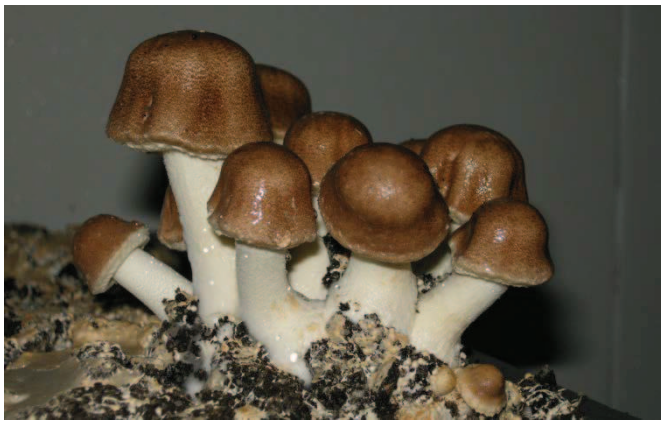

Figure 1.1. Sporophores d'A*garicus subrufescens* à chapeau brun doré caractéristique des cultivars (Photo INRA MycSA).

Figure 1.2. Voile floconneux caractéristique d'*Agaricus subrufescens* (Photo INRA MycSA).

1.1.2. Habitat et répartition géographique

Agaricus subrufescens est un champignon décomposeur de litières, qui pousse naturellement bien dans les sols riches en débris lignicoles, dans les bois mixtes, en bordure de forêts et dans les pâturages riches en débris et bois en décomposition mélangés avec du fumier animal (Firenzuoli et al., 2008 ; Pokhrel et Ohga, 2007 ; Horm et Ohga, 2008). Il est souvent présent dans les habitats domestiqués ou semi perturbés, y compris des tas de feuilles (Kerrigan, 2005). Il pousse naturellement dans les régions montagneuses de la forêt tropicale atlantique dans le sud de l'état de São Paulo, au Brésil. La littérature signale d'autres zones de production que le Brésil. Il est présent aux Etats-Unis, de la Floride à la Californie du Sud (Colauto et al., 2002 ; Fukuda et al., 2003). Il a été trouvé à l'état sauvage en Israël, à Taïwan et Hawaï (Kerrigan, 2005). En se basant sur des caractères morphologiques, Sabir et al. (2002) ont rapporté une collecte d'*A. subrufescens* dans les forêts de Changa Manga, au Pakistan, pendant les mois de mousson. Rishikesh (2003) a trouvé le champignon associé à des arbres forestiers dans la vallée de Siang, Arunachal Pradesh, en Inde. En Thaïlande, *A. subrufescens* pousse dans les forêts avec *Bambuseae* sp., *Dipterocarpus* sp. et *Samanea saman* (Wisitrassameewong et al., 2012a). Des champignons récoltés sur des tas de feuilles de bambou près de la ville de Bihu, dans la province de Zhejiang, en Chine, ont été identifiés comme étant *A. subrufescens* sur des bases morphologiques et la séquence ITS (Song et al., 2012). Le champignon se développe aussi dans les régions tempérées. Il est signalé la première fois en Espagne par Arrillaga et Para (2006). Il a été trouvé dans la forêt de Bercuit, dans le Braban wallon, en Belgique, sur un tas de feuilles, à côté de *Coprinus lagopus*, *Agrocybe rivulosa* et *Volvariella gloiocephala* (Ghyselinck, 2007), et en France, sur un compost d'herbes, dans une clairière (Guinberteau, comm. pers.). Dû à sa capacité à produire des fructifications à une température autour de 26 °C, *A. subrufescens* est considéré comme un Agaricus tropical ayant migré vers les zones tempérées (Zhao et al., 2011). Jusqu'à présent, très peu de mycologues ont prospecté pour ce champignon, aussi, sa répartition géographique est probablement plus étendue.

1.1.3. Zones de production commerciale et marché

La culture des champignons comestibles médicinaux est devenue une alternative économique intéressante au cours des dernières années, principalement en raison de l'augmentation de la demande et de leur valeur sur le marché. La caractéristique de la culture

d'*A. subrufescens* est de valoriser des déchets agricoles et agro-industriels locaux dans des petites entreprises familiales (Guzman et al., 1993). Les zones de production commerciales d'*A. subrufescens* et le marché pendant la période 2001-2009 sont présentés dans l'article 1. Récemment, Chang et Wasser (2012) ont rapporté une production en Chine de 42, 43,6 et 47 tonnes d'*A. brasiliensis* déshydraté dans les années 2003, 2005 et 2007 respectivement, ce qui correspond à une quantité plus élevée que celle indiquée pour le Brésil en 2006-2007 avec 40 tonnes de champignons déshydratés (Tomizawa et al., 2007).

1.1.4. Arôme, goût et valeur nutritionnelle

Les macromycètes comestibles constituent un élément de l'alimentation humaine dans de nombreux pays à travers le monde. Leur intérêt culinaire et commercial résulte principalement de leurs propriétés organoleptiques telles que l'arôme, le goût et la texture.

L'arôme, le goût et la valeur nutritionnelle des souches brésiliennes sont rapportés dans l'article 1. Dans une publication récente sur la composition chimique des sporophores d'*A. subrufescens* (Chang et Wasser, 2012) la valeur citée pour les hydrates de carbones (68,82%) correspond à un pourcentage plus élevé que la fourchette 38-45% rapporté dans l'article 1, ce qui semble montrer de la variabilité à l'intérieur de l'espèce.

Selon Ernesto N. Uryu, un agronome d'une organisation d'assistance technique (Coodenadoria de Assitência Técnica Integral – CATI, Sorocaba, São Paulo, Brazil) et contemporain de M. Furumoto, les champignons originaux cultivés par ce dernier étaient forts en arôme et saveur, ce qui les rendait difficiles à consommer, tandis que les souches renvoyées depuis le Japon avaient perdu plusieurs de ces caractéristiques originales et avaient acquis une odeur plus agréable, une coloration plus claire et un plus gros calibre (Dias et al., 2004).

A l'exception d'une saveur d'amande marquée chez *A. subrufescens* il existe très peu de travaux qui font mention du goût. Geoesel et al. (2011) ont fait un profil sensoriel selon les méthodes internationales d'évaluation de produits (ISO, 1994). Ces auteurs ont testé sept souches d'*A. subrufescens* en comparaison de deux souches d'*A. bisporus* (une variété claire et une crème) et n'ont pas trouvé de différence significative d'intensité de goût entre *A. subrufescens* et *A. bisporus*. Ils ont toutefois défini que le goût d'*A. subrufescens* est clairement différent de celui d'*A. bisporus* et conclu que cette caractéristique pourrait être un avantage ou un désavantage selon les demandes du marché. En plus de sa consommation sous forme déshydratée pour ses effets médicinaux, *A. subrufescens* peut avoir un grand potentiel

au niveau culinaire. Escouto et al. (2006) ont réalisé une enquête au Brésil auprès de 218 personnes âgées de 18 à 56 ans, par apport à l'acceptabilité de ce champignon cuisiné en un plat typique, et obtenu une acceptabilité de 68,3%.

1.1.5. Croissance mycélienne *in vitro* et conservation

Agaricus subrufescens fait partie des champignons médicinaux. Aussi, la majorité des publications sur les méthodes de culture est ciblée sur la définition de conditions de culture *in vitro* qui soient adaptées à la production de composés d'intérêt médicinal en milieu liquide. Ces études montrent que les conditions diffèrent (composition du milieu de culture, temps d'incubation) pour la production de biomasse et celle de neutraceutiques ou de substances à effet médicinal (Fan et al., 2007 ; Gao et Gu, 2007).

Les conditions de stockage de ce champignon et les problèmes liés à sa conservation à basses températures sont présentés brièvement dans l'article 1. En effet, *A. subrufescens* est la seule espèces dans le genre *Agaricus* qui est connue pour ne pas supporter une longue conservation à 4 °C ou à des températures inférieures (Kerrigan, 2005 ; Wasser et al., 2002). Dans le cas d'*A. bisporus*, il est possible de conserver à long terme, dans l'azote liquide (-196 °C), du mycélium développé sur des grains de seigle traités avec du diméthylsulfoxyde (DMSO) sans perte des caractéristiques agronomiques (San Antonio et Hwang, 1982). A la différence de la conservation d'*A. bisporus*, celle d'*A. subrufescens* est généralement faite par repiquage du mycélium en tubes, sur gélose nutritive, avec ajout d'huile minérale pour recouvrir et protéger le mycélium quand il a colonisé le milieu. Mais cette technique peut provoquer des dégénérescences génétiques, et d'autre part demande des repiquages fréquents. Des recherches ont été effectuées pour trouver d'autres méthodes de conservation. Récemment, Colauto et al. (2011) ont démontré que du mycélium développé sur grains de céréale est viable après un an de cryoconservation à -80 °C dans du glycérol à 10%. Par la suite Colauto et al. (2012) ont obtenu 100% de viabilité pour du mycélium sur gélose nutritive conservé 1 an à -80 °C dans du glycérol à 10%, et après 4 ans en utilisant du saccharose ou du glucose comme agent protecteur. Mata et Savoie (2013) ont évalué une conservation à 4 °C et -196 °C à partir de grains de sorgho envahis avec *A. subrufescens*, et récupération du mycélium. Ils ont déterminé une viabilité de 100% dans le traitement à 4 °C et de 84 à 100% avec le traitement à -196 °C, après 6 mois de conservation.

Par ailleurs, un système de conservation utilisant du riz colonisé par du mycélium dans de l'eau à température ambiante avec un taux de 100% de viabilité des souches après un an de stockage a été proposé par Maia et al. (2012).

1.1.6. Mode de culture d'*A. subrufescens*

Que ce soit pour la production de champignons frais destinés à l'alimentation ou pour celle de champignons séchés utilisés pour l'obtention de poudre (production de neutraceutiques), la culture d'*A. subrufescens* est effectuée en milieu solide.

La culture commerciale des champignons comestibles peut s'effectuer sur une large gamme de déchets agro-industriels : sciure de bois, papier, paille de céréales, bagasse de canne à sucre, pulpe de café, feuilles de bananier, déchets d'agave, pulpe de soja (Miranda Gern et al., 2010 ; Salmones et al., 2005), fumiers de bovins, d'équidés, de volailles, de porcs et d'autres animaux domestiques, pailles de blé ou de riz, coton, bois (Chang et Miles, 1984). Le choix du substrat est généralement déterminé par la disponibilité régionale en matériaux de base.

En général au Brésil, les techniques et les paramètres spécifiques à la culture d'*A. subrufescens* sont définis empiriquement sur la base de la culture d'*A. bisporus* (Eira, 2003 ; Mantovani et al., 2007). Cependant, en raison des différences écologiques entre les pays d'origine de ces deux espèces, il est encore nécessaire de développer une technologie propre pour la culture d'*A. subrufescens* puisque son rendement est toujours très faible comparé à celui d'*A. bisporus* (Siqueira et al., 2009). Par exemple, le rendement d'*A. subrufescens* au Brésil est autour de 3 à 25% du substrat humide (3 à 25 kg/100 kg) selon le système de culture (Eira, 2003).

Il existe peu de travaux sur la culture d'*A. subrufescens* à grande échelle. Actuellement, le mode de culture se développe encore (Zied et Minhoni, 2009). La culture est basée sur celle d'*A. bisporus* (Eira, 2003 ; Minhoni et al., 2005) en ce qui concerne la composition du substrat, sa préparation (par compostage) comportant une phase finale de conditionnement appelée pasteurisation, et les différentes étapes du processus (inoculation, incubation, ajout de la terre de couverture permettant la fructification) (Figure 1.1), sauf la température de fructification qui doit se situer entre 20 et 30 °C pour *A. subrufescens* (Almeida Amazonas et Siqueira, 2003) tandis qu'*A. bisporus* fructifie autour de 17 °C (Dias et al., 2004).

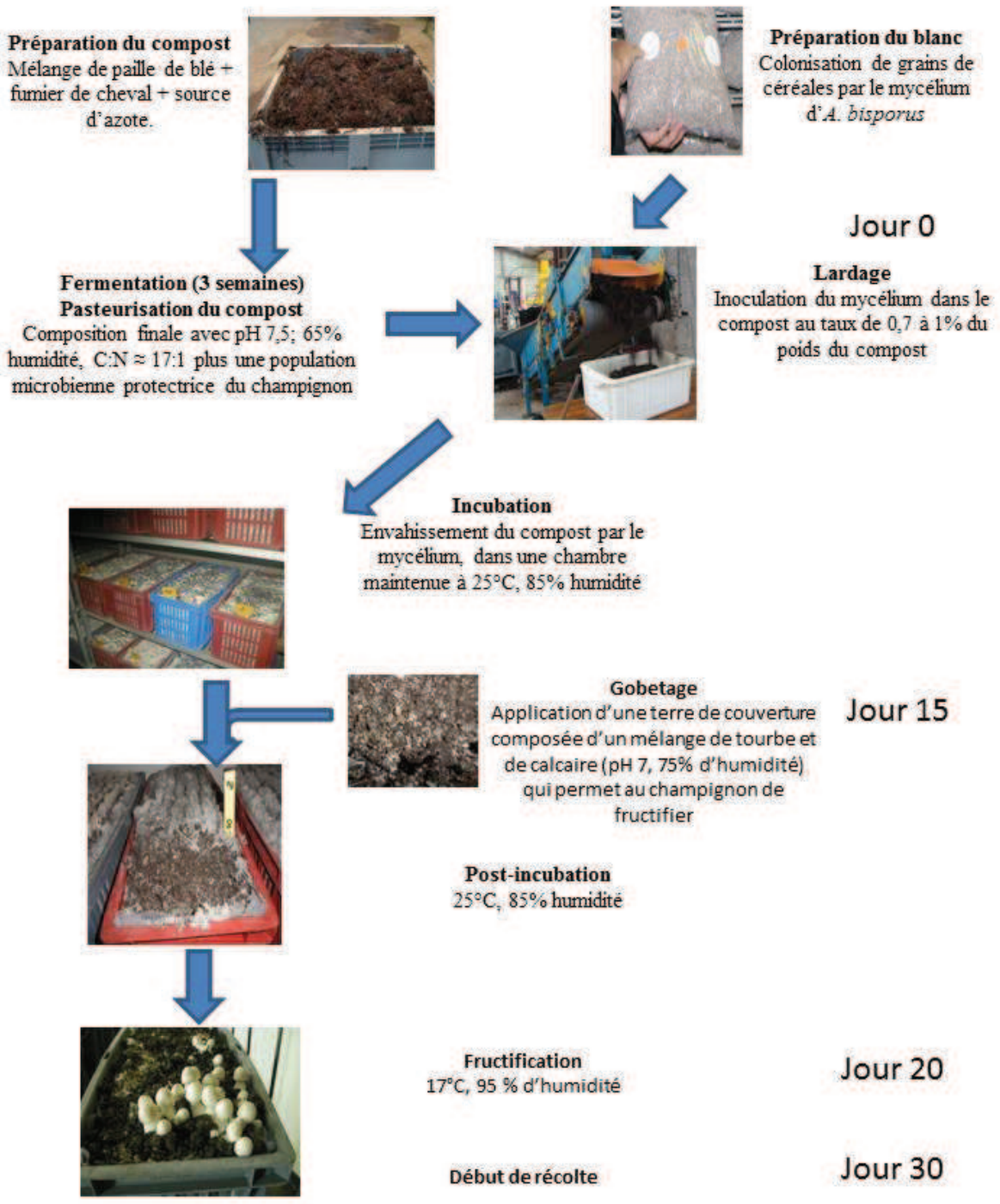

Figure 1.3. Diagramme représentatif du processus de production *d'Agaricus bisporus* (champignon de Paris). (Photos INRA, MycSA).

La culture de ce champignon dans différents pays, à partir de substrats et de mélanges de gobetage locaux est décrite dans le paragraphe 'Mushroom cultivation' de l'article 1. D'autre part, la littérature montre beaucoup de différences dans l'estimation du rapport C/N à obtenir lors de la préparation du substrat de culture. Le rapport C/N initial du compost est important, mais aussi la source d'azote qui agit significativement sur la production d'*A. subrufescens* (Minhoni et al., 2005).Tandis que Zaghi et al. (2010) observent une croissance mycélienne optimale avec un rapport C/N de 11/1, Faccin et al. (2007) ont évalué différents dosages et sources d'azote et ont trouvé une croissance mycélienne supérieure avec des valeurs de C/N entre 10/1 et 50/1, avec l'urée comme principale source d'azote. Andrade et al. (2007) ont obtenu de bons rendements avec des C/N de 29/1. Toutefois Eira et al. (2005) rapportent que des C/N élevés, de l'ordre de 150/1 donnent de meilleurs rendements que des rapports de 33/1 et 40/1, proches de ceux utilisés pour *A. bisporus*. D'autres auteurs ont observé que pour un rapport C/N initial de 37/1 et de 27 à 33/1 à la fin de la phase II, la meilleure productivité est obtenue quand le rapport azote apporté par l'urée/azote de farine de soja (N_u/N_s) est de 1,5/1 (Almeida Amazonas et Siqueira, 2003 ; Siqueira et al., 2011 ; Minhoni et al., 2005). Par comparaison avec les composts qui sont utilisés couramment par les producteurs brésiliens (C/N de 25-30/1 et N_s/Nu de 1/1,5), les composts avec un C/N plus élevé et un N_s/Nu de 1,5/1 ont tendance à être plus productifs (Kopytowski Filho et Minhoni, 2004). Ceci peut s'expliquer par le fait que l'urée est plus rapidement transformée en azote assimilable et ammoniac par les microorganismes du substrat, entrainant une perte d'azote importante pendant le compostage, tandis que l'azote apporté par le soja reste disponible pour le champignon. Récemment, Siqueira et al. (2011) ont signalé qu'une teneur initiale en azote de 1% à 1,5% doit être utilisée pour optimiser le substrat destiné à la production d'*A. subrufescens*.

Les travaux menés sur la culture d'*A. bisporus* ont montré que la microflore présente dans le substrat influence grandement le développement mycélien du champignon cultivé. La phase II du compostage (pasteurisation) favorise le développement de germes thermophiles conduisant à un compost sélectif pour *A. bisporus* (Ross et Harris, 1983 ; Sharma et Kilpatrick, 2000). Une analyse microbiologique d'un compost développé à partir de matériaux comme certains résidus de la canne à sucre a montré une quantité importante de microorganismes thermophiles présents dans le compost pour *A. bisporus*, mais aussi la présence de pathogènes comme *Enterobacter agglomerans*, *Escherichia coli* et *Klebsiella pneumoniae* (Ferreira Silva et al., 2009). Bilay et Ivashchenko (2011) ont observé une

croissance d'*A. subrufescens* deux fois plus rapide sur la surface d'une colonie de *Humicola insolens* dans un milieu de paille-agar que sur ce milieu sans le champignon thermophile. Il est à noter qu'un effet bénéfique de *H. insolens* a été observé vis-à-vis de la croissance mycélienne (Bilay et Lelley, 1997) et de la fructification d'*A. bisporus* (Vijay et al., 2002).

1.1.7. Pathologies affectant les cultures d'agarics

Les problèmes biotiques et abiotiques observés chez des cultivars d'*A. subrufescens* sont présentés dans l'article 1. Les principales maladies signalées chez *A. subrufescens* sont dues à la bactérie *P. tolaasii* et aux moisissures *Trichoderma aggressivum* et *Lecanicillium fungicola*, et font l'objet de peu de renseignements dans la littérature. Cependant, ces pathologies sont également les plus sévères chez *A. bisporus* et une abondante documentation existe (Berendsen et al. 2010 ; Largeteau et Savoie, 2010 ; Soler-Rivas et al. 1999, pour des revues).

1.1.7.1. La tache bactérienne due à *Pseudomonas tolaasii*

Bien que les pertes de récoltes varient en fonction de la sensibilité des souches de champignon et de l'agressivité de *P. tolaasii*, la maladie pose un sérieux problème dans les cultures commerciales d'*A. bisporus* mais aussi de *Pleurotus ostreatus* (Nguyen et al., 2012).

La bactérie peut être présente sous forme pathogène produisant une toxine (la tolaasine) et non pathogène ne produisant pas de toxine (Olivier et al., 1978). La forme pathogène peut être mise en évidence par confrontation de la bactérie avec *Pseudomonas reactans* sur milieu B de King (Figure 1.4). Une réaction se produit entre un composé synthétisé par *P. reactans*, le WLIP (*White Line Inducible Principle*), et la tolaasine, ce qui génère une ligne blanche entre les deux colonies (Goor et al., 1986).

P. tolaasii provoque des taches brunes sur les sporophores d'*A. bisporus* (Rainey et al., 1992) entraînant des pertes en culture pouvant atteindre 40%. La bactérie est responsable de taches jaunes chez *P. ostreatus* (Figure 1.5).

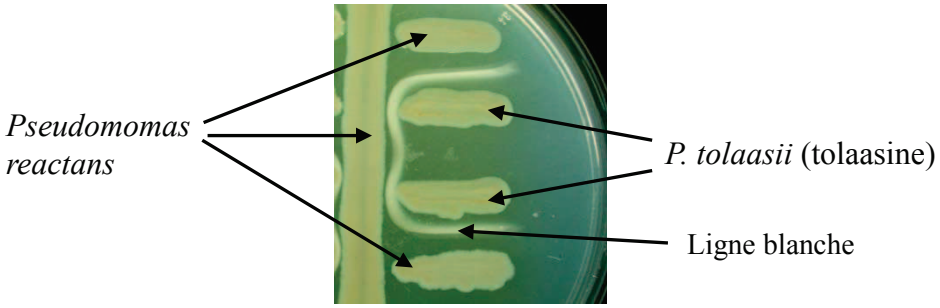

Figure 1.4. Generation d'une ligne blanche entre la tolaasine produite par *Pseudomonas tolaasii* et le WLIP produit par *Pseudomonas reactans.* (Photo INRA, MycSA)

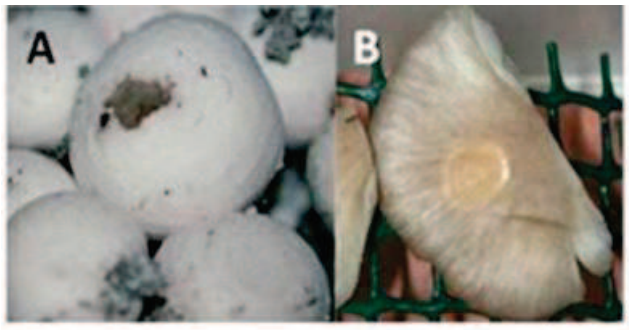

Figure 1.5. Taches brune et jaune produites par *Pseudomonas tolaasii* chez *Agaricus bisporus* (A) et *Pleurotus ostreatus* (B). (Photos INRA, MycSA).

1.1.7.2. La moisissure verte due à *Trichoderma*

Les moisissures sont des compétiteurs plus efficaces que les champignons cultivés pour utiliser les nutriments du substrat et le coloniser. Elles peuvent produire des métabolites secondaires toxiques, des enzymes extracellulaires, ainsi que divers composés organiques volatiles qui peuvent réduire considérablement ou même bloquer entièrement la production commerciale (Hatvani et al., 2012). La moisissure verte, causée par différentes espèces de *Trichoderma* spp., est un grave problème pour les producteurs de champignons à travers le monde. Chez *P. ostreatus*, la moisissure verte est causée par *Trichoderma pleurotum* et *T. pleuroticola* (Gea, 2009 ; Hatvani et al., 2012). Par contre, chez *A. bisporus*, les plus grosses pertes sont dues à *Trichoderma aggressivum* f. *europaeum* (anciennement Th2) et *T. aggressivum* f. *aggressivum* (Th4) respectivement responsables de la maladie en Europe et en Amérique du Nord (Figure 1.6 ; Ospina-Giraldo et al., 1998 ; Mamoun et al., 2000 ; Samuels et al., 2002). Ces dernières années, des pertes considérables dans les champignonnières d'Europe Centrale ont été causées par *T. aggressivum* f. *europaeum* (Hatvani et al., 2007). Cette moisissure se développe essentiellement pendant l'incubation des cultures d'*A. bisporus.* Elle entre en compétition avec l'Agaric pour l'utilisation des nutriments et colonise le compost. Les zones de compost contaminées deviennent rapidement vertes du fait de la sporulation de *T. aggressivum* (Figure 1.7 ; Rinker, 1996 ; Largeteau et al., 2002). Aucun sporophore ne pousse dans la zone contaminée, et ceux situés à la périphérie peuvent être recouverts par la moisissure.

Récemment, Andrade et al. (2011) ont inoculé des isolats de *Trichoderma* sp. dans deux types de compost pendant les premiers jours après le lardage. Ils ont observé un effet négatif sur le rendement d'*A. subrufescens* en présence de cet antagoniste.

1.1.7.3. La môle sèche causée par *Lecanicillium fungic*ola

La moisissure *Lecanicillium fungicola* (anciennement *Verticillium fungicola*) (Figure 1.8) est responsable de 8 à 10% de pertes lors d'attaques de cultures d'*A. bisporus*.

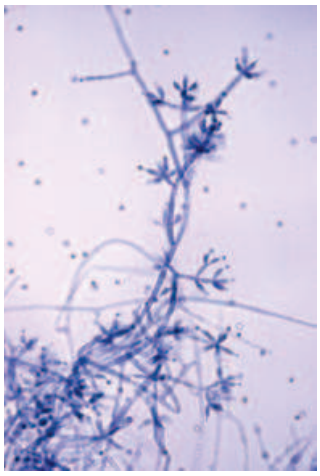

Figure 1.6. *Trichoderma aggressivum* f. *aggressivum* vu au microscope.
(Photo INRA, MycSA).

Figure 1.7. Compost envahi par *Trichoderma aggressivum*. (Photo INRA, MycSA).

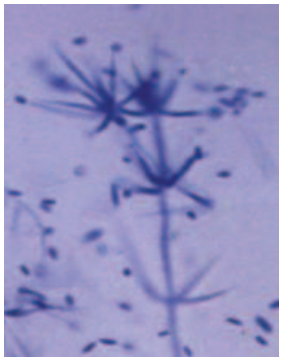

Figure 1.8. *Lecanicillium fungicola* vu au microscope. (Photo INRA, MycSA).

Le pathogène infecte les sporophores et produit trois types de symptômes : des taches, des champignons à pied fendus et qui pèlent, et des masses de tissus non différenciés appelées môles, qui sont le symptôme principal (Figure 1.9).

Figure 1.9.Culture d'*Agaricus bisporus* contaminée par *Lecanicillium fungicola*. (Photo INRA, MycSA).

1.1.8. Valorisation non médicinale d'*A. subrufescens*

L'utilisation du compost usé (en fin de production) comme alternative possible aux produits chimiques pour améliorer la croissance des plantes ainsi que le rôle potentiel d'extraits d'*A. subrufescens* en agriculture biologique, pour protéger des plantes contre des pathogènes sont décrits dans l'article 1, pagraphe '*Use in plant protection and other valorisation'*. Les mécanismes mis en jeu ne sont pas toujours identifiés, cependant une augmentation de l'activité peroxydase a été observée chez l'aubergine et le concombre.

Des travaux récents suggèrent que le β-glucane contenu dans la paroi cellulaire d'*A. subrufescens* est un bon substrat pour la production de β-1,3-glucanase chez l'agent de lutte biologique *Trichoderma harzianum* (Carneiro et al., 2011).

Des applications concernent l'industrie des cosmétiques. *Agaricus subrufescens* figure parmi les espèces utilisées actuellement ou bientôt autorisées pour la fabrication de cosmétiques à action neutraceutique, comme du shampoing maintenant la vitalité du cheveu, des crèmes pour la lutte contre le vieillissement de la peau et sa revitalisation (Hyde et al., 2010).

Article1

Appl Microbiol Biotechnol (2011) 92:897–907
DOI 10.1007/s00253-011-3630-7

MINI-REVIEW

The medicinal *Agaricus* mushroom cultivated in Brazil: biology, cultivation and non-medicinal valorisation

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Received: 30 June 2011 / Revised: 19 September 2011 / Accepted: 2 October 2011 / Published online: 18 October 2011

Abstract Sun mushroom is a cultivated mushroom extensively studied for its medicinal properties for several years and literature abounds on the topic. Besides, agronomical aspects were investigated in Brazil, the country the mushroom comes from, and some studies focus on the biology of the fungus. This review aimed to present an overview of the non-medicinal knowledge on the mushroom. Areas of commercial production and marketing trends are presented. Its specific fragrance, taste, nutritional value and potential use of extracts as food additives are compared to those of the most cultivated fungi and laboratory models. The interest of the mushroom for lignocellulosic enzyme production and source of biomolecules for the control of plant pathogens are shown. Investigation of genetic variability among cultivars is reported. Growing and storage of mycelium, as well as cultivation conditions (substrate and casing generally based on local products; indoor and outdoor cultivation; diseases and disorders) are described and compared to knowledge on *Agaricus bisporus*.

Keywords *Agaricus blazei* · *Agaricus brasiliensis* · *Agaricus subrufescens* · Biology · Cultivation · Genetic variability · Marketing · Non-medicinal use · Pathology · Production areas

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Introduction

Gourmet mushrooms may contribute to the development of a new agriculture by addressing the consumer demand and some of the non-nutritional uses of agricultural productions. Mushrooms have been consumed by humans since ancient times, not only as a part of the normal diet but also as a delicacy due to their desirable taste and aroma, and as nutraceuticals. Important cultivated edible and medicinal mushrooms belong to the *Agaricus* genera and are saprophytic fungi acting as secondary decomposers in forest litters. Once the value of recycling with gourmet and medicinal mushrooms is clearly understood, wastes can be viewed as positive products, at least in terms of providing new economic opportunities and positive environmental consequences. Among secondary decomposers, an *Agaricus* species is today widely used and studied for its medicinal and/or therapeutic properties. Kawagishi et al. (1989) were the first to separate an active anticancer compound from its fruiting bodies. Since that time, numerous works were performed on the medicinal properties of the mushroom. Several reviews gave an overall view on the topic (Wasser 2002; Firenzuoli et al. 2008; Oliveira Lima et al. 2011).

According to most of the published articles, the mushroom cultivated in Brazil is native to the São Paulo State, Brazil. It was formerly known in literature as *Agaricus blazei* Murrill (*sensus* Heinemann). In the last few years, several articles focused on the clarification of its botanical name (Kerrigan 2005, 2007; Wasser et al. 2002, 2005) and agreed that the former name was wrong. By studying other specimens in addition to the cultivated strains, two names have been proposed *Agaricus subrufescens* Peck or *Agaricus brasiliensis* Wasser et al. Currently, many publications refer to *A. brasiliensis* for the cultivated medicinal mushroom originating from Brazil.

Various vernacular names have been given to this cultivated medicinal mushroom. It is popularly known in Brazil as Piedade mushroom, medicinal mushroom (cogumelo medicinal), Sun mushroom (cogumelo do sol), God's mushroom (cogumelo de Deus), and, due to its fragrance and taste, as the Almond Portobello (Almond Mushroom; Portobello de amêndoa) (Colauto et al. 2002; Camelini et al. 2005; Dias et al. 2004; Gene 2009; Kopytowski Filho et al. 2006). The mushroom is called Himematsutake, Agarikusutake and Kawariharatake in Japan, and Ji Song Rong in China (Firenzuoli et al. 2008).

A. brasiliensis has reached the uppermost ranks among the best of all gourmet and medicinal mushrooms. There is an extensive literature on this mushroom in which food sciences, biotechnology, pharmacology and medicine are the main topics whilst agronomy, marketing and non-medicinal use are far less represented. This review deals with the biological characteristics of the Brazilian medicinal mushroom, its cultivation and non-medicinal valorisation.

Areas of commercial production and marketing

The medicinal mushroom *A. brasiliensis* was imported into Japan from Brazil in 1965. Since 1988, it is cultivated on a contract basis in various parts of Japan and in Indonesia (Mizuno et al. 1990). In Brazil, it is produced at a commercial scale since the early 1990s (Braga et al. 2006), in regions close to the Atlantic coast, of moderate to warm temperatures and of high humidity, from September to April. The production areas have expanded from São Paulo state (still the most important producing region) to other states such as Minas Gerais (Southeast), Paraná, Santa Catarina and Rio Grande do Sul (South), Bahia and Ceará (Northeast) (Mendonça et al. 2005; Neves et al. 2005).

Japan has become the greatest importer of *A. brasiliensis* produced in Brazil (Dias et al. 2004). Indeed, during 2001, approximately 37 tons of dehydrated mushrooms were exported from Brazil, principally to Japan (Eira 2003). Exportations sharply increased from 1996 to 1998 (from 7 to 30 tons a year), were steady until 2001 (33 tons), then decreased in 2002 and 2003 down to 20 tons, due to competition with other producers and the economic crisis in Japan (Mendonça et al. 2005). Decline in Japanese importations occurred for three consecutive years (2006–2008) and the market suffered a loss of 76% because of problems found in mushrooms imported from other countries (SECOM 2010). Although the Brazilian product was considered of higher quality, this did not prevent a decrease in Japanese consumption. In 2009, the market stabilized, with a slight increase in exportations, but it is not possible to predict whether the market will return to the levels of 2004 (Dias 2010). Brazilian growers export to several countries including Australia, Bolivia, Germany, Italy, Spain, South Africa, Thailand, the USA, India and Korea. At the same time as exportations have decreased, there has been a noticed improvement of the local market due to the interest of the Brazilian population for the mushroom benefits (Mendonça et al. 2005).

Mushrooms are harvested when they reached the highest biomass, which is the button stage with intact veil membrane enclosing the gills (Mendonça et al. 2005). This stage reaches the highest market value for exportation (Camelini et al. 2005). The mushroom is traditionally sold in dehydrated form, as it is mainly consumed as nutraceutical, after being ground to powder. The dehydration process includes washing and brushing to eliminate cap pigment, followed by anti-oxidant treatment (ascorbic acid, 3–5 gl^{-1} water) and drying. According to market standards, dried mushrooms are classified as grade Extra, A, B or C, following criteria of colour (straw colour, pale yellow), morphology (stalk aspect) and size (Mendonça et al. 2005; Minhoni et al. 2005).

In 2006–2007, the annual production in Brazil reached 40 tons of dehydrated mushrooms (Tomizawa et al. 2007). Sliced dehydrated mushrooms, paid R$60–180 per kilogramme to the producer, were commercialised by wholesalers, mainly to Japan (Eira 2003). Mushroom price is variable, depending on the region. Values ranged from US $28.6 to 71.4 per kilogramme of dried mushrooms, with average price of 50 US$ for grade A mushrooms (Mendonça et al. 2005). In São Paulo State, during 2004–2005, the average value for dried mushrooms was R$180–200 for grade Extra, R$150–180 for grade A, R$70–120 for grade B and R$30–50 for grade C (Minhoni et al. 2005). Commercialization prices, low investment costs and fast return of invested capital make cultivation financially attractive. In Brazil, in social terms, it creates jobs, setting families down to the land, as it demands a small cultivation area. It is adapted to family agriculture, based on small farms (Cavalcante et al. 2008).

Apart from the large development in Brazil, *A. brasiliensis* has been produced at large scale in China since 1990s (Wang et al. 2010), whilst in Korea, cultivation started in 1996 (Mizuno 2000). Nowadays, the mushroom is cultivated at the industrial level in Brazil, Japan, China, Taiwan and Korea (Gregori et al. 2008). In 2001, Takadu et al. (2001) estimated that the production in Japan reached approximately 100–300 tons of dried fruiting bodies every year. China, Japan and Korea are important competitors towards Brazil for commercial production (Braga et al. 2006).

A. brasiliensis benefits from soil microflora and warm temperature, making it an ideal candidate for outdoor cultivation in the subtropics. Due to the temperature needed for fruiting, it might be an alternative to *Agaricus bisporus* cultivation during the warm season in Europe, for reducing

cropping energetic cost. The almond taste might put a curb on its commercialisation as a gourmet mushroom, consumers being used to *A. bisporus* taste, but commercialization as nutraceutical can be expected because of the increasing demand in natural products improving human health and welfare.

Fragrance, taste and nutritional value

As indicated by one of its vernacular names, *A. brasiliensis* is characterised by a pleasant almond flavour. Benzaldehyde and its precursor benzoic acid are the major components of the volatile fraction; the other contributors to the flavour are benzyl alcohol, methyl benzoate and 4-hydroxybenzaldehyde. Benzoic acid (1,280–3,100 mg/kg dry weight), may contribute to the excellent shelf life of the mushroom and presumably, by conversion to benzaldehyde and benzyl alcohol, to the development of the almond flavour when reconstituting the commercial dried mushroom. Benzoic acid occurs also in significant amounts in several close relatives of *A. brasiliensis* suggesting that this compound could be a taxonomic marker (Stijve et al. 2003).

Analyses of the non-volatile taste components in three species of dried mushrooms commercially available in Taiwan showed highest contents of monosodium glutamate-like (MSG-like), sweet and bitter taste components in *A. brasiliensis* (4.40, 2.62 and 4.73 mg/g dry weight (DW), respectively) compared to *Agrocybe cylindracea* and *Boletus edulis*. The bitterness in *A. brasiliensis* could probably be masked by the sweetness, mainly from the high amount of soluble sugars and polyols (Tsai et al. 2008). Mannitol concentrations reached up to 22% on mushroom dry weight. Compared to the fruiting body, the mycelium exhibited no almond flavour and was found poor in flavour components, mannitol and MSG-like amino acids (Stijve et al. 2003). In another study, dried mycelia of *A. brasiliensis* showed lower content of sweet components and higher content of bitter components than *Antrodia camphorata* and *Cordyceps militaris* mycelia (Chang et al. 2001). Contents of flavour 5′-nucleotides (5′-GMP, 5′-IMP and 5′-XMP), which also gave the umami (meaty) or palatable taste, were higher in dried *A. brasiliensis* sporophores (5.15 mg/g) than in *A. cylindracea* and *B. edulis* (2.44 and 2.01 mg/g, respectively) (Tsai et al. 2008) and comparable to the 4.19–6.13 mg/g reported for *A. bisporus* by Tseng and Mau (1999). Considering the three ranges of flavour 5′-nucleotides defined by Yang et al. (2001) (low, <1 mg/g; middle, 1–5 mg/g and high, > 5 mg/g), the contents in *A. brasiliensis* and *A. bisporus* were in the high range. Using the equation derived from sensory evaluation (Yamagushi et al. 1971), the Equivalent Umami Content (EUC) value was high (135.90 g/100 g). The sensory EUC value of *A. brasiliensis* might be beneficial for its use as foods or food flavouring materials or in the formulation of nutraceuticals and functional foods with a palatable umami taste (Tsai et al. 2008).The content of flavour 5′-nucleotides was far lower in the mycelium of *A. brasiliensis* (7.0 mg/g) compared to those found in *A. camphorata* and *C. militaris* mycelia (38.0 and 29.2 mg/g) (Chang et al. 2001). Therefore, the balance between MSG-like and sweet components would be responsible for the specific taste of the Brazilian medicinal mushroom (Tsai et al. 2008).

Besides its medicinal interest, *A. brasiliensis* is a food of high nutritional value, rich in protein, fibre, minerals, with low lipid content. The protein content of 32.8–35.9% (veil closed) and 28.9–39.2% (veil open) measured by Eira (2003) in dried sporophores cultivated in Brazil (four strains) exceeded percentages reported for *Volvariella diplasia* (28.5%), *A. bisporus* (26.3%), *Pleurotus* spp. (18.7–23.3%) and *Lentinula edodes* (17.5–19.5%). Conversely, less fibre contents were found in *A. brasiliensis* (5.56–9.7%, veil closed; 6.9–11.8%, veil open) compared to *V. diplasia* (17.4%), *A. bisporus* (10.4%), *Pleurotus* spp. (10.4–15.6%) and *L. edodes* (8.0–12.4%). Other works performed on the Brazilian medicinal mushroom revealed similar levels of protein (30–45%) and crude fibre contents (6–14%), and showed carbohydrate contents of 38–45% and lipid contents of 1–5%. Fruiting bodies contained high amounts of potassium (2.34–2.97%), phosphorus (0.75–0.9%) and calcium (0.04–0.07%) (Mizuno et al. 1990; Oliveira et al. 1999; Eira 2003; Shibata and Demiate 2003). Compared to *A. bisporus* strain used as control, *A. brasiliensis* cultivars had lower K, P, Se and Na, but higher Bo, Cd, Cu, Sr and Zn contents, and the Co and V contents were below the limit of detection. There were no differences for Ba, Ca, Mg, Mn, Ni and Ti. Content in vitamin B1 was comparable in the Brazilian cultivars (0.48 mg/100 g DW) and *L. edodes* (0.40 mg) whilst that in *A. bisporus* was two times higher (1.14 mg). Similar contents in Niacin were found in *A. brasiliensis* (40.9 mg/100 g DW) and *A. bisporus* (36.2 mg) whilst only 11.9 mg were present in *L. edodes*. Vitamin B2 content ranged between *A. brasiliensis* (0.90 mg/100 g DW), *L. edodes* (0.90 mg) and *A. bisporus* (4.95 mg) (Eira 2003). Mushrooms cultivated in Japan showed similar content in vitamin B1 and niacin (0.3 mg and 49 mg/100 g DW) (Mizuno et al. 1990). Interestingly, in a recent study, *A. brasiliensis* contained far less agaritine (22–57 μg/g DW), a known carcinogen, than *A. bisporus* (341±32 μg/g DW) (Koge et al. 2011). Despite its low content in fibre, *A. brasiliensis* is an attractive food product characterised by vitamin content in the same range, protein content higher than those present in the other fungi and the most cultivated in the world.

As a consequence of the chemical composition, besides the medicinal effects, literature reports some investigations on commercial or potential use of mushroom extracts as food additives. Aqueous extract of *A. brasiliensis* have been approved in Japan as food additive to provide an agreeable bitter taste (Kuroiwa et al. 2005). Da Silva et al. (2009) identified a potential use of methanol/water extract of the mushroom as natural anti-oxidant in vegetal oil.

Production of lignocellulose degrading enzymes

Fungal laccases and manganese peroxidases serve several purposes in the industry. There is an increasing interest on the use of cellulases and hemicellulases for the valorisation of the plant biomass. Lignocellulases are produced in the cultivation substrates throughout the mushroom crop, but production is differently regulated depending on the development stage of the mushroom. This has been extensively studied for *A. bisporus* (Savoie 1998) but not for *A. brasiliensis*. Some works focused on comparisons between fungal species for the production of enzymes by the vegetative mycelium during several days following inoculation. Laccases produced by *A. brasiliensis*, *A. bisporus*, *Stropharia rugosoannulata* and *Volvariella volvacea* are similar; they are blue copper proteins with a molecular mass around 65 kDa and acidic pI between 3 and 4 (66 kDa and pI 4.0 for *A. brasiliensis* laccase). The Brazilian medicinal mushroom produced high amounts of laccase during growth on complex liquid medium based on tomato juice (TJM), but only traces of the enzyme on synthetic sugar-containing medium commonly used for the production of ligninolytic enzymes. Purified laccase was stable at 20°C, pH 7.0 and 3.0, but rapidly lost its activity at 40°C or pH 10. Whilst *A. bisporus* secretes manganese peroxydase along with laccase in solid-state cultures on compost, no manganese peroxidase was detected during growth of *A. brasiliensis* in TJM. (Ullrich et al. 2005).

A. brasiliensis CS1 and *Pleurotus ostreatus* H1 proved to be efficient producers of holocellulases when grown on solid-state medium supplemented with various proportions of agro-industrial wastes (banana stem, sugarcane bagasse, cotton, corn and soybean residues). Enzyme induction depended on the nature of the substrate and the mushroom species. Cotton residue (10%) was the best carbon source for xylanase, mannanase, endoglucanase and FPAse activities (1,349, 206, 315 and 180 IU/l, respectively) in crude extracts, whilst polygalacturonase activity (451 IU/l) was observed with 5% sugarcane bagasse, the most bulk material used in mushroom compost in Brazil. Both fungi released the highest quantities of reducing sugar after 72 h with sugarcane bagasse (10%) and produced the highest amount of protein with banana stems (10%), but higher values were obtained with CS1 (11.6 mg and 344 μg per milliliter of crude extract) compared to *P. ostreatus* (2.33 mg/l and 184 μg/ml) (Siqueira et al. 2010).

Industrial production of lignocellulolytic enzymes by *A. brasiliensis* in solid-state fermentation or by extraction from spent cultivation substrates is a new opportunity of use of this mushroom that is worthwhile to study.

Use in plant protection and other valorisation

Besides the well-known medicinal effects, *A. brasiliensis* extracts showed potential application in organic agriculture for the control of plant pathogens. Several works reported efficiency against various plant pathogens. Di Piero et al. (2010) compared the efficiency of aqueous extract from *L. edodes* and *A. brasiliensis* to protect passion flower (*Passiflora edulis*) and *Chenopodium quinoa* against *Cowpea aphid-borne mosaic virus* (CABMV). Pre-treatment of passion flower leaves with *A. brasiliensis* extracts before inoculation with CABMV reduced virus infection by 66% (extract from ABL 99/29) and by 80–100% (extracts from ABL 99/28 and ABL 99/26). Three *L. edodes* strains gave less or no efficient extracts. No significant systemic protection was observed whatever the extract tested. No protection of the extract from ABL 99/26 was observed in experiments involving CABMV transmission by aphid vectors. Other investigations, performed with isolates Abl-11 and Abl-28, showed potential to induce resistance in eggplant against bacterial wilt caused by the bacterium *Ralstonia solanacearum*. The mushroom aqueous extracts caused significant reduction in the occurrence of wilted leaves, when applied 2 days before inoculation. No protection was observed with aqueous extracts from two *L. edodes* isolates (Silva et al. 2008). Crude extracts from the isolate ABL 29/99 (before and after veil opening) showed inhibitory effect against spore germination of the *Triticum aestivum* pathogen *Puccinia recondita*, but were less efficient than extracts obtained from *L. edodes* LE95/01 (Fiori-Tutida et al. 2007). Aqueous extracts from *A. brasiliensis* and *L. edodes* sporophores reduced the formation of new lesions caused by the ascomycete *Guignardia citricarpa*, the causal agent of citrus black spots, in the sweet orange fruits (*Citrus sinensis* var. *Valencia*) (Pascholati et al. 2007). It is obvious from these investigations that *A. brasiliensis* isolates exhibited variability in their biocontrol effect and, in some cases, proved to be more efficient than *L. edodes*.

Besides, ethanolic extract obtained from compost after *A. brasiliensis* crop appeared as a potential biotic elicitor of resistance against *Corynespora cassiicola* in the cucumber plant (*Cucumis sativus*), showing a potential valorisation of the spent compost (Ueda et al. 2008). Spent compost also

appears an interesting alternative to chemicals to promote plant and animal growth. Fresh spent mushroom substrate (SMS) from *A. brasiliensis* production was a good source of N, P, and K and was an excellent supplement for lettuce growth promotion. At 10% supplementation, lettuce aerial dry weight increased 2.2 and 1.3 times compared to the control (without SMS) and NPK treatment. In contrast, supplementation with *L. edodes* SMS provided a drastic reduction in lettuce development. Therefore the use of *A. brasiliensis* SMS may be an excellent alternative to the chemical amendments when considering organic production of horticultural crops and Eucalyptus growing (pulp production) in artificial forests. SMS also showed potential for remediation of biocides possibly due to improved microbial diversity and enzymatic activity (Ribas et al. 2009). Substitution of antibiotics by *A. brasiliensis* SMS was evaluated in chicken farming. Diet with 0.2% SMS provided the highest weight gain and the best feed conversion (Machado et al. 2007).

Genetic variability

Several authors have investigated the genetic polymorphism among cultivated strains of the Brazilian medicinal mushroom. Neves et al. (2005) compared genetic variability among six strains provided by Brazilian spawn makers or isolated from fruiting bodies collected in mushroom farms from different States (São Paulo, Minas Gerais, Santa Catarina and Paraná). RFLPs of the ITS region did not show polymorphism among the six strains. Genetic distances obtained from RAPD profiles varied from 0% to 14%, and separated the strain 22, originating from Santa Catarina, from the other strains which grouped together. Strain 22 also differed from the others by its lowest in vitro mycelial growth rate and was considered by mushroom growers to have low productivity. Colauto et al. (2002) performed RAPD on five strains obtained from mushroom growers in São Paulo and Rio Grande do Sul states, and found that three isolates (ABL 97/11, ABL 99/25 and ABL 99/29) were probably clones, whilst ABL 99/28 and ABL 99/26 (re-isolated from Jun17-Japan) exhibited little genetic polymorphism. Strains 99/29 and 99/26 showed distinct morphological differences despite maintaining the characteristic pattern of the species. Later, using a far higher number of primers, Tomizawa et al. (2007) analysed nine Brazilian cultivars, including ABL 97/11, ABL 99/25 and ABL 99/29. A group of six isolates (CS1, CS3, CS4=ABL97/11, CS6, CS8 and CS9) showed a high genetic similarity and were considered isolates of the same origin or clones. Isolates CS7=ABL99/29, CS5=ABL99/25 and CS2 revealed 91.3%, 88.7% and 60.6% similarity with the group, respectively. Referring to PhD theses, the authors reported that cellulose degradation ability, cytological and physiological aspects of CS2 also distinguished this isolate from the others. RAPD performed by Marques et al. (2006) on three sets of cultivars, each from a different Brazilian producer, showed that only 2.7% of the 160 primers tested generated polymorphism but did not form separate groups. Small differences in morphology were attributed to environmental factors during cultivation. Molecular analyses performed on eight strains (SA515–520, SA 555 and SA527) provided by Japanese growers and strain SA514 from a Brazilian grower showed clear genetic differences between the Brazilian cultivar and the Japanese-cultivated strains; the latter grouped together (Fukuda et al. 2003; Mahmud et al. 2007). All these works revealed great homogeneity for commercial strains analysed either in Brazil or in Japan, but in each country, the strains currently cultivated probably derived from a single or a very few sporophores. Local cultivation conditions and strain improvement by laboratories or producers might explain differences found between Brazilian and Japanese cultivars despite the latter are of Brazilian origin.

Two needs were identified for improving the knowledge of biodiversity in cultivated strains of the medicinal mushroom. First, it is necessary to develop efficient markers. Since transposons of the same type were found in high number of copies in each genome of three basidiomycetes, they appear to be excellent markers for studying genetic structures of natural populations and for constructing linkage maps, especially in *Agaricus* species (Sonnenberg et al. 2004). Other markers like ISSR (Guan et al. 2008) and especially SSR that proved highly efficient in differentiating *A. bisporus* strains (Foulongne-Oriol et al. 2009) might also be developed. High genetic homogeneity was observed among cultivars, so there is a need to prospect for wild strains for trying to find genetic diversity subsequently available for the improvement of the cultivated strains through breeding programmes. Besides, samples of the Brazilian strains named *A. braziliensis* and formely *A. blazei* proved to be genetically similar to or very close to, and inter-fertile with, the North American population of *A. subrufescens* (Kerrigan 2005), showing that there is a potential for the production of new hybrids (Kerrigan and Wach 2008). Specimens of divers geographical origins belonging to this species complex were collected and studied (Kerrigan 2005; Peter-Valence et al. 2011) opening new opportunities for genetic improvement through the use of new sources of genetic variability. However, level of medicinal activities in hybrids must be analysed.

In vitro mycelial growth and storage conditions

Mycelial growth in vitro is well documented, although most of the publications refer to conditions adapted for the

leaded to only 2.7% yield, whilst outdoor (higher variation in maximal temperature and higher RH), cultures began to fruit earlier, and yielded 6.5%, which reached near the productivity obtained in the traditional production areas (Cavalcante and Gomes 2005; Cavalcante et al. 2008). Braga et al. (2006) compared cultivation in rough structure of wood fenced by bamboo sticks, plastic green house and climatic room (dark, 25±2°C, 80±5% RH, air renewal). The earliest mushroom initiation and shortest production cycle occurred in the climatic room, but the highest yield was recorded in the plastic greenhouse, where the mean daily temperatures were higher compared to those recorded in the two other cultivation environments. The rustic structure, with bamboo covering, was the least adapted for initiation, yield and production cycle. The authors confirmed their previous observation stating that the mushroom develops better under relatively high temperatures, close to 30°C. In contrast, Eira (2003) recommended falling environmental temperature when exceeding 28°C. Minhoni et al. (2005) observed higher productivity in plastic greenhouse with temperature ranging from 15°C to 35°C than in cultivation room set at a constant temperature of 25°C. But no primordium induction occurred under a constant temperature of 15°C. Zied et al. (2010) obtained higher yield (15.5–16.1%) in plastic greenhouse compared to controlled environment (12.4–12.9%) and considered that the weather during the experiment could explain the better result observed in greenhouse. These observations suggest that daily temperature variations are more favourable to *A. brasiliensis* mushroom yield than constant temperature.

Biotic and abiotic disorders during cultivation

A. brasiliensis is a fungus of hot climate and low technology systems. Temperature range (25–30°C throughout the production cycle) favours the emergence of pests and diseases (Eira 2003; Nascimento and Eira 2007), and contamination occurs frequently when adding supplement to the compost, either at spawning or before casing (Kopytowski Filho et al. 2008). Besides, frequent exchanges of compost and spawn among different Brazilian states facilitated the rapid spread of diseases and pests (Mendonça et al. 2005). The major fungal competitors observed during cropping were those affecting *A. bisporus* crops (Fletcher et al. 1989).

The use of pH around 7.0–7.5 for compost and casing layer preparation would favour the establishment of *A. brasiliensis* while reducing fungal competition, especially *Trichoderma* spp. (green mould) that is usually present under tropical temperature and proliferating at pH 5–6. But high percentage of composts contaminated by *Trichoderma* sp. (green mould) was observed when the temperature of the compost was raised above 32°C after spawning (Kopytowski Filho et al. 2008). As observed in *A. bisporus* cultivation, *Chaetomium olivaceum* (olive green mould) develops when the temperature overcomes 62°C during phase II of composting, leading to a decrease in thermophilic microflora. The false truffle (*Diehlomyces microsporus*) is one of the main problems in the production of the medicinal mushroom in Brazil, mainly when the appropriate technology is not used. The disease was observed in 2000 in the Brazilian states of São Paulo and Paraná, with crop losses up to 90%. In Spain, the first report of *D. microsporus* occurred in 2010 (Gea et al. 2010). When deformed mushrooms, commonly called 'pop corn' appeared, symptomless mushrooms no longer developed. *D. microsporus* needs high temperature (22–30°C) for ascospore germination. The maximum growth speed has been observed when the culture was incubated at 28–30°C (Eira 2003; Nascimento and Eira 2003, 2007). Species of *Aspergillus*, *Emericelle* and *Penicillium* genera have been identified from two kinds of compost (sugarcane bagasse/coastcross hay and cotton residue/coastcross hay). *Aspergillus fumigatus*, a human pathogen, was also found in both formulations, so preventive measures should be taken by workers involved in compost production (Dias et al. 2009). *Papulaspora* sp. (brown plaster mould), known to develop in wet compost has been observed (Minhoni et al. 2005). *Coprinus* sp., a competitor in compost, could develop during spawn run, revealing too high NH_3 concentration that is unfavourable to mycelial growth. *Coprinus* and *A. brasiliensis* sporophores cohabit in infected mushroom beds, but infection after casing could result in deterioration of the mushroom mycelium (Eira 2003). Other major competitors observed in *A. brasiliensis* crops are *Dactylium dendroides* (cobwed disease), *Arthrobotrys* sp. (brown mould), *Stemonitis* sp., *Peziza* sp. (cinnamon mould), *Alternaria* sp., *Mucor* sp. and *Cladosporium* sp. (Eira 2003; Andrade et al. 2007). Competitors generally delayed the time course for the flush and reduced the yield. In the major cases analysed, contamination became more severe on the second or third year of cultivation, as observed for *A. bisporus* (Eira 2003).

There is a high incidence of pathogenic bacteria (*Pseudomonas tolaasii*) in Brazil conditions of cultivation due the high air temperature and relative humidity. Bacterial rot associated to *Mycogone* or *Lecanicillium* (*Verticillium*) and damage caused by flies in too wet and insufficient aeration conditions was reported. In Brazil, at the beginning of the 2000s, dry bubble symptoms were observed in a single area of production, and were attributed to *Lecanicillium fungicola* (Eira 2003). Lately, some strains are being selected and tested by presenting greater resistance to *Lecanicillium* (Zied, personal communication).

Pests and mites can affect crops. Dipters from three families, Sciaridae (*Lycoriella* spp.), Phoridae, especially *Megaselia halterata* and Cecidomidae (*Mycophila speyeri*) are responsible for important qualitative and quantitative losses (Eira 2003). The parasite *Collembola entomobryidae* was observed in mycelium and sporophore. Mites (*Tarsonemus* spp. and *Pymaephorus stercoricola*) were detected in cultivation materials from various producers. They develop quickly at the environment conditions suitable for *A. brasiliensis* cultivation: RH above 90%, and temperature 25–30°C. Nematodes identified as mycophagous (*Aphelenchoides composticola* and *Ditylenchus myceliophages)* or not (*Rhabditus* spp., *Mesorhabditis* spp. especially) cause damage in cultures. Most of these nematodes also infest *A. bisporus* crops in Europe, USA and Asia (Eira 2003).

Environmental conditions and chemicals could be responsible for sporophore malformation. Poor aeration, excess of CO_2, result in too thin and elongated stipes. Cracked caps appear with insufficient aeration and too low RH (Eira 2003; Minhoni et al. 2005)

Conclusion

Literature shows valuable non-medicinal aspects of the cultivated strains of *A. brasiliensis* originating from Brazil, as its high nutritional value, its potential use for industrial production of lignocellulolytic enzymes and for application in organic agriculture for the control of plant pathogens and elicitation of resistance, and as an alternative to chemical amendments. The published works on genetic variability concern mostly cultivars. Mycological prospectings to enlarge collections of specimens should lead to advance in the study of phenotypic and genetic diversity of the *Agaricus* complex (*A. brasiliensis*, *A. subrufescens*) and provide, through breeding experiments, high producing strains for which the medicinal activities will have to be evaluated. Indeed, the *A. brasiliensis* is mainly produced in Brazil and Asian countries on local substrates, with poor yields compared to *A. bisporus*. Improvement of cultivation conditions is needed. Several parameters (raw material, casing, temperature) must be studied to increase yield and adapt cultivation to temperate countries.

Acknowledgement This work was supported by a France–Mexico Project (project 115790 CONACYT, and ANR-09-BLAN-0391-01). RC Llarena Hernández would like to thank CONACYT, Mexico, for scholarship.

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1.2. Les champignons médicinaux

1.2.1. Propriétés médicinales

Les champignons sont consommés depuis fort longtemps pour leur qualité nutritive – ils sont pauvres en lipides, sans cholestérol et constituent une excellente source de protéines – mais certains le sont aussi pour leurs propriétés médicinales. Depuis plus de 2000 ans, les champignons médicinaux sont utilisés en Chine comme suppléments alimentaires ou médicaments (Chang, 2001). Depuis plusieurs années il y a un intérêt industriel accru pour des composés, nommés par Chang et Buswell (1996) 'neutraceutiques fongiques', qui peuvent être extraits du mycélium ou du sporophore de ces champignons.

Les molécules avec des propriétés antitumorales pour l'homme les plus abondantes chez les champignons sauvages sont les sesquiterpènes, tri-terpenoides, glucanes et glycoprotéines (Ferreira et al., 2009a). Certains des composés les plus efficaces dans les champignons sont les (1,3) (1,6)-β-glucanes qui ont été signalés comme inhibiteurs de la croissance tumorale par stimulation du système immunitaire par l'activation des macrophages, par la voie de balance des populations de cellules T helper et des effets ultérieures sur des cellules tueuses naturelles (NK), et aussi par la production de cytokines (Hetland et al., 2011).

D'autres travaux ont mis en évidence le rôle des polysaccharides avec des sucres variés tels que les β-et α-glucanes. (Borchers et al., 2008). Ces polysaccharides de champignons, à effet stimulateur du system immunitaire, commencent à être évalués comme composés adjuvants de traitement du cancer aux côtés de traitements conventionnels (Standish et al., 2008). Les mécanismes par lesquels ces polysaccharides exercent leurs effets immunomodulateurs ne sont pas totalement élucidés, bien que la relation structure-fonction ait été décrite entre les activités antitumorales et les caractéristiques structurelles des β-D-glucanes. Ces polysaccharides de champignons n'ont généralement pas d'effets cytotoxiques sur les cellules tumorales, et amélioreraient les défenses de l'organisme par un effet immunomodulateur (Wong et al., 2011). Dans une revue sur les effets anticancéreux des champignons médicinaux, de Silva et al. (2012b) présentent un mécanisme hypothétique d'action des β-glucanes sur les cellules cancéreuses, impliquant un ensemble de réactions complexes qui inclue les systèmes immunitaire innés et acquis. Toutefois, ces auteurs soulignent que ce mécanisme est basé sur des données concernant l'animal, et restent prudents

quant à son extrapolation chez l'homme. Récemment, Roupas et al. (2012) ont fait une analyse de l'état actuel des connaissances sur les champignons comestibles et leurs composants bénéfiques pour la santé, avec un accent sur l'évaluation des essais cliniques.

Parmi de nombreux champignons connus pour leurs propriétés médicinales, *Ganoderma lucidum*, *Grifola frondosa*, *Lentinula edodes*, *Schizophyllum commune* et *A. subrufescens* sont à l'origine de plusieurs produits commerciaux, médicaments ou compléments alimentaires (Wasser et Weis, 1999).

1.2.2. Propriétés antitumorales et antidiabétiques d'*Agaricus subrufescens*

Agaricus subrufescens a été utilisé comme champignon médicinal depuis longtemps. Kawagishi (1989) a été le premier à séparer un composé actif contre le cancer à partir de sporophores. Depuis ce temps, de nombreux travaux ont été réalisés sur les propriétés médicinales du champignon. Plusieurs revues ont fait une large révision sur ce sujet et montrent une activité antitumorale d'*A. subrufescens* (Wasser, 2002, 2010 ; Firenzuoli et al. 2008 ; Oliveira Lima et al., 2011 ; Ramberg et al., 2010).

Les extraits d'*A. subrufescens* peuvent être cytotoxiques pour les cellules tumorales *in vitro* et inhiber la croissance de cellules leucémiques par induction de l'apoptose (Jin et al., 2007), laquelle inhibe directement la croissance *in vitro* de cellules tumorales (Kim et al., 2009). Les travaux de Akiyama et al. (2011) et de Roupas et al. (2010) suggèrent que l'agaritine, une phenyl hydrazine présente dans *A. subrufescens*, peut induire l'apoptose dans les cellules leucémiques U937 par la voie d'activation de caspases par libération de cytochrome C de la mitochondrie. Mais la substance antitumorale principale d'*A. subrufescens* est formée d'extraits riches en polysaccharides et de protéines liées aux polysaccharides complexes (Itoh et al., 1994 ; Kawagishi et al., 1989b). Les polysaccharides des champignons sont connus comme préventifs de l'oncogenèse. Ils ont montré une activité antitumorale indirecte contre plusieurs types de tumeurs et préventive contre les métastases tumorales (Kimura et al., 2004). La fraction majeure polysaccharidique des extraits d'*A. subrufescens* correspond aux β-glucanes (Ohno et al., 2001 ; Peter-Valence et al., 2011) crédités de la plupart des activités antitumorales et des effets antimutagènes en partie en raison de leurs propriétés antioxydantes (Camelini et al., 2005 ; Firenzuoli et al., 2008 ; Mizuno, 1995 ; Oliveira Lima et al., 2011). De plus, des extraits méthanoliques de ce champignon sont considérés comme une source alternative d'antioxydants naturels (Da Silva et Jorge, 2011). Récemment, Vinhal Costa Orsine et al. (2012) ont présenté une revue mettant en évidence le fort potentiel antioxydant

de la famille *Agaricacae*, et plus particulièrement d'*A. subrufescens*. Une revue récente (De Silva et al., 2012a) rapporte l'utilisation d'*A. subrufescens* comme antidiabétique en médicine traditionnelle et cite des travaux suggérant que la répression du stress oxydante serait impliqué dans l'action du champignon contre le diabète sucré.

1.3. Le stress oxydant

La littérature fournit de nombreux travaux sur les propriétés antioxydantes d'extraits de plusieurs champignons sauvages (comestibles ou non) et cultivés (Barros et al., 2008 ; Choi et al., 2004 ; Dubost et al., 2007 ; Huang et Mau, 2006 ; Jayakumar et al., 2011 ; Lee et al., 2007 ; Mau et al., 2004 ; Reis et al., 2012 ; Savoie et al., 2008).

1.3.1. Mécanismes d'action

Les Eucaryotes et les Procaryotes produisent des espèces d'oxygène réactif (*reactive oxygen species*, ROS) comme sous-produits de leur métabolisme, essentiellement par la respiration aérobie. Les ROS sont de l'oxygène dans sa forme de singlet excité ($^1O_2$), de radical anion superoxyde ($O_2^{-\cdot}$), de radical ($HO_2^{-\cdot}$) et d'ion peroxyde (HO_2^-), de radical ($HO^\cdot$) et d'ion hydroxyle (HO^-), de peroxyde d'hydrogène (H_2O_2). Chez un organisme sain, un faible niveau de ROS est maintenu par un équilibre entre leur production et leur élimination (Savoie, 2008).

Le stress oxydant peut être défini comme tout déséquilibre entre la production et la détoxification de ROS (Hekimi et al., 2011). Il provoque l'augmentation du nombre de radicaux libres, principalement sous la forme de ROS qui jouent un rôle important dans les pathologies humaines. La production non contrôlée de radicaux libres a été reliée à plus d'une centaine de maladies, y compris plusieurs types de cancers et le diabète, entre autres (Ferreira et al., 2009), et aux maladies dégénératives associées au vieillissement. La théorie mitochondriale des radicaux libres du vieillissement (MFRTA) propose que le vieillissement soit causé par des dégâts provoqués aux macromolécules mitochondriales par les espèces réactives de l'oxygène (ROS). Elle est basée sur l'association observée du taux de vieillissement et du phénotype vieilli avec la production de ROS et les dommages oxydatifs. Cependant, des études récentes, en particulier chez *Caenorhabditis elegans*, mais aussi chez

les rongeurs suggèrent que la génération de ROS n'est pas la cause primaire ou initiale du vieillissement. Ils joueraient un rôle de médiateur dans la réponse aux dommages liés à l'âge (Hekimi et al., 2011).

Un régime riche en antioxydant est supposé aider l'organisme à lutter contre les maladies et le vieillissement. Une fois produits, la plupart des radicaux libres sont neutralisés par des défenses cellulaires de type antioxydant (enzymes et molécules non enzymatiques). La conservation de l'équilibre entre la production de radicaux libres et les défenses par action d'antioxydants est une condition essentielle pour le fonctionnement normal de l'organisme (Ferreira et al, 2009). Bien que presque tous les organismes soient équipés de systèmes de défense de type antioxydant et de systèmes de réparation pour les protéger contre les dommages oxydatifs, ces systèmes sont souvent insuffisants pour empêcher complètement le stress oxydant induit par les dommages.

Par conséquent, des compléments antioxydants ou des produits naturels contenant des composés bioactifs peuvent être utilisés pour aider à réduire les dommages oxydatifs chez l'être humain (Kanter, 1998). L'effet bénéfique des fruits et légumes est bien connu. L'effet défensif de leurs antioxydants naturels est lié à trois groupes majeurs : les vitamines, les phénols et les caroténoïdes. L'acide ascorbique et les phénols sont des antioxydants hydrophiles, tandis que les caroténoïdes sont des antioxydants liposolubles (Halliwell, 1996). Les champignons sont également des sources d'antioxydants particulièrement intéressantes comme aliments fonctionnels ou neutraceutiques.

1.3.2. Les systèmes de défenses antioxydants

La fonction première des propriétés antioxydantes est de protéger les cellules contre les dommages provoqués par les ROS. Le système de défense des champignons comprend à la fois des enzymes qui détoxifient les ROS (superoxyde dismutase, catalase, peroxydases) et des antioxydants (glutathion, ascorbate, pigments et composés phénoliques, proline), (Belozerskaya et Gessler, 2007). Le potentiel antioxydant d'un composé peut être attribué à plusieurs caractéristiques, mais les plus importantes sont le piégeage et la réduction de radicaux libres, la catalyse par chélation d'ions de métaux de transition, l'inhibition de la peroxydation lipidique (Kumar et al., 2005). Des extraits alcooliques de champignons se sont révélés être d'efficaces pièges de radicaux superoxyde.

1.3.3. Indicateurs de l'activité antioxydante

Une grande variété de méthodes d'évaluation *in vitro* de la capacité de piégeage de radicaux ont été développées. Différentes molécules de synthèse sont utilisées, comme l'acide 2,2'-azibinobis-3-ethylbenzotiazoline-6-sulfonic (ABTS), le 1,1-diphenyl-2-picrylhydrayl (DPPH) et le N, N-dimethyl-*p*-phenylendiamine (DMPD). Les conditions de mesure peuvent varier de l'un à l'autre (ex. les solvants, la longueur d'onde choisie), et donnent différents résultats. Ces méthodes présentent l'avantage d'utiliser un radical libre stable et commercialement disponible. Elles ont été largement utilisées pour analyser des aliments, tels que l'huile d'olive, les jus de fruits et les vins (Villano et al., 2007) en raison de leur stabilité, leur simplicité et leur reproductibilité (Kitts et al., 2000).

La capacité de réduction d'un composé peut servir d'indicateur significatif de son potentiel d'activité antioxydante (Meir et al., 1995). Les propriétés réductrices d'une substance sont généralement associées à la présence de réductones (Duh, 1998) telles que l'acide ascorbique (un fort agent réducteur) connu pour exercer une action antioxydante par la rupture de la chaine de radicaux libres par le don d'un atome d'hydrogène. Le pouvoir réducteur chez les champignons pourrait être dû à leur capacité de donneur d'hydrogène (Shimada et al., 1992). *P. ostreatus* présente une bonne activité antioxydante *in-vivo* en réduisant l'intensité de la peroxydation lipidique et en augmentant l'activité enzymatique et le niveau des antioxydants non-enzymatiques. Les principes antioxydants tels que l'acide ascorbique, l'α-tocophérol, le β-carotène et des composés flavonoïdes (rutine et chrysine) identifiés dans l'extrait de ce champignon peuvent contribuer aux effets observés (Jayakumar et al., 2011).

1.4. Conclusion

Le principal intérêt d'*A. subrufescens* dans la littérature porte principalement sur ses propriétés médicinales. Cependant, cette revue bibliographique met aussi l'accent sur la variabilité de l'espèce, la composition et les propriétés chimiques du champignon, les aspects de la culture en pays tropical (qui ne sont pas transposables en pays tempérés), la qualité post-récolte.

Ces informations concernent principalement les cultivars originaires du Brésil et qui sont actuellement produits et commercialisés comme champignon médicinal dans différents pays. Les quelques articles faisant référence à des origines sauvages ne concernent que l'identification d'*A. subrufescens* dans ces régions. De nouvelles souches d'*A. subrufescens* sauvages sont indispensables pour apporter de la biodiversité.

A partir de cet état de l'art nous pouvons définir les objectifs de notre travail.

Chapitre 2. Objectifs et démarche

L'étude bibliographique présentée dans le chapitre précédent montre que les travaux publiés portent essentiellement sur la biologie, la culture et l'aspect thérapeutique de cultivars d'*A. subrufescens* d'origine brésilienne.

L'amélioration quantitative et qualitative de la culture de ce champignon peut s'appuyer sur l'amélioration génétique qui fait appel au développement de connaissances sur la variabilité intra-spécifique pour le rendement, la résistance aux maladies, la qualité nutritionnelle et la teneur en composées bioactifs.

La "Collection du Germoplasm des Agarics à Bordeaux" (BGCM) comprend des cultivars brésiliens d'*A. subrufescens* mais aussi des isolats sauvages provenant du continent américain, d'Europe, et récemment d'Asie. Cette collection offre une opportunité pour l'étude de la diversité à l'intérieur de l'espèce afin d'améliorer les connaissances sur la biologie et la reproduction qui pourraient être mises en pratique plus tard pour la création de nouvelles souches commerciales.

Les objectifs de ce travail sont de contribuer à la connaissance de la biologie d'*A. subrufescens* pour l'adaptation de la culture en régions tempérées à partir des souches de différentes origines géographiques, et d'identifier celles qui présentent un intérêt agronomique et thérapeutique.

Pour atteindre les objectifs fixés, la démarche comporte trois axes :

- ✓ L'estimation de la diversité génétique et phénotypique des cultivars et des souches sauvages.
- ✓ L'optimisation de la culture d'*Agaricus subrufescens.*
- ✓ La caractérisation chimique des souches et la recherche de propriétés antioxydantes.

Le premier axe vise à déterminer la variabilité génétique et phénotypique des souches pour identifier le potentiel des isolats européens d'*A. subrufescens* comme une source de nouvelles caractéristiques pour améliorer le rendement, la qualité et les propriétés des champignons. La littérature fait référence à un nombre limité de cultivars commerciaux

disponibles, mais génétiquement similaires. Cette absence de diversité génétique représente un risque d'épidémies dans les cultures en cas d'attaque par des pathogènes en raison d'une réponse semblable des cultivars face à l'infection, et du fait de l'absence de souches de substitution moins sensibles. L'étude de la variabilité génétique et phénotypique doit permettre d'identifier des souches présentant des caractères d'intérêt. La capacité de fructification dans différents conditions de culture (présentées dans le deuxième axe) été prise en compte pour la comparaison des souches. En complément, des études ont été réalisées *in vivo* et *in vitro* sur l'interaction entre *A. subrufescens* et des pathogènes d'agarics pour connaitre la réponse physiologique des isolats aux maladies. La démarche pour cette partie a été la suivante:

- Etude de la variabilité génétique par microsatellites pour la détermination du polymorphisme.
- Evaluation des souches *in vitro* avec incubation à différentes températures afin de déterminer la température optimale et létale pour la croissance mycélienne.
- Comparaison de l'aptitude à la colonisation et à la fructification lors de cultures sur un compost commercial.
- Détermination de la variabilité morphologique des sporophores.
- Etude sur l'interaction *in vitro* et *in vivo* d'*A. subrufescens* face à des pathogènes.

Le deuxième axe consiste à rechercher des conditions de culture facilement adaptables par les champignonnistes européens, c'est-à-dire le plus proche possible de celle utilisée pour cultiver le champignon de couche *Agaricus bisporus*. Une étude préliminaire a montré qu'*A. subrufescens* est capable de fructifier à 23-25°C sur le substrat produit pour la culture commerciale d'*A. bisporus*, avec un gobetage commercial enrichi en sable (Callac, com. pers.). L'ajout de sable a été décidé d'après des observations de terrain sur les zones de récolte des souches sauvages (Guinberteau, com. pers.). Ces conditions de culture servent de base pour l'étude des paramètres permettant d'optimiser la production d'*A. subrufescens*. Pour répondre à cette question, un dispositif de culture qui permet de contrôler et d'enregistrer les paramètres physiques (lumière, humidité, température, ventilation, CO_2, O_2) a été utilisé ainsi que le pourcentage de semence dans le substrat, la présence ou l'absence de lumière, la quantité et la composition de la terre de couverture.

Le premier et le second axe sont présentés dans les chapitres 4 et 5.

Le troisième axe concerne la caractérisation chimique et l'estimation des propriétés antioxydantes chez *A. subrufescens*. Ce champignon est connu comme un champignon médicinal mais aussi comme un aliment fonctionnel grâce à la qualité et à la quantité de polysaccharides. L'analyse en résonnance magnétique nucléaire (RMN) du carbone 13 offre une approche globale permettant d'observer les échantillons d'*A. subrufescens* dans leur état brut et de donner la proportion des groupes fonctionnels. Cette analyse donne également la possibilité d'identifier des marqueurs pour la détermination de certains polymères d'intérêt médicinal, par exemple les polysaccharides.

Les cultivars d'*A. subrufescens* sont considérés comme une source riche en antioxydants naturels, mais à notre connaissance il n'existe pas de travaux sur l'activité antioxydante de souches sauvages. L'activité antioxydante de différentes souches a été comparée pour mettre en évidence les caractéristiques des ressources sauvages, en comparaison avec celle des cultivars.

Cette étude est présentée dans le chapitre 6.

Les réponses attendues à partir des voies d'approches choisies devraient permettre :

- d'apporter de nouvelles connaissances sur la biologie d'*A. subrufescens*, en particulier du fait de l'analyse de souches sauvages,
- de déterminer les paramètres culturaux permettant une production commercialement rentable pour un développement commercial en France,
- d'identifier des souches possédant des caractéristiques agronomiques (rendement, morphologie et texture) et d'intérêt comme aliment fonctionnel d'après leur composition chimique et leurs propriétés antioxydantes.

Tableau 3.1. Liste des souches d'*Agaricus subrufescens*

Code CGAB	Nature [1]	Origine		Code d'origine	Collection d'origine
		Pays	Région/Ville ou compagnie		
CA276		Taiwan	-		Coll. W Chen
CA438-A	S	Espagne	-	ARAN559	Coll. LA Parra
CA 454	S	Brésil	-	WC837 [2]	PSUMCC [3]
CA 455	C	Brésil	-	WC838	PSUMCC
CA 462	?	USA	Hawaï	ER-1 [2]	Coll. RW Kerrigan
CA 487	S	France	Gironde, Saint Léon	PA93	CGAB
CA 516	S	France	Gironde, Saint-Léon		CGAB
CA 536	?	Italie	Comacchio	24b-01	Coll. Lanconelli
CA 560	S	Brésil	Sao Paulo/ Botucatu	ABL99/28	FCA/UNESP [4]
CA 561	C	Brésil	Sao Paulo/ Piedade	ABL99/30	FCA/UNESP
CA 562	C	Brésil	Sao Paulo/ LençoisPaulista	ABL03/44	FCA/UNESP
CA 563	C	Brésil	Sao Paulo/ Sao José Rio Preto	ABL04/49	FCA/UNESP
CA 564	C	Brésil	Sao Paulo/ Bariri	ABL05/51	FCA/UNESP
CA 565	C	Brésil	Sao Paulo/ Boituva	ABL03/48	FCA/UNESP
CA 566	C	Brésil	Brazilia	ABL-06/53	FCA/UNESP
CA 567	S	Belgique	Dion de Val, Bois de Berquit		CGAB
CA 570	C	Brésil	Rio de Janeiro/ Rio de Janeiro	ABL01/29	FCA/UNESP
CA 571	C	Brésil	Sao Paulo/Mogi-da-cruzes	ABL98/11	FCA/UNESP
CA 572	C	Brésil	Sao Paulo/Suzano	ABL07/58	FCA/UNESP
CA 574	C	Brésil	Sao Paulo/Atibaia	ABL07/60	FCA/UNESP
CA603	S	Mexique	Tlaxcala		CCGAB, IneCol [5]
CA 643	S	France	Gironde, Le pian médoc		CGAB
CA 646	C	?	Soc. Mycelia	m7700	
CA 647	C	?	Soc. Mycelia	m7703	
CA918	S	Thailande			CGAB, MFLU [5]

[1] S = sauvage, C = cultivée. [2] selon la PSUMCC, WC837 est similaire à ATCC 76739, qui selon l'ATCC, est la souche fournie par T. Furumoto. [3] PSUMCC = Pennsylvania State University Mushroom Culture Collection ; [4] FCA/UNESP = Centre de recherche sur les champignons, Collège des sciences agronomiques, Université de Sao Paulo, Brésil. [5] Inecol = Institut d'écologie de Xalapa, Mexique. [6] MFLU = Mae Fah Luang University, Chiang Rai, Thailande.

Chapitre 3. Matériel et méthodes

3.1. Le matériel biologique

3.1.1. *Agaricus subrufescens*

Les souches d'*A. subrufescens* utilisées dans ce travail appartiennent à la collection de Germoplasm des Agarics de Bordeaux (CGAB), INRA-Bordeaux. Les origines des souches et leur code sont présentés dans le tableau 3.1. L'hybride CA454-3 x CA487-100 (Tableau 3.2) a été créé par E. Huang et P. Callac à partir de deux isolats monospores homocaryotiques obtenus à partir respectivement de la souche brésilienne CA454 et de la souche française CA487.

L'ensemble de ces souches a été utilisé pour vérifier leur aptitude à fructifier (petite échelle), estimer leur croissance mycélienne *in vitro* et leur variabilité génétique. Par la suite, les différents critères ont été étudiés sur un groupe formé de 3 cultivars (CA561, CA565, CA570), la souche CA454 considérée à l'origine des cultivars, trois souches sauvages européennes (CA438-A, CA487 et CA643) génétiquement différentes (cf. chapitre 4), et l'hybride CA454-3 x CA487-100.

Des hybrides intercontinentaux entre la souche française CA487 et la souche brésilienne CA454, et des hybrides européens entre CA487 et la souche espagnole CA438-A (Tableau 3.2) ont été mis en culture pour des premiers essais de recherche d'amélioration variétale.

Tableau 3.2. Liste des hybrides d'*A. subrufescens*.

Hybrides intercontinentaux			Hybrides européens
487-42 x 454-43	487-100 x 454-43	487-125 x 454-43	438-A -12 x 487-100
487-42 x 454-3	487-100 x 454-3	487-125 x 454-3	438-A -12 x 487-42
487-42 x 454-4	487-100 x 454-4	487-125 x 454-4	438-A -12 x 487-125
			438-A -12 x 487-35

Les souches sont conservées à l'état mycélien à 12 °C, en tubes sur milieu extrait de compost et recouvertes avec de l'huile minérale. Pour la réactivation des souches, un implant est mis dans une boîte de Petri contenant de l'extrait de compost et l'ensemble est incubé à 25 °C. Après développement de la colonie mycélienne, deux repiquages successifs suivis d'une incubation à 25 °C pendant 15 jours sont effectués avant utilisation pour les diverses expérimentations.

3.1.2. Les agents pathogènes

La moisissure *Trichoderma aggressivum* (souche TriB) et la bactérie *Pseudomonas tolaasii* (souche CNBP2152, collection CNBP, INRA, Angers, France) font partie de la collection des micro-organismes de l'UR MycSA, INRA, Bordeaux. Ils sont conservés dans l'azote liquide, et une semaine avant l'expérimentation, ils sont décongelés à 36 °C pendant 5 minutes, placés sur gélose nutritive et incubés à 25 °C, puis repiqués. La moisissure est cultivée sur milieu malt gélosé et la bactérie sur milieu B de King gélosé (King et al. 1954). Avant utilisation des micro-organismes, l'aptitude de la moisissure à sporuler est vérifiée sous loupe binoculaire, et la fluorescence de *P. tolaasii* est vérifiée sous lumière UV, en plus de la production d'une ligne blanche en présence de *P. reactans*, ces deux propriétés étant indicatrices de la production de toxine par la bactérie.

3.2. Culture d'*A. subrufescens*

3.2.1. Préparation du blanc

Le blanc (ou semence) est produit sur des grains de seigle fournis par la société Euromycel. Des barquettes munies d'un filtre d'aération (Microbox, Combiness, Belgique) (Figure 3.2) sont remplies avec 160g de grain, stérilisées une heure à 120 °C et laissées refroidir à température ambiante. Le grain est inoculé avec du mycélium d'*A. subrufescens* développé sur milieu cristomalt solide (le contenu d'une boîte de Petri par barquette). L'ensemble est incubé à 25 °C pendant environ 3 semaines jusqu'à envahissement du grain. Une fois prêt, le blanc est conservé à 19 °C et utilisé dans un délai inférieur à 3 semaines.

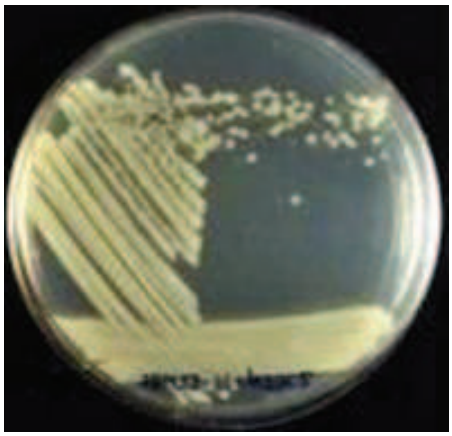

Figure 3.1. Culture de *Pseudomonas tolaasii* sur milieu B de King (Photo INRA, MycSA).

Figure 3.2. Barquette contenant 160 g de seigle envahi par le mycélium d'*Agaricus subrufescens* (Photo INRA, MycSA).

3.2.2. Le substrat de culture

Le substrat utilisé pour la culture d'*A. subrufescens* est un compost préparé pour la production commerciale du champignon de Paris (*Agaricus bisporus*) par la société Renaud, Pons, France. Les principaux ingrédients pour le processus de compostage sont la paille de blé et le fumier de cheval. Le compostage est réalisé en salle (*indoor*). La composition moyenne du substrat est donnée dans l'article 3, chapitre 4. La teneur en eau est comprise entre 69 et 71%.

3.2.3. Les paramètres de culture

Dans un premier temps, toutes les souches sont cultivées à petite échelle, en barquette contenant 500 g de compost (Figure 3.3) selon le protocole décrit dans l'article 2, chapitre 4. Par la suite, le groupe de souches retenu pour l'ensemble des expérimentations est cultivé à moyenne échelle, en paniers de 8 kg de compost (Figure 3.4). Ensuite, des variations sont appliquées à différents paramètres de culture (quantité et composition du gobetage, photopériode et variations de température pendant la fructification) selon les modalités décrites dans les articles 2 et 4 (chapitre 4), afin d'optimiser la production.

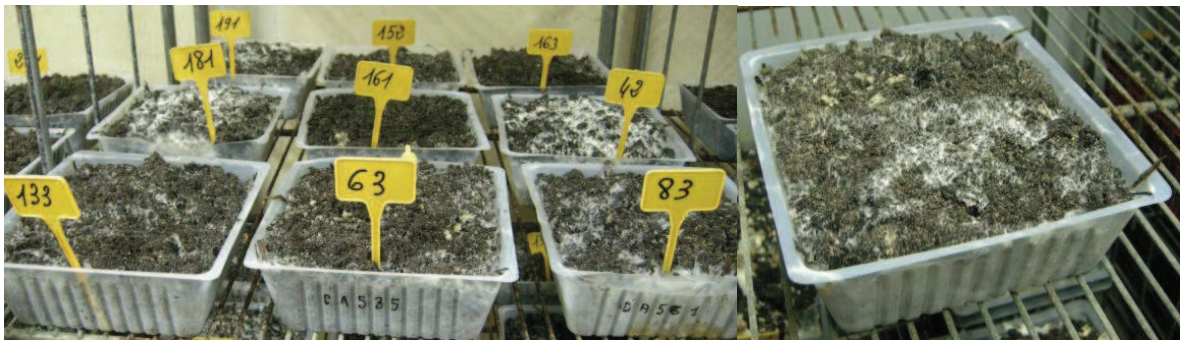

Figure 3.3. Barquettes contenant 500 g de compost, utilisées pour la production d'*Agaricus subrufescens* à petite échelle (Photo INRA, MycSA).

Figure 3.4. Paniers contenant 8 kg de compost, utilisés pour la production d'*Agaricus subrufescens* à moyenne échelle (Photo INRA, MycSA).

3.3. Génotypage des souches

L'ensemble des souches (Tableau 3.1) est utilisé dans cette étude. Les souches sont cultivées sur milieu cristomalt. L'extraction d'ADN génomique des échantillons de mycélium lyophilisé est réalisée avec le kit Nucleon Phytopure (GE Healthcare) en suivant les instructions du fabricant. La qualité et la quantité de l'ADN sont évaluées en utilisant un spectrophotomètre Nanodrop® ND-1000. Pour la PCR, les échantillons d'ADN sont calibrés à une concentration de 25 ng μl^{-1}. Les marqueurs microsatellites ou SSR (*single sequence repeat*) utilisés dans ce travail ont été développés par Foulongne-Oriol et al. (2012b).

Tableau 3.3. Caractéristiques des 22 marqueurs microsatellites utilisés pour le génotypage des souches (d'après Foulongne-Oriol et al. 2012b).

Locus Name	Accession à Genbank	Motif répété	Amorce F	Amorce R	Nombre d'allèles
SubSSR14	JQ901239	(AG)5	TCGGGGTAGGATACAGATGC	GAAACCCTTTTATTCGTCACTG	5
SubSSR16	JQ901195	(AC)5	ACACCCTTATGTTCACCCCA	CCTCAGCAGTCACTCCATCA	2
SubSSR21	JQ901126	(ACC)6	CTCAAACCAAACCTCCCAAA	AAGCCGATGCGATATTTACG	6
SubSSR22	JQ901144	(ACG)5	ACACCTCCAAGATGTCGTCC	GATGGAGAGGAGGGATAGGG	9
SubSSR36	JQ901145	(AGT)7	GGGATCCGGAGATAGGACTT	GGGATGACGGTTTGGTTTTT	6
SubSSR41	JQ901377	(AG)6	CAATGGCTGCTAGGTACGTG	GAAGGGAGTGGAGCTGTTTG	4
SubSSR44	JQ901213	(AG)5	GAAGAACACACTTCGCGTCA	TGACTGTCTTGGAGTCGTCG	2
SubSSR46	JQ901240	(AG)5	GAGGTTCGAAGCATTGCCTA	CCTTCTCCATCCCACTCTGA	3
SubSSR47	JQ901326	(AG)5	GTCGTACGCCGTCTCCTATC	GCCGTCGAAGTGAATACCAT	3
SubSSR50	JQ901140	(AC)5	GACAAGAAGCGAAGGAGTGC	ATGGCAGTAATCGGGAACAG	2
SubSSR51	JQ901142	(ACG)5	TAGGACTCGACTGTCCACCC	CTCTCAGGTCGAATGCTGGT	2
SubSSR52	JQ901191	(AAG)9	CTTCGCCCAGCTTTGTAGTC	ACAGAATCACCGCAATCCTC	6
SubSSR54	JQ901316	(ACC)5	ACCGTCATCCAGTCGTTAGG	CCATGGTCGGCTTCTACACT	4
SubSSR56	JQ901170	(ACTCAG)6	AAGTGACTCGCTCAATGCCT	TACCCATAGGTGAAAGGGCA	6
SubSSR59	JQ901372	(AGC)6	TGGCTGCATCTTGATCTGAC	TGTAGCAGTGGAAGGTGGTG	5
SubSSR66	JQ901204	(ACG)5	CGCCATACTCTGCTTCACCT	GGCGAAGTCTGAGGAGTCAA	4
SubSSR68	JQ901370	(AC)5	AACATCCTTTCCAAACACGC	GTTGATTGATTTCCAACGCA	6
SubSSR80	JQ901208	(AC)7	CCGCACTCATTTAATTTTGC	GAGGGCTGAGCATAAAGCAC	4
SubSSR83	JQ901374	(CCG)5	CATCGAATCGGAAGACGAAT	CTCAACTCCCACACCCACTT	10
SubSSR84	JQ901135	(AAT)5	CGTATCGTAGCGACACCTGA	CAACCGATGATTCACAGACG	3
SubSSR86	JQ901182	(AGC)5	GTGTCTCTATAGCGGCCTCG	AGGACAGCTGTGTCTGCTCA	5
SubSSR93	JQ901115	(AGT)9	AACGTCGTCGACAACAACC	GCTGAAGATAGAGGCGATGG	6

Vingt-deux marqueurs SSR (Tableau 3.3) ont été utilisés pour le génotypage des souches.

Les conditions d'amplification et d'électrophorèse sur séquenceur ABI 3130 (Applied Biosystems) ont été définies par Foulongne-Oriol et al. (2012b). Les profils des électrophérogrammes (Figure 3.5) sont lus manuellement et à l'aide du logiciel GENEMAPPER™ version 4.0 pour attribuer les pics aux allèles correspondants.

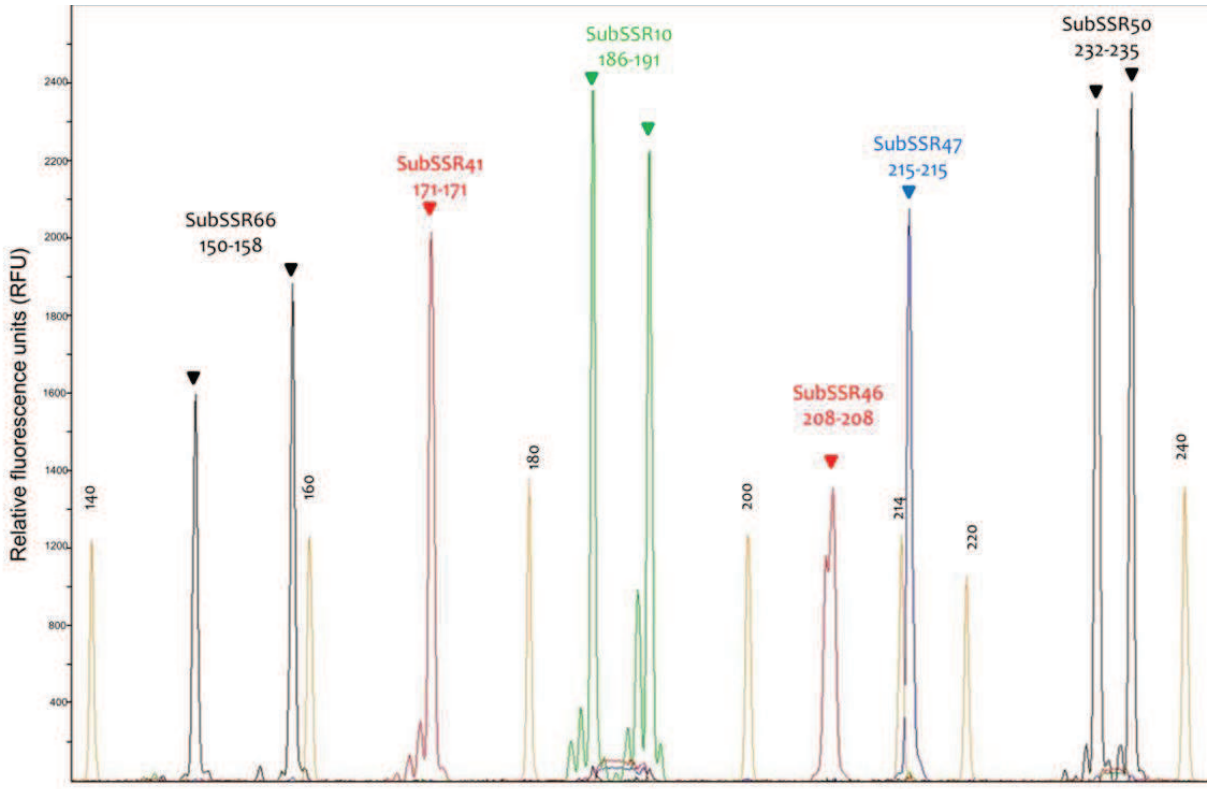

Figure 3.5. Exemple d'électrophérogramme d'un ensemble multiplex SubSSR obtenu avec le logiciel GeneMapper pour un génotype d'*Agaricus subrufescens* (d'après Foulongne-Oriol et al. 2012).

Six microsatellites (SubSSR66, SubSSR41, SubSSR10, SubSSR46, SubSSR47, SubSSR50) sont amplifiés simultanément en fonction de l'ordre de leur taille d'allèles et de la couleur de leur fluorescence (6-FAM = bleu, PET = rouge, VIC = vert et NED = jaune (pics noirs)). Le standard interne GeneScanTM-600 LIZ (pics orange) est utilisé pour estimer la taille des allèles.

Les données sont analysées avec le logiciel Power Marker V3.25. Les relations génétiques entre les 25 souches ont été analysées par la méthode UPGMA (*Unweighted Pair-Group Method with Arithmetic mean*), à l'aide du logiciel Treeview, en utilisant les distances génétiques basées sur la proportion des allèles partagés (DAS, *Shared Allele Distance*) avec $\mathrm{DAS} = 1 - \frac{\sum_{\mu} S}{2}$ où S est le nombre d'allèles partagés et μ le nombre de loci analysés (Bowcock et al., 1994).

3.4. Croissance mycélienne

3.4.1. Développement mycélien *in vitro*

La croissance mycélienne d'*A. subrufescens* sur milieu gélosé (Figure 3.6) est mesurée à différentes températures (22, 25, 28, 30, 32, 35, 38 et 40 °C) pour déterminer la température optimale et la température létale. La souche d'*A. bisporus,* Bs057*,* qui est la souche commerciale 30A (France Mycelium) conservée dans la collection CGAB, est utilisée, en comparaison, comme témoin externe. Les détails du protocole sont présentés dans les articles 2 et 3 (chapitre 4).

3.4.2. Développement mycélien dans le substrat de culture

Le peroxyde d'hydrogène (H_2O_2) est impliqué dans la dégradation de la lignine et de la cellulose du substrat de culture par les basidiomycètes de pourriture blanche (Savoie et al., 2007). La mesure des concentrations de H_2O_2 a été proposée comme un moyen d'estimer les changements de l'activité de biomasse mycélienne depuis l'initiation jusqu'à la fin de la culture d'*A. bisporus*. Pendant la croissance végétative d'*A. bisporus*, la concentration en peroxyde d'hydrogène augmente selon une relation directe entre celle-ci et la biomasse active du champignon (Savoie et al., 2007). La culture d'*A. subrufescens* est effectuée dans des barquettes (Microbox, Combiness, Belgique) avec 150 g de compost, dont toute la surface du substrat a été recouverte de mycélium développé sur milieu MEA (Figure 3.7). Après 21 jours d'incubation à 25 °C et 85% d'humidité, le substrat a été lyophilisé et la colonisation par le mycélium a été estimée par la mesure de H_2O_2 comme décrit par Savoie et al. (2007).

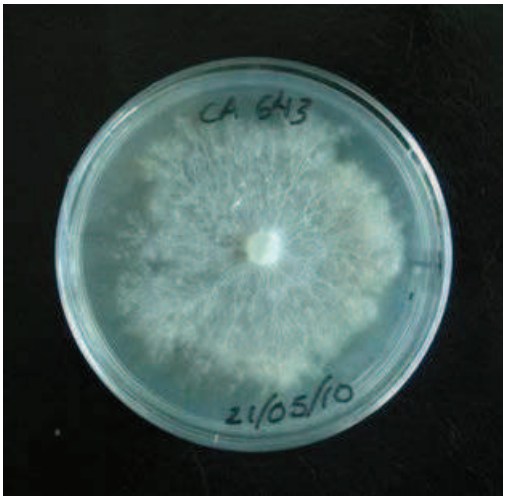

Figure 3.6. Développement du mycélium d'*Agaricus subrufescens* sur milieu cristomalt gélosé (Photo INRA, MycSA).

Figure. 3.7. Barquettes (Microbox, Combiness, Belgique) avec filtre d'aération pour le développement du mycélium (Photo INRA, MycSA)

Pour la préparation d'extraits bruts, 150 mg de substrat de culture lyophilisé sont mélangés avec 4 ml de tampon phosphate (0,05 mol l^{-1}, pH 6,0), et l'ensemble est incubé à 25 °C pendant 15 minutes. Après centrifugation pendant 10 min à 12 000 *g* et 4 °C, le surnageant, constituant l'extrait brut, est prélevé et utilisé immédiatement pour le dosage de H_2O_2. La concentration de H_2O_2 est déterminée après l'inactivation des enzymes par chauffage des extraits bruts à 80 °C pendant 40 min pour limiter l'interférence des polyphénols oxydases dans les mesures. Après refroidissement, les extraits sont mélangés avec 40 µl de polyéthylènimine (PEI) à 1%, puis centrifugés à 1000 *g* pendant 5 min pour éliminer les polymères qui pourraient interférer dans le dosage. Le mélange standard contient 250 µl de solution à doser, 250 µl de solution de peroxydase de raifort (Sigma) à 20 $U \cdot ml^{-1}$, et 625 µl de solution de chromophore contenant 0,6 mmol l^{-1} de chlorhydrate de 3-methyl-2-benzothiazolinone hydrazone (MBTH) et 7,5 mmol l^{-1} d'acide 3-dimethylamino benzoique (DMAB) dans du tampon phosphate 0,05 mmol l^{-1}, pH 6,0. Le mélange est incubé pendant 3 min à 30 °C et l'oxydation des colorants est mesurée à 590 nm. Les mesures sont effectuées en absence (témoin) et en présence (échantillon) de peroxydase. La concentration en H_2O_2 est donnée par la différence entre le témoin et l'échantillon et exprimée en µmol de H_2O_2 par mg du substrat.

3.5. Caractérisation chimique d'*A. subrufescens* par Résonance Magnétique Nucléaire du Solide sur le Carbone 13

La haute qualité des polysaccharides chez *A. subrufescens* représente un des aspects importants comme aliment fonctionnel.

L'analyse par résonance magnétique nucléaire offre une approche holistique qui permet d'analyser les échantillons dans leur état natif. Cet outil permet d'identifier des marqueurs chimiques (Pizoferrato et al., 2000) pour déterminer l'effet du type de biomasse (mycélium ou corps fructifère), le stade de développement du corps fructifère, les conditions de culture et la diversité génétique sur les concentrations de groupes fonctionnels associés à des polymères constituants de la biomasse fongique.

3.5.1. Résonance Magnétique Nucléaire du ^{13}C par polarisation croisée et rotation à l'angle magique (*^{13}C CP/MAS NMR*).

Des analyses chimiques du pourcentage de groupes carboxyliques ont été faites à partir de mycélium et de sporophores d'*A. subrufescens*. La préparation des échantillons et les paramètres utilisés pour l'analyse par Résonance Magnétique Nucléaire du ^{13}C par polarisation croisée et rotation à l'angle magique (^{13}C CP/MAS NMR,) sont décrits dans l'article 6 (chapitre 5). La technique de rotation à l'angle magique (rotor incliné de $\theta = 54{,}74°$ par rapport au champ magnétique) et à une fréquence élevée (10 kHz dans notre travail) permet d'obtenir des spectres qui séparent les espèces chimiques en fonction de leur déplacement chimique (Figures 3.8). Dans les solides, les spins abondants (^{1}H) sont proches des spins rares (^{13}C) et sont couplés par interaction dipolaire. Le principe de la ^{13}C CP/MAS NMR est le suivant : les spins abondants (^{1}H) sont excités, leur magnétisation est transférée aux spins rares (^{13}C) via le couplage, et le signal est enregistré.

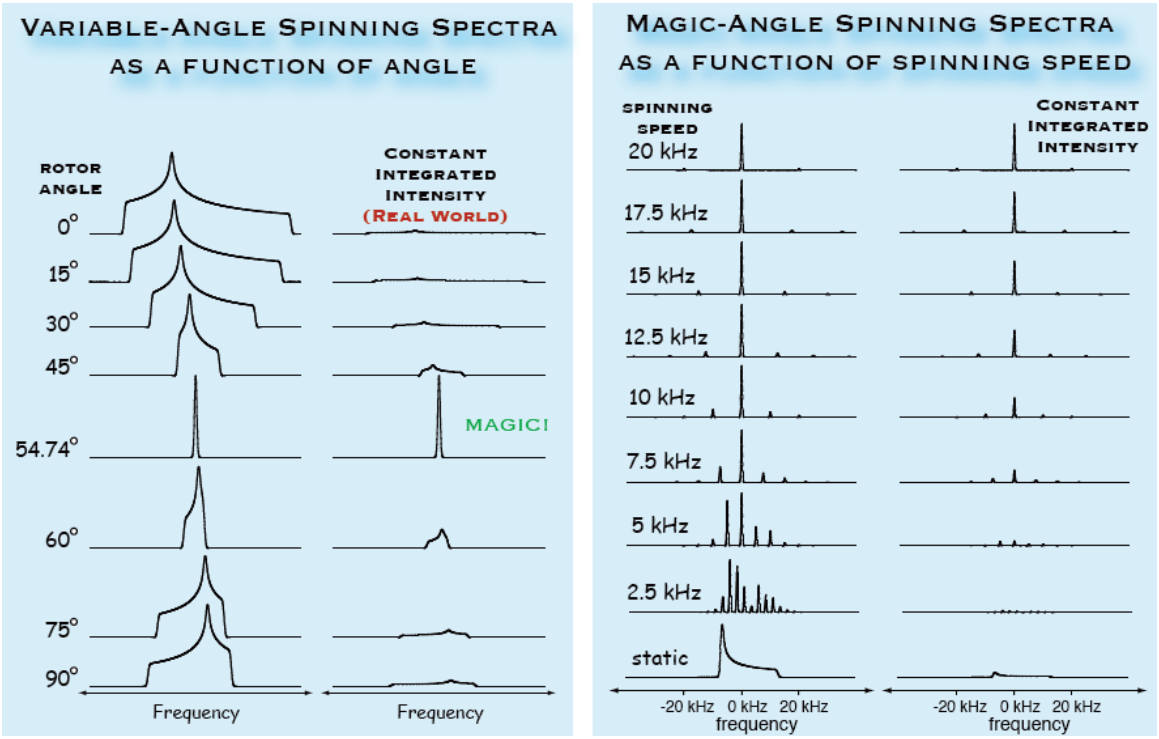

Figure 3.8. Effet de l'angle et de la vitesse de rotation du rotor sur la qualité du spectre RMN obtenu. (d'après Grandinetti, www.enc-conference.org/portal/0/Tutorial Grandinetti.pdf)

3.6. Effet d'extraits hydrophiles d'*Agaricus subrufescens* sur des pathogènes d'agarics

La sensibilité de quatre souches sauvages et trois cultivars d'*A. subrufescens* a été testée face aux pathogènes *Trichoderma aggressivum* et *Pseudomonas tolaasii*. L'information concernant l'origine et la culture des pathogènes *T. aggressivum* et *P. tolaasii* et les protocoles utilisés sont décrits dans l'article 5 (chapitre 4).

Deux types d'extraction hydrophile ont été réalisés, l'une à 100 °C, l'autre à 4 °C, à partir de sporophores lyophilisés pour être testées vis-à-vis de la multiplication de *P. tolaasi*, et à partir de mycélium lyophilisé pour étudier leur effet sur l'aptitude de *T. aggressivum* à coloniser le compost. Les sporophores sont produits selon la condition standard décrite dans l'article 3, (chapitre 4). La production de mycélium, le mode d'extraction et les protocoles des tests sont décrits dans l'article 5 (chapitre 4).

3.6.1. Dosage de l'activité d'hydrolyse du FDA

Il a été démontré que presque tous les microorganismes libèrent de la fluorescéine à partir de diacétate de fluorescéine (FDA) grâce à des estérases qui sont liées au métabolisme primaire. Ainsi la mesure de cette activité *in situ* permet d'estimer les variations de biomasse microbienne totale présente dans un milieu naturel. On estime en fait une activité métabolique associée à une biomasse (Lestan et al., 1996).

Le dosage de l'activité d'hydrolyse du FDA est utilisé pour mesurer le développement des pathogènes en présence d'extrait d'*A. subrufescens*. La méthode de dosage est adaptée du protocole de Inbar et al. (1991). Après 48 heures d'incubation, 1 ml de solution de FDA à une concentration de 0,01 µg ml^{-1} dans du tampon phosphate 60 mM à pH 7,6 (8,7 g de K_2HPO_4 et 1,3 g de KH_2PO_4) est ajouté dans les tubes contenant les échantillons et 1 ml de tampon phosphate 60 mM à pH 7,6 pour les témoins. Les tubes sont placés dans un bain-marie à 30 °C pendant 30 min, puis 1 ml d'acétone est ajouté. Après 5 min à température ambiante, les tubes sont centrifugés à 11 000 rpm, à 15 °C pendant 5 min. La densité optique de 1 ml du surnageant est mesurée à 490 nm à l'aide d'un spectrophotomètre (UVIKON, Bio-tek Instruments). L'activité d'hydrolyse du FDA est exprimée en µg équivalent de FDA hydrolysé/implant/ min, en utilisant l'équation suivante :

Activité = (DO mesurée *1/n) * (1/tps)

avec n = nombre d'implants, et tps= temps d'incubation en minutes.

Les extraits froids ont été soumis à une incubation dans un bain-marie à 80 °C pendant 20 min. Au moment de faire la lecture au spectrophotomètre, des témoins contenant l'extrait d'*A. subrufescens* et la solution de FDA ont été mesurés, et une légère activité a été détectée. La valeur de densité optique obtenue pour le témoin a été soustraite de celle des échantillons.

3.7. Activités antioxydantes

L'activité antioxydante des souches d'*A. subrufescens* a été déterminée à partir de sporophores cultivés sur deux lots de compost. L'aptitude au piégeage des radicaux de DPPH ou d'ABTS et le pouvoir réducteur des extraits méthanoliques ont été étudiés. Les protocoles d'extraction et les méthodes utilisés sont décrits dans les articles 3 (chapitre 4) et 7 (chapitre 5).

Chapitre 4. Diversité chez *Agaricus subrufescens* et optimisation de la culture

Ce chapitre décrit la variabilité génétique et phénotypique observée au sein d'un groupe de souches d'*A. subrufescens* disponibles dans la collection CGAB, et l'adaptation de la culture aux conditions européennes (matériaux de base, environnement) tout en permettant une production de niveau commercial.

4.1. Introduction

La culture industrielle d'*A. subrufescens* s'est développée au Brésil, principalement pour l'exportation, et ce pays est considéré comme le premier producteur de ce champignon. *Agaricus subrufescens* est également cultivé à l'échelle industrielle au Japon, en Chine, à Taiwan et en Corée (Gregori et al. 2008). A notre connaissance, il n'existe pas de méthode standardisée de culture pour ce champignon. Les conditions de culture rapportées dans la littérature concernent presque uniquement des expérimentations effectuées au Brésil à partir de matériaux locaux. Dans ce pays, la culture d'*A. subrufescens* est relativement récente ; la productivité est variable et dépend principalement du type de substrat utilisé (Ferreira Silva et al., 2009). A ce jour, les seules autres données culturales disponibles concernent le Japon.

La culture d'*A. subrufescens* présente plusieurs similitudes avec celle d'*A. bisporus,* comme le substrat (obtenu par compostage de matériaux lignocellulosiques et de fumiers), les phases d'inoculation et d'incubation, et l'ajout d'une terre de gobetage permettant la fructification. La principale différence est qu'*A. subrufescens* n'a pas besoin d'une réduction de la température pour la fructification, alors qu'*A. bisporus* nécessite une température en dessous de 20 ºC pendant la période de fructification (Dias et al., 2004 ; Eira et al., 2005). Ainsi, *A. subrufescens* pourrait être une option saisonnière pour les producteurs de champignons dans les pays occidentaux où la production est effectuée en salles climatisées. Les champignonnistes pourraient économiser de l'énergie en produisant ce champignon

pendant l'été, en raison de ses exigences de températures de fructification (23 – 29 °C) qui sont plus élevées que celles d'*A. bisporus* (16 – 18 °C).

Il est nécessaire de développer une technologie appropriée pour cultiver ce champignon car son niveau de production est encore très faible par rapport à celui d'*A. bisporus* (Siqueira et al., 2009). Par conséquent, plusieurs projets de recherche ont été développés au Brésil afin de trouver de meilleurs procédés de compostage et matériaux pour le substrat et la terre de couverture (Colauto et al., 2010; Rocha Cavalcante et al., 2008; Silva et al., 2007; Siqueira et al., 2009). Mais ces projets ont été mis en œuvre avec des matériaux locaux (par exemple la bagasse de canne à sucre et la pulpe de café pour le substrat, des sols pour le gobetage) qui ne sont pas disponibles en Europe.

L'étude bibliographique a montré qu'*A. subrufescens* a été découvert dans une région particulière du Brésil puis a été envoyé au Japon où l'identification de propriétés antitumorales a retenu l'attention d'industriels pour ce champignon (Mizuno, 1995). La culture industrielle, principalement pour l'importation, a alors commencé au Brésil et ce pays est considéré comme le plus grand producteur de ce champignon (Dias et al., 2004). Plusieurs auteurs ont cherché à déterminer la diversité génétique de différentes souches d'*A. subrufescens* utilisées par les producteurs (Colauto et al., 2002; Fukuda et al., 2003; Neves et al., 2005; Tomizawa et al., 2007). Leurs travaux montrent une grande homogénéité des souches commerciales du Brésil, et il est très probable que les souches actuellement cultivées dérivent d'un seul spécimen ou d'isolats de la même population, ce qui laisse supposer une disponibilité limitée de diversité biologique pour la culture. Récemment divers travaux ont signalé de nouveaux spécimens d'*A. subrufescens* dans d'autres pays que le Brésil (Arrillaga et Parra, 2006 ; Kerrigan et al., 2005 ; Wisitrassameewong et al., 2012). Depuis les années 2000, un germoplasme d'*A. subrufescens* est en cours de réalisation au sein de la collection CGAB. Il rassemble une vingtaine de souches d'origines diverses, dont certaines correspondent à une aire de répartition nouvelle pour cette espèce. L'étude de ce nouveau matériel génétique peut conduire à l'identification d'une diversité pour des caractéristiques d'intérêt agronomique et de qualité comme aliment fonctionnel.

Une haute productivité, qui est liée à une bonne efficacité du champignon pour la conversion d'un substrat en biomasse fongique, est un intérêt prioritaire pour les champignonnistes. Il a été démontré dans des études antérieures, en particulier sur *A. bisporus* (Manolopoulou et al., 2007) que la productivité des cultures et la qualité des champignons frais dépendent du type de souche. Les critères de qualité considérés sont principalement le calibre, le degré de brunissement, la forme et la couleur du chapeau. Ils sont sous contrôle

génétique, et tous ces caractères sont pris en compte dans les programmes de sélection de champignons (Foulongne et al. 2012a et b).

Le premier objectif du travail présenté dans ce chapitre est de déterminer la variabilité génétique et phénotypique disponible dans la collection CGAB d'*A. subrufescens*. L'identification de variabilité génétique est de première importance dans les programmes d'amélioration de champignons afin d'éviter les risques de dérive génétique dus à l'utilisation de cultivars fortement apparentés. Le polymorphisme génétique a été estimé pour l'ensemble des souches disponibles.

Plusieurs critères ont été retenus pour analyser la variabilité phénotypique :

- La température optimale et la température létale pour optimiser la propagation *in vitro* (précultures et préparation du blanc) et pour éviter des risques de dégénération des souches par stress thermique.
- L'aptitude à coloniser le substrat de culture et à fructifier, ainsi que la couleur des champignons, qui ont été évaluées à petite échelle.

Le deuxième objectif est d'optimiser les conditions de culture d'*A. subrufescens* en partant du substrat et de la terre de couverture utilisés pour la production commerciale d'*A. bisporus* en France. Les cultivateurs d'*A. bisporus* utilisent généralement pour le gobetage une épaisseur de 2,5 cm d'un mélange standardisé. Au Brésil plusieurs travaux ont été réalisés pour identifier le mélange et la hauteur de gobetage qui permettent le meilleur rendement d'*A. subrufescens* (Largeteau et al., 2011). Cependant tous ces travaux sont faits à partir de matériaux non disponibles en France. L'étude portera sur la quantité et la composition de la terre de couverture, car ces paramètres peuvent déterminer le rendement d'*A. subrufescens*.

Plusieurs champignons cultivés ont besoin de lumière pour l'initiation fructifère et pour le développement des sporophores. Il est essentiel de connaitre les besoins en lumière pour une espèce donnée afin d'avoir de bonnes pratiques culturales (Stamets et Chilton, 1983). Par exemple, dans la culture d'*A. bisporus* la lumière n'a pas d'impact sur la production et la qualité. Cependant pour la culture de *Pleurotus ostreatus* la présence de lumière est indispensable pour l'induction fructifère et le développement normal des sporophores.

Par ailleurs, une température doit être fixée pour la colonisation du substrat par le mycélium ; elle est de 25 °C pour *A. bisporus* et pour *A. subrufescens*. D'autre part, Stamets et Chilton, (1983) rapportent que chaque espèce a sa température optimale pour le développement des sporophores. Cependant, la littérature indique plusieurs conditions de températures pour la fructification d'*A. subrufescens*. Considérant l'étude préliminaire effectuée par Callac (com. pers.) qui montre qu'*A. subrufescens* est capable de fructifier à 20-30°C sur le substrat et le gobetage (enrichi en sable) utilisés par les producteurs d'*A. bisporus*, ces conditions ont servi de point de départ pour optimiser la culture d'*A. subrufescens* en apportant des changements faciles à réaliser par les champignonnistes.

Plusieurs paramètres de culture ont été évalués :

- des facteurs environnementaux (lumière, choc froid et variation ou non de la température pendant la période de fructification),
- des paramètres techniques agronomiques (pourcentage de semence, composition et quantité de gobetage).

Les résultats attendus devraient permettre de proposer des conditions de culture facilement utilisables en pays tempérés ainsi que des souches adaptées à ces paramètres culturaux.

4.2. Résultats et discussion

4.2.1. Variabilité génétique

Vingt-deux marqueurs microsatellites (SSR) ont été utilisés pour estimer la variabilité génétique des souches d'*A. subrufescens*. Le dendrogramme obtenu (Figure 4.1) montre que les groupes de cultivars se distinguent bien des groupes de souches sauvages. Un groupe est constitué de 11 cultivars du Brésil génétiquement homogènes avec une distance génétique nulle. Ce résultat confirme l'absence de polymorphisme génétique rapporté dans la littérature pour des souches du Brésil (Neves et al., 2005 ; Tomizawa et al., 2007). Une deuxième branche, la plus proche du groupe précédent, comprend cinq souches, un cultivar supposé (CA455, acheté sur un marché chinois) qui montre une DAS (*distance of allele shared*) ou

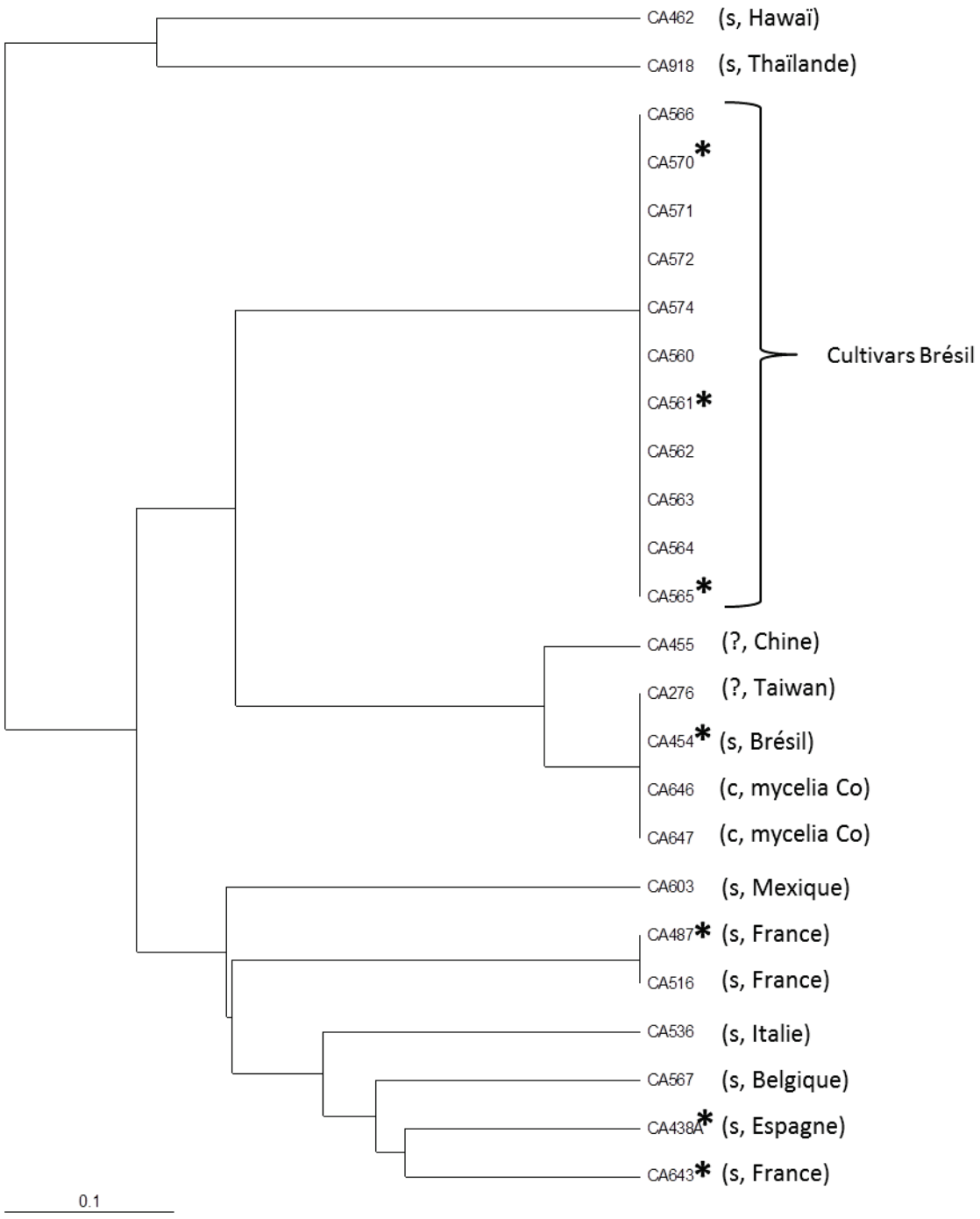

Figure 4.1. Dendrogramme des distances génétiques entre 25 souches d'*A. subrufescens,* basées sur la méthode d'analyse UPGMA (Unweighted Pair-Group Method Analysis) à partir des résultats des marqueurs SSR.

s = souche sauvage, c = cultivar, ? = cultivar supposé.

* Souches retenues pour l'ensemble des études de variabilité et de production.

distance d'allèles partagés) de 0,11 avec un groupe non polymorphe comprenant deux cultivars (CA646 et CA647) de la société Mycelia (industrie productrice de blanc), un cultivar supposé, acheté sur un marché asiatique, et la souche CA454 qui, d'après la littérature, serait à l'origine des cultivars brésiliens. La DAS entre les cultivars brésiliens et les souches CA454 et Mycelia est de 0,45.

Les souches sauvages sont séparées en deux branches, l'une est composée de sept souches dont six sont d'origine européenne et une d'origine mexicaine (CA603). La DAS entre ces souches sauvages varie de 0 (entre les souches françaises CA487 et CA516 provenant d'un même site) à 0,55 (entre CA567 isolée en Belgique et CA487), avec une moyenne de 0,42. La DAS entre les souches brésiliennes et les souches sauvages d'Europe et du Mexique varie de 0,52 à 0,61 avec une moyenne de 0,56. L'autre branche de souches sauvages comprend CA462, originaire d'Hawaï, et CA918 de Thaïlande, qui montrent une DAS entre elles de 0,57. Les plus grandes distances génétiques sont observées entre les cultivars brésiliens et les souches CA462 (DAS = 0,83) et CA 918 (DAS = 0,77).

Les souches utilisées pour l'ensemble des études sont identifiées sur la figure 4.1. Elles sont représentatives de la variabilité génétique observée avec l'ensemble des souches. Leur variabilité phénotypique est présentée dans l'article 3. La souche CA918, récoltée tardivement, n'a été utilisée que pour les études de pathologie décrites dans le chapitre 5.

4.2. 2. Variabilité phénotypique

L'étude sur la variabilité phénotypique (température optimale et létale pour la croissance mycélienne, aptitude à coloniser le substrat de culture, rendement et précocité en culture à petite échelle, morphologie des sporophores) est présentée dans les articles 2 et 3.

4.2.3. Optimisation de la culture

Les différents paramètres analysés pour optimiser la culture d'*A. subrufescens* dans des conditions applicables en France sont présentés dans l'article 2 (pourcentage de blanc, effet de la lumière et d'un choc froid sur la fructification) et dans l'article 4 (quantité et nature du gobetage, variations de température pendant la fructification).

Article 2.

Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products (ICMBMP7) 2011

Phenotypic variability in cultivars and wild strains of *Agaricus brasiliensis* and *Agaricus subrufescens*

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ABSTRACT

In recent years, there is continued commercial interest in the cultivation of mushrooms with medical and pharmacological value. In Brazil, research and development of cultivation techniques, selection of strains to increase mushroom yield and production of bioactive molecules is needed as the industry is relatively young (1990's) and is focused on the mushroom formerly known as *Agaricus blazei* or *A. brasiliensis*. Recent studies have clarified the taxonomic status of these fungi and they are considered to be synonyms of *A. subrufescens* however the name *A. brasiliensis* is used in many publications on the Brazilian medicinal mushroom. In this paper we evaluate medicinal strains presently cultivated in Brazil, strain ATCC 76739 and wild strains of *A. subrufescens* from various countries for i) mycelial growth at different temperatures, ii) mycelium efficiency to colonize the substrate, and iii) mushroom yield under various cultivation conditions (spawn rate, light, cold shock). Most of the medicinal cultivars showed higher mycelial growth rates than the wild *A. subrufescens*. A temperature of 35°C was not lethal for the cultivars, but seemed so for two *A. subrufescens* strains. Cultivation experiments were performed using commercial compost used for *A. bisporus*. The efficiency of compost colonization was estimated by measuring H_2O_2 after 20 days of incubation. Cultivars produced variable concentrations of H_2O_2 (39-217 nmoles/g of compost) whilst low concentrations were found in the group of wild *A. subrufescens* (47-91 nmoles/g). There was no relationship between compost colonization and others parameters studied (yield, time to fruiting and sporophore mean weight). When taken as a whole, the group of cultivars differed from the group of wild strains for the time taken to first fruiting, yield and sporophore mean weight. Cap colour also separated the cultivars from the wild strains. ATCC 76739 grouped with the cultivars for the various traits analysed. Light and cold shock had no significant effect on the time to first fruiting and yield. Valuable wild material useful for productivity and breeding was identified.

Keywords: Mycelial growth; Compost colonization; Yield; Morphology.

INTRODUCTION

Edible mushrooms are appreciated for their gastronomic, nutritional and medicinal values. The mushroom cultivated in Brazil, formerly known as *Agaricus blazei* Murrill, is widely used and

studied for its medicinal and/or therapeutic properties [1]. Several works have been published to clarify its taxonomic status [2-6], and two new species names were proposed, *Agaricus brasiliensis* and *Agaricus subrufescens*. Kerrigan [2] considers *A. brasiliensis* Wasser et al. and *A. blazei* Murrill to be synonomous with *A. subrufescens* as they interbreed and produce fertile offspring. Wasser [3, 4] claimed that they are different species and proposed *A. brasiliensis* for the medicinal mushroom. Currently many publications refer to the Brazilian cultivar as *A. brasiliensis* and it is believed to originate from Brazil [7]. Wild mushrooms referred to as *A. subrufescens* have been found in the wild in California, Israel, Taiwan and Hawaii [2, 6], Mexico and European countries. These mushrooms are not currently cultivated at commercial scale, but recent work performed by Moukha *et al.* (this issue) proved that wild strains of *A. subrufescens* can show medicinal properties.

Several studies describe specific techniques and parameters for cultivating the Brazilian commercial strains [8-14] but substrates, casing materials and procedures are adapted for tropical countries. Brazilian strains have been reported to have a high genetic similarity [15-18] and a typical morphology [3, 19, 20].

Our target was to assess phenotypic variability among currently cultivated Brazilian strains and wild strains of *A. subrufescens* from various origins. We also wanted to identify individuals giving good yields when cultivated using substrates and procedures similar to those used for *Agaricus bisporus* production in France. With this aim, we evaluated both groups of strains for: i) mycelium growth rate at different temperatures, ii) mycelium efficiency to colonize the substrate, and iii) morphology, time to fruiting and yield under various cultivation conditions.

MATERIALS AND METHODS

Fungal material. Twenty five strains from the "Collection du Germoplasm des Agarics à Bordeaux" (CGAB), including 14 cultivars, the Brazilian strain ATCC 76739, 9 wild *A. subrufescens* and a hybrid between ATCC 76739 and a French *A. subrufescens* were evaluated in this study (Table 1). The strains were preserved in tubes on compost extract medium submerged with mineral oil. Before being used for the experiments, the strains were sub-cultured on malt agar (MEA) medium (pH 5.75) for 20 days at 25°C.

Table 1: Commercial and wild strains with reference to origin and code in collection.

Cultivars		Wild strains	
Origin	Code	Origin	Code
Brazil	CA 455	Brazil	ATCC 76739
Brazil	CA 560 = ABL-99/28	Mexique	CA 603
Brazil	CA 561 = ABL-99/30	Taiwan	CA 276
Brazil	CA 562 = ABL-03/44	USA	CA 462
Brazil	CA 563 = ABL-04/49	Belgium	CA567
Brazil	CA 564 = ABL-05/51	France	CA 487
Brazil	CA 565 = ABL-03/48	France	CA 516
Brazil	CA 566 = ABL-06/53	France	CA 643
Brazil	CA 570 = ABL-01/29	Spain	CA 438-A
Brazil	CA 571 = ABL-98/11	Italy	CA 536
Brazil	CA 572 = ABL-07/58	Hybrid	ATCC 76739-3 x CA487-100
Brazil	CA 574 = ABL-07/59		
Mycelia Co	CA 646 = 7700		
Mycelia Co	CA 647 = 7703		

Radial mycelial growth. Inoculum plugs (7 mm diameter) were removed from edge of 20-day-old cultures and placed at the centre of Petri dishes filled with MEA medium. The strains were grown in the dark for 14 days at 25°C, 28°C, 30°C, 32°C and 35°C. Three replications per strain were made for each treatment. Radial mycelial growth was estimated by two perpendicular measurements of the colony diameter. The linear growth period common to all strains (d5 to d10) was identified from the kinetics of radial growth and used to calculate the mycelial growth rate (mm day^{-1}). At the end of the experiment (d14), the strains of the 35°C treatment were changed to 25°C and measurements were performed as previously described.

Compost. The substrate used to assess mycelial colonization and mushroom yield was commercial compost prepared for *A. bisporus* cultivation, and provided by Renault SA, Pons, France.

Ability of mycelium to colonize commercial compost. Small crates were filled with 150 g of compost and the whole surface of the substrate was covered with mycelium of the studied strains on agar medium (content of three Petri dishes per crate). After 21 days of incubation at 25°C and 85% humidity, the substrate was freeze-dried and colonization by the mycelium was estimated by measuring H_2O_2 as described by Savoie et al. [21]. Compost samples from 12 cultivars, ATCC 76739 and four wild strains of *A. subrufescens* were analysed, with 2 replicates per strain.

Small scale cultivation. Crates filled with 500 g of compost were inoculated as described for compost colonization. After incubation for 20 days at 25°C and 85% humidity, a casing layer (1/3 limestone, 1/3 peat, 1/3 thin sand) was added, and the crates were left under the same environmental condition for a 7-day post-incubation period. To initiate fruiting, the room temperature was maintained at 22-25°C with 95-97% humidity and low CO_2 concentration. Time to fruiting was calculated as the time period between casing and the first pick of mushrooms. The number and fresh weight of the fruiting bodies were recorded for up to 65 days after casing. Nineteen strains (12 cultivars, ATCC 76739, 5 wild strains of *A. subrufescens* and the hybrid) were cultivated in duplicate in a completely randomised design experiment. Yield data are mean values of the total weight of biomass produced per kilogram of substrate.

Medium scale cultivation. The substrate (8 kg) was inoculated with 2% spawn and incubated at 25 °C, 85 % humidity, for 15 days. Standard conditions for casing, post-incubation, fruiting conditions, and collection of data were as described above. Fourteen strains (8 cultivars, ATCC 76739 and 5 wild strains of *A. subrufescens*) randomly chosen among those screened in the small scale experiment were cultivated in a completely randomised design experiment with four replicates per strain.

Effect of climatic and biological factors. Strains were cultivated on 8 kg substrate according to standard conditions, except where otherwise stated. The experiments were performed according to a completely randomised design with four replicates per strain.
Light and cold shock: At the end of post-incubation, three strains (ATCC 76739, CA 487 and the hybrid) were submitted to four different treatments, namely A: 12 h light / 24h, cold shock (4 h at 18 °C twice a week); B: 12 h light / 24h, no cold shock; C: no light, cold shock (4 h at 18 °C twice a week); D: no light, no cold shock.
Spawn rate: Spawning at 1% and 2% were compared for their effect on the time to fruiting and biomass production for three Brazilian cultivars (CA561, CA565 and CA 570), ATCC 76739 and *A. subrufescens* CA 487.

Data representation and statistical analyses. The box-plot representation [22] was used to show data distribution for radial mycelial growth. The Cramer–Von Mises's and Kolmogorov-Smirnov's non parametric tests were performed to compare data distributions.

Data recorded for time to fruiting, biomass production and mean weight were analysed using ANOVA followed by Duncan's test to identify statistical differences. The Pearson coefficient was calculated to find correlations between treatments.

RESULTS AND DISCUSSION

Mycelial growth rate. When all Brazilian cultivars were considered as a whole, no significant differences were observed between the distributions of radial growth rates at 25, 28 and 30 °C. A significant move toward slower growth rates was observed at 32°C, and to a greater extent, at 35 °C. Similar results were obtained with the group of wild strains. Although distributions at 28 and 30 °C did not differ significantly, growth rate data for the wild isolates tended to be highest at 30 °C (Fig. 1). At each temperature, except for 30°C and 35°C the distributions for the group of cultivars and the group of wild strains differed significantly. The fastest growth rates were observed among the cultivars and the slowest among the wild strains. The mycelial growth rates of the Brazilian strain ATCC 76739 were in the range of those observed for the cultivars, whatever the incubation temperature, whilst those of the hybrid differed from the cultivar distributions.

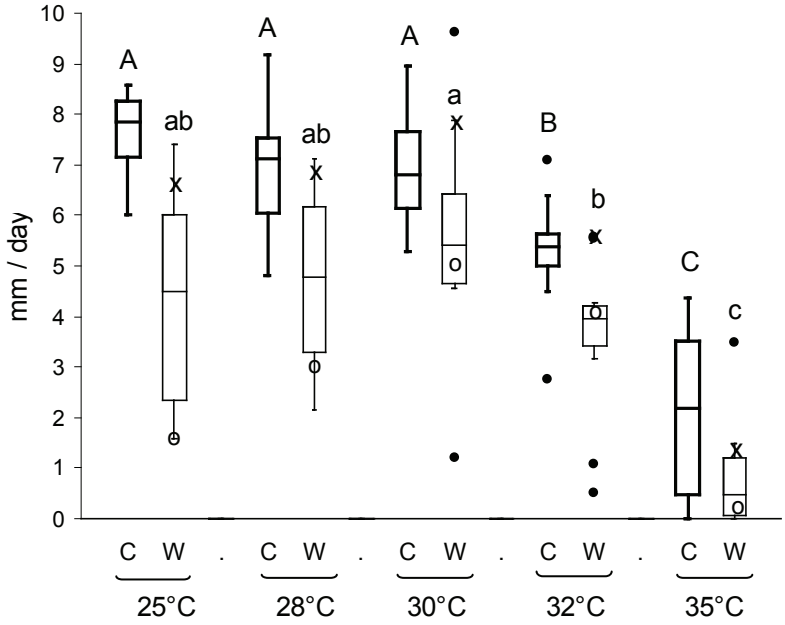


Figure 1: Distribution of the mycelium growth rate at five incubation temperatures.
C: Brazilian cultivars (C), W: wild strains. ●: data outside the distribution, x : ATCC 76739, o : hybrid.
Within a same group of strains, growth rate distributions with a same letter did not differ significantly at $p = 0.05$.

In the treatment at 35°C, the cultivar CA 574 failed to grow, 4 cultivars had non-significant growth ranging from 0.23 to 0.90 mm d^{-1}, and the others showed poor to medium growth rates ranging from 1.47 to 4.37 mm d^{-1}. The wild strains were more severely affected by incubation at 35 °C. Three strains failed to grow, four strains had non-significant growth ranging from 0.10 to 0.53 mm d^{-1}, and three others showed poor growth rates of 1.17 - 1.50 mm d^{-1}. The only one with a medium growth of 3.47 mm d^{-1} was outside the distribution (Table 2 and Fig. 1).

Neves et al. [16] reported that all but one of six strains provided by spawn makers, or isolated from fruiting bodies collected in Brazilian mushroom farms, showed optimal growth temperatures of 28 or 30 °C. More recently, Colauto et al. [8] observed that five strains can develop mycelium at temperatures between 22 and 34 °C. The temperatures tested in our experiments did not enable us to identify optimal growth temperatures for the cultivars but we found that 25–30 °C was suitable for their mycelial development; this is in accordance with the observations described above. The growth rate of the Brazilian ATCC 76739 did not differ from the cultivars whilst the wild *A. subrufescens* strains tended to grow better at 30 °C.

Table 2: Mycelial growth rate of the commercial and wild strains at 35°C

Radial growth rate (mm day^{-1}) *					
Cultivars			Wild strains		
CA570	4.37	A	CA516	3.47	A
CA563	3.90	AB	CA438	1.50	B
CA647	3.63	ABC	ATCC 76739	1.23	BC
CA562	3.57	ABC	Hybride	1.17	BC
CA646	3.37	ABC	CA487	0.53	BCD
CA565	3.00	BC	CA462	0.47	BCD
CA572	2.70	CD	CA643	0.33	CD
CA560	1.67	DE	CA276	0.10	CD
CA561	1.47	EF	CA567	0.00	D
CA566	0.90	EFG	CA536	0.00	D
CA571	0.33	FG	CA603	0.00	D
CA564	0.30	G			
CA455	0.23	G			
CA574	0.00	G			

* Within a column, data followed by a same letter did not differ at $p = 0.05$.

When placed at 25°C after 14d incubation at 35°C, all the cultivars began or continued to grow. With the exception of CA 563, growth rates at 35 °C and growth rates after change to 25°C were significantly correlated (r = 0.770, $p = 0.002$) (Fig. 2A). However, the growth rates of cultivars at 25°C after a first incubation period at 35 °C did not correlate with the growth rates at 25°C with no pre-incubation (r = -0.491, $p = 0.075$; line not shown on graph) and they were always lower than those observed for direct incubation at 25°C (Fig. 2B). Similarly, ATCC 76739 developed a slower growth rate when changed from 35°C to 25 °C compared to direct incubation at 25 °C.

When placed at 25 °C after incubation at 35 °C, two of the three *A. subrufescens* wild strains that failed to grow at 35 °C developed no mycelium; the other strains began or continued to grow, but growth rates were not correlated to those observed at 35 °C (r = 0.170, p = 0.663; line not shown on graph) (Fig. 2A). However, there was a significant correlation (r = 0.860, p = 0.001) between growth rates of *A. subrufescens* wild strains at 25 °C, and growth rate at 25 °C after a first incubation at 35 °C, although mycelial development was slower in the latter treatment (Fig. 2B).

Incubation at 35°C proved that this temperature was not lethal for the cultivars, but was lethal for two wild strains. Similarly, this temperature was lethal for *in vitro* mycelial development of several strains of *A. bitorquis* [23] but only rendered inactive four strains of this species [24]. Experiments with more strains and temperature (35 °C and above) are necessary before conclusions on the lethal temperature for both Brazilian cultivars and wild *A. subrufescens* can be drawn. However, both groups of strains were less susceptible to high

temperature than *A. bisporus*. Indeed, all the six *A. bisporus* strains tested by Lemke [24] failed to develop mycelium at this temperature.

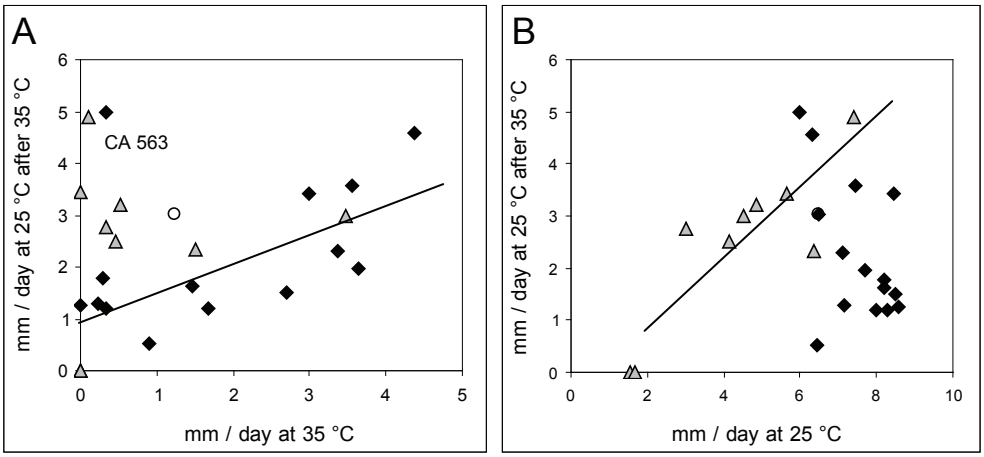


Figure 2: Radial growth rate observed at 25°C after previous 14-day incubation at 35°C compared to (A) radial growth at 35 °C and (B) radial growth at 25°C with no pre-incubation.
♦ Cultivars, ○ ATCC 76739, △ *A. subrufescens*

Compost colonization. Five cultivars showed high ability to colonize and transform the substrate with H_2O_2 concentrations ranging from 217.5 to 560 nmol g^{-1}, whilst low concentrations (39.5 - 78 nmol g^{-1}) were measured in substrates colonized by the other cultivars. A similar range of low H_2O_2 concentrations was obtained with the *A. subrufescens* strains (47.5 – 91.5 nmol g^{-1}) and the hybrid (74 nmol g^{-1}). ATCC 76739 produced a medium concentration (129 nmol g^{-1}). The H_2O_2 levels observed are in the same range to those measured for another Agaricus species, *A. bisporus*, producing 300 - 600 nmol g^{-1} substrate 15 days after spawning [21]. In contrast to *A. bisporus* [21], neither the group of cultivars nor the group of wild strains showed a correlation between the H_2O_2 concentration and the mushroom yield (Fig. 3), time to fruiting or sporophore mean weight (not shown).

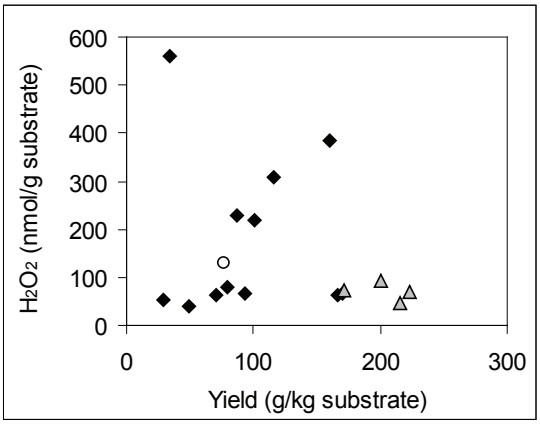


Figure 3: Concentrations in hydrogen peroxide and biomass produced by the Brazilian cultivars and the wild strains.
♦ Cultivars, ○ ATCC 76739, △ *A. subrufescens*

Mushroom yield. The cultivars and the wild strains showed a wide range of mushroom yield in commercial compost commonly used to grow *A. bisporus*. In the small scale experiment, the cultivars yielded 29 - 171 g kg^{-1} substrate showing a great variation in adaptation to commercial

compost produced for *A. bisporus* cultivation (Fig. 4A). The Brazilian ATCC 76739 was comparable to cultivars showing average biomass production. *Agaricus subrufescens* from Spain and France showed little differences in mushroom yield and were highly productive (197.5 – 215.7 g kg^{-1} substrate). The yield of the hybrid was between those observed for its two parents (Fig. 4A).

The fourteen strains screened in medium scale experiments under the standard conditions confirmed the high variability in biomass production found in the small scale experiment. Mushroom yields at both experiment scales were significantly correlated, either for the cultivars (r=0.796, $p = 0.018$) or the wild strains (r = 0.870, $p = 0.024$) (Fig. 4B).

Four medium scale experiments confirmed that the hybrid yield (116.7 ± 32.4 g kg^{-1} substrate) fell between the yields of its two parents, ATCC 76739 (41.9 ± 12.2 g kg^{-1}) and CA 487 (207.9 ± 47.9 g kg^{-1}).

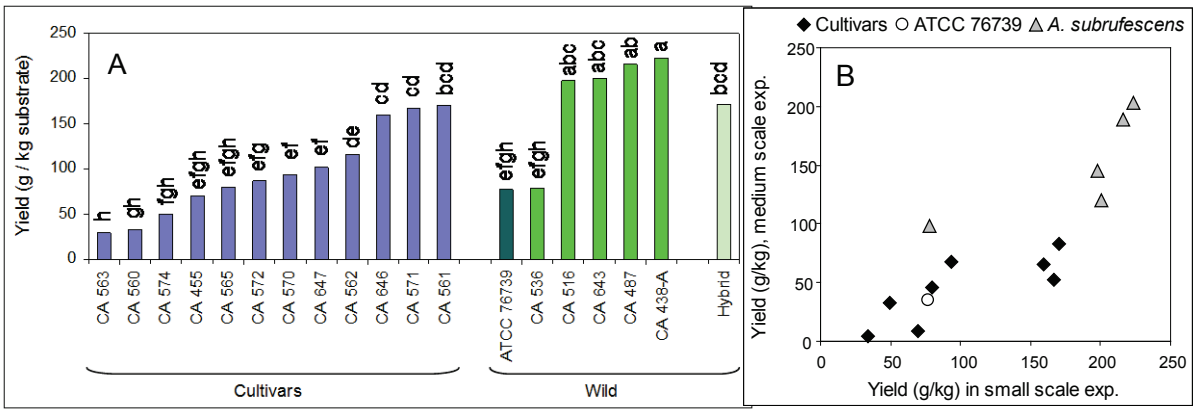


Figure 4: Mushroom yield after a 65-day fruiting period.
A: small scale experiment; B: comparison of the small and medium scale experiment.
Data for medium scale are means of 2 to 4 experiments of 4 replicates

Compared to other cultivated *Agaricus*, the productivity of the Brazilian cultivars reported in the literature is low and depends to a high degree on the strain and the cultivation conditions. Eira [1] reported yield from 3 to 25 kg of fresh mushroom /100 kg of substrate based on local material. More recently, the average production in Brazil was estimated at 8 - 16% after 120 days of cultivation [25]. In this study, three cultivars showed productions between 6.5 and 8.2% after the 65-day fruiting period and would therefore be considered valuable material for cultivation using commercial compost produced for *A. bisporus* cultivation. The French and Spanish *A. subrufescens* strains and the hybrid adapted well to this substrate.

Time to fruiting. In the small scale experiment, the time to first fruiting varied from 26 to 40 days after casing for the group of cultivars. ATCC 76739 began to fruit on day 34, and consequently did not differ from the group of cultivars for this trait. Four of the wild strains were early fruiting (19 - 25 d) (Fig. 5A). The medium scale experiment confirmed these observations (Fig. 5B). The literature indicates that cultivar first flush occurs approximately 15-20 days after casing. Under our cultivation conditions, cultivars began to fruit later. However, time to primordial onset is dependent on the casing materials [26]. We used a single casing mixture, derived from that prepared for *A. bisporus* cultivation. The casing composition seemed suitable for *A. subrufescens* cultivation, but the time to first fruiting of cultivars might be improved by the use of different casing mixtures.

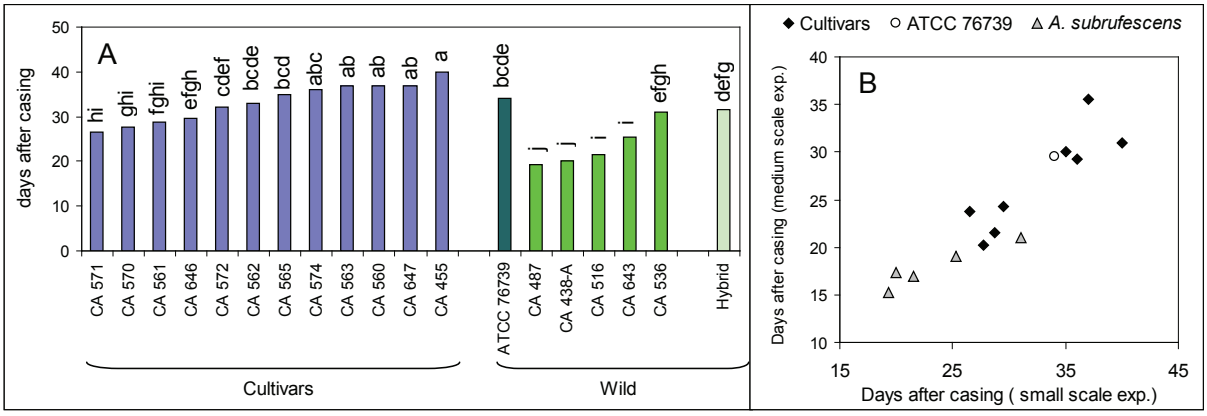
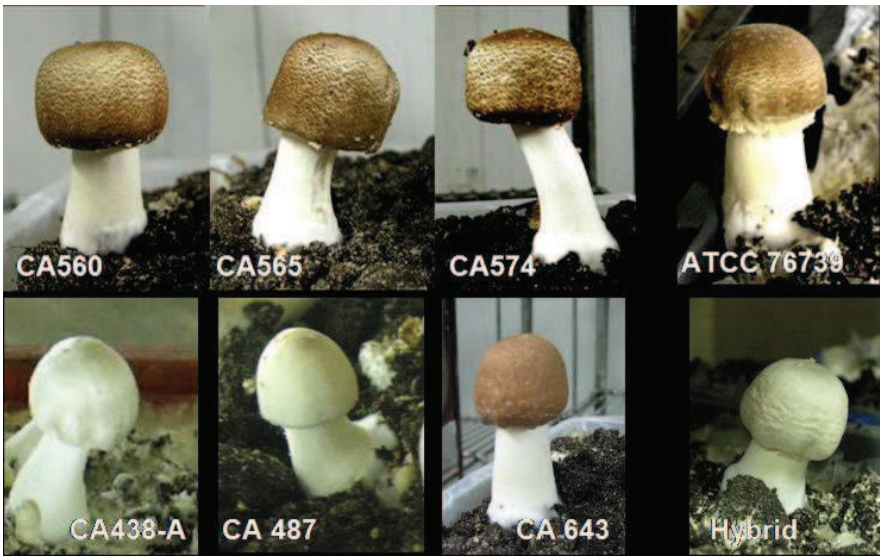


Figure 5: Time to fruiting (in days after casing) of the cultivars and the wild strains: (A) small scale experiments; (B) comparison of small scale and medium scale experiments.

Sporophore morphology. Under the standard cultivation conditions described above, Brazilian cultivars showed a cylindric, brownish-gold cap, as described in the literature for the cultivated strains [3, 20]. The Brazilian wild strain ATCC 76739 showed the cultivar morphology, whilst the wild *A. subrufescens* strains exhibited different morphology. The French strain CA487 and the Spanish strain CA438-A exhibited a cream cap whilst the French strain CA643 showed a brown cap, but without the gold appearance characteristic of the Brazilian cultivars (Fig. 7). All strains bore a white stipe, and an elastic flocculent veil (Fig. 8).



Fgure 7: Examples of aspects of young sporophores of cultivars (CA 560, CA565, CA574), ATCC 76739, wild *A. subrufescens* (CA 438-A, CA 487, CA 643), and the hybrid ATCC 76739-3 x CA 487-100)

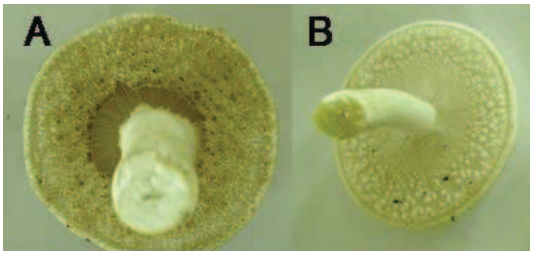


Figure 8: Veil of cultivar (A) and wild strain (B).

Sporophore mean weight was used as a rough estimation of sporophore size. In the small scale experiment, the cultivars showed a wide range of sporophore mean weight (14.0 – 36.6 g) whilst the wild strains produced small sporophores (11.0 – 19.1 g) (Fig. 6A). This observation was confirmed with the medium scale experiment. When taken as a whole, the group of cultivars produced larger sporophores compared to the group of wild strains. The Brazilian ATCC 76739 did not differ from the cultivars (Fig. 6B). The morphological traits of mushrooms grown on the same substrate clearly separated the Brazilian cultivars and the ATCC 76739 from the wild *A. subrufescens*.

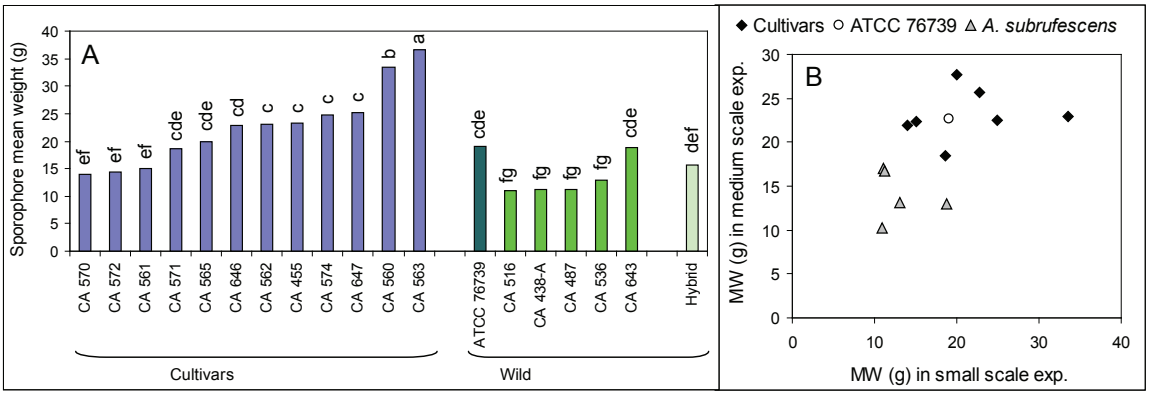


Figure 6: Mean weight of sporophores: (A) produced in small scale experiment and (B).comparison of small scale and medium scale experiments

Effect of light and cold shock. Neither the light period nor the cold shock had a significant effect ($p = 0.05$) on the biomass production and time to fruiting of the three strains tested (ATCC 76739, CA 487 and the hybrid). As previously observed, ATCC 76739 was later fruiting and produced less biomass but larger sporophores compared to CA 487. The time to fruiting, yield and sporophore size of the hybrid were between those of its two parents.
The literature reports the production of Brazilian cultivars in the dark [13], with light/dark photoperiod [27], under day/night periods in glasshouse [9] and outdoor [10], but no study focused on the effect of light on the mushroom yield. From our observations, it is clear that the Brazilian ATCC 76739, the *A. subrufescens* CA 487 and the hybrid developed perfectly in the dark. The three strains studied by Zied et al (This issue) on 3 different composts with variations in temperature during the cropping period showed the same levels of yields and time to fruiting.

Effect of spawn quantity. The time to first fruiting was significantly affected by the reduction of spawn from 2% to 1%, but the effect was highly dependent on the strain. On substrate spawned at 1%, average time between casing and first fruiting increased significantly by 5 and 8 days for CA 561 and CA 570, respectively. No significant difference was observed for the other strains (Tables 3 - 4). Despite the effect on the time to first fruiting, no significant variation in biomass production was detected in relation to spawn rate (Table 3).

Table 3: Effect of spawn rate on fruiting earliness and mushroom yield.

Source	DDL	Fruiting earliness			Yield		
		Mean squares	F	Pr > F	Mean squares	F	Pr > F
Strain	4	264.013	61.237	< 0,0001	4501115.055	32.793	< 0,0001
Spawn rate	1	53.635	12.440	0.002	425483.469	3.100	0.092
Replicate	3	0.522	0.121	0.947	192902.776	1.405	0.268
Strain*spawn rate	4	19.864	4.607	0.007	93777.654	0.683	0.611

Table 4: Comparison of the time to fruiting at the two spawning rates.

Strains		Days after casing [1]	
		1% spawn	2% spawn
Cultivars	CA561	28 A	20 B
	CA 565	26.2 A	26.7 A
	CA 570	24.2 A	18.5 B
Wild	ATCC 76739	29.5 A	29.7 A
A. subrufescens	CA 487	14.2 A	13.7 A

[1] Data were means of four replicates.

Spawning at 1-2% is commonly used for commercial production of Brazilian cultivars [10, 13, 28, 29]. Spawning at 2% rate increased the yield of strain 7700 (Mycelia Co) by 26% on average, compared to yields obtained with 1% spawn, in a cultivation substrate composed of wheat straw and chicken manure [30]. Such yield improvement were not observed with the three cultivars and the two wild strains cultivated on French commercial compost based on horse manure reported here.

CONCLUSION

We evaluated 14 Brazilian cultivars, ATCC 76739 – the presumed source material of many strains cultivated in Brazil, and 9 wild strains of *A. subrufescens* from different geographic origins for phenotypic variability. All the studied traits (mycelium growth rate, compost colonization, time to first fruiting, yield, sporophore macro-morphology) clearly separated the cultivars and the wild *A. subrufescens*, whilst ATCC 76739 did not differ from the cultivars.

Significant phenotypic diversity was found among each group, cultivars and wild *A. subrufescens*. Several strains appear to have valuable characteristics for cultivation on commercial compost. Future work with selected strains will focus on investigating the following biological and climatic conditions: spawning at 1 % for economic consideration as this rate did not reduce yield, no light to reduce energy cost and no cold shock to limit manipulations. Different casing mixtures may improve yield of the selected strains. This species is of interest for cultivation during the warmest months in Europe to reduce energy costs,

The medicinal properties of *A. subrufescens* strains is also an important consideration. Detection of biomolecules in *A. subrufescens* (Moukha et al., this issue) and the results presented here suggest that interesting strains among wild *A. subrufescens* may be identified that have both medicinal properties and high yield on commercial compost for *A. bisporus.*

ACKNOWLEDGEMENTS

The authors are grateful to Diego Zied for the Brazilian cultivars, and Ludoviq Devaux and Christiane Coldefy for help in mushroom cultivation. This work is part of a research project funded by a bilateral cooperation between Mexico (project 115790 CONACYT) and France (ANR-09-BLAN-0391-01) in the "AgaSub" project: "Biology of the gourmet and medicinal mushroom *Agaricus subrufescens*, development of its cultivation and of new products of therapeutic interest or for diseases prevention"

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Article 3

World J Microbiol Biotechnol
DOI 10.1007/s11274-013-1287-3

ORIGINAL PAPER

Potential of European wild strains of *Agaricus subrufescens* for productivity and quality on wheat straw based compost

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Received: 7 September 2012 / Accepted: 9 February 2013

Abstract The Brazilian almond mushroom is currently cultivated for its medicinal properties but cultivars are suspected all to have a common origin. The objective of this work was to assess the potential of wild isolates of *Agaricus subrufescens* Peck (*Agaricus blazei, Agaricus brasiliensis*) as a source of new traits to improve the mushroom yield and quality for developing new cultures under European growing conditions. The wild European strains analysed showed a good ability to be commercially cultivated on wheat straw and horse manure based compost: shorter time to fruiting, higher yield, similar antioxidant activities when compared to cultivars. They have a valuable potential of genetic and phenotypic diversity and proved to be interfertile with the original culture of the Brazilian almond mushroom. Intercontinental hybrids could be obtained and combine properties from both Brazilian and European germplasm for increasing the choice of strains cultivated by the mushroom growers.

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Keywords Antioxidant activity · Biomass production · Mycelial growth · Optimal temperature · SSR polymorphism

Introduction

The almond mushroom cultivated in Brazil is today widely used for its medicinal and/or therapeutic properties. It was formerly known as *Agaricus blazei* Murill, but two new species names were proposed in the 2000s, *Agaricus subrufescens* Peck (Kerrigan 2005) and *Agaricus brasiliensis* Wasser (2011). Currently, many publications refer to the Brazilian cultivar as *A. blazei* or *A. brasiliensis*, and it is believed to originate from Brazil (Mizuno 1995). Recently, Wisitrassameewong et al. (2012) stated that the correct name is *A. subrufescens* Peck but excluded neither the existence of infraspecific taxa, nor the fact that *A. subrufescens* might be a complex of species. The almond mushroom might be a seasonal option for the *Agaricus* growers in western countries. They can save energy by producing it efficiently during summer, due to its higher optimal temperature requirements when compared to the button mushroom, *Agaricus bisporus*. However, the culture of the almond mushroom is very scarce.

Agaricus bisporus and *A. subrufescens* are both cultivated on compost. In Brazil, the raw materials commonly used for the traditional process of composting are: sugar cane bagasse, various grasses (e.g. *Braquiaria* sp., *Cynodon dalactylon*, *Panicum maximum*), cereal straw (*Triticum aestivum*, *Avena sativa*, *Oryza sativa*) and manure supplemented with nitrogen sources such as soybean, wheat, corn and cotton meal, urea, ammonium sulphate, and sources of phosphorus and calcium (superphosphate, calcium carbonate and gypsum) (Zied et al. 2011).

Published online: 16 February 2013

Table 1 Commercial and wild strains with reference to origin and code in collections

	Origin	Code
European wild isolates	Spain	CA438-A
	Saint-Léon, 33, France	CA487
	Le Pian Médoc, 33, France	CA643
Original cultivated strain	Brazil	CA454 (=WC837)[b]
Brazilian cultivars	Piedade, SP, Brazil	CA561 (=ABL-99/30)[a]
	Boituva, SP, Brazil	CA565 (=ABL-03/48)
	Rio de Janeiro, RJ, Brazil	CA570 (=ABL-01/29)
Hybrid	Obtained at INRA	CA454-3 × CA487-100
Agaricus bisporus	Cultivar 30A, France Mycelium	Bs0527

[a] Code ABL = code in the collection of the Mushroom Research Centre of the College of Agronomic Sciences, Sao Paulo State University (FCA/UNESP)

[b] According to PSUMCC WC837 is similar to ATCC 76739, which, according to ATCC, was originally provided by T. Furumoto

In Europe, the main raw materials are horse or poultry manures and wheat straw to which are added various nitrogen sources and calcium carbonate. The differences in compost qualities might affect the possibilities to have high yielding cultures of the almond mushroom under European cultivation conditions.

The medicinal properties of the Brazilian mushroom are known for more than three decades. Kawagishi et al. (1989) were the first to detect polysaccharides with apparent antitumor activity in the mushroom fruiting body. Then, many works have focused on these polysaccharides, especially β-glucans credited with most of the antitumor and antimutagenic effects partly due to antioxidant properties (see Camelini et al. 2005; Firenzuoli et al. 2008; Mizuno 2002; Oliveira Lima et al. 2011 for non-exhaustive literature). In addition, methanolic extracts of this mushroom are regarded as an alternative source of natural antioxidants (Da Silva and Jorge 2011). Attention was paid both to liquid and solid state fermentation procedures to improve mycelium and fruiting body production of Brazilian cultivars (see Largeteau et al. 2011a for a review) and to extraction technology of its polysaccharides. All these works concern the cultivated strains of the Brazilian almond mushroom suspected by Neves et al. (2005) to have a common origin based on genetic studies. Few commercial cultivars are currently available and there are risks inherent to a nearly monolineage crop. Fortunately, several new wild isolates of different origins have recently been collected (Kerrigan 2005; Zhao et al. 2011) and could be used for introducing genetic and phenotypic diversity in cultures of the almond mushroom. Here, we investigated the potential of three European isolates of *A. subrufescens* to be cultivated on compost made from horse manure and wheat straw as it is prepared for the cultivation of the button mushroom, *A. bisporus*, with the objective of good yield and quality under European cultivation conditions.

Materials and methods

Agaricus strains

Eight strains were analysed: three cultivars of almond mushroom (CA561, CA565 and CA570) kept in the collection of Germplasms of Agarics in Bordeaux (CGAB), INRA-Bordeaux, since 2007; CA454, a subculture of the collection strain *A. blazei* WC837, kept in the CGAB collection since 2006; three wild European strains of *A. subrufescens* (CA438-A, CA487 and CA643); and a hybrid provided by E. Huang and P. Callac who crossed two homokaryotic single-spore isolates, CA454-3 from the Brazilian strain CA454 and CA487-100 from the French strain CA487 (Table 1). Haploid status of the homokaryotic isolates and heterokaryotic status of the hybrid were confirmed using CAPS co-dominant markers, multi-locus genotype tests and methods previously described in Kerrigan et al. (1994) and Kerrigan and Wach (2008). Previous experiments have shown that the intercontinental hybrid is fertile. Bs0527, the commercial *A. bisporus* 30A (France Mycelium) kept in the CGAB collection since 1997 was used as external species in studies on mycelial growth, mushroom production, chemical composition and antioxidant activities.

SSR genotyping

Total DNA was extracted from freeze dried mycelium with a classical CTAB-chloroform-isoamyl alcohol protocol. In routine use, DNA concentration was adjusted to 25 ng μl^{-1}. Microsatellite markers (SSR markers) had previously been developed in our laboratory. The 14 SSR loci used for genotyping were chosen on the basis of their unambiguous allele scoring, their level of polymorphism and their multiplex compatibility. Primer sequences, amplification conditions, and capillary electrophoresis on ABI 3130

sequencer (Applied Biosystems) are described in Foulongne-Oriol et al. (2012). Electropherogramme profiles were read manually with GENEMAPPER™ software version 4.0 to assign peaks to the corresponding alleles. Data were analysed with Power Marker software and the unweighted pair-group method analysis (UPGMA) was used to obtain trees with Treeview software.

Mycelial growth

Inoculum plugs (7 mm Ø) were removed from the edge of 20-day-old cultures obtained on malt extract agar (MEA) medium at 25 °C, and placed at the centre of Petri dishes filled with MEA medium. The strains were grown in the dark for 14 days at 22, 25, 28, 30, 32, 35, 38 and 40 °C. Radial mycelial growth was estimated by two perpendicular measurements of the colony diameter. The linear growth period common to all strains (d5–d10) was identified from the kinetics of radial growth and used to calculate the mycelial growth rate (mm day^{-1}). Data are means of two independent experiments, each having three replicates per strain and treatment. The optimal temperature for mycelial growth was calculated by non-linear regression. At the end of the experiment (d14), the strains of the 38 and 40 °C treatments were changed to 25 °C and mycelial growth rate measurements were performed as previously described.

Cultivation substrate

The substrate used for mushroom cultivation was compost prepared for commercial production of the button mushroom *A. bisporus*, and provided by Renaud SA, Pons, France. The main ingredients for composting were wheat straw and horse manure. Composting was performed indoor. The mean composition of the compost was as following: Minerals 303.3 g kg^{-1} with K = 32.1, Mg = 6.4, Ca = 55.8, Na = 2.8, S = 35.8, organic C = 348 and N (Kjeldahl) = 23.1 g kg^{-1} (C/N = 15.1). The water soluble organic matter (OM) and the OM insoluble in acid detergent represented 32.5 and 48.8 % of total OM, respectively. Hemicelluloses, celluloses and lignin+humic compounds accounted for 18.7, 2.9 and 45.9 % total OM, respectively.

Mycelium ability to colonize commercial compost

Small crates were filled with 150 g of compost and the whole surface of the substrate was covered with mycelium of the studied strains developed on MEA medium. After 21 days of incubation at 25 °C and 85 % humidity, the substrate was freeze-dried and the level of colonization by the mycelium was estimated by measuring H_2O_2 concentration in extracts, as described by Savoie et al. (2007). Data are means of three sample replicates per crate, with two crates per strain.

Fructification

The eight strains were cultivated in two independent experiments, each with a different batch of compost. Based on previous results (Llarena-Hernandez et al. 2011), trays filled with 8 kg of compost were inoculated with 1 % spawn and incubated at 25 °C, 85 % humidity, for 15 days. After casing layer was added, the trays were left under the same environment conditions for a 7-day post-incubation period. To initiate fruiting, the room temperature was set at 23–25 °C with 95–97 % humidity and low CO_2 concentration. Time to fruiting was the number of days between casing and the first picking. The fresh weight of the fruiting bodies was recorded until 65 days after casing. The experiments were performed according to a completely randomised design experiment with four replicates per strain and batch of compost. The commercial *A. bisporus* strain used as external species was cultivated in a different room with temperature set at 17 °C for the fruiting phase.

Colour and morphology

Pictures of the mushrooms were taken in situ under the same conditions to assess cap colour by reference to the Munsell Book of Colour (Munsell 1976) as in Delú et al. 2006. The Munsell scale defined colour by hue (R = red, Y = yellow, G = green, B = blue, P = purple), value (from 0 for black to 10 for white) and chroma. Data were expressed as [hue value/chroma]. To assess morphology, freshly harvested sporophores (veil closed) were cut by the middle and the inner face was photocopied. The cap height (H), widest cap diameter (D1) and diameter at the cap margin (D2), found informative in preliminary analyses, were measured on the photocopies. Parameters were defined to characterize the sporophore morphology (veil closed) which varied between type A (ovoid cap) and type B (cylindrical cap), (Fig. 1) depending on the strain. Two ratios were calculated: H/D1 and D2/D1. Stipe was not measured because ratios including stipe diameters were not informative in previous analyses.

^{13}C CP/MAS NMR

Mycelium and sporophore tissue were analysed by Cross-Polarization Magic Angle Spinning ^{13}C Nuclear Magnetic Resonance (^{13}C CP/MAS NMR) procedure. Solid state ^{13}C NMR data were acquired on a Bruker Avance-400 MHz spectrometer operating at ^{13}C and ^{1}H resonance frequencies of 101.6 and 400.3 MHz, respectively, using a commercial broker double-bearing probe as described in

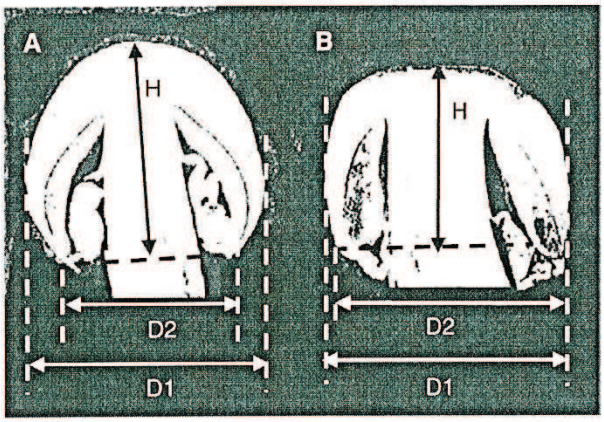

Fig. 1 Parameters used to analyse sporophore morphology. Examples of morphology: ovoid cap (**a**) and cylindrical cap (**b**). H = cap height, D1 = widest cap diameter, D2 = diameter at the cap margin

Peter-Valence et al. (2011). Lyophilised tissue samples were prepared from at least four sporophores and mycelium samples from 20 different cultures. Relative intensity of the O-alkyl-C group (polysaccharide moiety) which extend from 45 to 110 ppm was calculated.

Antioxidant activities

Immediately after harvest the fruiting bodies were put in a freezer at −80 °C and kept at this temperature until being freeze dried and ground to powder (particle diameter <0.1 mm). Methanolic extracts were prepared and the scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals ($DPPH^{\bullet}$) and 2,2- azino-bis-(3-ethyl-benzothiazoline-6-sulfinic acid) radical cations ($ABTS^{\bullet+}$), and the reducing power were measured as described in Savoie et al. (2008). The percent ABTS or DPPH radical scavenging effect was calculated according to the following equation:

$$\text{Scavenging ability}\,(\%) = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100$$

where $A_{control}$ and A_{sample} are the absorbance of control without sample and sample, respectively. The effective concentration at which DPPH and ABTS radicals were scavenged by 50 % (EC_{50} value expressed as mg mushroom powder ml^{-1}) was obtained by interpolation from linear regression analysis; the absorbance was 0.25 for reducing power. Ascorbic acid was used as a standard antioxidant for comparison with DPPH and reducing power whilst butylated hydroxyanisole (BHA) was used for ABTS. $DPPH^{\bullet}$, $ABTS^{\bullet+}$, BHA, potassium ferricyanide and trichloracetic acid were purchased from Sigma (Sigma-Aldrich, Saint-Quentin Fallavier, France). Ferric chloride was obtained from Merck (Darmstadt, Germany).

Data representation and statistical analyses

Analyses of variance (ANOVA) were performed and followed, when necessary, by the Duncan's test to identify statistical differences. The box plot representation was used to show data distribution of the morphology parameters. The Cramer–Von Mises's and Kolmogorov–Smirnov's non parametric tests were performed to compare data distributions. Data recorded for phenotypic variability were submitted to principal component analysis (PCA).

Results

Genetic polymorphism

The genetic distance tree showed that the Brazilian cultivars (CA561, CA565 and CA570) and CA454 belong to the same cluster whilst the European *A. subrufescens* (CA487, CA438-A and CA643) belong to another cluster (Fig. 2). The distances of alleles shared (DAS) between the seven strains ranged from 0 (between the three cultivars showing no polymorphism between themselves) to 0.71 (between CA454 and CA643), with an average value of 0.45. The cultivars showed DAS of 0.43 with CA643, 0.57 with CA487 and 0.61 with CA438-A. A significant polymorphism was detected among the European *A. subrufescens* with DAS = 0.50 between CA438-A and CA643, and 0.54 between CA438-A and CA487. Strains CA 438-A and CA643 were closer related, with DAS = 0.21.

In vitro mycelial growth rate and ability to colonize compost used for the cultivation of *A. bisporus*

The seven *A. subrufescens* strains and the hybrid exhibited substantial growth rates when incubated at 22–32 °C,

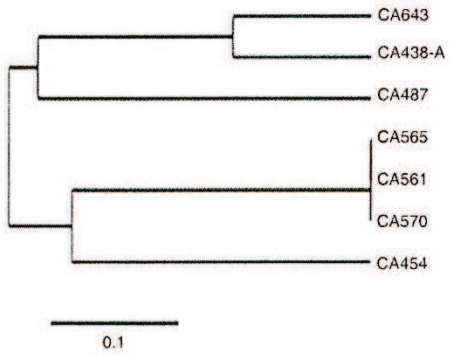

Fig. 2 Dendrogram of genetic distances between the seven strains, based on the UPGMA analysis resulting from SSR fingerprints

Table 2 Effect of incubation at 38 °C on mycelial development, and estimation of growth rate at the optimal temperature

Strain	Optimal temperature (°C)	Optimal growth rate (mm d^{-1})	Mycelium development at 25 °C after 14 days at 38 °C	
			Latent period (days)[a]	Mycelial growth recovery (%)[b]
CA438-A	26.8	8.6	6	14.9
CA487	28.1	5.0	2	102.5
CA643	30.2	4.6	4	114.2
CA454	29.4	8.0	–	0
CA561	25.8	8.6	4	32.4
CA565	26.9	9.3	4	49.8
CA570	29.6	8.5	4	65.2
CA454-3 × CA487-100	26.6	5.3	4	48.0

[a] Number of days between change to 25 °C and the start of mycelial growth

[b] Growth rate at 25 °C after 14 days incubation at 38 °C as percentage of growth rate at 25 °C without incubation at 38 °C

whilst mycelium development was dramatically reduced at 35 °C for all strains except for CA570. The optimal temperatures determined by non-linear regression differed between strains and varied from 26 to 30 °C, with similar range of variation for cultivars (CA561, CA565 and CA570) and wild strains. Estimated values for the optimal growth rate were close together (8.0–9.3 mm day^{-1}) for Brazilian cultivars, CA454 and the Spanish wild isolate CA438-A. The two other wild strains (CA487 and CA643) and the hybrid showed far lower optimal growth rates (Table 2).

No strain was able to grow at 38 and 40 °C, and the latter temperature was lethal for all the strains. When changed to 25 °C after incubation at 38 °C, all strains but CA454 developed mycelium after a 2–6 day latent period. Growth rate of CA487 and CA643 did not differ significantly ($p = 0.05$) from that observed with direct incubation at 25 °C whilst it was reduced for the other strains (Table 2).

Optimal temperature and growth rate of 24.5 °C and 4.5 mm day^{-1}, respectively were found for *A. bisporus* Bs0527, and incubation at 32 °C dramatically reduced mycelium development of the strain, whilst incubation at 35 °C was lethal.

Measurements of H_2O_2 in the substrate showed that all strains colonized significantly the compost during incubation for 14 days. Two strains differed significantly from the others, the ancient strain in collection (CA454) with the higher ability to colonize the compost leading to a concentration of 129 nmol H_2O_2 g^{-1} compost and CA487 which was the less performing (47.5 nmol H_2O_2 g^{-1}). Significant differences were observed among the three cultivars (CA561, CA565 and CA570) and among the three wild isolates (CA438-A, CA487 and CA643), but two cultivars (CA565, CA570), the Spanish wild isolate CA438-A and the hybrid showed similar abilities. The hybrid ability was between those of its parents (Table 3).

Differences observed for mycelial growth rate and temperature sensitivity or compost colonisation ability cannot be related to the genetic distance estimated with the SSR markers (Fig. 2) or the geographic origin of the strains.

Mushroom production

The eight strains showed important variability in the time to fruiting (14–33 days). The group of cultivars (CA561, CA565 and CA570) and CA454 were significantly less early fruiting than the group of wild strains (CA438-A, CA487 and CA643), and the hybrid placed itself between its parents (Table 3).

At the end of the experiment, despite their lower growth rate and their lower ability to colonize the substrate, two wild strains (CA438-A and CA487) were significantly more productive than the others and reached yields similar to that obtained with *A. bisporus* Bs0527 (240 g kg^{-1}) cultivated on the same compost, but at 17 °C. Mushroom biomass produced by the French isolate CA643 and the hybrid was of the same range than that recorded for the best cultivars. The three cultivars did not differ between themselves for mushroom yield. CA454 showed the lowest yield but did not differ significantly from the least productive of the cultivars, CA565 (Table 3). Considering that the harvest of the least early-fruiting strain (CA454) lasted 31 days, yields at 30 days after the first picking (d1–d30) was used as parameter for strain comparison. The classification of the strains for mushroom biomass production during d1–d30 was close to that observed at the end of the

Table 3 Comparison of the eight strains for H_2O_2 concentration in compost, time to fruiting and mushroom yield

Strain	H_2O_2 concentration (nmol g^{-1} substrate)	Time to fruiting (days)	Total yield[a] (g kg^{-1} substrate)	Yield d1–d30[b] (g kg^{-1} substrate)
CA438A	69.3 D	16.0 EF	227.3 A	157.0 B
CA487	47.6 E	14.4 F	241.4 A	231.8 A
CA643	91.5 C	20.0 DE	121.7 B	110.0 C
CA454	129.2 A	28.3 B	41.0 D	36.1 E
CA561	103.6 B	24.9 BC	96.3 BC	87.7 CD
CA565	78.0 D	33.3 A	60.1 CD	54.9 DE
CA570	66.3 D	25.1 BC	84.7 BC	75.4 CDE
CA454-3 × CA487-100	73.9 D	21.8 CD	128.8 B	109.1 C

[a] Yield at the end of the experiment, 65 days after casing; [b] d1 = first day of harvest, d30 = 30 days after the beginning of harvest
Within a column, values followed by the same letter are not different at $p = 0.05$ by the Duncan's test

experiment, but CA643 grouped with the cultivars and CA487 was clearly more productive than CA438-A (Table 3).

Mushroom quality

Colour and shape

The seven strains and the hybrid bore a white stipe and an elastic flocculent veil. The cap colour clearly separated the cultivars (CA561, CA565 and CA570) and CA454 (colour code 7.5YR 3/6 to 7.5YR 4/6) showing the typical brownish gold colour described in the literature (Firenzuoli et al. 2008), from the wild isolates of *A. subrufescens*. CA438-A and CA487 (2.5Y 9/2 to 2.5Y 8/2) exhibited a cream to light beige cap, whilst CA643 showed a brown cap (approx. 2.5YR 3/6). The hybrid was close to its wild parent for the colour (5Y 8/4 to Y 7/4). Colour photos of the strains can be seen in Llarena-Hernandez et al. (2011).

The parameters used to describe sporophore shape (Fig. 1) revealed also differences between the cultivars and European wild strains CA438-A, CA487 and CA643. The latter showed significantly more elongated caps (distribution of H/D1 ratios moving toward high values) compared to the cultivars and CA454 (Fig. 3a). The cultivars CA565 and CA570 exhibited almost exclusively cylindrical caps (ratio D2/D1 = 1). The cultivar CA561 and the wild isolate CA643, each exhibited 50 % mushrooms with cylindrical cap and 50 % with nearly cylindrical cap, and did not differ significantly from CA565 and CA570 for this ratio. CA454 showed only nearly cylindrical caps and differed significantly from CA565 and CA570. The wild strains CA438-A and CA487 differed significantly from the other strains, with all caps more or less tightening at the bottom (ratio D2/D1 < 1, ovoid type, Fig. 3b).

O-alkyl-C group and antioxidant properties

Relative intensities of the O-alkyl-C group measured in the mycelium did not separate the group of the studied cultivars (CA561, CA565 and CA570) from CA454 and the wild strains CA438-A, CA487 and CA643 (Table 4). In sporophores, the value of CA487 was the highest and close to that of *A. bisporus*.

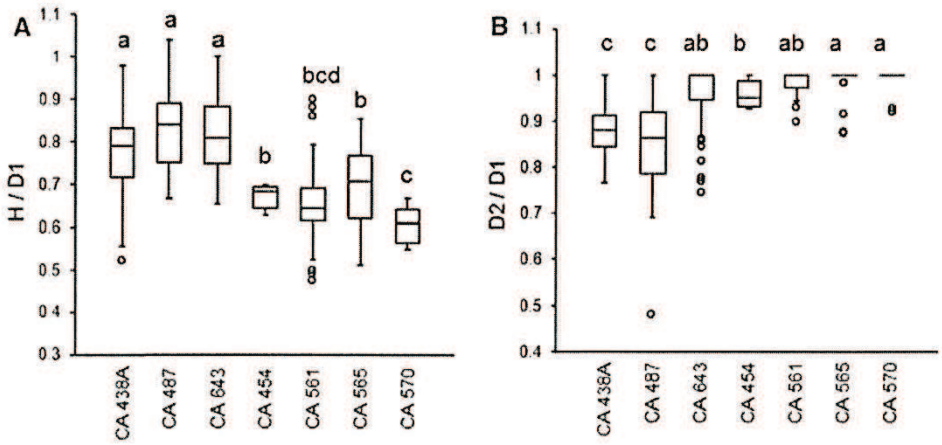

Fig. 3 Representation of the mushroom cap morphology. **a** Distribution of the ratios cap height (H)/widest cap diameter (D1). **b** Distribution of the ratios D1 (widest cap diameter)/ D2 (diameter at the cap margin)

Table 4 Comparison of the eight strains and *A. bisporus* for the relative intensity of the O-alkyl-C group in vegetative mycelium and sporophore tissue

Strain	O-alkyl-C group	
	Mycelium	Sporophore
CA438-A	67.55	56.99
CA487	79.99	62.61
CA643	72.59	nd[a]
CA454	77.67	52.91
CA561	71.08	54.18
CA565	81.14	56.30
CA570	72.52	51.39
CA454-3 × CA487-100	77.48	54.68
Bs0527	59.95	60.22

[a] Not determined

Methanolic extracts showed similar antioxidant properties on DPPH and ABTS radicals. Significant variability in radical-scavenging activity was detected (Table 5). The three cultivars exhibited significantly higher activities compared to CA438-A and CA487. Strains CA643 and CA454 did not differ from CA570 which showed the lowest activity among the cultivars. The hybrid exhibited medium scavenging activity, ranging between those of its two parents. Radical-scavenging activity of *A. bisporus* Bs0527 was intermediate between those measured in cultivars and wild strains. By comparison, EC_{50} values for ascorbic acid were 0.016, 0.010, and 0.008 mg ml^{-1} for reducing power, DPPH, and ABTS scavenging activities, respectively. The reducing power did not separate the cultivars and the wild strains.

Confirmation of the interest of the European wild isolates

The overall interest of the European wild isolates (CA438-A, CA487 and CA643) as source of phenotypic diversity for the cultivation of the almond mushroom on horse manure and wheat straw was stated with a PCA (Fig. 4). Variables showing the major contribution to the first component (F1 axis), contributing for 54 % of the total variability, were cap colour (14.01 %), mushroom yield (13.97 %), time to fruiting (12.23 %), cap morphology H/D1 (10.32 %) and scavenging ability on ABTS radicals (9.79 %). The three studied cultivars (CA561, CA565 and CA570) and CA454 grouped together with very little variability on this principal axis. The three wild strains showed variability and separated clearly from the cultivars. This distribution is in agreement with the genetic polymorphism identified with SSR markers. On this first component, the hybrid was located between its two parents, but it was closer to the French wild parent CA487 than to the original cultivated strain, its second parent. The major contributors to axis 2 (17 % of the total variability) were those linked to the thermotolerance of vegetative mycelium: optimal temperature for mycelial development and growth recovery at 25 °C after 14 days at 38 °C. This axis did not separate cultivars from wild *A. subrufescens* strains confirming the direct observations of these traits. On this second component, the hybrid is very close to its parent CA454 and different to its parent CA487.

Table 5 Antioxidant properties of methanolic extracts prepared from sporophores of the eight strains and *A. bisporus* Bs0527

Strain	% antioxidant activity[a]		EC_{50} values (mg extract ml^{-1})[b] for scavenging ability on		Reducing power[c]
	DPPH	ABTS	DPPH	ABTS	
CA438-A	37.80 D	43.94 D	2.41 A	1.71 A	3.02 A
CA487	36.48 D	48.88 CD	2.67 A	1.57 AB	2.70 AB
CA643	68.22 B	80.58 B	1.22 D	0.99 D	1.63 CD
CA454	39.39 B	75.77 B	1.09 DE	0.97 D	1.53 D
CA561	90.07 A	98.47 A	0.81 E	0.69 E	1.09 D
CA565	60.64 BC	60.66 C	1.61 C	1.30 C	2.35 ABC
CA570	75.59 AB	82.74 B	1.07 DE	0.93 D	1.57 CD
CA454-3 × CA487-100	43.75 CD	55.23 CD	1.73 BC	1.42 BC	2.69 AB
Bs0527	nd[d]	nd	2.02 B	1.26 C	2.30 BC

Data are means of three biological replicates. Means with different letters within a column are significantly different at $p = 0.05$

[a] Percentage of the radical scavenging of ascorbic acid at 1.5 mg ml^{-1}

[b] EC50 values: the effective concentration at which 1,1-diphenyl-2-picrylhydrazyl radicals ($DPPH^{\bullet}$) or 2,2-azino-bis (3-ethylbenzothianizoline-6-sulfonic acid cation radicals ($ABTS^{\bullet+}$) were scavenged by 50 %. EC_{50} values were obtained by interpolation from linear regression analysis

[c] Concentration of extract (mg ml^{-1}) for which the absorbance was 0.25

[d] Not determined

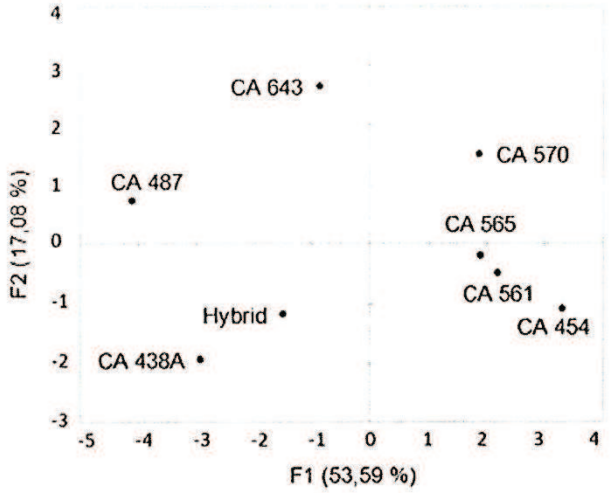

Fig. 4 Principal component analysis of the phenotypic variation among the eight strains, namely the Brazilian cultivars (CA561, CA565, CA570), CA454, the wild *A. subrufescens* (CA438-A, CA487, CA643) and the hybrid

Discussion

In mushrooms, as in plants, the progressive loss of genetic diversity in cultivated lines or the absence of initial diversity due to a specific history of the cultivated species raises the issue of the sanitary and economic risks related to a monolineage crop. A survey of the literature revealed a great homogeneity for commercial strains analysed either in Brazil or in Japan (Largeteau et al. 2011a). The absence of genetic polymorphism between the three Brazilian cultivars analysed herein along with the close genetic relationship among other strains provided by Brazilian spawn makers or mushroom growers (Neves et al. 2005; Tomizawa et al. 2007) are consistent with a common origin for the present cultivars, as already proposed by Neves et al. (2005). Fukyda et al. (2003) observed no somatic incompatibility reaction with seven strains cultivated in Japan, suggesting that they derived from cultures with little genetic variations. The genetic basis of the Japanese cultivated strains may also be narrow. The authors also showed that genetic variation may be small between these Japanese strains and a Brazilian cultivar.

Consequently, wild types are important sources of breeding material to restore or create genetic variability as well as to improve the characteristics of commercially cultivated varieties. Compared to strains cultivated in Brazil and Japan, the few wild isolates studied here showed significant SSR polymorphism and proved to be a potential source of genetic diversity that might introduce interesting new traits. Traits of interests for the development of the culture of this mushroom in western countries on wheat straw based substrate are those relating to both its ability to grow on compost made for the button mushroom and the quality of the fruiting bodies. The present work completes the study on phenotypic variability by Llarena Hernandez et al. (2011).

Optimal temperature for mycelial growth rate is a trait of significance for this mushroom having probably a tropical origin (Zhao et al. 2011). Llarena Hernandez et al. (2011) have shown important variability in mycelial growth rate between 25 and 35 °C. In the present study, the range of temperatures tested was extended to lower (22 °C) and higher temperatures (38 and 40 °C) allowing the estimation of the optimal temperature for mycelial growth. The optimal temperatures identified for the eight strains was in the same range than temperatures reported in the literature for Brazilian cultivars: 28–31 °C (Colauto et al. 2008) and up to 30 °C (Neves et al. 2005), or 22–26 °C for Japanese cultivars (Eguchi et al. 1994). It is noteworthy that with no difference between the group of the studied cultivars originating from Brazil and the group of European wild strains, the optimal temperature for mycelial growth rate is a strain dependent trait not correlated to overall adaptation to a dominant climate. Growth rates measured at the optimal temperature were in accordance with our previous observations of the faster growth rates among the 12 cultivars and the slowest among the 7 wild strains of *A. subrufescens* at each temperature tested (Llarena Hernandez et al. 2011). The mycelial growth rate is the first trait to be selected, explaining the higher values in cultivars than in wild isolates.

In addition to the optimal growth rate, the highest temperature without any effect on the longevity of the mycelium is of interest. Eguchi et al. (1994) observed that maximal temperature for in vitro growth of a Japanese cultivar was 35 °C, but from our knowledge, the present work is the first report on the lethal temperature for the cultivated almond mushroom, although more strains must be screened before to conclude. Optimal and lethal temperatures are higher than those of the button mushroom. Adaptation of *A. subrufescens* to higher temperature is an advantage for the cultivation in summer seasons by comparison with *A. bisporus*.

The ability to colonize the cultivation substrate is considered a criterion for strain selection of cultivated mushrooms. In the present study, measurement of hydrogen peroxide as a value correlated to the presence of mycelium in compost (Savoie et al. 2007) was used to assess the colonisation of compost by mycelium from an agar culture. Mycelial growth rate and temperature sensitivity could not be related to the genetic distance or the geographic origin of the isolates. Similar observations had been reported for *A. bisporus* (Largeteau et al. 2011b). Strains exhibited individual behaviour irrespective of their origin.

The delay for fruiting is a trait of economic significance for mushroom growers. The Japanese strain KS-72 cultivated on substrates based on cattle bedding compost

showed time to fruiting ranging from 54 to 77 days after spawning (Pokhrel and Ohga 2007). The Brazilian cultivar ABL-99/30 had a time to fruiting of 24.7–27 days after casing (Zied et al. 2012) on sugar cane based composts. Compared to these observations and the cultivars used as control in the present study, the European wild strains, with 14.4–20 days after casing (37–42 days after spawning) are very attractive for this parameter. A high production in a short time period is also regarded as profitable.

Under the present cultivation conditions, yields obtained with Brazilian cultivars were comparable to those reported for commercial strains in Brazil (e.g. 80–110 g kg^{-1} after 65 days for the strain AB97/12 (Braga et al. 2006), and 88 g kg^{-1} after 70 days for the commercial strain ABL04/49 (Zied et al. 2010), but lower than in Japan where Pokhrel and Hoga (2007) and Horm and Ohga (2008) reported 155 g kg^{-1} after 60 days in their experiments on less than 1 kg of substrate with KS-72. However it is frequent to record higher yields when cultivations are performed in small cultivation units. For instance, with the cultivars grown on compost of same composition and origin but in boxes containing 500 g of substrate, we measured yields from 67 to 185 g kg^{-1} compost. However, the cultivars were far less interesting than the wild strains for adaptation to cultivation on wheat straw based compost, as the latter combined both a shorter time to fruiting and a higher yield.

The absence of correlation between mycelial growth rate, compost colonisation and mushroom yield was congruent with the observations of Horm and Ohga (2008). Although, an intensive mycelial development corresponds to a high consumption of nutrients present in the compost, which could lead to too few nutrients to ensure high yield. That might explain the opposite behaviour of CA454 and CA487.

Although mushroom biomass production is the prime trait to the phenotypic variability of a crop, quality of the harvested mushrooms is also important. The shape and colour are the first criteria for quality of fresh mushroom for market. Quality might be affected by both the genetic background and the cultivation conditions. Stamets (2000) wondered about the taxonomic significance of cap pigmentation, as cap colour varies in relation to the compost composition. Under our controlled fructification conditions (climatic chamber, no light, horse manure and straw-based mushroom compost), cap colours recorded in the same experiments were in agreement with the genetic data. The same results concerning mushroom shape and colour were obtained during previous experiments with different batches of compost showing the prevalence of the genetic background on the definition of morphotypes.

The almond mushroom is a medicinal mushroom and its composition in active ingredients is a major quality trait. Polysaccharides such as (1-6)-β-glucan, (1-3)-β-glucan, (1-4)-β-glucan, or (1-2)-β-mannopyranosyl residues from the Brazilian almond mushroom have shown medicinal effects (Camelini et al. 2005; Mizuno et al. 1998). ^{13}C CP/MAS NMR had been used to estimate the polysaccharide contents in mushrooms. The signal of functional group O-alkyl-C is assigned to polysaccharides. We previously observed with an overall sample of strains that the average percentage of O-alkyl-C group was significantly higher in vegetative mycelium than in fruiting bodies (Peter-Valence et al. 2011). This is confirmed here with the data obtained from each strain. Relative intensities of the O-alkyl-C group suggested close compositions in putative active ingredients in Brazilian cultivars and wild strains of *A. subrufescens*. The fact that the most productive strain (CA487) was not negatively affected in its functional group O-alkyl-C content which was the highest is encouraging. Further work on a higher number of wild strains of *A. subrufescens* should assess the variability in polysaccharide contents and compare their medicinal properties. Recently, Moukha et al. (2011) observed that crude extracts of CA454 and CA487 mushrooms induced preventive effect at 72 and 64 %, respectively, against Wistar rat intestinal carcinogenesis, confirming a suitable medicinal potential of the European wild strains.

The Brazilian almond mushroom is claimed a natural source of antioxidant compounds other than polysaccharides, but to our knowledge, no reports are currently available on the variability of this trait in studies including wild strains of *A. subrufescens*. Preparing methanolic extracts from vacuum freeze dried samples is a reliable way to compare the antioxidant activity of the strains. Da Silva and Jorge (2011) compared different extraction solvents and concluded that methanolic extracts of *A. blazei* present the highest antioxidant activity. Similarly, Mourão et al. (2011) found methanol the best solvent for extraction of antioxidant compounds from five *A. brasiliensis* strains including ABL99/30 (=CA561). The literature provides several works on the antioxidant properties of cultivars but data varied greatly. Similar EC_{50} values for DPPH scavenging activity (0.30 and 0.26 mg extract ml^{-1}) were reported for strains cultivated in Brazil and Taiwan, respectively (Carvajal et al. 2012; Huang and Mau 2006), whilst a far higher EC_{50} value of 3.00 mg extract ml^{-1} was reported for a Brazilian cultivar (Soares et al. 2009). Mourão et al. (2011) analysed other Brazilian cultivars and found DPPH scavenging activity of 81 % for ABL99/30 (=CA561) and 92–98 % using 60 µM quercetin solution as reference of 100 %. Scavenging activity of the eight evaluated strains ranged among values reported in the literature. It is noteworthy that the wild strain CA643 exhibited a scavenging activity as high as the cultivars. Besides, the two other wild strains showed a higher scavenging activity than the cultivar

analysed by Soares et al. (2009). Values obtained for the reducing power ranged between those measured by Huang and Mau (2006) and Soares et al. (2009) for cultivars produced in Taiwan and Brazil (EC_{50} = 0.89 and 8.05 mg extract ml^{-1}). Although the cultivation conditions differed and could affect mushroom properties, our results suggest that the wild European strains of *A. subrufescens* possess, in general, antioxidant activities as effective as present cultivars.

It has been shown that *A. bisporus* has antioxidant properties at least equal to those of mushroom species the most commonly studied and valued as commercial pharmaceutical products (Öztürk et al. 2011; Savoie et al. 2008). Thus the *A. subrufescens* strains studied here had valuable activities and pharmaceutical potential.

Face to the lack of genetic diversity in cultivars and with the increasing interest on this mushroom, a new hybrid was recently patented in USA (Kerrigan and Wach 2008). It has been obtained from single spore isolates of an American wild isolate and a strain cultivated in Japan, developed from Brazilian germ plasm. One of the objectives of the present study was to reveal the possibility to change some phenotypic traits in a reference strain from Brazil by outcrossing with a new European isolate. The hybrid selected for this study exhibited middle values between its two parents for most of the traits. It can be considered as an improved strain comparing to its Brazilian parent for all the traits related to the mushroom yield, whilst its antioxidant activity in methanol extracts was as the same level than in cultivars. Kerrigan and Wach (2008) also observed significant yield improvements in their hybrid compared to its cultivar parent. Interestingly, the cream colour of the French wild parent was transmitted to the hybrid whilst in the button mushroom *A. bisporus* the brown colour is dominant (Callac et al. 1998). The hybrid biomass production was 47 % that of its parent CA487. In another experiment, this percentage was similar (41 %), and another hybrid between CA454 and CA487 produced 90 % of the yield of CA487 (unpublished results).

In conclusion, the wild European strains analysed showed a good ability (time to fruiting, yield, antioxidant properties) to be commercially cultivated on wheat straw and horse manure based compost. They have a valuable potential of genetic and phenotypic diversity. *A. subrufescens* is mostly used for its medicinal properties and sold as dried powder after mushrooms are washed and brushed to eliminate cap pigment (Mendonça et al. 2005). Consequently the cap colour which differs from that of the known cultivars is not a problem. Intercontinental hybrids could be obtained and combine properties from both Brazilian and European germplasm for increasing the choice of strains cultivated by the mushroom growers.

Acknowledgments This work was supported by a research project funded by a bilateral cooperation between Mexico (project 115790 CONACYT) and France (ANR-09-BLAN-0391-01). RC Llarena Hernández would like to thank CONACYT, Mexico, for scholarship. The authors gratefully thank D.C. Zied for providing the Brazilian cultivars and D. Royse for providing strain WC837.

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Article 4

Research Article

SCI

Received: 15 January 2013 Revised: 8 April 2013 Accepted article published: 30 April 2013 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.6200

Optimization of the cultivation conditions for mushroom production with European wild strains of *Agaricus subrufescens* and Brazilian cultivars

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Abstract

BACKGROUND: The almond mushroom *Agaricus subrufescens* (formerly *Agaricus blazei* or *Agaricus brasiliensis*) is cultivated at commercial level in Brazil and some Asian countries on local substrates and casing mixtures. Despite its tropical origin, *A. subrufescens* might be a seasonal option for mushroom growers in western countries, where some wild strains have been isolated. For this purpose, cultivation conditions were developed starting from the substrate and casing mixture commonly used for commercial production of the button mushroom *Agaricus bisporus* in France.

RESULTS: The commercial compost, based on wheat straw and horse manure, used for *A. bisporus* and the casing mixture (peat and limestone) supplemented with fine sand proved efficient to grow *A. subrufescens*. Increasing the depth of the casing layer improved significantly the yield and time to fruiting. Daily variations in temperature did not markedly modify the yield. Significantly higher mushroom biomass was obtained with three wild European strains compared with three Brazilian cultivars. The very productive wild strain CA438-A gave mushrooms of size and dry matter content comparable to those of a cultivar.

CONCLUSION: Commercial production of *A. subrufescens* can be developed in western countries on the wheat straw-based substrate commonly used for *A. bisporus* in these regions, by a simple modification of the casing mixture and maintaining the incubation temperature throughout the crop, which is expected to save energy during summer. Good yields were obtained cultivating European strains under optimised parameters.

Keywords: casing; dry matter; mushroom size; substrate; temperature

INTRODUCTION

Important cultivated edible and medicinal mushrooms belong to the *Agaricus* genus. Among them is the almond mushroom formerly known as *Agaricus blazei* Murrill. In the 2000s, two new species names, *Agaricus subrufescens* Peck[1] and *Agaricus brasiliensis* Wasser *et al.*,[2] were proposed for this fungus, which is believed to originate from Brazil.[3] Currently, many publications refer to the Brazilian cultivars as *A. blazei* or *A. brasiliensis*. Recently, *A. subrufescens* Peck was declared the correct name,[4] but the authors excluded neither the existence of infraspecific taxa nor the fact that *A. subrufescens* might be a complex of species. The mushroom has been produced on a commercial scale in Brazil since the early 1990s[5] and exported to several countries. Nowadays it is also cultivated at the industrial level in Japan, China, Taiwan and Korea.[6] These cultures rely on local agroindustrial waste-based substrates. The majority of articles on *A. subrufescens* cultivation available in the literature refer to experiments in Brazil. The raw materials commonly used to prepare the substrate are sugar cane bagasse, various grasses (e.g. *Brachiaria* spp., *Cynodon dactylon*, *Panicum maximum*), cereal straw (*Triticum aestivum*, *Avena sativa*, *Oryza sativa*) and manure supplemented with nitrogen sources (soybean, wheat, corn and cotton meal, urea, ammonium sulfate) and sources of phosphorus and calcium.[7] Experiments performed in China showed the possible use of cottonseed hulls, rice hulls, asparagus straw and soybean cake.[8,9] Efficient *A. subrufescens* production was also obtained with substrate compositions closer to that used to grow the button mushroom *Agaricus bisporus* in temperate countries, such as cattle bedding compost/sawdust/cereal bran[10] and chicken manure/wheat straw.[6] Local soils, with or without the addition of vegetal charcoal, have been tested as an alternative to peat in the casing layer used for *A. subrufescens* cultivation.[11–14]

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The casing soil composition is important, regardless of the substrate formulation.[13] The physical characteristics of the soil contribute greatly to the mushroom yield.[12] These cultivation conditions are well adapted to tropical countries, although yields are significantly lower than those obtained with *A. bisporus*, but *A. subrufescens* might be a seasonal option for mushroom growers in western countries. They can save energy by producing the almond mushroom efficiently during summer, owing to its higher optimal temperature requirements when compared with *A. bisporus*. Our aim was to define cultivation conditions suitable for *A. subrufescens* starting from the substrate and casing mixture used for *A. bisporus* commercial production and making changes easy to perform by producers of button mushroom in western countries.

MATERIAL AND METHODS

Agaricus subrufescens

Three strains, CA561, CA565 and CA570, were cultivars from Brazil kept in the Collection of Germplasms of *Agaricus* in Bordeaux (CGAB), INRA (Bordeaux, France) since 2007; CA454 was a subculture of the strain *A. blazei* ATCC 76739 kept in the CGAB since 2006; CA438-A, CA487 and CA643 were wild European strains; the hybrid was obtained between CA454 and a French wild isolate (Table 1). Previous experiments have shown that the hybrid is fertile. Spawn was prepared as follows. Mycelium grown on malt agar medium was transferred to sterilized rye grain purchased from a spawn maker (Euromycel, Ile-Bouchard, France) and incubated at 23 ± 2 °C until completely colonized. The spawn was stored at 11 °C and placed at room temperature the day before spawning. Spawn storage never exceeded 2 weeks.

Cultivation substrate

The substrate was compost prepared for commercial production of *A. bisporus* and provided by Renault SA (Pons, France). The main ingredients for composting were wheat straw and horse manure. Composting was performed indoors. The characteristics and mean composition of the compost were as follows: 66.8% humidity; pH 7; minerals 303.3 g kg^{-1}, with K = 32.1, Mg = 6.4, Ca = 55.8, Na = 2.8, S = 35.8, organic C = 348 and N (Kjeldahl) = 23.1 g kg^{-1} (C/N = 15.1). The water-soluble organic matter (OM) and the OM insoluble in acid detergent represented 32.5 and 48.8% of total OM respectively. Hemicelluloses, celluloses and lignin + humic compounds accounted for 18.7, 2.9 and 45.9% of total OM respectively. The analyses were performed by LCA (La Rochelle, France) using normalized methods.

Standard conditions for fructification

Based on previous results,[15] trays filled with 8 kg of compost were inoculated with 1% spawn and incubated for 15 days in a climatic chamber at 23 ± 0.5 °C with 98 ± 2% relative humidity. Then 2.5 cm of casing layer C1 (casing used for commercial production of *A. bisporus* + fine sand, corresponding to 45% peat/20% limestone/35% fine sand (v/v/v)) was added and the trays were left under the same environmental conditions for a 7 day post-incubation period. After that the climatic chamber was set at 23 ± 0.5 °C with 97 ± 2% humidity and 1400 ± 100 ppm CO_2 concentration. Time to fruiting was the number of days between casing and the first picking. The numbers and fresh weight of fruiting bodies were recorded until 65 days after casing. The experiments were performed according to a completely randomised design with four replicates per strain and cultivation condition.

Variations in casing parameters

Casing quantity

Two depths of casing layer, 2.5 cm (quantity used for *A. bisporus*) and 5 cm, were compared in two independent experiments, each performed with a different batch of compost.

Casing composition

Fruiting was compared using the following casing mixtures: C1 as described for standard cultivation conditions; C2 = 15% peat/23% limestone/37% fine sand/25% vegetal charcoal (v/v/v/v); C3 = 15% peat/23% limestone/42% fine sand/20% spent compost (v/v/v/v); C4 = 80% peat/20% spent compost (v/v) + $CaCO_3$ (100 g L^{-1} casing mixture); C5 = 25% peat/25% limestone/50% fine sand (v/v/v). Spent compost was obtained from previous experiments performed with compost provided by Renault SA, as for the present experiments: after the last harvest of *A. subrufescens* mushrooms (65 days after casing), the casing layer was removed and the spent compost was collected, submitted to thermal treatment (70 °C, 12h) and matured for 2 months[16] before being used. No casing inoculum was used, whatever the casing mixture. Owing to the capacity of the climatic chamber, four to six strains were used to compare the casing mixtures (see 'Results'). All strains were compared under the best casing conditions in two independent experiments.

Variations in temperature

The eight strains were inoculated and incubated following the standard conditions. After incubation, the trays were removed from the climatic chamber for addition of the best casing mixture. The trays were returned to the climatic chamber with the same setting for 2 days. Then the temperature was increased to 30 °C and the following temperature cycle, set automatically (Fancom BV system, Panningen, The Netherlands), was begun: decrease in temperature over 10 h 40 min at a rate of 1.4 °C h^{-1} and holding at this temperature for 1 h 20 min; increase in temperature over 10 h 40 min at a rate of 1.4 °C h^{-1} and holding at this temperature for 1 h 20 min. Standard conditions were applied for relative humidity and CO_2 concentration. The air temperature in the climatic chamber was recorded. The temperature was also measured inside the compost of four trays, each inoculated with a different strain, namely CA565, CA454, CA487 and CA643. Measures were recorded automatically every 30 min with F-central for Windows Version B4.4 (Fancom BV).

Parameters of yield components of *A. subrufescens*

Strains were compared for time to fruiting (number of days between casing and the first picking day) and total fresh weight of fruiting bodies at the end of the experiment. Because the strains showed great variation in time to fruiting, they were also compared for biomass production during three periods extending from d1 to d10, from d1 to d20 and from d1 to d30, where d1 is the day of the first picking for the strain and d10, d20 and d30 are the 10th, 20th and 30th days following the first picking respectively. Because flushes were not always well differentiated, biomasses obtained during d1–d10, d11–d20 and d21–d30 were compared to give an idea of the kinetics of production of each strain. Mean fresh weight and dry matter content were estimated for sporophores obtained with variations in temperature. The size of fruiting bodies was measured as g per mushroom.[10]

Statistical analysis and graphical representation

Analyses of variance (ANOVA) were performed followed by Duncan's test to identify statistical differences. Box plot

Table 1. Cultivars and wild strains with reference to origin and code in collection

Type	Origin	Code CGAB[a]	Code FCA/UNESP[b]
European wild isolates	Spain	CA438-A	
	Saint-Léon, Gironde, France	CA487	
	Le Pian Médoc, Gironde, France	CA643	
Original cultivated strain	Collection, subculture of ATCC 76739	CA454	
Brazilian cultivars	Piedade, SP, Brazil	CA561	ABL-99/30
	Boituva, SP, Brazil	CA565	ABL-03/48
	Rio de Janeiro, RJ, Brazil	CA570	ABL-01/29
Hybrid	Obtained at INRA	CA454-3 × CA487-100	

[a] Collection of Germplasms of *Agaricus* in Bordeaux, INRA.
[b] Collection of the Mushroom Research Centre of the College of Agronomic Sciences, Sao Paulo State University, Brazil.

Table 2. Comparison of casing depth effect on time to fruiting and mushroom biomass

		Biomass mean value (g kg^{-1} substrate)[a]			
Casing depth (cm)	Mean time to fruiting (days)[a]	End of exp.	d1–d10	d1–d20	d1–d30
2.5	32.725A	57.710B	22.417B	39.642B	51.189B
5	28.100B	93.248A	39.053A	65.216A	83.738A

[a] Within a column, values followed by the same letter are not different at $P = 0.05$ by Duncan's test.

representation was used to show the data distribution of mushroom fresh weight and dry matter content. Cramer–Von Mises and Kolmogorov–Smirnov nonparametric tests were performed to compare data distributions.

RESULTS

Effect of casing quantity

At the end of the post-incubation period, *A. subrufescens* mycelium had fully colonized the casing, irrespective of the quantity used. The ANOVA showed that variation in casing depth significantly modified time to fruiting ($P = 0.021$) and mushroom yield ($P < 0.001$). The absence of interaction between strain and casing ($P = 0.575$, 0.107, 0.104 and 0.266 for biomass recorded at the end of the experiment and during periods d1–d10, d1–d20 and d1–d30 respectively) indicated the same classification of the casing treatments for all strains. Higher mushroom biomass was obtained with the 5 cm casing layer (Table 2). This casing depth was used in the following experiments.

Effect of casing composition

The ANOVA revealed a significant effect ($P < 0.0001$) of casing composition on time to fruiting and mushroom biomass, but the significant interaction ($P < 0.0001$) between strain and casing composition meant that the best composition varied with the strain. No variation in time to fruiting was observed with CA 487, while the other three strains showed the shortest time to fruiting with C1 and C2. CA 565 failed to fruit with C3 and C4. Casing mixtures C1 and C2 gave the best biomass at the end of the experiment. When considering biomass obtained 30 days after the first picking, C1 remained the most efficient (Table 3). The ANOVA showed that the percentage of sand in the casing mixture (C5 *vs* C1) had no significant effect ($P = 0.05$) on time to fruiting and biomass production. Variations related to the mushroom strains cultivated with C1 are presented in Table 4. Based on these results, further experiments were performed with casing mixture C1.

Strain comparison on substrate covered with 5 cm of casing mixture C1

The ANOVA showed a significant effect ($P < 0.001$) of the experiment (substrate effect), but no interaction ($P = 0.05$) between strain and experiment was detected, meaning that the classification of the strains for the different parameters tested was the same in the two experiments.

Important variability in time to fruiting was observed among the eight strains (15–48 days). The wild European strains and the hybrid began to fruit significantly earlier than CA454 and the cultivars (Table 5).

Mushroom biomass produced at the end of the experiment or during the same period of time since the first picking (d1–d10, d1–d20 or d1–d30) clearly separated the wild European strains and the hybrid from the Brazilian strains and showed that the latter were significantly less productive (Table 5). Mushroom biomass obtained during the three successive periods of 10 days after the first picking showed that the productivity of the strains decreased significantly after the first period, except for CA561. For all strains but CA438-A and CA561, mushrooms harvested during the first period (d1–d10) represented more than 50% of the biomass collected during the d1–d30 period (Table 5). Mushroom biomass was significantly and negatively correlated with time to fruiting ($r = 0.751$, $P = 0.032$ for d1–d10; $r = 0.948$, $P = 0.001$ for d1–d20; $r = 0.963$, $P < 0.001$ for d1–d30).

Effect of variations in temperature

During incubation and the couple of days following casing, minimum, maximum and mean air temperatures ranged from 20 to 21.5 °C, from 21.2 to 23.4 °C and from 20.8 to 22.4 °C respectively (Fig. 1). Until the 10th day of incubation, composts of the four strains showed similar small variations in temperature. Thereafter the temperature kinetics varied dramatically with the strain. Small variations were observed with CA643, for which the compost temperature ranged from 23.9 to 24.1 °C during the day before casing. In compost inoculated with CA487,

Table 3. Comparison of effect of four casing compositions on time to fruiting and mushroom biomass of four strains

Parameter	Strain	Casing[b] C1	C2	C3	C4
Time to fruiting (days)[a]	CA487	16A	16A	17A	17A
	CA643	20C	22C	28B	32A
	CA565	41A	43A	—[c]	—
	CA570	27BC	25C	29B	40A
Biomass, end of experiment (g kg^{-1} substrate)[a]	CA487	206.2A	152.4AB	77.8B	202.5A
	CA643	122.9A	97.3A	15.9B	16.6B
	CA565	21.8A	24.1A	0.0B	0.0
	CA570	62.6A	30.3AB	3.5B	21.8AB
Biomass, d1–d30 (g kg^{-1} substrate)[a]	CA487	193.6A	126.4B	72.6C	163.7AB
	CA643	112.1A	84.9B	15.9C	11.7C
	CA565	—[d]	—	—	—
	CA570	62.6A	29.2AB	3.5B	—

[a] Within a row, values followed by the same letter are not different at $P = 0.05$ by Duncan's test.
[b] C1, 45% peat/20% limestone/35% fine sand (v/v/v); C2, 15% peat/23% limestone/37% fine sand/25% vegetal charcoal (v/v/v/v); C3, 15% peat/23% limestone/42% fine sand/20% spent compost (v/v/v/v); C4, 80% peat/20% spent compost (v/v) + $CaCO_3$ (100 g L^{-1}).
[c] No record: strain did not fruit on this casing mixture.
[d] No record: strain began to fruit too late.

Table 4. Comparison of six strains grown with casing mixture C1

Strain	Time to fruiting (days)[a]	Biomass (g kg^{-1} substrate)[a] End of experiment	d1–d30
CA438-A	23.2B	262.1A	227.8A
CA487	16.5C	244.8A	196.4A
CA643	22.7B	236.8A	193.1A
CA561	33.2A	75.7B	73.9B
CA565	32.7A	12.8C	12.6C
CA570	24.3B	75.1B	64.4B

[a] Within a column, values followed by the same letter are not different at $P = 0.05$ by Duncan's test.

the temperature reached 27.7 °C on day 11 then declined gradually to 24.1 °C. In contrast, the temperature increased from 24 to 28 °C from day 12 in compost inoculated with CA454. Moderate variations ranging from 25.9 to 27.7 °C were recorded in the CA 565 compost (Fig. 2). The mycelium of all strains except CA454 and CA565 developed intensively in the compost. Homogeneous but less dense colonization was observed with CA454 and CA565 at the end of the incubation step.

Rapidly after daily variations in temperature were applied, minimum, maximum and mean air temperatures ranged from 14.3 to 18.3 °C, from 25.5 to 28.4 °C and from 19.9 to 23.1 °C respectively. Despite computer control, the maximum air temperature recorded in the climatic room showed a peak centred on the d42–d53 period (Fig. 3) because of a dramatic increase in the outdoor temperature, which reached 31.6 °C on d50. Similar daily fluctuations in compost temperature occurred for the four strains until the end of the experiment. Minimum temperatures varied from 17.5 to 19.4 °C and maximum temperatures from 23.7 to 26.8 °C (Fig. 4). Around d50, the temperature measured in the compost showed a peak that followed the variations in air temperature. The small volume of compost per tray (0.018 m^3) could explain such variations. No explanation can be proposed for the peaks of temperature recorded around d24 and d35 in the compost of CA454.

The wild European strains and the hybrid began to fruit significantly earlier than CA454 and the cultivars (Table 6). Except for CA 565, no marked difference in time to fruiting was detected compared with cultivation under constant air temperature presented in Table 5, which was confirmed by the Pearson correlation of 0.777 ($P = 0.023$). As regards mushroom biomass production, the Pearson correlation of 0.939 ($P = 0.021$) showed that variations in temperature did not markedly modify the classification of the strains. Significantly higher mushroom biomass was obtained with the three European strains compared with the Brazilian strains and the hybrid either at the end of the experiment or for periods of 10, 20 or 30 days since the first picking. Biomass produced during the d1–d10 period represented 47–63% of that recorded during the d1–d30 period for all but the two poorly productive strains CA454 and CA565 (Table 6). Amounts of biomass produced during the same period of time were significantly and negatively correlated with times to fruiting ($r = 0.947$, $P < 0.001$ for d1–d10; $r = 0.956$, $P < 0.001$ for d1–d20; $r = 0.959$, $P < 0.001$ for d1–d30).

Sporophore fresh weight and dry matter content

The wild strains and the cultivar CA 561 differed significantly from CA454, the two other cultivars and the hybrid in the distribution of sporophore fresh weight, which moved towards higher values in the second group of strains (Fig. 5A).

Data distributions of fresh weight moved significantly toward low values after the d1–d10 period for the wild strains CA438-A and CA487, while values varied in the same range throughout the crop for the third wild strain, CA643. No significant differences in the distributions obtained for the first two periods were detected for CA570 and the hybrid (Fig. 6).

Lower dry matter contents were found in the sporophores of the wild strains CA487 and CA 643 than in those of the other strains. Distributions obtained for CA454 and two cultivars (CA561 and CA570) did not differ among themselves. High dry matter contents were measured in sporophores of CA565. The hybrid showed higher dry matter content compared with its parent

Table 5. Comparison of eight strains for time to fruiting and biomass production on substrate covered with 5 cm of casing mixture C1

Strain	Time to fruiting (days)[a]	Biomass (g kg^{-1} substrate)[a]				Biomass[b]		
		End of exp.	d1–d10	d1–d20	d1–d30	d1–d10	d11–d20	d21–d30
CA438-A	18.5DE	139.2AB	49.0C	81.5B	102.6B	49.0[d]a (47.7)[e]	32.5b (31.7)	21.2c (20.6)
CA487	15.1E	166.2A	95.3A	139.5A	157.5A	95.3a (60.5)	44.3b (28.1)	18.0b (11.4)
CA643	20.8CD	119.8AB	77.1AB	88.4B	109.2B	77.1a (70.6)	20.8b (19.0)	11.4b (10.4)
CA454	30.4B	11.5C	7.2D	7.4C	11.5C	7.2a (63.0)	4.1ab (35.5)	0.1b (1.5)
CA561	27.0B	38.6C	13.8D	24.3C	34.9C	13.8a (39.7)	10.7a (30.5)	10.4a (29.8)
CA565	48.8A	9.4C	4.5D	—[c]	—	4.5a (100)	—	—
CA570	26.8B	24.1C	14.2D	18.2C	20.5C	14.2a (69.5)	4.0b (19.3)	2.3b (11.2)
Hybrid	22.8C	117.4B	58.6BC	91.3B	99.4B	58.6a (59.0)	32.6b (32.8)	8.1c (8.2)

[a] Within a column, values followed by the same capital letter are not different at $P = 0.05$ by Duncan's test.
[b] Within a row, values followed by the same lowercase letter are not different at $P = 0.05$ by Duncan's test.
[c] No record: strain began to fruit too late.
[d] As g kg^{-1} substrate.
[e] As % biomass (d1–d30).

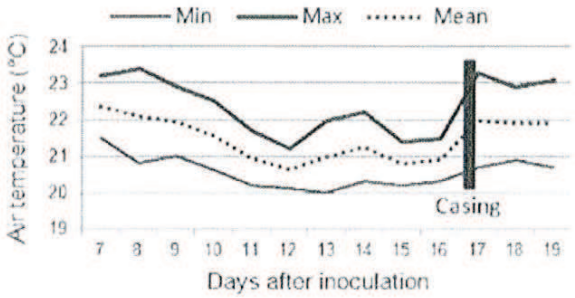

Figure 1. Minimum, maximum and mean air temperatures measured daily in climatic room during incubation and couple of days following casing.

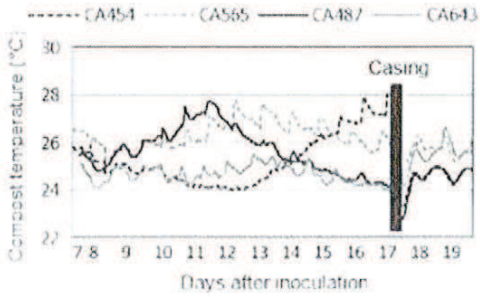

Figure 2. Temperature measured in substrate of four strains during incubation and couple of days following casing.

CA487 but did not differ significantly from its parent CA454 for this trait (Fig. 5B).

DISCUSSION

Works aimed at comparing the productivity of different mushroom strains have generally considered total biomass obtained during the same period of time starting at casing. The eight strains studied in the present work showed very different time to fruiting, meaning harvests lasted for very different periods of time in experiments analysed 65 days after casing. Comparing biomass production during the same period of time is a better representation of the

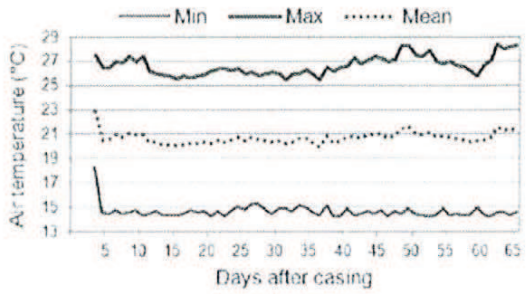

Figure 3. Minimum, maximum and mean air temperatures measured daily in climatic room from day 3 after casing to end of experiment.

ability of strains to mobilise nutrients to fruit. Consequently, strains were compared for mushroom biomass 10, 20 and 30 days after the first picking.

Under our cultivation conditions (straw- and horse manure-based compost for *A. bisporus*, incubation and fructification in a climatic chamber), using a 5 cm casing layer instead of the 2.5 cm conventionally used in experiments with *A. bisporus* significantly improved biomass production of the strains tested. A similar effect of casing depth was obtained in Brazil with different casing mixtures and substrate compositions. Both 5 and 8 cm of casing mixture based on Brazilian soils were better than 3 cm for cultivating ABL-97/12 in a greenhouse on substrate prepared with local Brazilian wastes.[17] Increasing casing quantity led to higher yield in strain M7700 grown in a climatic chamber on substrate made of chicken manure, wheat straw and gypsum.[6] Besides the effect observed in a greenhouse, no improvement occurred in a bamboo-covered structure.[17] The quantity of casing material proved important for biomass production of *A. subrufescens*, irrespective of the strain, casing and substrate materials, but its effect could be affected by the cultivation environment.

Peat use causes the loss of non-renewable resources and participates in the greenhouse effect by the liberation of CO_2 through the aerobic decomposition of carbon,[18] thus generating a worldwide demand for alternatives in agriculture. In Brazil the most utilised casing for both *A. bisporus* and *A. subrufescens* is subsoil or subsoil mixtures with charcoal,[12] and several works have shown various effects of charcoal on *A. subrufescens*. Rhodic Hapludox/eucalyptus charcoal 4:1 (v/v) led to a better yield of strain

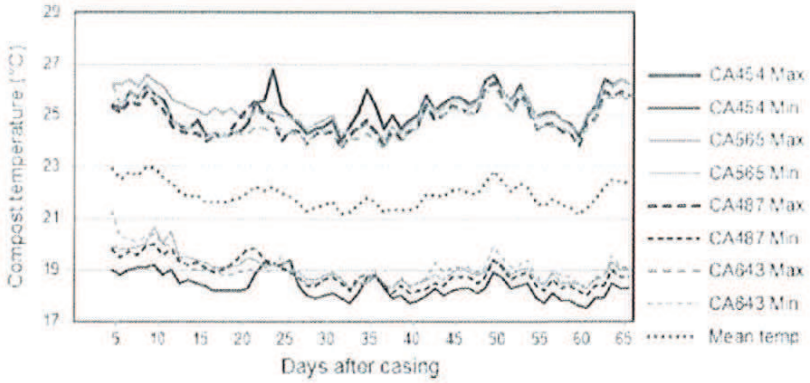

Figure 4. Minimum, maximum and mean temperatures recorded daily in substrate of four strains from day 3 after casing to end of experiment.

Table 6. Effect of variations in temperature on time to fruiting and biomass production of eight strains

Strain	Time to fruiting (days)[a]	Biomass (g kg^{-1} substrate)[a]				Biomass[b]		
		End of exp.	d1–d10	d1–d20	d1–d30	d1–d10	d11–d20	d21–d30
CA438-A	21.5CD	114.6C	54.5B	98.4C	110.1C	54.5[d]a (49.5)[e]	44.0a (39.9)	11.7b (10.6)
CA487	18.5D	188.9A	89.2A	154.4A	171.1A	89.2a (52.1)	65.2b (38.1)	16.7c (9.8)
CA643	21.0D	149.0B	83.7A	130.5B	145.2B	83.7a (57.7)	46.8b (32.2)	14.7c (10.1)
CA454	33.7A	7.1E	6.5D	6.5E	7.1E	6.5a (91.5)	0b (0)	0.6b (8.5)
CA561	34.7A	16.3E	10.3D	13.9E	16.3E	10.3a (63.0)	3.7b (22.5)	2.3b (14.5)
CA565	34.0A	7.5E	7.5D	—[c]	—	7.5a (—)	—	—
CA570	29.3B	27.9E	12.7D	16.1E	27.1E	12.7a (46.7)	3.4b (12.7)	11.0a (40.6)
Hybrid	25.0C	74.4D	38.4C	60.0D	62.5D	38.4a (61.5)	21.6ab (34.6)	2.5b (3.9)

[a] Within a column, values followed by the same capital letter are not different at $P = 0.05$ by Duncan's test.
[b] Within a row, values followed by the same lowercase letter are not different at $P = 0.05$ by Duncan's test.
[c] No record: strain began to fruit too late.
[d] As g kg^{-1} substrate.
[e] As % biomass (d1–d30).

CS1 compared with Xanthic Hapludox or Humic Haplaquox soil.[13] In contrast, casing made of peat or shale increased the productivity of ABL-99/26 and ABL-99/29 by 10–20% compared with a mixture of 70% ravine soil/30% charcoal (v/v),[19] and lime schist or peat led to better biological efficiency of the same strains compared with a mixture of subsol/charcoal 7:3 (v/v).[12] In outdoor cultivation, strain BZ-04 showed better yield on a substrate covered with local soil horizon A compared with casing mixtures composed of 30% eucalyptus charcoal/70% local soil horizon B (v/v).[11] Our aim was to replace the standard casing used for *A. bisporus* cultivation with a casing mixture efficient for growing *A. subrufescens* and easy to implement by European mushroom growers. Peat/limestone was far less efficient than peat/limestone/sand casing for time to fruiting and biomass production of CA487.[20] Based on this result, our reference casing mixture was *A. bisporus* casing (peat/limestone) supplemented with fine sand. Under our cultivation conditions, not only did the partial replacement of peat with vegetal charcoal (casing C2 *vs* C1) cause no improvement in mushroom biomass at the end of the experiment, it also reduced the d1–d30 yield of the two wild strains.

Spent mushroom substrate has also been evaluated as a substitute for peat in *Agaricus* cultivation. A casing mixture composed of *Sphagnum* peat/spent mushroom substrate (wheat straw and poultry manure ingredients) 4:1 (v/v) + $CaCO_3$ was as efficient as a peat/lime mixture for *A. bisporus* productivity.[16] When all strains were taken as a whole, we obtained the worst biomass production with the two casing mixtures (C3 and C4) containing spent compost. In contrast to C3 that reduced the yield of the four strains, the C4 effect varied with the strain. Despite the yield of CA487 being similar with C1 and C4, the casing mixture C1 was chosen because it gave the best yield for all strains. Indeed, our objective was to find the best casing mixture to grow *A. subrufescens*, irrespective of the strain. Besides, C4 has the same peat/spent substrate/$CaCO_3$ proportion as the casing mixture with which Pardo-Giménez *et al.*[16] obtained a good performance for an *A. bisporus* strain. The difference in *Agaricus* species, the use of several strains and possibly the peat origin and the characteristics of the spent substrate could contribute to explaining why spent compost was not a good material to prepare a standardized casing for *A. subrufescens*.

The C1 casing mixture used for the different experiments allowed CA487 to produce 171–228 g kg^{-1} substrate in the d1–d30 period, which fell between the values of 443 and 1315 g per 4 kg substrate that Mata *et al.*[20] obtained for this strain 30 days after pin formation with casing made of sand/peat/limestone 2:1:1 and 1:1:1 (v/v/v) respectively and substrate based on wheat straw supplemented with sugar cane bagasse. In contrast to their observation, we did not improve the mushroom biomass by increasing the percentage of sand in the casing mixture. The substrate we used, based on wheat straw and horse manure, might explain this difference.

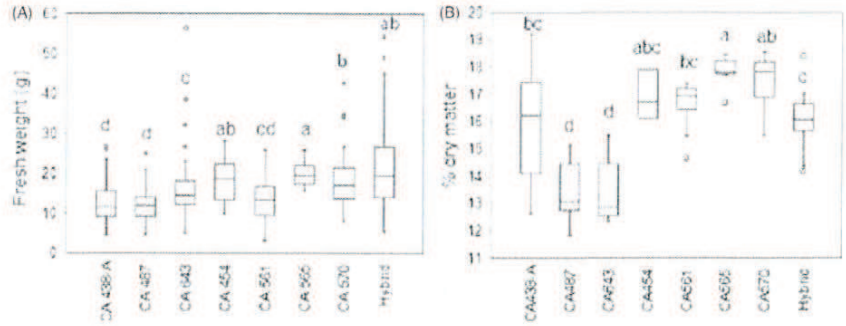

Figure 5. Sporophore (A) fresh weight and (B) dry matter content of eight strains.

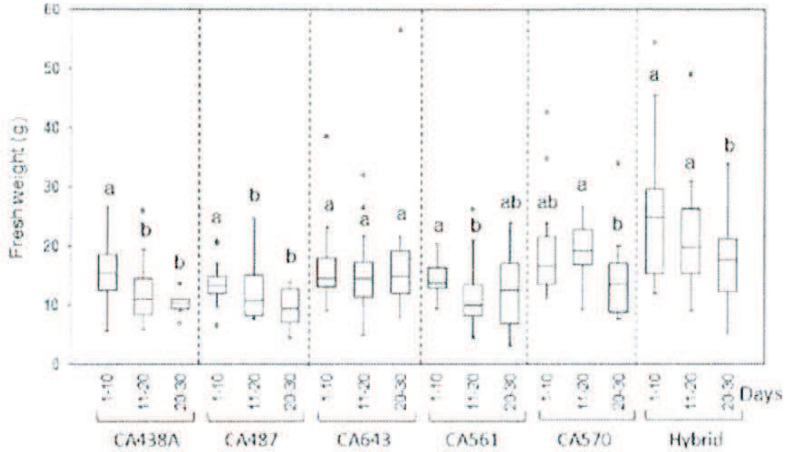

Figure 6. Variations in sporophore fresh weight during three successive time periods under variations in temperature.

Most air temperatures during incubation reported for experiments performed in Brazil ranged between 25 ± 2 and 28 ± 1 °C.[5,11,12,14] Incubation at a minimum temperature of 8 °C during the night and a maximum temperature of 26 °C during the day was also applied.[21] Our incubation conditions were close to the 22 ± 1 °C used in a Slovenian facility.[6] These temperatures are suitable for the incubation of *A. subrufescens* and more adapted to cultivation in temperate countries with moderate energy expenditure. The variations in temperature measured in the compost during incubation reflected neither the propensity of the strain to colonize the substrate nor its ability to produce mushroom biomass.

The mushroom can fruit at a temperature between 20 and 30 °C.[22] Although, in contrast to *A. bisporus*, the almond mushroom does not need a decrease in temperature for fruiting,[23] a reduction in air temperature to 17–20 °C to induce primordial formation followed by an increase to 22–28 °C for the development of the fruiting body was reported.[6,12,14,24–26] However, various experiments performed in Brazil showed that large and uncontrolled variations in temperature also proved suitable. A mushroom yield of 9.6% (strain BZ-04) was obtained after 90 days under natural conditions in an area bordering the Guaramiranga forest, Ceará State (climate predominantly warm and wet) with minimum temperature around 20 °C and high variations in maximum temperature ranging from approximately 20 to 32 °C.[11] Strain CS1 cultivated at Lavras (Minas Gerais State) in a room under natural conditions with the temperature ranging from 17 to 28 °C had a yield of 13.3% at 101 days after casing.[21] More interesting are the Brazilian works showing valuable production in a greenhouse where local climatic conditions directly influenced mushroom yield. Cultivating ABL-97-12 in a plastic greenhouse where minimum, maximum and mean temperatures ranged from 13 to 21 °C, from 23 to 36 °C and from 14.3 to 25.6 °C respectively led to the same yield as in a climatic chamber set at 25 ± 2 °C.[5] Strain ABL-04/49 cultivated in a greenhouse where the air temperature varied between 20.4 and 36.8 °C and the compost temperature between 20.3 and 30.8 °C showed 20% higher yield compared with that obtained in a climatic chamber with primordial induction.[14,26] Starting from these observations, we decided to investigate whether variations in temperature could improve the productivity of the eight studied strains. The air temperature was regulated to mimic variations between night and day. Minimum and mean temperatures recorded were in the same range as those reported by Braga *et al.*,[5] but the maximum temperature did not exceed 28.4 °C, while it reached 36 °C in the Brazilian greenhouse. The lack of high temperatures during our experiment could explain the absence of yield improvement. However, the lowest temperature recorded in the compost was 17.5 °C and the highest 26.8 °C, which did not differ markedly from the 20.3 and 30.8 °C measured by Zied *et al.*[14] in a greenhouse. The only notable difference related to variations in temperature was that CA565 began to fruit earlier than at constant temperature and no longer differed from the other two cultivars for this trait. The Brazilian strains remained far less productive than the European strains, regardless of the experiment.

Precocity is defined as yield at mid-cycle of harvest expressed as a percentage of total harvest and depends on the type of compost and casing.[14] Zied *et al.*[14] observed precocity ranging from 50 to 62% in a first set of crops and from 69 to 75% in a second set. We chose to assess the percentage of biomasss production during three successive periods of 10 days following the first picking of the strain. The major part of the biomass was obtained during the first 10 days, and production was very low during the last period (d20–d30). The kinetics of production differed dramatically from that known for cultivars of *A. bisporus* and observed under our cultivation conditions for this species. These observations suggest that the commercial compost used for *A. bisporus* is probably not the optimal substrate to grow *A. subrufescens*, but yields obtained with the wild European strains were much better than those reported for commercial strains in Brazil (e.g. 80–110 g kg^{-1} after 65 days for the strain ABL-97/12[5] and 88 g kg^{-1} after 70 days for the commercial strain ABL-04/49[14]). The average production in Brazil was estimated to be 8–16% after 120 days of cultivation.[7]

As Colauto *et al.*[24] reported for two Brazilian cultivars, we observed that changes in mushroom fresh weight during harvest depended on the strain, although all strains except CA643 produced less heavy mushrooms during the d20–d30 period. As regards the whole harvest, Brazilian strains were generally more interesting than wild European strains for individual fresh weight and dry matter content, but this was offset by the far higher yield of the wild strains. In particular, the Spanish strain CA438-A proved to be a good material owing to its classification for fresh weight and dry matter, which did not differ from those of the Brazilian cultivar CA561.

CONCLUSIONS

The experiments described herein demonstrate that commercial production of the almond mushroom can be developed in Europe with the substrate commonly used for *A. bisporus*, a simple modification of the casing mixture (addition of peat and sand) and maintaining the incubation temperature throughout the crop. The use of new strains selected among wild European isolates is promising.

ACKNOWLEDGEMENTS

This work was supported by the research projects 115790 CONACYT (Mexico) and ANR-09-BLAN-0391-01(France). RC Llarena-Hernández would like to thank CONACYT, Mexico for a scholarship. The authors gratefully thank DC Zied for providing the Brazilian cultivars and Ludovic Devaux for mushroom production.

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4.2.4. Recherche d'amélioration par création d'hybrides

Des hybrides intercontinentaux (Tableau 4.1) et européens (Tableau 4.2) ont été obtenus par P. Callac et coll. à partir de croisements entre des isolats monospores homocaryotiques issus des souches CA454 (Brésil), CA487 (France) et CA438-A (Espagne). Les méthodes d'obtention sont décrites dans l'article 3 de ce chapitre.

Les hybrides intercontinentaux ont montré une augmentation significative du rendement en comparaison du parent brésilien, et un hybride (487-42 x 454-43) a montré le même rendement (p=0,05) que le parent français identifié comme bon producteur. Un autre critère observé est la couleur du chapeau, qui varie de crème à brun foncé chez les hybrides intercontinentaux, chez lesquels la couleur crème a été transmise à 5 hybrides. Ceci montre que chez aucun des deux parents la couleur du chapeau est un caractère récessif, contrairement à ce qui est observé chez *A. bisporus* où la couleur blanche est récessive (Callac *et al*., 1998).

Tableau 4.1. Rendement et couleur du chapeau des hybrides intercontinentaux.

Agaricus subrufescens		Rendement		Couleur
Hybrides	487-42 x 454-43	126,0	ab [1]	Crème à beige rosé
	487-42 x 454-3	94,5	bc	Crème
	487-42 x 454-4	41,5	cde	Brun à brun foncé
	487-100 x 454-43	88,2	bc	Crème à beige
	487-100 x 454-3	41,5	cde	Crème à beige rosé
	487-100 x 454-4	14,3	de	Beige à brun
	487-125 x 454-43	2,9	e	Beige à brun
	487-125 x 454-3	76,6	bcd	Crème à beige
	487-125 x 454-4	22,4	de	Brun foncé
Parents	CA454	11,0	de	Beige ± foncé
	CA487	148,8	a	Crème à beige

[1] Les valeurs suivies de la même lettre ne sont pas différentes entre elles (p=0,05) d'après le test de Student Newman Keuls.

Les quatre hybrides européens et les deux parents présentent tous le même rendement (p = 0,05) (Tableau 4.2). Toutefois un hybride (438-A-12 x 487-42) montre une tendance à produire plus que son parent le plus producteur.

Tableau 4.2. Rendement et couleur du chapeau des hybrides européens

Agaricus subrufescens		Rendement		Couleur
Hybrides	438 -12 x 487-100	174,6	ab [1]	Beige
	438 -12 x 487-42	198,1	a	Beige
	438 -12 x 487-125	70,8	b	Beige ± foncé
	438 -12 x 487-35	129,1	ab	Crème à beige
Parents	CA438	148,8	ab	Crème à beige
	CA487	142,1	ab	Crème à beige

[1] Les valeurs suivies de la même lettre ne sont pas différentes entre elles (p=0,05) d'après le test de Student Newman Keuls.

Les rendements obtenus montrent le potentiel des souches sauvages pour introduire du matériel génétique d'intérêt (production de biomasse) avec l'objectif d'obtenir un fort rendement mais aussi des champignons de bonne qualité. Des analyses de la composition des champignons pourraient être envisagées pour l'amélioration de la qualité avec pour objectif de développer un aliment fonctionnel.

4.3. Conclusions

Le faible nombre de souches d'*A. subrufescens* disponibles, en particulier d'origine sauvage, ne permet pas d'avoir une estimation de la variabilité au sein de l'espèce. Le but de cette étude était de rechercher les meilleures souches pour une production commerciale et des sources de diversité utilisables pour une amélioration génétique. Les cultivars évalués sont génétiquement homogènes avec les outils moléculaires utilisés. Ce résultat confirme la grande homogénéité des souches commerciales du Brésil rapportée dans la littérature. Le plus intéressant est le haut niveau de polymorphisme génétique observé parmi les souches sauvages, ce qui ouvre la voie à des programmes d'amélioration du champignon.

Les souches étudiées, aussi bien cultivées que sauvages, ont montré une large diversité phénotypique quel que soit le critère étudié (accroissement mycélien, capacité de colonisation du substrat, morphologie et aptitude à fructifier). Cependant, les souches européennes (CA438-A, CA487 et CA643) révèlent davantage de variabilité. Les cultivars (CA561 et CA570) montrent d'avantages de qualités au niveau chimique, comme l'activité antioxydante. Ce point est approfondi dans le chapitre 5.

Le groupe de souches retenu pour l'optimisation des conditions de cultures est représentatif de la variabilité génétique observée avec l'ensemble des souches.

Les conditions permettant d'optimiser la production d'*A. subrufescens* en France ont été définies. Les modifications identifiées par rapport au mode de culture utilisé pour *A. bisporus* (pour le gobetage, ajout de sable et augmentation de la hauteur ; fructification à 23-25 °C au lieu de 17-18 °C) sont facilement applicables par les champignonnistes.

Le rendement des cultivars d'*A. subrufescens* a été similaire à celui rapporté dans la littérature pour des cultures effectuées au Brésil avec des substrats locaux. Cependant, ce rendement est encore très faible par rapport aux rendements d'*A. bisporus*. Par contre, dans les conditions définies lors de cette étude, les souches sauvages ont montré une meilleure performance que les cultivars, et un rendement proche de celui d'*A. bisporus*. Les souches européennes testées, en particulier CA487 et CA438-A, constituent un matériel pouvant être proposé pour une culture commerciale en France. D'autre part, l'évaluation des hybrides a démontré le potentiel de croisements entre des souches intercontinentales, avec transmission des aptitudes culturales des souches sauvages.

Ces différents points seront repris et commentés plus en détail dans la discussion générale.

Chapitre 5. Sensibilité d'*Agaricus subrufescens* aux pathogènes

Ce chapitre présente la sensibilité d'*A. subrufescens* à des pathogènes majeurs en champignonnières et l'effet d'extraits hydrophiles du champignon sur ces micro-organismes.

5.1. Introduction

La sensibilité aux maladies est un problème majeur dans l'industrie du champignon. Du fait des modes de cultures proches, les compétiteurs et pathogènes majeurs redoutés, et observés, dans les cultures d'*A. subrufescens* sont les même que ceux d'*A. bisporus* qui sont présentés dans le chapitre 1. Les conditions de forte humidité et de températures (entre 25 et 30 °C) maintenues pendant la culture d'*A. subrufescens* favorisent le développement de moisissures comme *Trichoderma aggressivum* et *Lecanillium fungicola* ou de bactéries comme *Pseudomonas tolaasii* connues pour provoquer les plus importantes pertes dans les cultures d'*A. bisporus*. Les quelques données disponibles dans la littérature sur les contaminants des cultures d'*A. subrufescens* sont données dans le paragraphe '*Biotic and abiotic disorders during cultivation*' de l'article 1 (chapitre 1).

Au Brésil, des dégâts importants dus à *P. tolaasii* ont été signalés chez *A. subrufescens*, et au début des années 2000 des symptômes attribués à *L. fungicola* ont été observés dans une zone de production (Eira 2003). La littérature signale des contaminations de culture d'*A. subrufescens* par *Trichoderma* sp. (Andrade et al. 2007 ; Kopytowski Filho et al. 2008). Cependant aucune donnée n'est disponible sur le niveau de sensibilité des souches et l'aspect des symptômes. La résistance des souches commerciales aux pathogènes constitue une forte demande de la part des producteurs de champignons comestibles. L'introduction de la culture d'*A. subrufescens* en Europe amène de nouveaux défis, aussi, la prévention de risques de maladies par la sélection de souches tolérantes est un facteur important.

D'autre part, *A. subrufescens* montre du potentiel comme agent de contrôle biologique. Des extraits du champignon ont permis de réduire le niveau d'infection de plusieurs plantes

par des pathogènes et un effet éliciteur a été détecté. Ces points sont développés dans le paragraphe '*Use in plant protection and other valorisation*' de l'article 1 (chapitre 1).

Ce chapitre présente la sensibilité d'*A. subrufescens* à deux maladies, la moisissure verte et la tache bactérienne, avec pour premier objectif la comparaison du degré de sensibilité des cultivars et des souches sauvages dans le but d'identifier du matériel permettant de limiter les risques de pertes en culture. Le deuxième objectif est de rechercher si des extraits d'*A. subrufescens* peuvent avoir un effet inhibiteur vis-à-vis de ces pathogènes en vue d'une utilisation en lutte biologique.

5.2. Résultats

Le projet d'article 5 présente la sensibilité de trois cultivars et quatre souches sauvages d'*A. subrufescens* à *T. aggressivum* et *P. tolaasii* ainsi que la mise en évidence d'effets inhibiteurs ou stimulants vis-à-vis de ces pathogènes dans des extraits hydrophiles de mycélium et de sporophore (Figure 5.1).

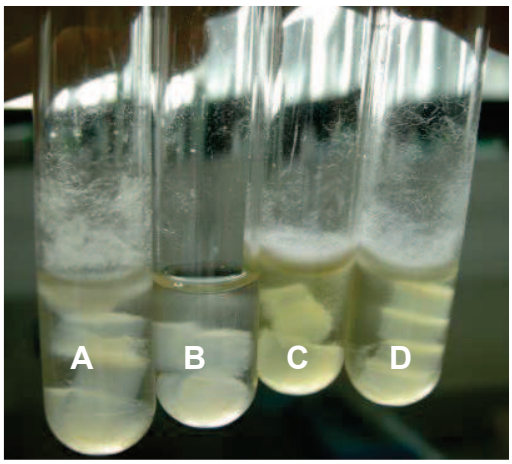

Figure 5.1. Développement mycélien de *T. aggressivum* en absence d'extrait d'*A. subrufescens* (A), en présence d'amphotéricine (antifongique commercial) (B) et d'extrait chaud (C) et froid (D) de CA487. (Photo INRA, MycSA).

Projet d'article 5.

Susceptibility of *Agaricus subrufescens* to *Trichoderma aggressivum* and *Pseudomonas tolaasii*

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Abstract Three cultivars and four wild strains of *Agaricus subrufescens* were analysed for their susceptibility to *Trichoderma aggressivum* and *Lecanicillium fungicola,* two pathogens known to cause severe crop losses in *Agaricus bisporus* crops. The seven strains of *A. subrufescens* were highly susceptible to *T. aggressivum*, but contrary to *A. bisporus* they were poorly affected by *P. tolaasii*, and no relationship was observed between the cap colour of the mushroom and its susceptibility to the bacterium. Compounds present in hydrophilic extract prepared from sporophores could explain the weak susceptibility of the *A. subrufescens* strains to *P. tolaasii*.

Key words Bacterial blotch · Green mould · Hydrophilic extract · Symptoms

INTRODUCTION

Brown blotch disease due to *Pseudomonas tolaasii* and green mould disease caused by *Trichoderma aggressivum* are still major diseases in mushroom growing regions worldwide. The literature provides extensive information on the two diseases in the button mushroom *Agaricus bisporus* (see Soler-Rivas et al. 1999, and Largeteau and Savoie 2010 for reviews). Mushrooms contaminated by *Pseudomonas tolaasii* develop brown spots on the cap making them unmarketable. Many *Trichoderma* sp. that occur in the button mushroom cultivation cause problem but *T. aggressivum* is by far the most aggressive and can totally wipe out crop production (Baars et al. 2011). *T. aggressivum* competes with *A. bisporus* for space and nutrients (Savoie et al. 2001) and no mushroom develops in the contaminated area. *Agaricus bitorquis*, a species cultivated at higher temperature (24-28 °C) than *A. bisporus*, is also affected by *P. tolaasii* (Fletcher et al. 1989) and shows significant reduction in yield in the presence of *T. aggressivum* (Sobieralski et al. 2010). *Agaricus subrufescens* is a fungus of

hot climate and low technology systems. Temperature range of 25 to 30 °C throughout the production cycle favours the emergence of pests and diseases (Eira 2003). *Trichoderma* sp. is common in *A. subrufescens* culture beds when the compost temperature was raised above 32 °C after spawning (Kopytowski Filho et al. 2008), and in environments with deficient fresh air intake and circulation, in addition to high moisture either in the compost, casing soil, or the air (Andrade et al. 2007). By inoculating *Trichoderma* sp. at spawning, Andrade et al. (2011) reduced significantly the yield of mushroom production, but the strain and the species of *Trichoderma* used was not specified. Currently, there is no report comparing *A. subrufescens* strains for competition with *T. aggressivum*. The same remark can be made about the susceptibility of the mushroom to *P. tolaasii*. The aim of this work was to assess the susceptibility of Brazilian cultivars and wild European strains of *A. subrufescens* to both pathogens.

MATERIAL AND METHODS

Agaricus subrufescens

Three strains (CA561, CA565, and CA570) were cultivars of almond mushroom from Brazil kept in the Collection of Germplasms of Agaricus in Bordeaux (CGAB), INRA, since 2007; CA438-A, CA487 and CA643 were wild European strains of the CGAB collection; CA918 was a Thai strain from the CGAB/MFLU (Mae Fah Luang University, Chiang Rai, Thailande) collection (Table 1).

Table 1: Commercial and wild strains with reference to origin, cap colour and code in collection.

	Origin	Code CGAB	Code FCA/UNESP [1]	Cap colour
Wild isolates	Spain	CA438-A		Cream to light beige
	Saint-Léon, 33, France	CA487		Cream to light beige
	Le Pian Médoc, 33, France	CA643		Brown
	Thailand	CA918		Red brown
Brazilian Cultivars	Piedade, SP, Brazil	CA561	ABL-99/30	Brownish gold
	Boituva, SP, Brazil	CA565	ABL-03/48	Brownish gold
	Rio de Janeiro, RJ, Brazil	CA570	ABL-01/29	Brownish gold

[1] Code in the collection of the Mushroom Research Centre of the College of Agronomic Sciences, Sao Paulo State University.

Pathogens

Trichoderma aggressivum isolate B (formerly Th2 B) was isolated in 1997 at a French mushroom farm from *Agaricus bisporus* culture beds showing severe green mould symptoms (Mamoun et al. 2000). It was preserved in liquid nitrogen at INRA-Bordeaux. *Pseudomonas tolaasii* CNBP2152 and *Pseudomonas reactans* CNBP2156 (CNBP collection, INRA, Angers, France) were kept in liquid nitrogen. In the presence of *P. reactans*, the *P. tolaasii* strain generated the typical white line reaction characteristic of its toxin production.

Production of fruiting bodies

The substrate used to grow *A. subrufescens* was compost prepared for commercial production of the button mushroom, and provided by Renault SA, Pons, France. The main ingredients for composting had been wheat straw and horse manure. Composting had been performed indoor. Trays filled with 8 kg compost inoculated with 1% spawn were incubated for 15 days in a climatic chamber with 25 ± 0.5 °C in compost and 98 ± 2% relative humidity. Then, 5 cm of casing layer corresponding to 45% peat, 20% limestone, 35% fine sand (v/v/v) was added and the trays were left under the same environment conditions for a post-incubation period of seven days. After that, the climatic chamber was set at 23 ± 0.5 °C for air temperature with 97 ± 2 % humidity and 1200 ± 100 ppm CO_2 concentration.

Confrontation *A. subrufescens* - *T. aggressivum*

Inoculum was made by rolling a sterile rye grain onto the surface of a sporulating colony of *T. aggressivum*. Polypropylene boxes with a volume of 540 mL (Microbox, Combiness, Belgium) were filled with 150 g of compost (batch used to produce fruiting bodies) and inoculated with 2% spawn (16 grains of *A. subrufescens* spawn per tray, uniformly distributed into the substrate). On one side of the box, a sterile rye grain coated with *T. aggressivum* spores was introduced into the compost, at 1 cm from the surface (Fig. 1). Lids with a specific air filter were added (7.44 gaz exchange per day) and the cultures were incubated at 23 ± 0.5 °C, for 20 days. Sporulation areas of *T. aggressivum* were plotted daily on tracing paper. Sporulation surfaces were measured using the software Adobe Photoshop CS5. Data are means of six replicates.

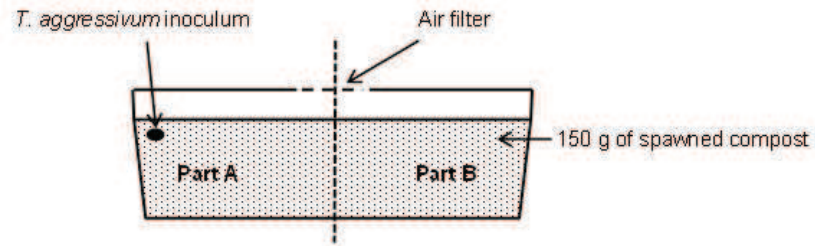

Figure 1. Experiment design for confrontation between *T. aggressivum* and *A. subrufescens*.

Susceptibility to *P. tolassii*

The experimental design was adapted from that used by Olivier et al. (1997) to assess the susceptibility of *A. bisporus* to *P. tolaasii*. To prepare the inoculum, *P. tolaasii* was cultivated during 48 h at 25 °C on liquid King's medium B (King et al. 1954) and cells were suspended in sterile water. The suspension was adjusted to 0.3 absorbance at 450 nm. Ten mushroom caps, veil closed, were arranged inside plastic boxes used as moist chamber. A 20 µL aliquot of the bacterial suspension at 0.3 absorbance (approx. 10^8 cfu mL^{-1}) was placed at the surface of each cap surface. After 48 h of incubation at 20-23 °C, symptoms were rated on a scale of 0 to 3: 0 = no symptom; 0.5 = level, yellowish lesion, 1 = level, beige-yellow lesion; 1.5 = slightly sunken, beige-yellow lesion; 2 = sunken, yellow-brown lesion; 2.5 = extensive yellow-brown sunken lesion; 3 = extensive brown sunken lesions.

Hydrophilic extracts of *A. subrufescens*

The mycelium is produced in 1 L flasks containing 200 mL of liquid medium (cristomalt 20 g L^{-1}) inoculated from liquid sub-cultures. After incubation for 20 days at 25 °C on a rotary shaker (200 rpm), the mycelium was recovered by filtration, washed with ice water, and immediately frozen at -80 °C. Frozen mycelium from 20 liquid cultures was grouped, lyophilised and ground to fine powder (< 0.1 mm). Sporophores (veil closed) were frozen at –80 °C immediately after harvest and lyophilised. Mushroom powder was obtained from 10 sporophores. The same protocol was applied to prepare extract from mycelium and mushroom powder. To obtain the hot extract, 2.5 g of powder were suspended in 50 mL of ultrapure sterile water at 65 °C and incubated at 100 °C for 1 h. After homogenization at room temperature for 6 h on a rotary shaker (30 rpm), the suspension was centrifuged for 30 min at 6000 *g*, 20 °C. The supernatant was collected, freeze dried and the powder was diluted in sterile water to obtain a solution at 15 mg mL^{-1}. The pH was adjusted to 5.0-6.0 if necessary. To obtain cold extracts, 2.5 g of powder were suspended in 50 mL of ultrapure sterile water and incubated for 1 h at room temperature with strong agitation every 15 min, then for 48 h at 4°C,

30 rpm. The solution at 15 mg mL^{-1} was prepared as described above. Extracts were stored at -80 °C and thawed at 36 °C for 5 min just before use. Cold and hot extracts were prepared from mycelium and sporophores of the wild strain CA487 and the cultivar CA565. The cold extract was incubated 20 min at 80 °C for deactivation of enzymes which could interfere with the FDA hydrolysis.

Effect of the mycelium hydrophilic extracts on mycelial growth of *T. aggressivum*

The isolate Tri B was cultivated on malt agar medium at 25 °C, in the dark to avoid sporulation. Four inoculum plugs (7 mm diam.) were cut at the edge of the colony and placed into tubes containing 960 mL of liquid medium (cristomalt, 15 g L^{-1}). The medium was supplemented with either 40 µL of hydrophilic extract (sample), or 40 µL of Amphotericine Gibco™ at 2.5 µg mL^{-1} (commercial fungicide), or 40 µL sterile water (control without extract), each with two replicates. Control without Tri B consisted in 960 mL of liquid medium supplemented with 40 µL of hydrophilic extract. The active microbial biomass was assessed by the measurement of FDA hydrolysis activity adapted from Inbar et al. (1991). Within each replicate tubes, one received 1 mL of FDA at a concentration of 0.01 mg mL^{-1} in 60 mM phosphate buffer pH 7.6 (FDA), and 1 of mL 60 mM phosphate buffer pH 7.6 was added to the other tube (Buffer). Immediately (t0) and after incubation for 48 h at 25 °C (t48h), the tubes were placed at 30 ° C for 30 min, then the reaction was stopped by the addition of 1 mL of acetone. After 5 min at room temperature, the tubes were centrifuged at 11 000 rpm at 15 °C for 5 min and the supernatant collected for lecture of the absorbance at 490 nm (UVIKON, Bio-Tek Instruments). The FDA hydrolysis activity was expressed in mg equivalent of FDA hydrolysed $implant^{-1}$ min^{-1}, following the equation:

$$\text{FDA hydrolysed} = [((A_{490}*1/n) * (1/t))_{FDA} - ((A_{490}*1/n) * (1/t))_{Buffer}]_{t48h} - [((A_{490}*1/n) * (1/t))_{FDA} - ((A_{490}*1/n) * (1/t))_{Buffer}]_{t0}$$

with n = number of implants, and t = incubation time at 30 °C in minutes.
Data are means of three replicates per treatment.

Effect of the sporophore hydrophilic extracts on *P. tolaasii* growth

P. tolaasii suspension was prepared as described for the susceptibility test and adjusted to 0.3 absorbance at 450 nm. King's medium B (950 µL) was supplemented with either 50 µL of hydrophilic extract (sample), or 50 µL of antibiotic mixture providing 10 ng Clortetracyclin, 50 ng Streptomycin sulfate, 25 ng Penicillin G sodium salt at 1MU (antibiotic treatment), or 50 µL of sterile water (control). The antibiotics were purchased from Sigma-Aldricht. After incubation for 24 h at 24 ± 1 °C, the bacterial biomass was assessed by the measurement of FDA hydrolysis as described above. Data are means of three experiments, each with two replicates per treatment.

Results and Discussion

Susceptibility of *A. subrufescens* to *T. aggressivum*

Whatever the incubation time and the variable analysed in the confrontations between *A. subrufescens* and *T. aggressivum*, variations between replicates were not significant (p=0.05).

Mycelia of *A. subrufescens* and *T. aggressivum* grew simultaneously, as observed with *A. bisporus* confronted to *T. aggressivum* (Mamoun et al. 2000).

Mean values for symptom appearance ranged between 12.7 and 14 days after inoculation, and did not differ significantly with the strain (p = 0.05). On day 13 after inoculation, all strains showed green mould symptoms.

In part A of the substrate (with *Trichoderma* inoculum), the surface covered by sporulating mycelium of *T. aggressivum* increased from d13 to d17 then little to no increase occurred between d17 and d20. No change was observed on d30 (not shown). The kinetics of sporulation surfaces was identical for the substrates spawned with the three cultivars, and of the same type for substrates spawned with the wild strains CA438-A and CA487. In presence of the wild strains CA643 and CA918, kinetics revealed a slower extension of the surface covered by sporulating *T. aggressivum* (Fig. 2).

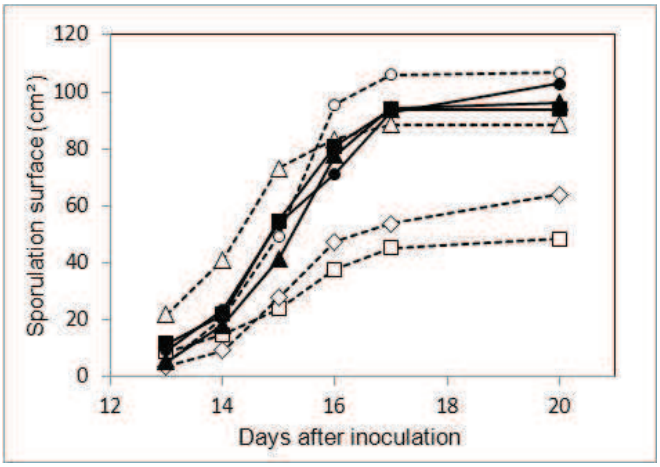

Figure 2. Kinetics of surface covered by sporulating mycelium of *T. aggressivum* in part A of the substrate (with *T. aggressivum* inoculum).

Substrate spawned with ---○--- CA438A, ---Δ--- CA487, ---□--- CA643, ---◊--- CA918, —●— CA561, —▲— CA565, —.— CA570.

These observations were confirmed by the ANOVAs followed by the student's test performed on measurements of sporulation surfaces. From d13 to the end of the experiment (d20), substrates spawned with the three Brazilian cultivars, CA438-A and CA487 did not differ significantly ($p = 0.05$) between themselves. Since d15-16, substrates inoculated with the wild strains CA643 and CA918 were significantly less contaminated than substrates contaminated with the other strains. On part B of the substrate, kinetics of sporulation surfaces did not differ markedly (not shown). At each time of measurement, substrates spawned with the seven strains showed similar surfaces with sporulating *T. aggressivum* ($p = 0.05$) except some differences observed with CA918 since d16 (Table 2).

These observations and the percentage of surface of the substrate covered with spores (Table 2) showed that *A. subrufescens* is highly susceptible to *T. aggressivum* but identification of two wild strains less susceptible than the others suggested the possibility to find more tolerant strains in the wild. The absence of significant difference between the three cultivars might be related to their genetic homogeneity (Llarena-Hernández et al. 2013) which was consistent with the common origin of the present Brazilian cultivars proposed by Neves et al. (2005).

Table 2. Comparison of *T. aggressivum* sporulation surface from 13 to 20 days after inoculation in substrate spawned with seven strains of *A. subrufescens*.

Substrate part analysed	Mushroom strains	Sporulation of *Trichoderma aggressivum*									
		Surface (cm^2)								% total surface	
		d15		d16		d17		d20		d17	d20
Part A	CA438-A	49.3	ABC	95.6	A	106.0	A	106.6	A	60.4	60.4
	CA487	73.1	A	83.4	A	88.5	A	88.5	A	50.1	50.1
	CA643	23.9	C	37.8	C	45.3	B	48.4	C	25.7	27.4
	CA918	27.7	BC	47.4	BC	53.6	B	64.0	B	30.4	36.3
	CA561	53.8	AB	71.2	AB	92.8	A	102.9	A	52.6	58.3
	CA565	41.3	BC	77.7	A	94.2	A	96.2	A	53.4	54.5
	CA570	54.7	AB	81.0	A	93.8	A	93.8	A	53.1	53.1
Part B	CA438-A	61.7	b	125.5	ab	142.3	a	143.9	ab	80.6	81.5
	CA487	123.1	a	134.5	a	141.3	a	141.3	ab	80.0	80.0
	CA643	90.4	ab	125. 9	ab	132.0	ab	135.6	ab	74.8	76.8
	CA918	46.4	b	81.1	b	95.8	b	127.1	b	54.3	72.0
	CA561	75.3	ab	110.8	ab	118.7	a	139.5	ab	67.2	79.0
	CA565	82.0	ab	139.3	a	151.3	a	152.9	a	85.7	86.6
	CA570	56.7	b	124.5	ab	141.2	ab	141.2	ab	80.0	80.0

For each part of the substrate, within a column, values followed by the same letter are not different at p=0.05 by the Duncan's test.

The sporulation surface was identical on parts A and B on days 13 and 14 whatever the strain of *A. subrufescens* present. At that time spawn run was achieved. Then the surface covered by spores of *T. aggressivum* was significantly extended ($p<0.05$) on part B compared to part A since d15 in substrates spawned with CA487 and CA643, d16 in presence of CA561 and CA565. On d17, part B showed the higher surface whatever the mushroom strain. No hypothesis was postulated to explain this observation which suggested a different behaviour compared to the stimulation of the *Trichoderma* sporulation observed in confrontations with *A. bisporus* as soon as both mycelia were in contact (Mamoun et al. 2000). Indeed, in the present study the contact between *A. subrufescens* and *T. aggressivum* mycelia cannot occur later in part A than in part B.

Effect of hydrophilic extracts of *A. subrufescens* mycelium on mycelial growth of *T. aggressivum*

CA487 hot extract reduced significantly the growth of the pathogen whilst the cold extract was without effect. Preparations from CA565 had no effect (hot extract) or stimulated significantly (cold extract) the mycelial growth of *T. aggressivum* (Table 3). These observations were in accordance with the poor competitiveness of *A. subrufescens* against *T. aggressivum* observed in the confrontation experiment.

Table 3. Effect of hydrophilic extracts of *A. subrufescens* on the development of the pathogens.

Treatment		Mycelial growth of *T. aggressivum* (ng FDA min^{-1} μL^{-1})		*P. tolaasii* growth (ng FDA min^{-1} μL^{-1})	
Control without extract		6.61	B	2.91	A
Commercial inhibitor		1.27	C	0.38	F
Cold extract	CA487	5.70	B	2.12	D
	CA565	11.62	A	1.87	E
Hot extract	CA487	2.20	C	2.79	B
	CA565	6.31	B	2.30	C

Susceptibility to *P. tolassii*

Lesions observed at the surface of the mushroom caps were yellowish to yellow-brown, never brown to dark brown (Fig. 3). The discolouration observed was closer to the yellowish brown to orange colour developed on *Pleurotus eryngii* (González and González-Varela 2009) than to the pale to dark

brown colour reported for *A. bisporus* (Soler-Rivas et al. 1999). *A. subrufescens* belong to the group of the yellowing agaricus (the flesh becomes yellow to orange after injury and at the base of the stipe after harvest) whilst *A. bisporus* is a reddening agaricus. That explains the difference in the bloch colour in the two species.

Significant variability in susceptibility was observed between *A. subrufescens* strains but the group of wild strains did not differ from that of cultivars for the trait (Table 4). Symptoms are at low levels (score < 1.5) except for CA643 and some caps of CA565, and never extensive (no score 2.5 or 3). Based on observations in *A. bisporus*, it must be mentioned that strains showing no symptom in this experiment could exhibit slight symptoms in other experiments. The mushrooms analysed exhibited various cap colours (Table 1, Llarena-Hernández et al. 2011) but, contrast to observations on *A. bisporus* (Moquet et al. 1999), the susceptibility of *A. subrufescens* to *P. tolaasii* was not linked to cap colour.

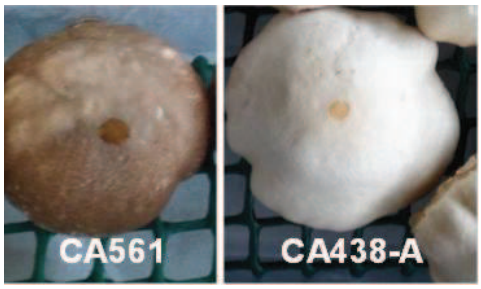

Figure 3. Blotch symptoms developed on the cap surface of two *A. subrufescens* strains. (Photo INRA, MycSA)

Table 4. Susceptibility of the *A. subrufescens* strains to *P. tolaasii*.

Strain	Mean score	
CA438-A	0.4	CD
CA487	0	D
CA643	1.9	A
CA918	0	D
CA561	0.7	C
CA565	1.3	B
CA570	0	D

Effect of hydrophilic extracts of *A. subrufescens* sporophores on *P. tolaasii* growth

A significant inhibition of *P. tolaasii* growth was observed with all the extracts although it was low compared with the antibiotic cocktail (Table 3). The cold extracts reduced the bacterial growth by 27.1 and 35.7% and were more efficient than the hot extracts. Further investigations with more strains and partition of the extract could confirm the weak susceptibility of *A. subrufescens* to *P. tolaasii* and inform on the components reducing the development of the bacterium.

Conclusion

The susceptibility of *A. subrufescens* strains to two pathogens was analysed in this work. Cultivars and wild strains revealed a low susceptibility to *P. tolaasii,* but more strains must be screened before to extrapolate the poor susceptibility to the *A. subrufescens* species. Hydrophilic extracts of *A. subrufescens* were less effective than a commercial antibiotic as inhibitor of *P. tolaasii.* However, bacterial disease control seemed not of prime importance considering the results of the susceptibility test. In contrast, confrontation between *A. subrufescens* and *T. aggressivum* mycelia on compost revealed a great susceptibility of the mushroom to green mould, and the hydrophilic extracts obtained from two mushroom strains failed to reduce *T. aggressivum* mycelial growth. But two wild strains were significantly less susceptible to green mould than the others, and might offer a means of partial control, and their hydrophilic extracts must be tested.

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5.3. Conclusions

L'article 5 montre que la surface de compost colonisée par *T. aggressivum* a été légèrement inférieure lorsque la moisissure était en compétition avec deux souches sauvages alors que les mêmes niveaux très élevés d'occupation de l'espace ont été observés en présence du mycélium des deux autres souches sauvages et des cultivars. *Trichoderma aggressivum* peut donc potentiellement causer des pertes importantes dans la culture.

Globalement les extraits aqueux d'*A. subrufescens* n'ont pas eu un effet protecteur vis-à-vis du développement mycélien de la moisissure, à part un extrait qui a montré une inhibition similaire à l'inhibiteur commercial. Toutefois, l'effet d'extraits des deux souches sauvages les moins sensibles, qui n'ont pas pu être obtenus dans le temps imparti pour ce travail, doit être connu avant de confirmer l'absence d'effet protecteur de tels extraits, ce que la sensibilité des souches laisse supposer. Cette étude est en cours.

La sensibilité des sporophores à l'agent de la tache brune a été variable selon les souches, mais globalement faible. Des différences par rapport à la réponse chez *A. bisporus* (couleur de la tache, absence de relation entre la couleur du chapeau et la sensibilité à la bactérie) sont analysées dans la discussion générale.

Les extraits aqueux d'*A subrufescens* ont montré une inhibition de *P. tolaasii* par apport au traitement témoin, mais à un niveau plus faible qu'un cocktail d'antibiotiques. Cependant, la forte tolérance de l'ensemble des souches étudiées laisse supposer qu'il pourrait s'agir d'un caractère intrinsèque chez *A. subrufescens*. Il ne semble donc pas nécessaire d'envisager la recherche de moyens de lutte contre la tache bactérienne pour les cultures d'*A. subrufescens*, mais l'effet antibiotique observé pourrait être utilisé, après concentration des extraits, pour une protection des cultures d'autres champignons contre cette maladie.

La souche CA918 est l'une des moins sensibles à *T. aggressivum* et s'est montrée résistante dans le test *in vivo* face à *P. tolaasii*. Même si cette souche pourrait révéler une faible sensibilité à la bactérie dans d'autres conditions de culture, elle représente un premier matériel intéressant pour la culture. L'identification de nouvelles souches moins sensibles à *T. aggressivum* est nécessaire. Le comportement des quelques souches sauvages testées ici supporte l'hypothèse qu'il sera possible de trouver du matériel sauvage présentant une meilleure tolérance à la moisissure verte.

Chapitre 6. Potentiel d'*A. subrufescens* comme source de composés bioactifs

Ce chapitre présente une analyse chimique d'*A. subrufescens* par résonance magnétique nucléaire du solide et l'estimation de l'activité antioxydante dans les corps fructifères.

6.1 Introduction

Depuis plusieurs années il y a un intérêt industriel accru pour des composés, nommés neutraceutiques fongiques, qui peuvent être extraits du mycélium ou du sporophore des champignons. Les polysaccharides avec des sucres variés tels que les α- et surtout les β-glucanes ont été reconnus comme agents anti-tumoraux (Angeli et al., 2009 ; Borchers et al., 2008 ; Chen et Seviour, 2007 ; De Silva et al., 2012 a et b ; Poncheret et al., 2006).

Dans le cas d'*A. subrufescens* la caractérisation de la biomasse fongique par des marqueurs chimiques, en particulier la quantité et la qualité des polysaccharides est un aspect important pour le développement de méthodes de criblage permettant d'identifier rapidement des souches ou des conditions de cultures optimisées pour la production de substances bioactives à visées thérapeutiques. La résonance magnétique nucléaire (RMN) du solide a été largement utilisée pour étudier les structures des polymères parce qu'une extraction préliminaire n'est pas nécessaire et de ce fait cette approche globale permet l'analyse d'échantillons dans leur état d'origine. Cette technique permet d'obtenir directement le rapport entre protéines et polysaccharides (Pizzoferrato et al., 2000).

Par ailleurs, *A. subrufescens* est considéré comme une source naturelle de composés antioxydants différents des polysaccharides, mais à notre connaissance il n'existe pas de données disponibles sur la variabilité de ces métabolites incluant des souches sauvages d'*A. subrufescens*. Des études sur l'activité biologique pendant le développement des sporophores ont révélé qu'il peut y avoir des changements de l'activité anti-oxydante chez un sporophore à l'état commercial (chapeau fermé) en comparaison avec l'état mature (chapeau ouvert) (Soares et al., 2009 ; Mourao et al., 2011), mais il n'existe pas d'étude au niveau primordium.

L'objectif de ce chapitre est de révéler le potentiel de la RMN du solide pour caractériser le mycélium et les sporophores d'*A. subrufescens* comme source de polysacharides diversifiés et d'évaluer la variabilité du potentiel antioxydant de souches sauvages et cultivées à deux stades de développement du sporophore.

6.2 Résultats

6.2.1. Caractérisation chimique

La caractérisation chimique des biomasses fongiques d'*A. subrufescens* par RMN du solide du carbone 13 est présentée dans l'article 6. Ce travail décrit l'analyse chimique de la biomasse mycélienne et des corps fructifères d'*A. subrufescens* à l'état commercial (chapeau fermé). Ma contribution à ce travail a consisté d'abord à préparer les échantillons à partir de sporophores puis une fois les spectrogrammes des échantillons obtenus, à participer à leur lecture (calcul des surfaces des pics, en particulier dans la région des polysaccharides, Figure 6.1) ainsi qu'aux analyses statistiques des données, et ultérieurement, à rechercher la bibliographie pour discuter les rapports des surfaces des pics protéines / polysaccharides dans les échantillons.

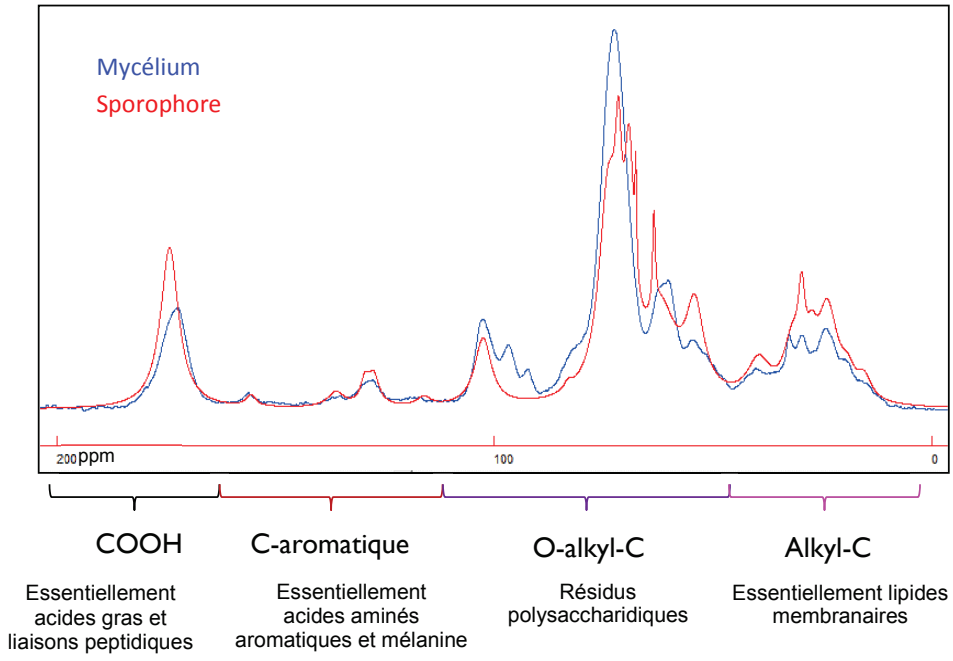

Figure 6.1. Exemple de spectres RMN-^{13}C en phase solide obtenus avec du mycélium et des sporophores d'*Agaricus subrufescens*.

Article 6

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Chemical Characterization of the Biomass of an Edible Medicinal Mushroom, *Agaricus subrufescens*, via Solid-State ^{13}C NMR

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ABSTRACT: The biomass of 18 strains of *Agaricus subrufescens* and of 13 strains of *Agaricus bisporus* was chemically analyzed using solid-state ^{13}C NMR. The study focused on polysaccharides because they can play a major role as antitumor molecules. The study also examined whether biomass chemical properties varied between the vegetative mycelium and the fruiting bodies of *A. subrufescens*, and these data were compared with the mycelium of *A. bisporus*. Qualitative differences between vegetative mycelia and fruiting bodies were observed, whereas quantitative differences were measured between the two species with a higher percentage of polysaccharides in the biomass of *A. subrufescens*. This *Agaricus* species is thus an interesting potential source of polysaccharides with medicinal properties, both from vegetative mycelium obtained in liquid cultures and from fruiting bodies produced on composts.

KEYWORDS: *Agaricus subrufescens*, *Agaricus bisporus*, fruiting bodies, vegetative mycelium, $\beta(1-3)$ polysaccharides

INTRODUCTION

The edible mushroom *Agaricus subrufescens* Peck., formerly known as *Agaricus blazei* or *Agaricus braziliensis*, is a Basidiomycete that has long been widely consumed in Brazil and Japan as a health food because of its nutritional properties. Furthermore, this mushroom is believed to prevent or to fight various diseases such as diabetes, hepatitis, hyperlipidemia, and cancer, although both the bioactive molecules it produces and their physiological roles are still not clear. An overall view of the question is available in reviews.[1,2] Nevertheless, polysaccharides such as (1−6)-β-glucan, (1−3)-β-glucan, (1−4)-α-glucan, or (1−2)-β-mannopyranosyl residues[1,2] from this fungal species have been demonstrated to inhibit the growth of cancer cells and increase the immune activity in patients.[3,4] The major carbohydrate structure in the water-soluble part of the fruiting bodies of *A. subrufescens* is (1−6)-β-glucan, and the yield and structural diversity of glucans are supposed to increase as the fruiting bodies mature.[5] Apart from the use of fruiting bodies, attempts have also been made for exopolysaccharide production in submerged cultures of mycelium[5] or polysaccharide production in solid cultures of mycelium.[6]

It thus seems of great importance to identify chemical markers that would reveal the presence of these polysaccharides in fungal biomass, that is, vegetative mycelium or fruiting body. These indicators would be useful to determine the effects of (i) the type of fungal material considered (vegetative mycelium or fruiting bodies), (ii) the age of the fruiting bodies, (iii) the culture conditions, and (iv) the genetic diversity on the concentrations of such polymers of medicinal interest in the fungal biomass.

Our study consequently focused on the chemical characterization of the fungal biomass, using solid-state ^{13}C NMR. This method has been widely used to study polymer structures because no preliminary extractions are required and this holistic approach thus allows for analysis of samples in their native state. This technology is efficient for various fields such as environmental research, to monitor organic matter transformation,[7] or the food industry, for instance, to characterize proteins and free amino acids in cheese.[8] Here, the fungal biomass of *A. subrufescens* is chemically described using both vegetative mycelium and fruiting bodies from a large number of strains from various geographical origins (Europe, North, South, and Central America, Asia). Vegetative mycelium from different strains of the button mushroom *Agaricus bisporus* is also analyzed to compare their specificities with those of *A. subrufescens*.

MATERIALS AND METHODS

Samples. The 18 strains of *A. subrufescens* used were strains cultivated in Brazil by various mushroom growers (CA454, CA455, CA560, CA561, CA562, CA563, CA564, CA565, CA570, CA571, CA572 and CA574), a strain obtained on the market in Taïwan (CA276), two cultivars from the Belgium company Mycelium (CA646 and C647), three European wild strains (CA438A from Spain; CA487 and CA643 from France), and a hybrid strain (CA487 × CA454). Thirteen wild strains of *A. bisporus* belonging to the three known varieties were isolated from different areas: USA (Bs094 and Bs738, variety *burnettii*; Bs10 variety, *bisporus*), France (Bs423, variety *eurotetrasporus*; Bs261, Bs419B, Bs518, Bs527, and Bs550, variety *bisporus*); Greece (Bs514, variety *eurotetrasporus*; Bs571 and Bs594, variety *bisporus*), Russia (Bs475, variety *bisporus*), Spain (Bs680, variety *bisporus*), Canada (Bs483, variety *bisporus*),

Received: May 5, 2011
Revised: July 5, 2011
Accepted: July 19, 2011
Published: July 19, 2011

and Mexico (Bs739, variety *bisporus*). All of the strains were from the CGAB collection (INRA, UR MYCSA, France).

Production of the Vegetative Fungal Biomass. The vegetative fungal biomass was produced using 500 mL flasks containing 100 mL of liquid medium (Cristomalt 20 g/L, $CuSO_4$ 5 mg/L) inoculated with a 1 cm wide plug of solid cultures (Cristomalt 20 g/L, agar 15 g/L). The flasks were incubated at 25 °C with continuous shaking (200 rpm) for 15 days. Then the cultures were transferred in 1 L flasks containing 200 mL of liquid Cristomalt medium under the same conditions for 15 days. The mycelium was recovered by filtration on gauze and frozen before being lyophilized for solid-state NMR analyses.

Production of Fruiting Bodies. The compost used for the cultures of 13 strains of *A. subrufescens* was provided by a company producing button mushrooms (Renaud SA, Pons, France). Eight kilograms of compost was inoculated with 2% of spawn (mycelium developed on rye grains), placed in trays, and incubated at 25 °C and 85% relative humidity during 20 days to allow the invasion of the compost by the mycelium. Then a casing layer (one-third ground limestone, one-third peat, one-third thin sand) was added. The ambient air temperature was maintained between 22 and 25 °C with 95% relative humidity and low concentrations of CO_2. Two and 4 weeks after the addition of the casing layer, the trays were placed at about 18 °C for 8 h. The fruiting bodies were harvested young, tough, and medium size. A composite sample of 10 fruiting bodies for each strain was frozen immediately after harvest and lyophilized before the NMR analyses.

Cross-Polarization Magic Angle Spinning ^{13}C Nuclear Magnetic Resonance (^{13}C CP/MAS NMR) Procedure. The solid-state ^{13}C NMR spectra were obtained on a Bruker Avance-400 MHz NMR spectrometer operating at ^{13}C and ^{1}H resonance frequencies of 101.6 and 400.3 MHz, respectively, using a commercial Bruker double-bearing probe. About 60 mg of samples was placed in zirconium dioxide rotors of 4 mm o.d. and spun at a magic angle spinning rate of 10 kHz. The CP technique was applied with a ramped ^{1}H pulse starting at 100% power and decreasing until 50% during the contact time (2 ms) to circumvent Hartmann−Hahn mismatches. To improve the resolution, a dipolar decoupling GT8 pulse sequence[9] was applied during the acquisition time. To obtain a good signal-to-noise ratio in ^{13}C CPMAS experiment 6000 scans were accumulated using a delay of 3 s (enough to ensure the complete ^{1}H relaxation). The ^{13}C chemical shifts were referenced to tetramethylsilane and calibrated with glycine carbonyl signal, set at 176.03 ppm. The ^{13}C NMR spectra were divided into seven chemical shift regions according to Dignac et al.[7] Dmfit 2003 software was used to determine each chemical shift region intensity.[10] Protein/polysaccharide ratios (COOH−C CONH−C/O-alkyl-C) were calculated as described by Pizzoferrato et al.:[11] 170 ppm/105 ppm, and 20 ppm/105 ppm.

Statistical Analysis. The nonparametric Mann−Whitney U-test[12] was used to separate significantly different means ($P < 0.05$) for each NMR signal (COOH−C, O-alkyl-C, aromatic + phenolic-C, and alkyl-C). To compare vegetative mycelia and fruiting bodies, only the data from the 13 common strains were used. To compare the mycelium of *A. bisporus* and *A. subrufescens*, the data from all of the strains were available.

RESULTS AND DISCUSSION

This study reports for the first time the chemical comparison of fungal biomass from vegetative mycelium and fruiting bodies of a fungal species, *A. subrufescens*, using solid-state ^{13}C NMR. Interspecific chemical differences of vegetative mycelium biomass have been also investigated between two *Agaricus* species, *A. subrufescens* and *A. bisporus*. A large sample of both cultivated and wild strains from various origins has been studied, and thus the data can be considered as representative of each species.

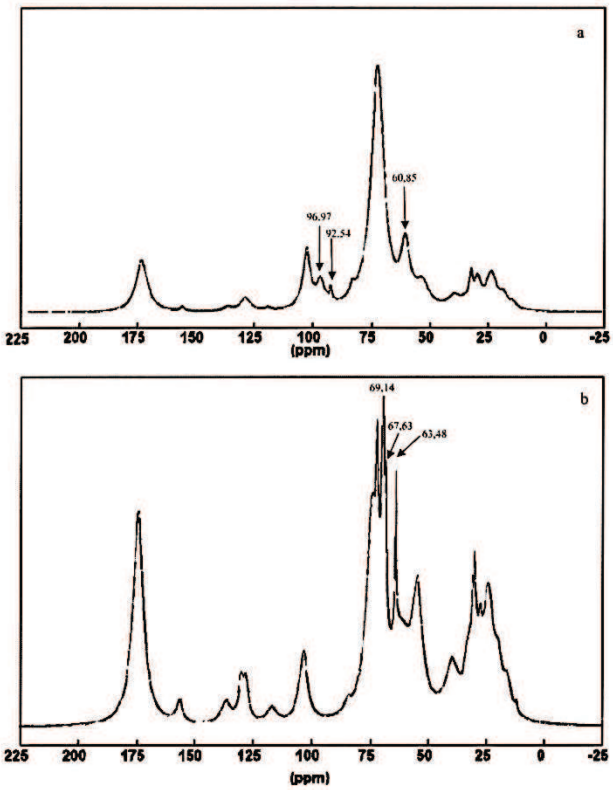

Figure 1. ^{13}C NMR spectra of vegetative mycelium (a) or fruiting body (b) biomass from strain CA570 of *Agaricus subrufescens*. The numbers (ppm) indicate the peaks from the O-alkyl region, which differ in the ^{13}C NMR spectrum of the vegetative mycelium or the fruiting body.

The ^{13}C CP/MAS NMR data have been analyzed using each area of the spectrum that can be assigned to different categories of biomolecules: from 0 to 45 ppm, alkyl-C (mainly lipids from membranes); from 45 to 110 ppm, O-alkyl-C (polysaccharide moiety); from 110 to 160 ppm, aromatic-C (mainly aromatic amino acids and melanin); from 160 to 200 ppm, COOH−C/CONH−C (mainly fatty acids and peptide bonds).[7] Furthermore, whatever the spectra considered (either from the vegetative mycelium or from the fruiting body), certain peaks may be more specifically assigned to certain functions from biopolymers. With regard to proteins, several peaks can be linked to carbons from side chains of amino acids: Cα at 54 ppm; Cβ at 40 ppm (from Phe, Ile, Tyr, Asn, Asp) or 30 ppm (from Arg, Gln, Glu, His, Lys, Trp); Cζ at 155 ppm from Arg and Tyr.[13] Polysaccharide resonances can be found in the O-alkyl region (45−92 ppm) and di-O-alkyl region (90−110 ppm): the main peak at 72 ppm can be assigned to C2, C3, and C5 of carbohydrates, and the region from 99 to 105 ppm is linked to anomeric carbons with either α or β configuration.[14,15]

Chemical Differences in the Fungal Biomass of Vegetative Mycelia and Fruiting Bodies. The ^{13}C CP/MAS NMR spectra of 13 strains of *A. subrufescens* have been compared between the vegetative mycelium biomass obtained in liquid cultures and the fruiting bodies. Figure 1 shows an example of two NMR spectra obtained from the vegetative mycelium and the fruiting bodies of strain CA570. This first result reveals significant differences for the functional groups COOH/CONH−C, O-alkyl-C, and alkyl-C (Table 1). The fruiting bodies contain a higher percentage in

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Table 1. Means of Relative Intensities of Chemical Groups from ^{13}C NMR Data from the Vegetative Mycelium or the Fruiting Bodies of 13 Strains of *Agaricus subrufescens*[a]

	type of mycelium	
chemical functions, ppm	vegetative mycelium	fruiting bodies
COOH/CONH−C, 160−200 ppm	8.70 ± 1.54*	15.13 ± 0.85*
aromatic-C, 110−160 ppm	2.54 ± 0.87	5.60 ± 0.76
O-alkyl-C, 45−110 ppm	74.84 ± 4.86*	55.91 ± 2.72*
alkyl-C, 0−45 ppm	13.92 ± 2.54*	23.36 ± 1.34*

[a] The asterisks (*) indicate significant differences between means ($p < 0.05$) using the non-parametric Mann−Whitney U-test.

Table 2. Means of Ratios of Peaks Assigned to Proteins or Polysaccharides (Proteins/Polysaccharides) As Described by Piazzoferrato et al.[11a]

	A. subrufescens		*A. bisporus*
ratio	fruiting body	vegetative mycelium	vegetative mycelium
173 ppm/102 ppm	2.93 ± 0.49	1.27 ± 0.35	2.14 ± 0.38
19 ppm/102 ppm	0.31 ± 0.06	0.17 ± 0.07	0.23 ±0.07

[a] These ratios were calculated from ^{13}C NMR data of 18 strains of *Agaricus subrufescens* (for the vegetative mycelium and the fruiting body) and 16 strains of *A. bisporus* (for the vegetative mycelium).

Table 3. Comparison of Relative Intensities of Different Regions from the ^{13}C NMR Spectra of Fruiting Bodies of *Pleurotus ostreatus* and *Agaricus subrufescens*

	fungal species	
chemical function	*Pleurotus ostreatus*[a]	*Agaricus subrufescens*
CONH−C	18.4 ± 5.59 (170 ppm)	15.09 ± 0.83 (173 ppm)
CH_3 groups from proteins	26.8 ± 7.5 (20 ppm)	1.59 ± 0.20 (19 ppm)

[a] Data from Piazzoferrato et al.[11]

COOH/CONH−C and alkyl-C, both signals being mainly assigned to lipids, than the vegetative mycelium. On the other hand, the vegetative mycelium shows a higher level of O-alkyl-C, assigned to polysaccharides.

Qualitative differences between peaks are mainly observed in the functional region assigned to polysaccharides. In the ^{13}C CP/MAS NMR spectra from the vegetative mycelium, three peaks at 96.97, 92.54, and 60.85 ppm have been recorded that were not in the spectra of the fruiting bodies. On the other hand, three other peaks at 69.14, 67.63, and 63.48 ppm have only been obtained in the ^{13}C CP/MAS NMR spectra of the fruiting bodies (Figure 1).

To compare with more accuracy the results obtained from both the vegetative mycelia and the fruiting bodies, only certain peaks have been selected in the regions assigned to polysaccharides and proteins in the solid-state ^{13}C NMR spectra. A protein moiety can be detected in the spectrum by signals at 175−173 ppm (CONH), at 20−50 ppm (C−N, C−H from peptide bonds), and at 120−165 ppm (aromatic side chains of amino acids). A polysaccharide moiety can also be described more precisely: 60−90 ppm signals can indeed be assigned to carbons C2, C3, C4, C5, and C6 from the glycosidic ring,[14] whereas anomeric carbon can be linked to a signal from 99 to 105 ppm.[15] Piazzoferato et al.[11] compared ^{13}C NMR spectroscopy of different mushroom species and focused on certain peaks for the proteins (170 ppm for C=O and 20 ppm for CH_3 groups) and for the polysaccharides (105 ppm for anomeric CH and 90 ppm for other CH groups). To estimate the relative percentage of proteins and polysaccharides in the fungal biomass, we have thus used peaks at 173 and 19 ppm for proteins and at 102 ppm for polysaccharides. Because the latter peak at 92 ppm is present only in the mycelium but not in the fruiting bodies, we did not calculate the ratios 19 ppm/92 ppm and 173 ppm/92 ppm. The results are presented in Table 2. For both ratios, it is more than twice as high in the fruiting bodies as in the vegetative mycelium. From the data of Piazzoferato et al.,[11] the lowest ratio 19 ppm/102 ppm was obtained for the fruiting bodies of *Lentinula edodes* (0.69) and the highest for that of *Volvariela volvacea* (8.98). However, these results were based on data from only one strain per species. On the other hand, the same authors used five different strains of *Pleurotus ostreatus* in their study. Comparison of the average peak intensities at 173, 102, and 19 ppm between these 5 *P. ostreatus* and the 13 *A. subrufescens* strains we studied shows that only the difference in the peaks corresponding to the CH group of the protein moiety (19 ppm) was significant, with a 10 times lower value for *A. subrufescens* compared with *P. ostreatus* (Table 3). These data stress the relative low concentration in proteins of *A. subrufescens* biomass by comparison with other edible mushrooms.

Furthermore, the observed differences between the vegetative mycelia and the fruiting bodies may be taken into consideration for the choice of the biomass to be used, depending on nutritional or medicinal objectives, to favor either high protein or high polysaccharide concentrations, respectively.

In this study, we also aimed to identify certain peaks that may be assigned to the polysaccharides of medical interest. This information is indeed of importance because it can be used as a chemical indicator of the presence of β glucans in fungal biomass. The availability of such a chemical marker is essential to determine which type of culture (solid-state fermentation or liquid culture) is the most relevant to obtain the best production of these molecules of medical interest. Furthermore, this would indeed be helpful to find the best culture conditions (either in liquid or solid medium) to obtain significant concentrations of these polysaccharides of interest in the fungal biomass of *A. subrufescens*. As described above, the anomeric carbon signal from glycosides ranges from 99 to 105 ppm. It should be noted that various data about the assignation of NMR signals to chemical functions are available and greatly depend on the material studied. For instance, Synytsya et al.,[16] assigned the peak at 103.3−103.6 ppm to anomeric-C with β configuration, whereas Chenghua et al.,[17] assigned this function to the peak at 102.6 ppm. In our study, whatever the NMR spectrum considered, we found a signal at 102.7 ppm: this signal from the vegetative mycelia (average percentage of relative intensity = 7.0) was significantly different ($P < 0.05$) from that in the fruiting bodies (average percentage of relative intensity = 5.3), confirming the higher content in polysaccharides in the vegetative mycelium than in the fruiting body as found for total polysaccharides (Table 1). However, to accurately identify chemical indicators of polysaccharides of medical interest (with β configuration) from *A. subrufescens* fungal biomass, further studies should focus on extracting these polysaccharides to obtain pure polymers, which would be further analyzed via solid-state NMR.

dx.doi.org/10.1021/jf2017622 |J. Agric. Food Chem. 2011, 59, 8939–8943

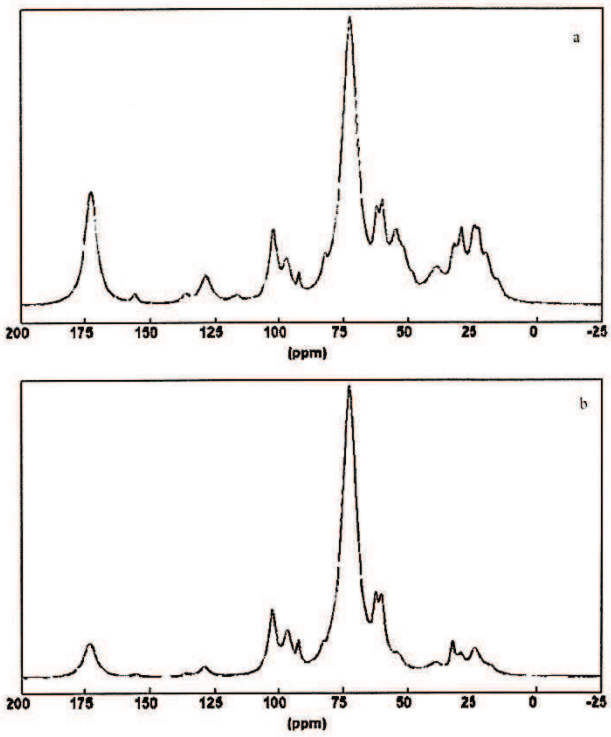

Figure 2. ^{13}C NMR spectra of strain CA565 of *Agaricus subrufescens* (a) and of strain Bs514 of *Agaricus bisporus* (b).

No quantitative differences were observed for the aromatic-C signals where differences, partially due to various degrees of melanization, may have been found. However, other regions of the spectrum can be used as markers of melanization: Fuji et al.[18] have found that different signals from 128.9 to 134.6 ppm can be assigned to olefinic carbons (CH_2=CH_2—CH_2=CH_2) from melanin. In our study, a peak at 129 ppm was found only in the fruiting body biomass. This result can be explained by the fact that the fungal cap of *A. subrufescens* is particularly colored, clearly indicating melanization. However, Knicker et al.[19] have found that the solid-state ^{13}C NMR spectra of melanin from various fungal species showed large and significant interspecies differences. Thus, extracting melanin from the fungal biomass of *A. subrufescens* would be useful to clearly define the peaks that can be assigned to this polymer.

Chemical Differences in the Vegetative Mycelium of *A. subrufescens* and *A. bisporus*. The ^{13}C CP/MAS NMR spectra from the vegetative mycelium biomass of the 18 strains of *A. subrufescens* have been compared to those of the 16 strains of *A. bisporus* to test the hypothesis of interspecific chemical differences in the biomass of *Agaricus* spp. The spectra from the vegetative mycelium of both species do not show qualitative differences between the two species studied (Figure 2), whereas significant differences in the relative intensity are observed except for the signals assigned to aromatic-C. NMR signals in this region of the spectrum are the weakest: in fungal biomass they may be assigned to the aromatic amino acids or to melanin, which is not observed in the vegetative mycelium of *Agaricus* species. The results indeed show a significantly higher intensity for the O-alkyl-C region with *A. subrufescens*, whereas higher intensities for both the COOH/CONH—C and the alkyl-C regions are found for *A. bisporus* (Table 4). These differences suggest that

Table 4. Means of Relative Intensities of Chemical Groups from ^{13}C NMR Data in Vegetative Mycelium from 18 Strains of *Agaricus subrufescens* and 16 Strains of *Agaricus bisporus*[a]

	fungal species	
chemical functions, ppm	*A. subrufescens*	*A. bisporus*
COOH/CONH—C, 160—200 ppm	8.48 ± 1.39*	12.35 ± 1.22*
aromatic-C, 110—160 ppm	2.39 ± 3.63	3.92 ± 0.93
O-alkyl-C, 45—110 ppm	75.52 ± 4.39*	65.25 ± 4.50*
alkyl-C, 0—45 ppm	13.61 ± 2.28*	18.48 ± 2.53

[a] The asterisks (*) indicate significant differences between means ($p < 0.05$) using the nonparametric Mann—Whitney U-test.

A. subrufescens biomass contains more polysaccharides, whereas that of *A. bisporus* contains a higher proportion of lipids. The polysaccharide moiety of the fungal biomass is an important chemical characteristic because this fraction potentially contains polymers of medical interest as previously mentioned. Many fungi such as *L. edodes*,[20] *P. ostreatus*,[21] or *A. subrufescens*[22] possess antitumor activities related to the presence of (1—3)-β-glucan[23] and/or a β-glucan—protein complex.[24] Thus, finding that the relative intensity of the polysaccharide fraction from *A. subrufescens* species is higher than that from *A. bisporus* is noteworthy. However, *A. bisporus* mushrooms have been shown to have a potential breast cancer chemopreventive agent, as they suppress aromatase activity and estrogen biosynthesis. This is mainly due to unsaturated fatty acids,[25] and vegetative mycelia may also be a source of such fatty acids.

The ratios from Piazzoferato et al.[11] have also been used to compare the fungal biomass of the two species of *Agaricus*. With both ratios 173 ppm/102 ppm and 19 ppm/102 ppm, no significant differences in protein and polysaccharide are found between both species (Table 2). In their study, Piazzoferato et al.[11] calculated protein/polysaccharide ratios from the different fungal species, and their results indicated that the chemical characteristic of fungal biomass can strongly vary depending on the species. However, more data are needed to draw conclusions because a small number of strains of *P. ostreatus* (five strains) were analyzed in their study.

Edible mushrooms can be considered as a source of active molecules, and thus investigating whether certain factors (such as intra- and interspecies diversity or culture conditions) can enhance this potential is of importance. This study reveals that a high proportion of polysaccharides is found in *A. subrufescens* biomass. This warrants further investigations to purify certain polysaccharides of therapeutic interest from this fungus to clearly identify solid-state NMR chemical markers that would be helpful to test the effects of culture conditions on such polysaccharide concentrations.

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Funding Sources

C.L.-H. thanks CONACYT, Mexico, for a Ph.D. fellowship. This work was supported by the French ANR, Project ANR-09-BLAN-0391.

ACKNOWLEDGMENT

We thank Diego Cunha Zied for purchasing Brazilian *A. subrufescens* strains and ARP for some *A. bisporus* strains. We are grateful to Christiane Coldefy and Ludovic Devaux for mushroom production.

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6.2.2. Analyse des activités antioxydantes

Une première analyse de l'activité antioxydante, effectuée sur des sporophores entiers, est donnée dans l'article 3 (chapitre 4).

L'activité antioxydante des extraits methanoliques des primordia et des sporophores (chapeau fermé) d'*A. subrufescens* a été comparée chez le groupe de souches analysées pour l'ensemble des caractères étudiés dans les chapitres précédents. Cette étude est présentée dans l'article 7 soumis à *Journal of Agricultural and Food Chemistry*.

Le contenu de chaque numéro de la revue est accompagné, sur le site Web, d'une figure donnant une impression visuelle du travail effectué. La figure 6.2 est proposée pour illustrer l'article 7.

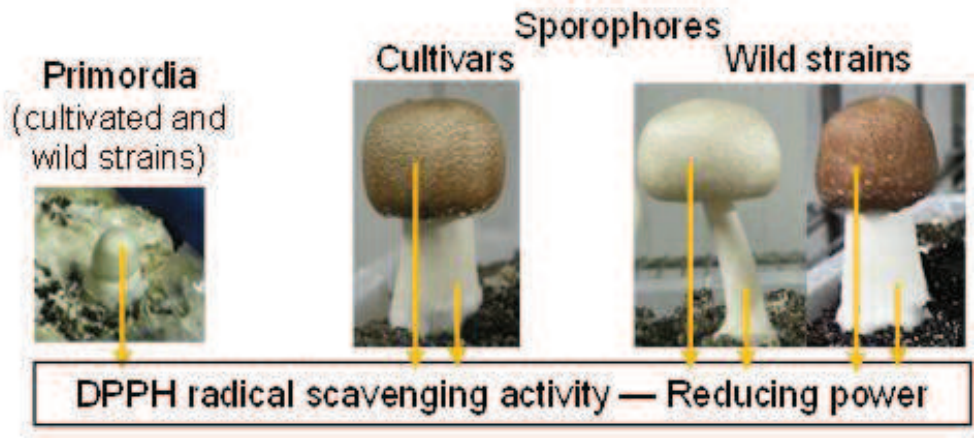

Figure 6.2. Echantillons analysés dans l'article 7 pour l'activité antioxydante chez *Agaricus subrufescens*. (Photos INRA, MycSA)

Article 7

Antioxidant activities in primordia, stipes and caps of cultivars and wild strains of the almond mushroom *Agaricus subrufescens*.

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ABSTRACT.

This work compared the DPPH radical scavenging activity and reducing power of methanolic extracts from eight strains of *Agaricus subrufescens*. Primordia had better antioxidant activity than sporophores (veil closed) in all the strains but one. DPPH radical scavenging activity and, to a lesser extent, reducing power were higher or similar in the cap than the stipe depending of the strain. No relationship was found between cap colour and antioxidant activities. Two wild strains characterized by good productivity and antioxidant capacities that varied little with the batch of substrate showed advantages compared to the tested cultivars for development as functional food or nutraceuticals.

KEY WORDS: *Agaricus brasiliensis*, *Agaricus blazei*, cultivar, wild strain, harvest.

INTRODUCTION

Mushrooms are widely recognized as a functional food and a source of various physiologically active compounds for the development of new drugs and nutraceuticals. [1] There have been recent investigations on the antioxidant properties of extracts from various cultivated and wild edible species. [2-8] Among the antioxidant properties, various phenolic

compounds, [3,4,9] flavonoids, ascorbic acid [3] and polysaccharides [5] are recognized as antioxidant in mushrooms.

Agaricus subrufescens Peck is formerly known in the literature as *A. blazei* Murril *sensu* Heinemann, or *Agaricus brasiliensis* Wasser et al. and is also called Almond Mushroom, Himematsutake and Cogumelo do Sol. [10] The medicinal properties of the culinary-medicinal mushroom *A. subrufescens* are known for more than three decades and several reviews analysed its importance as functional food or for medicinal purposes. [11-14]

Reactive oxygen species (ROS) are by-products of normal metabolism or are produced during stresses. They may cause extensive damage to DNA, proteins and lipids, and interfere with vital cellular functions. Complex antioxidant defense mechanisms within the organisms have evolved to limit the levels of reactive oxidants and the damage they inflict, [15] but these mechanisms are often inadequate to completely prevent oxidative stress-induced damage. Antioxidant supplements, or natural products containing bioactive compounds, may be used to reduce oxidative damage to the human body. [16] Edible mushrooms are good candidates for purchasing such natural products. Works have been done about their antioxidant activity, but there are few published data for *A. subrufescens.* [17-20]

The choice of the optimal stage of harvest and the part of the fruiting body containing the higher amount of bioactive compound is important to improve the use of *A. subrufescens* as a functional food. The content of bioactive compounds may vary within a species with differences between strains, cultivation techniques and maturity of harvest. [21] To our knowledge, no reports have been done about changes in antioxidant activity during the development of *A. subrufescens* from primordia to fruiting bodies. On another hand, antioxidant activity had been measured in *A. subrufescens* fruiting bodies with closed caps compared to mushrooms with open caps, [22,23] and between cap and stipe, [24] but all these works concerned cultivars which are suspected by Neves et al. [25] to have a common origin, based on genetic studies.

In a previous work we studied a group of Brazilian cultivars and a group of wild *A. subrufescens* and observed a large variability between the groups in antioxidant activity measured in fruiting bodies. [20] The aim of the present work was to identify strains and mushroom tissue with high potentials as source of antioxidant activities. Both cultivars and wild strains of *A. subrufescens* were cultivated on compost and antioxidant activities of methanolic extracts were analysed for clarifying whether they varied with the development stage (primordium *vs* fruiting body with closed cap) and the parts of the mushroom (stipe *vs* cap).

MATERIALS AND METHODS

***A. subrufescens* strains.** Eight strains were analysed: three cultivars (CA561, CA565 and CA570) kept in the Collection of Germplasms of Agarics in Bordeaux (CGAB), INRA-Bordeaux, since 2007; CA454 kept in the CGAB collection since 2006; three wild European strains of *A. subrufescens* (CA438-A, CA487 and CA643); and one hybrid provided by E. Huang and P. Callac who crossed homokariotic single spore isolates of the Brazilian strain CA454 and the French strain CA487. CA454 is a subculture of the collection strain WC837 in PSUMCC assumed to be similar to ATCC 76739, which, according to ATCC, was originally provided by T. Furumoto, used by Mizuno et al., [26] and would be at the origin of the new cultivation of this mushroom. The three Brazilian strains and CA454 showed brownish gold caps, CA 643 brown caps, CA487, CA438-A and the hybrid light cream caps.

Cultivation conditions. The substrate used to cultivate the strains was compost prepared for commercial production of the button mushroom *Agaricus bisporus*, and provided by Renaud SA, Pons, France. The main ingredients for composting were wheat straw and horse manure. Composting was performed indoor. The casing soil was a mixture of 45% peat, 20% limestone, 35% fine sand; v / v / v. The eight strains were grown in trays of 0.09 m² filled with 8 kg of compost inoculated with 1% spawn. Incubation was performed at 25 °C, 85% humidity, for 15 days. After casing, the trays were left under the same environmental conditions for a 7-day post-incubation period. To initiate fruiting, the room temperature was set at 23-25 °C with 95-97% humidity and CO_2 concentration lower than 1200 ppm. Four replicates were prepared for each strain and the yields were estimated by collecting all the mushrooms produced until 65 days after casing.

Samples. Experiment 1 was performed with a single batch of compost. The mushrooms were harvested during the first flush, at two development stages: primordium (P, 10-15 mm high, before gill development) and fruiting body at commercial stage considered as stage 3 for *A. bisporus*. [27] The mushrooms were placed on plates on ice and immediately conditioned. Each fruiting body was cut by the middle to obtain three types of samples: one half represent the whole fruiting body (SP), the other half was separated in stipe (S) and cap (C). Samples P were composed of 7-14 primordia, samples SP, S and C were constituted from 3-6 mushrooms each. To analyse a putative effect of the cap pigmentation on the antioxidant

activities, the pileipellis was removed from caps of CA643 and CA438 using a scalpel (sample PC). The samples were immediately frozen at -80 °C and kept at this temperature until being freeze dried. In experiment 2, the culture was performed on a different batch of compost provided 9 months later by the same company and samples SP were prepared as described above.

Antioxidant activity. For *A. subrufescens*, methanol is considered a good extraction solvent for antioxidant activity test compared to ethanol or water. [22] Antioxidant activity was determined in methanolic extracts as radical scavenging activity on 1,1-diphenyl-2-picrylhydrazyl radicals ($DPPH^{\bullet}$) and reducing power using the methods previously described in Savoie et al. [28] Freeze dried samples were ground to powder (particle diameter < 0.1 mm). Methanolic extracts were prepared from 3 g subsamples of lyophilised powder in 100 mL of methanol HPLC gradient grade. The effective concentration at which DPPH radicals were scavenged by 50 % (EC_{50} value expressed as mg extracted mushroom powder mL^{-1} reactive mixture) was obtained by interpolation from linear regression of absorbance measured with different concentrations of extracts. The percent DPPH radical scavenging effect was calculated according to the following equation:

Radical scavenging activity (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100$

where $A_{control}$ and A_{sample} are the absorbance of control without and with sample, respectively. The reducing power was reported as the effective concentration of extract at which the absorbance was 0.25. Ascorbic acid was used as a standard.

Statistical analyses. Data were analysed by variance analyses (ANOVA) followed, when necessary, by the Duncan's test to identify statistical differences. ANOVA with contrast was performed to compare the wild strains to CA454 and the cultivars for antioxidant activities.

RESULTS

Change of antioxidant activity with the development stage

All strains but CA565 showed higher antioxidant activities in the primordia compared to the whole sporophores, although differences were not significant for CA487 ability to scavenge DPPH radicals (Table 1) and for the reducing power of CA561 (Table 2). In contrast to the other strains, CA565 exhibited a higher activity in the whole sporophore whatever the method used.

Table 1. DPPH radical scavenging activities of methanolic extracts from eight *A. subrufescens* strains harvested at the primordium and sporophore stages.

strain	EC_{50} values (mg mushroom mL^{-1})			
	primordium	whole sporophore	cap	stipe
CA438-A	1.49 c[a] BCD[b]	2.17 b BC	1.72 c BC	2.84 a B
CA487	1.74 b AB	2.06 ab BC	1.88 b BC	2.37 a BC
CA643	1.56 c BC	2.48 b B	2.19 b AB	2.86 a B
Hybrid	1.04 d CD	1.86 b C	1.49 c CD	2.16 a C
CA454	2.12 b A	3.17 a A	2.47 b A	3.49 a A
CA561	0.95 b D	1.38 a D	1.42 a CD	1.35 a D
CA565	1.43 a BCD	1.13 b D	1.13 b D	1.13 b D
CA570	1.00 b CD	1.95 a C	1.75 a BC	2.12 a C

[a] Within a line, values followed by the same lower case letter are not different at $p = 0.05$ by the Duncan's test.
[b] Within a column, values followed by the same capital letter are not different at $p = 0.05$ by the Duncan's test.
EC_{50} of radical scavenging activity for ascorbic acid was 0.023 mg mL^{-1}

Table 2. Reducing power of methanolic extracts from eight *A. subrufescens* strains harvested at the primordium and sporophore stages.

strain	Concentration (mg mushroom mL^{-1}) leading to 0.25 absorbance			
	primordium	Whole sporophore	cap	stipe
CA438-A	2.64 c[a] BC[b]	3.87 b CD	2.75 c D	5.50 a BCD
CA487	2.90 c BC	4.30 b BCD	3.18 c CD	5.89 a B
CA643	4.20 c A	6.57 b A	4.29 c AB	9.49 a A
Hybrid	2.65 d BC	4.77 b BC	3.60 c BC	5.69 a BCD
CA454	3.63 c AB	5.33 a B	4.48 b A	5.73 a BC
CA561	2.24 b C	3.23 ab D	2.96 ab CD	3.65 a CD
CA565	3.67 a AB	3.17 b D	2.96 c CD	3.30 b D
CA570	2.85 c BC	4.20 ab BCD	3.70 bc ABC	4.65 a BCD

[a] Within a line, values followed by the same same lower case letter are not different at $p = 0.05$ by the Duncan's test.
[b] Within a column, values followed by the same capital letter are not different at $p = 0.05$ by the Duncan's test.
The reducing power measured with ascorbic acid was 0.018 mg mL^{-1}.

Caps of the wild strains, CA454 and the hybrid showed higher DPPH radical scavenging activity compared to stipe whilst similar activity was measured in caps and stipes of cultivars. The reducing power in the caps was higher compared to that found in the stipes in all strains but CA561.

The type of sample showing the highest antioxidant activities was primordium for the hybrid, primordium or cap for the wild strains CA438-A and CA487, and cap for CA565. In strains CA561, CA570 and CA643, the highest radical scavenging activity was measured in the cap, but similar reducing power was found in primordium and cap. The opposite was observed with CA454.

Variability of antioxidant activity between the strains

ANOVAs with contrast showed that the group of wild strains did not differ from the group consisting of CA454 and cultivars for scavenging activity and reducing power in primordia (p = 0.355 and 0.761, respectively) and in caps (p = 0.312 and 0.772, respectively). When compared to the other strains, the cultivars CA561 and CA565 exhibited significant higher DPPH scavenging activities in the sporophore and the stipe. Irrespective of the sample, the hybrid did not differ significantly from its parent CA487 (Table 1).

Radical scavenging activity and reducing power of the light cream strains (CA438-A, CA487 and the hybrid) were in the same range than those of the brown CA643 and the brownish gold cultivars CA561, CA565 and CA570 (Tables 1 - 2). In addition, no significant difference in DPPH scavenging activity was detected between caps with or without pileipellis (Table 3) either in the light cream strain CA438-A or in the brown strain CA643. The reducing power was reduced when the pileipellis of CA438-A was removed.

Variations in antioxidant activities in mushrooms collected in different experiments

The eight strains showed significant differences in total mushroom biomass produced in two different crops despite the control of the climatic conditions. Mushroom yield of wild strains ranged from 149 to 189 g kg^{-1} substrate in experiment 1, and from123 to 227 g kg^{-1} in experiment 2, whilst cultivars and CA454 produced 7.1 to 28 g kg^{-1} in experiment 1 and 13 to 91 g kg^{-1} in experiment 2. The wild strains were early fruiting compared to the other strains, irrespective of the experiment. Antioxidant capacities varied significantly with the experiment. The DDPH scavenging activity was higher in experiment 1 than in experiment 2 for CA438-A, CA487 and CA 565 whilst it was the opposite for the other strains but the hybrid, and greater differences between experiments were observed (Fig. 1).

Table 3. Effect of the pileipellis on antioxidant activities of methanolic extracts from a cream (CA438-A) and a brown (CA643) strain of *A. subrufescens*.

treatment	radical scavenging activity EC_{50} values (mg mushroom mL^{-1})		reducing power (mg mushroom mL^{-1}) leading to 0.25 absorbance	
	CA438-A	CA643	CA438-A	CA643
peeled	1.720 a	2.187 a	3.87 a	4.94 a
not peeled	1.364 a	2.219 a	2.75 b	4.29 a

Within a column, values followed by the same letter are not different at $p = 0.05$ by the Duncan's test

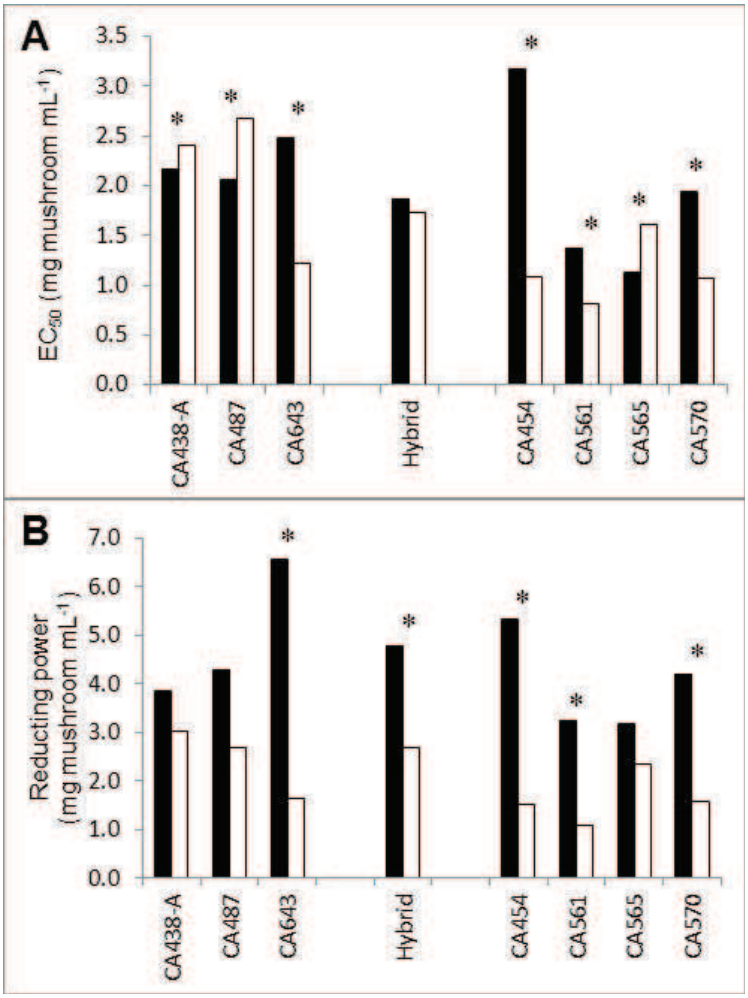

Figure 1. Comparison of DPPH radical scavenging activity (A) and reducing power (B) of the *A. subrufescens* strains on two different batches of compost.
Dark bars = experiment 1, white bars = experiment 2. * = significant difference between the two experiments at $p<0.05$, Duncan's test.

The Pearson coefficient between mushroom yields and DPPH EC_{50} values (r = 0.785 p = 0.037 for experiment 1 without CA454 (poorly yielding, 7.1 g kg^{-1}), and r = 0.753, p = 0.031 for exp. 2, all strains) showed that the more the strain was productive, the more its scavenging activity of DPPH radicals was low. No significant correlation (p = 0.05) was observed between mushroom yield and the level of reducing power. A common effect of the experiment was observed; all the strains had higher reducing power in experiment 2, but the differences between experiments were not significant for CA438-A, CA487 and CA565.

DISCUSSION

Radical-scavenging activity in the sporophore of the eight evaluated strains ranged among EC_{50} values of 1.13 to 3.17 mg mushroom mL^{-1}. Some works on the antioxidant properties of *A. subrufescens* cultivars have been made, but data varied greatly depending on the strain, technique of extraction and method of antioxidant activity measurement. Carvajal et al. [29] determined EC_{50} value of 0.305 mg of extract mL^{-1} for DPPH radical scavenging using a mix of ethanol:water (70:30) for extraction. On the other hand, Soares et al. [23] have observed EC_{50} values of 3.0 and 3.2 mg of extract mL^{-1} for young and mature sporophores respectively, using methanol as extraction solvent. In the present work we expressed the activities as EC_{50} per mg of dry powder of mushroom used for obtaining the extract, because it takes into account the extraction rate and it is more representative of the use of mushrooms as functional food. By a rapid conversion of the published data, we stated that the ranges of antioxidant activity observed herein among the eight strains showed efficient abilities compared to other works.

Variations in antioxidant activity during the *A. subrufescens* sporophore ripening are reported. These works concern the mushroom biology and its use as a nutritional source of antioxidant. Soares et al. [23] found no significant difference in antioxidant properties comparing DPPH radical scavenging activity and reducing power in young (cap closed) and mature (cap opened) sporophores of a Brazilian cultivar. Mourão et al. [22] analysed five Brazilian cultivars and measured higher DPPH radical scavenging activity in mushrooms with

closed cap compared to aging mushrooms with cap completely turn apart. Nevertheless, no information on the antioxidant activities in the first stage (primordium) of the *A. subrufescens* fruiting body was available in the literature. Tsai et al. [30] analysed ethanol extracts of *Agaricus bisporus* fruiting body at five development stages: pin head, veil intact (tight), veil intact (stretched), veil opened and gills well-exposed. They found the highest radical DPPH scavenging activity and reducing power in the sporophore veil intact (stretched) stage. The present work with *A. subrufescens* showed that primordia had better antioxidant activities than sporophores with closed veil in all the studied strains but one. Savoie [31] explained that growth and differentiation process during normal cellular metabolism have a balance between ROS generation and ROS elimination. At fruiting initiation in *A. bisporus* a transitory oxidative burst implies a finer regulation of the redox balance by increases in the antioxidant activities. Consequently, the highest antioxidant activity observed in primordia could result from antioxidant mediated defences necessary to protect the cells from the high levels of ROS generation at this critical stage of mushroom differentiation.

Beyond the development stage of sporophores, the antioxidant activity can differ according to the sporophore part. For DPPH radical scavenging activity and to a lesser extent for reducing power, activities in the cap were found either higher or similar to those present in the stipe, depending on the strain. Geosel et al. [24] analysed two cultivars of *A. bisporus* and seven cultivars of *A. subrufescens* by the FRAP method and found all strains but two *A. subufescens* cultivars with higher antioxidant activity in the cap than in the stipe. Investigations on wild edible mushrooms revealed that the cap contributed better to the DPPH radical scavenging activity than the stipe in *Boletus edulis*, *Suilus granulatus* and *Russula cyanoxantha* whilst it was the opposite in *Amanita rubescens*. [32] Caps of the wild edible mushrooms *Lactarius deliciosus* and *Tricholoma portentosum* exhibited greatest antioxidant activities than stipes. [33] Despites some variability between strains and species, the presence of higher antioxidant capacities in the cap compared to the stipe is probably common in edible fungi. This is in agreement with the results of Savoie et al., [28] who found the highest antioxidant activities in the gills, since the gills are an organ where the redox reactions are important and consequently the antioxidant defences are higher.

A. subrufescens cultivars are known to be characterised by a brownish gold colour of the cap, which explains that there is no report in the literature on antioxidant capacity in *A. subrufescens* in relation to cap colour. In our work using wild strains with different cap

colours, no relationship was observed between cap colour and antioxidant activities. Similarly, analysing *A. bisporus* mushrooms at commercial stage, Savoie et al. [28] found higher radical-scavenging activity and reducing power in a cream wild strain than in a brown wild strain and a white cultivar. Removal the pileipellis by cutting it off produced a yellowish colour that could explain the decrease in antioxidant capacity we observed with CA438-A. The antioxidant activity of methanolic extracts of *A. subrufescens* appeared to depend on the strain, and probably not on the cap colour. However, more studies including a greater number of strains are needed to clarify this point.

Cultivation condition is another source of variability as well as strain, development stage or mushroom part. The base ingredients of the commercial compost used in the present work were wheat straw and horse manure, whose nutrient content varied with batches. Results from experiments 1 and 2 showed that the best batch of substrate for mushroom antioxidant activities depended on the strain. Similarly, Geosel et al. [24] reported antioxidant capacity variations between cultivars and years and suggested that substrate fluctuation may influence the polyphenol biosynthesis pathway of the mushroom. These studies performed with different substrate origins and strains pointed out the importance of the genetic and phenotypic background of the strain when the objective is to produce functional food or bioactive components.

A. subrufescens is a culinary-medicinal mushroom mainly sold as powder, extracts or tea. From a scientific point of view, the primordium and the cap of *A. subrufescens* were the samples the most interesting for antioxidant properties, but processing primordia (weak biomass, time consuming harvest) or caps (one more step in preparation compared to whole sporophore) seemed not the best choice for commercial production when antioxidant properties were taken as a whole. But it would be different if particular compounds beneficial to health could be detected in primordium and/or cap.

Antioxidant properties of *A. subrufescens* compounds soluble in methanol varied with the strain, development stage, part of the fruiting body studied and cultivation conditions. All samples showed important radical scavenging activity but primordia and caps were particularly more active. The rapid production of a large biomass easy to transform is of prime importance for commercial valorisation of *A. subrufescens* as food or dietary supplement with antioxidant properties. Besides, the antioxidant capacities of the strain should vary little with the batch of commercial substrate. This point might be the most difficult to solve, although strains like CA438-A and CA487 could have this potential. Taking into account their short time to fruiting, high yield and good sporophore antioxidant

capacities, the wild strains, especially CA438-A and CA487, showed advantages compared to the cultivars for development as functional food or nutraceutical. This potential of wild strains can be useful to improve cultivated strains through breeding programmes.

ACKNOWLEDGMENTS

We thank Diego Cunha Zied for purchasing Brazilian *A. subrufescens* strains.
Funding Sources: R.C. L.-H. wishes to thank CONACYT, Mexico, for a Ph.D. fellowship. This work is part of research funded by a bilateral cooperation between Mexico (CONACYT project 115790) and France (ANR-09-BLAN-0391-01) in the "AgaSub" project.

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6.3. Conclusions

Ce travail est le premier rapport sur la comparaison de la composition chimique de la biomasse mycélienne et du sporophore d'*A. subrufescens* en utilisant l'analyse en phase solide par Résonance Magnétique Nucléaire du ^{13}C (RMN ^{13}C).

Dans cette étude, le mycélium a été identifié comme une biomasse plus riche en polysaccharides que les sporophores. Cette information peut être d'utilité pour la recherche de composés à effet thérapeutique. Certains polysaccharides comme les β-glucanes sont des composés d'intérêt thérapeutique comme l'indique la littérature (Angeli et al., 2009 ; Camelini et al., 2005 ; Cha et al., 2011). Les produits d'*A. subrufescens* vendus dans le commerce peuvent être d'origine mycélienne ou provenir de sporophores. Plusieurs travaux ont été focalisés sur la production de biomasse mycélienne en fermenteur (milieu liquide) pour l'obtention de polysaccharides d'*A. subrufescens* (Chin-Hang et al., 2004, 2007 ; Ha-Yull et al., 2005 ; Shu et Xu, 2007).

Que ce soit pour le mycélium ou pour le sporophore, les spectres RMN n'ont pas montré de différences qualitatives au niveau des pics entre les différentes souches étudiées. Par contre, comparé au mycélium d'*A. bisporus*, celui d'*A. subrufescens* a montré un pourcentage significativement plus élevé au niveau du groupe COOH/CONH (lipidique) et moindre pour le groupe O-alkyl-C (polysaccharides).

Les niveaux d'activités antioxydantes observés dans les extraits methanoliques des primordia et de sporophores identifient ce champignon comme une source valable de composées antioxydants. Le primordium a montré l'activité antioxydante la plus élevée. Bien que le primordium ne représente pas une biomasse commercialement intéressante, ce résultat contribue à la connaissance de la biologie de l'espèce.

D'autre part, les activités antioxydantes obtenues ont montré une variabilité intra spécifique et séparent les souches sauvages des cultivars. Les trois cultivars analysés sont plus actifs que les souches sauvages. Cependant, la souche d'*A. bisporus* analysée a montré un niveau d'activité se situant dans la moyenne de ceux observés pour l'ensemble des souches d'*A. subrufescens*. *A. bisporus* a été rapporté dans la littérature comme possédant un fort pouvoir antioxydant par rapport à d'autres champignons comestibles (Liu et al., 2013 ; Savoie et al., 2008). Par conséquent, les cultivars comme les souches sauvages d'*A. subrufescens* montrent un potentiel antioxydant intéressant.

L'hybride a montré une activité antioxydante moyenne, intermédiaire entre celles de ses deux parents (CA487 et CA454). Ce résultat représente la possibilité d'améliorer la qualité chimique des sporophores par le transfert de ce caractère d'intérêt à des individus d'une descendance.

Des études complémentaires sont nécessaires pour identifier les composés responsables de cette activité antioxydante, et voir s'ils diffèrent en fonction du stade de développement du champignon.

D'après la quantité de polysaccharides contenue dans le mycélium ou le sporophore et le niveau d'activité antioxydante dans les sporophores, nous pouvons considérer *A. subrufescens*, y compris les souches sauvages, comme un aliment de qualité. En effet, les rapports protéines/polysaccharides observés sont proches de ceux de *Lentinula edodes*, un autre champignon réputé pour ses vertus médicinales.

Chapitre 7. Discussion générale

Les résultats obtenus dans ce travail contribuent à la connaissance de la biologie d'*A. subrufescens* et à l'évaluation de souches de différentes origines géographiques pour leur diversité génétique et phénotypique en vue d'identifier des caractères d'intérêt agronomiques et des propriétés d'aliment fonctionnel après adaptation de la culture en France.

7.1. Diversité et optimisation de la culture

La diversité intra spécifique est capitale pour les programmes d'amélioration de la culture d'un champignon et pour son utilisation comme aliment fonctionnel. En effet, la diversité du matériel génétique est d'un grand intérêt pour éviter les risques liés à un '*monolineage'* (lignée de même génotype). Avec cette uniformité génétique des souches, la culture des champignons est plus vulnérable aux pathogènes et aux stress environnementaux. Ainsi, l'identification et l'exploitation des ressources génétiques sauvages pour élargir la variabilité génétique est prometteuse pour développer de nouvelles souches résistantes à des maladies ou leur adaptation à des conditions culturales ou aux changements climatiques (Callac et al., 2002 ; Foulongne-Oriol et al., 2009).

Dans la culture d'*A. bisporus*, il a été rapporté que les hybrides et cultivars commerciaux existants sont proches d'une culture '*monolineage'* (Foulongne-Oriol et al., 2009). Les travaux présentés dans le chapitre 4 confirment l'absence de polymorphisme chez les cultivars et montrent, avec les marqueurs microsatellites, un haut niveau de polymorphisme génétique chez les souches sauvages. Ce matériel ouvre la voie à une amélioration génétique chez *A. subrufescens*. Ce travail est le premier rapport d'évaluation des souches sauvages. Le seul cas d'amélioration signalé dans la littérature concerne un hybride entre une souche de Californie, USA, et une de Sao Paolo, Brésil, qui fait l'objet d'un brevet (Kerrigan et Watch, 2007).

L'ensemble des souches caractérisées génétiquement ne pouvait pas être étudié pour la variabilité phénotypique pour des raisons pratiques (capacité de la salle de culture) et de temps imparti pour ce travail. Un groupe de souches représentatives de la variabilité génétique

a été sélectionné et a fait l'objet de l'ensemble des études de variabilité phénotypique. Cependant, certains caractères ont pu être analysés sur un plus grand nombre de souches. Le groupe sélectionné constitué de sept souches (trois d'origine sauvage européenne, une considérée à l'origine de la culture au Brésil, et trois cultivars) ainsi qu'un hybride intercontinental, a été étudié pour la variabilité de réponse à des critères prioritaires pour l'introduction de la culture d'*A. subrufescens* en France :

1) l'accroissement *in vitro* à différents températures pour l'identification de la température optimale et létale,

2) la capacité de colonisation du substrat pour évaluer l'aptitude enzymatique de dégradation du compost,

3) la capacité à fructifier en fonction du pourcentage de blanc, de la quantité et de la composition de la terre de gobetage, et des conditions environnementales (nécessité ou non d'un choc froid et de lumière pendant l'incubation, et de variations de la température pendant la fructification), en faisant, à partir de la culture d'*A. bisporus*, des adaptations simples, facilement applicables par des producteurs,

4) la réponse à deux maladies répandues en champignonnières, la moisissure verte et la tache bactérienne.

La croissance *in vitro* des champignons dépend des conditions environnementales (intensité de la lumière, température, humidité et aération) et du milieu de culture (tel que pH, sources de carbone et d'azote, facteurs de croissance). Dans cette étude, sous les conditions testées, de la variabilité a été observée pour le taux d'accroissement. Il est plus élevé chez les cultivars (6,6 – 10,7 mm jour^{-1}) que chez les souches sauvages (2,2 – 8,6 mm jour^{-1}). Chez *A. bisporus,* les taux d'accroissement *in vitro* sont assez variables, mais en général, ils sont plus faibles (3,2 – 5,4 mm jour^{-1}) (Nogueira de Andrade et al., 2010) que ceux observés dans ce travail. La température optimale pour l'ensemble des souches a varié de 25 à 30 °C, avec des écarts de même amplitude dans le groupe de cultivars et celui de souches sauvages, mais en général une température de 28 °C pourrait être utilisée pour l'ensemble des souches sans une baisse significative de la vitesse moyenne de croissance. La connaissance de la température optimale *in vitro* est une condition préalable pour la préparation du blanc des champignons comestibles au niveau commercial. Bien que le milieu solide synthétique soit différent des grains utilisés pour la préparation du blanc, tous les deux permettent un bon développement du mycélium, ce qui permet d'extrapoler les résultats observés pour l'optimisation de la préparation du blanc.

Quelques travaux sur l'effet néfaste d'une basse température sur le mycélium d'*A. subrufescens* sont disponibles dans la littérature, mais aucune donnée n'existe sur l'effet de fortes températures. Ce travail établi, pour la première fois, la température létale pour le développement mycélien *in vitro* chez un ensemble de souches d'*A. subrufescens.* Cet acquis, d'un côté permet la prévention des risques liés à une exposition à des températures extrêmes pendant la multiplication des souches, et d'un autre côté contribue à la connaissance de la biologie de l'espèce.

Dans ce travail, cinq cultivars ont montré une capacité pour la colonisation du compost (estimation *via* la mesure de la concentration en H_2O_2) plus élevée que l'ensemble des souches. Farnet et al. (2013) ont évalué six souches d'*A. subrufescens* de la collection CGAB, dont cinq communes avec les souches de ce travail, et ont montré que trois cultivars avaient une capacité plus élevée pour la colonisation du compost d'après leurs activités lignocellulosiques (dont les laccases), mais que les valeurs de H_2O_2 mesurées étaient similaires pour l'ensemble des souches étudiées. Ces auteurs ont suggéré que le dosage enzymatique est plus efficace pour estimer l'aptitude à coloniser le substrat au niveau intra spécifique. Cependant, ils ont utilisé un lot de compost et une quantité (450 g) différents et effectué les analyses après une incubation de 15 jours au lieu de 21 jours pour des unités de 150 g dans notre étude où les cinq souches donnant les plus fortes concentrations en H_2O_2 comprennent les trois cultivars possédant les fortes activités laccases (CA572, CA646 et CA647), un cultivar qui ne diffère pas statistiquement de CA646 et CA647 pour cette activité, et un cultivar non étudié par Farnet et al. (2013). Ceci suggère que les mesures enzymatiques permettent de détecter de la variabilité au début de la colonisation mais que la mesure de H_2O_2 permet de détecter des différences d'envahissement du compost après quelques semaines, comme montré précédemment par Savoie et al. (2007) chez *A. bisporus* et *Pleurotus* spp.

La variabilité pour la colonisation du compost a été observée sous une seule température d'incubation, 25 °C, alors que la température optimale varie de 25,8 à 30,2 °C. Malgré tout, les huit souches sélectionnées pour les tests d'amélioration de la culture ont montré une colonisation active après 20 jours d'incubation à 25 °C, donc une bonne aptitude à utiliser les ressources nutritives du compost. Il y a plusieurs facteurs qui affectent la production d'enzymes lignocelulosiques dans le compost, d'une part la nature des sources d'azote, le matériel lignocelulosique, le niveau d'humidité (important pour la dégradation de la lignine)

et d'autre part, la souche étudiée (Colauto et al., 2010 ; Elisashvili et al., 2008 ; Sharma et Arora, 2010).

Les substrats (compost et terres de couverture) élaborés au Brésil pour la culture d'*A. subrufescens* sont faits à partir de matières premières locales qui ne sont pas disponibles en France. *A. subrufescens* est un champignon saprophytique, qui pousse naturellement dans des litières forestières en décomposition. Face à la nécessité d'un substrat homogène et standardisé pour l'obtention de sporophores, afin d'observer leurs caractères phénotypiques chez les souches étudiées, la première étape a été d'évaluer si le compost commercial et la terre de gobetage préparés pour la culture d'*A. bisporus* peuvent convenir (conditions standard de culture). La fonction du compostage est de préparer un milieu nutritif sélectif pour le champignon de manière à assurer le développement du mycélium et l'exclusion des organismes compétiteurs (Stamets et Chilton, 1983). Dans un premier temps, un essai de culture à petite échelle en conditions standard a montré que l'ensemble des cultivars et des souches sauvages d'*A. subrufescens* était capable de fructifier sur ce compost commercial et présentait de la variabilité phénotypique (précocité, rendement, couleur du chapeau). Cette variabilité a été confirmée lors du passage à une culture à moyenne échelle, en conditions standard. Une importante variabilité phénotypique dans le rendement et la précocité a été observée chez les cultivars qui sont génétiquement identiques. Ce phénomène a été rapporté pour *A. bisporus* et des explications avancées. Moquet et al., (1998) ont démontré chez *A. bisporus* var. *bisporus*, qui possède un cycle pseudohomothallique prédominant, que l'auto- reproduction (obtention d'une souche à partir d'une spore hétérocaryotique binuclée) conserve l'hétérozygotie mais induit de la variabilité phénotypique due au réarrangement de la distribution des loci entre les deux noyaux lors de la méiose. Plusieurs laboratoires ont ainsi généré de « nouvelles souches » à partir de spores des lignées pseudo-clonales d'*A. bisporus* qui conservent toute l'hétérozygotie parentale (Kerrigan, 1990). D'autre part, des mutations somatiques peuvent se produire pendant la propagation végétative du mycélium, comme par exemple lors de sa conservation en collection. Le cycle de vie d'*A. subrufescens* est mal connu, cependant Kerrigan et Wach (2008) considèrent qu'il s'agirait d'un cycle amphithallique, en partie hétérothallique et en partie pseudohomothallique. De ce fait les observations rapportées pour *A. bisporus* peuvent s'appliquer à *A. subrufescens*. Les analyses génétiques et phénotypiques sont deux approches complémentaires pour la gestion optimale du matériel génétique (Foulongne-Oriol et al., 2011).

La variabilité phénotypique observée chez *A. subrufescens* dans cette étude peut être utilisée pour l'amélioration de la production. Dans les conditions standard de culture utilisées au départ, les cultivars ont montré une faible production tandis que les souches sauvages avaient un meilleur rendement, proche de celui d'*A. bisporus*. Une optimisation de la production, tout en maintenant des conditions facilement adaptables par les producteurs de champignons de Paris, a été entreprise. En plus de l'aspect production, les conditions optimisées devraient favoriser l'expression des caractères, permettant ainsi une meilleure analyse de la diversité phénotypique**.**

Deux voies d'amélioration culturales sont possibles : en modifiant les facteurs agronomiques et/ou les facteurs environnementaux.

Les paramètres agronomiques déterminés (pourcentage du blanc, composition et épaisseur de la terre de couverture) ont permis d'augmenter le rendement et la précocité. Ces paramètres ont eu un effet plus ou moins marqué sur le rendement des souches. Le pourcentage du blanc utilisé au Brésil pour l'inoculation du compost oscille entre 1 et 3% du poids du compost en matière humide (Eira, 2003). Cette quantité reste élevée par rapport au 0,7% utilisé pour *A. bisporus*. Dans cette étude, le lardage à 1% a conduit au même rendement que 2 ou 3%. La terre de gobetage C1 (mélange utilisé pour *A. bisporus* avec ajout de sable) apportée en couche de 5 cm (le double de la quantité utilisée pour *A. bisporus*) est le facteur qui a contribué le plus à l'augmentation significative du rendement chez toutes les souches.

Les résultats des facteurs environnementaux testés (lumière, choc froid pendant l'incubation et variation de température pendant la fructification) ont permis d'optimiser la culture. La lumière peut agir comme un inducteur de la fructification dans la culture d'espèces comme *Pleurotus* sp. et *Lentinula edodes* (shiitake). Toutefois, *A. subrufescens* comme *A. bisporus*, n'a pas besoin de lumière pour l'induction et le développement des fructifications. Un choc froid et des variations de températures sont utilisés pour la production d'*A. bisporus*. D'abord, une réduction drastique de la température de 25 °C à 17 °C induit la fructification, ensuite, une courte période à 25 °C après les récoltes est appliquée par des champignonnistes pour resynchroniser les volées. La même étude a été appliquée chez *A. subrufescens* (Zied et al., 2012). D'autres études ont été réalisées en serre avec des conditions semi contrôlées (ventilation) ou non contrôlées. Dans ce travail nous avons testé pendant la fructification des variations de température similaires aux oscillations jour/nuit mentionnées pour les cultures en serre au Brésil. Ces variations de température n'ont pas

montré d'effet significatif sur le rendement et n'ont amélioré la précocité que pour un cultivar. Cependant, cette étude a montré que des variations entre 17 et 28 °C n'affectaient pas la fructification, ce qui peut permettre au producteur une économie d'énergie en faisant un contrôle de température moins strict que celui appliqué pour *A. bisporus* pendant la période estivale. Les matériaux à utiliser et les conditions définies pour l'incubation et la fructification sont facilement transposables pour une culture dans des installations utilisées pour produire *A. bisporus*.

Le groupe de souches sélectionnées (3 souches sauvages, CA454, 3 cultivars et 1 hybride intercontinental) pour l'ensemble des essais culturaux permet d'évaluer la variabilité des souches entre elles, mais aussi leur sensibilité aux variations culturales.

Les précocités observées parmi les souches évaluées d'*A. subrufescens* se situent entre 14 et 33 jours après le gobetage. Cette variabilité est déterminée par la diversité des souches, mais certains paramètres agronomiques comme le mélange de gobetage améliorent notablement la précocité.

Les matériaux utilisés pour la préparation du compost peuvent varier dans leur composition chimique, affectant les éléments disponibles pour le champignon à la fin du compostage. Le lot de compost était différent pour chaque essai, mais plusieurs essais comportant le même traitement (1% de blanc, 5 cm de mélange de gobetage C1, température constante, pas de lumière) ont mis en évidence une variation dans le rendement qui a été attribuée principalement au compost. Cet effet a été observé globalement pour l'ensemble des souches.

Le rendement des cultivars d'*A. subrufescens* observé dans cette étude a été similaire à ceux rapportés dans la littérature pour des cultures effectuées au Brésil avec des substrats locaux (Minhoni et al., 2005 ; Zied et al., 2009a). Plusieurs travaux réalisés au Brésil ont été menés avec les cultivars pour augmenter leur rendement en modifient le compost et la terre de gobetage (Colauto et al., 2010 ; Zied et al., 2009b, 2010 et 2011), mais ils sont encore très faibles par rapport aux rendements d'*A. bisporus*. Dans toutes les conditions testées ici, les souches sauvages ont montré une meilleure performance que les cultivars, avec un rendement de 17 à 24% proche du rendement commercial d'*A. bisporus* qui est de 25 à 41% selon Kariaga et al. (2005). Cependant, les cultivars ont montré un rendement plus élevé que celui rapporté pour les cultures brésiliennes (8-16%) (Zied et al., 2010). La variabilité phénotypique observée chez *A. subrufescens* dans cette étude peut être utilisée pour l'amélioration de la production.

L'hybride intercontinental CA487-100 x CA454-3 a été testé dans la majeure partie des essais. Il a montré des caractéristiques intermédiaires par rapport à celles de ses deux parents pour la croissance mycélienne *in vitro* et *in vivo*, la précocité, le rendement et l'activité antioxydante. Il représente la possibilité d'améliorer la qualité et la performance en culture. L'hybride intracontinental H1X1, crée par Kerrigan et Wach (2008) entre une souche du Brésil (I-101) et une des Etats Unies (SBRFG), a montré une amélioration de 4 jours pour la précocité et de 257% pour le rendement par rapport au parent SBRFG. Dans notre étude, l'hybride testé est entre 270 et 300% plus productif que son parent brésilien (CA454), mais moins productif que son parent français. Par contre, un autre hybride (CA487-42 x CA454-43) est aussi productif que son parent français, qui a un fort rendement. Cette étude sur des hybrides constitue un travail préliminaire qui montre qu'une amélioration des souches cultivées actuellement est possible grâce à du matériel nouveau d'origine sauvage. Le groupe de cultivars et des souches sauvages peuvent apporter des caractères différents. Par exemple, les souches sauvages montrent une précocité et un rendement plus élevés que les cultivars, tandis que les cultivars produisent des sporophores de poids moyen plus important. Les caractères de qualité (fermeté, calibre) sont recherchés pour la création d'hybrides, en conséquence, des hybrides intercontinentaux pourraient être plus intéressants que des hybrides intracontinentaux.

7.2. Sensibilité face aux pathogènes

La résistance des souches aux maladies est parmi les caractères d'intérêt à améliorer pour la culture des champignons comestibles. De plus, dans des conditions proches d'une culture monovariétale, comme c'est le cas pour *A. subrufescens* au Brésil, il existe un risque accru face aux maladies en raison d'une possibilité d'attaque massive liée à la faible biodiversité et à l'absence de matériel de remplacement. La recherche de nouveau matériel génétique est capitale pour le développement de souches moins sensibles.

Les souches d'*A. subrufescens* ont montré de la variabilité dans la sensibilité à *P. tolaasii*, avec des réponses allant de résistante à moyennement sensible. Le nombre de souches testées est trop faible pour pouvoir généraliser et conclure à une sensibilité plus faible que celle observée chez *A. bisporus*. Des différences par rapport à la réponse chez *A. bisporus* ont été observées. La couleur de la tâche (jaune et non brune) s'explique par le fait qu'*A. bisporus* est un champignon rougissant, chez lequel la voie des mélanines intervient

dans le brunissement après une blessure ou lors de la sénescence des tissus, tandis qu'*A. subrufescens* est un champignon jaunissant. D'autre part, chez *A. bisporus*, les souches brunes sont plus résistantes que les souches blanches du fait qu'un QTL de résistance à *P. tolaasii* est situé très près de l'allèle de la couleur brune du chapeau (Moquet et al. 1999). Par contre, aucune relation entre la couleur du chapeau et la sensibilité à la bactérie n'est observée chez *A. subrufescens*.

Une importante colonisation du compost par *Trichoderma aggressivum* a été observée après 16 jours de confrontation, avec toutes les souches d'*A. subrufescens*. Mamoun et al. (2000a) ont observé que *T. aggressivum* colonise plus fortement le substrat et produit plus de spores en présence qu'en absence d'*A. bisporus* et que cette sporulation a un effet inhibiteur vis-à-vis du développement mycélien du basidiomycète. Dans le cas d'*A. subrufescens*, cette interaction n'est pas observée. Mais le plus important est que deux souches sauvages ont présenté une sensibilité moins forte que les autres, ce qui laisse supposer la possibilité de trouver du matériel intéressant dans la nature. Anderson et al. (2001) ont observé une résistance plus élevée chez des hybrides d'*A. bisporus* à chapeau brun en comparaison avec des hybrides à chapeau clair. Par contre, Mamoun et al. (2000b) n'ont pas observé de relation entre la couleur du chapeau et la sensibilité chez 25 souches sauvages d'*A. bisporus*. De même, dans cette étude, nous n'avons pas observé de relation liée à la couleur du chapeau. Ceci n'est pas surprenant, car *T. aggressivum* entre en compétition avec le mycélium végétatif pour la colonisation du compost, et n'est qu'un pathogène de faiblesse pour le sporophore. Le nombre de souches étudiées par Anderson et al. (2001), 3 brunes et 4 claires, et le fait que ces dernières soient apparentées, pourraient expliquer les résultats observés par ces auteurs.

7.3. Analyse chimique et pouvoir antioxydant

Le terme aliment fonctionnel attribué aux champignons comestibles est dû à leur valeur nutritive riche et leur teneur en élément(s) possédant des propriétés neutraceutiques ou thérapeutiques. Pour être considéré comme aliment fonctionnel, les conditions d'utilisation et la valeur nutritionnelle, la composition chimique ou la caractérisation moléculaire du produit doivent être connues (Vinhal Costa Orsine et al., 2012).

A partir des résultats des analyses chimiques par RMN du ^{13}C en phase solide et des activités antioxydantes, les souches d'*A. subrufescens* étudiées peuvent être considérées comme un aliment fonctionnel riche en antioxydants et polysaccharides.

Les analyses par RMN ont montré des différences qualitatives et quantitatives entre le mycélium et les sporophores. Le mycélium est plus riche en polysaccharides que les sporophores. Plusieurs études ont montré l'intérêt des polysaccharides et en particulier des β – glucanes extraits d'*A. subrufescens* comme produits neutraceutiques et leur bénéfice pour la santé humaine (Smiderle et al., 2013 ; Angeli et al., 2009 ; Blafkova et al., 2004 ; Camelini et al., 2005 ; Ha-Yull et al., 2005), ce qui rend intéressante la quantité de ce polysaccharide contenu dans le champignon. Cependant, les cultivars ont montré une vitesse d'accroissement mycélien plus élevée que celle des souches sauvages, tandis que ces dernières ont eu un rendement plus important. Par conséquent, plus d'études sont nécessaires pour identifier la meilleure source de polysaccharides de qualité.

Les activités antioxydantes des échantillons ont montré une variabilité intra spécifique et séparent les cultivars des souches sauvages, les premiers ayant une activité plus élevée. Malgré cette différence d'activité, toutes les souches ont une activité antioxydante importante en considérant que des valeurs EC_{50} au-dessus de 10 mg mL^{-1} sont considérées comme importantes (Mau et al., 2004).

L'activité antioxydante est un critère important pour identifier un aliment thérapeutique ou une source de composés antioxydants naturels. Les souches les plus productives comme CA438-A et CA487 représentent un matériel intéressant pour ses propriétés antioxydantes. Les fortes activités antioxydantes observées dans les primordia représentent une avancée au niveau cognitif. Cependant, au niveau pratique, ils peuvent être moins intéressants car ils ne représentent pas une biomasse aussi importante que les sporophores.

Dans ce travail, une variation de l'activité antioxydante a été identifiée en fonction des lots de compost, c'est-à-dire que la qualité des matériaux de base peut avoir un effet sur les activités antioxydantes. D'autres études sont nécessaires pour préciser les composés qui induisent ces modifications. Les activités antioxydantes n'ont pas été mesurées dans le mycélium. Cette estimation permettrait de voir si le mycélium, qui se produit très bien en fermenteur (Han 2004) est un meilleur matériau que le sporophore pour valoriser cette propriété.

Conclusions et perspectives

L'amélioration quantitative et qualitative de la culture du champignon comestible et médicinal *Agaricus subrufescens* Peck devrait pouvoir s'appuyer sur l'amélioration génétique qui pourra être mise en œuvre suite au développement de connaissances sur la variabilité intra-spécifique pour le rendement, la résistance aux maladies, le potentiel comme aliment de haute qualité nutritionnelle et la teneur en des composées bioactifs.

Des cultivars et des souches sauvages d'*Agaricus subrufescens* Peck de différentes origines géographiques ont été caractérisés génétiquement et phénotypiquement. Une importante variabilité intra spécifique a été observée pour plusieurs caractères d'intérêt pour la biologie de l'espèce et son utilisation. Les souches cultivées au Brésil ont montré une absence de polymorphisme, ce qui confirme leur origine commune probable. Elles ont montré de la variabilité phénotypique, mais les risques liés à une culture monovariétale demeurent. D'autre part cette étude a permis d'identifier un haut niveau de polymorphisme parmi les souches sauvages. L'introduction, dans les cultivars, de ce nouveau matériel génétique permettra d'obtenir des souches qui limiteront les risques de pertes massives de production causées par des maladies ou des stress environnementaux.

Les approches génétiques et phénotypiques sont complémentaires pour la gestion optimale du matériel fongique avant d'entreprendre des travaux de création variétale avec pour objectif l'amélioration de l'espèce du point de vue agronomique et comme aliment fonctionnel.

L'analyse phénotypique a montré que le potentiel pour les différents caractères d'intérêt varie avec la souche. Globalement, les souches sauvages ont montré une meilleure précocité et un plus fort rendement en culture que les cultivars, tandis que ces derniers produisent des champignons plus fermes et généralement plus gros. Par contre, le groupe de souches sauvages et le groupe de cultivars ne diffèrent pas significativement pour le pouvoir antioxydant et la sensibilité à la tache bactérienne.

La création des hybrides européens ou intercontinentaux a démontré la capacité de transférer dans une descendance des caractères d'intérêt agronomiques et/ou biologique comme le rendement ou la couleur. Cependant, *A. subrufescens* est un champignon culinaire

et médicinal, par conséquent, l'amélioration devra porter non seulement sur le rendement, mais aussi sur les propriétés telles que le pouvoir antioxydant et la teneur en composés d'intérêt neutraceutique et/ou thérapeutique.

La caractérisation chimique d'*A. subrufescens* par RMN du solide (^{13}C) a montré qu'entre le mycélium et le sporophore, il existe des différences quantitatives et qualitatives au niveau des polysaccharides. Une purification des extraits devra être faite pour identifier ces polysaccharides et isoler ceux qui peuvent avoir une activité neutraceutique.

Dans le cas d'*A. subrufescens,* la production est en général ciblée sur des champignons déshydratés pour l'élaboration de produits à intérêt médicinal. L'optimisation de la culture a été réussie à partir de modifications des paramètres agronomiques et environnementaux définis pour la culture d'*A. bisporus*. Le compost commercial utilisé pour *A. bisporus* a servi de substrat standard pour la production des sporophores d'*A. subrufescens* en donnant des résultats satisfaisants. La biologie de l'espèce (fructification à température plus élevée qu'*A. bisporus*) et les techniques d'adaptation pour sa culture offrent une alternative pour les cultivateurs de champignon de Paris en France pendant la saison estivale. L'effet de différents niveaux de CO_2 pendant la fructification devra être testé car il peut avoir une influence sur la qualité finale des sporophores (vitesse de maturation, élongation du pied), comme dans la culture d'*A. bisporus*.

Cependant, les souches les plus productives présentent des caractères, comme la fermeté des sporophores et la résistance à *T. aggressivum*, qui devront être améliorés avant un transfert de la technologie de culture vers l'industrie française du champignon. D'autres caractères n'ont pas fait l'objet de ce travail, mais devront impérativement être étudiés avant de sélectionner des souches pour un programme d'amélioration d'*A. subrufescens*. Il s'agit de la durée de vie après récolte et de la sensibilité à *Lecanicillium fungicola*, un pathogène causant des pertes dans les cultures d'*A. bisporus*.

A. subrufescens, considéré comme un champignon bénéfique pour la santé mais aussi comme un aliment exotique peut être intéressant pour le marché en frais. La forme, la couleur du chapeau et le calibre des sporophores sont des critères de qualité pour les consommateurs. Chez le champignon de Paris, de nombreux travaux ont été faits pour améliorer la forme et la couleur du chapeau. La diversité des formes et des couleurs de chapeaux observée parmi les souches de la collection montre un potentiel pour l'amélioration d'*A. subrufescens* pour le

marché en frais. Cependant des études de marché seront nécessaires étant donné que le champignon a un goût d'amande auquel le consommateur n'est pas habitué.

Les données obtenues dans ce travail contribuent à la connaissance de la biologie d'*A. subrufescens*. Le matériel sauvage offre des avantages pour la mise en marche d'un programme d'amélioration de l'espèce. Les caractères agronomiques identifiés et le potentiel comme aliment fonctionnel montrent l'intérêt de l'introduction de la culture en France en remplacement d'*A. bisporus* pendant la période estivale.

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Biologie, caractérisation chimique et activités antioxydantes du champignon comestible et médicinal *Agaricus subrufescens*

RESUME

Récemment, *Agaricus subrufescens* est devenu un champignon d'intérêt de par ses propriétés médicinales. Sa production commerciale s'est développée dans des pays tropicaux à partir de matériaux locaux. Cependant, les données disponibles dans la littérature ne concernent que des cultivars qui sont génétiquement similaires. Notre étude sur un ensemble de cultivars et de souches sauvages a conduit à une meilleure connaissance de la biologie de l'espèce. Contrairement aux cultivars, les souches sauvages présentent un haut niveau de polymorphisme génétique. Une variabilité phénotypique élevée a été mise en évidence pour l'accroissement mycélien, la productivité et la morphologie. Des analyses chimiques par RMN du solide et l'estimation des activités anti-oxydantes ont montré que les souches sauvages comme les cultivars sont une source à exploiter comme aliment fonctionnel pour prévenir les maladies cardio-vasculaire, les cancers et le diabète. L'adaptation des conditions culturales à partir du substrat utilisé pour la culture d'*A. bisporus* a permis d'obtenir des fructifications d'*A. subrufescens*, puis d'augmenter le rendement et d'optimiser des caractères agronomiques d'intérêt. Du matériel sauvage avec une activité antioxydante et un rendement élevés a été identifié. L'évaluation d'hybrides intercontinentaux a montré la possibilité de transférer des caractères d'intérêt à une descendance. *Agaricus subrufescens* pourrait être proposé aux champignonnistes français comme alternative à *Agaricus bisporus* pendant la saison estivale.

Mots clés : Antioxydant, biodiversité, qualité, rendement.

Biology, chemical characterization and antioxidant activities of the culinary medicinal mushroom *Agaricus subrufescens*

SUMMARY

Agaricus subrufescens is becoming a mushroom of interest because of its medicinal properties. Commercial production had developed in tropical countries using local materials. However, data available in the literature referred to cultivars that are genetically similar. Our study of a set of cultivars and wild strains led to a better understanding of the biology of the species. Contrary to cultivars, wild strains exhibited a high level of genetic polymorphism. High phenotypic variability was identified in mycelial growth, productivity and morphology. Chemical analyses by solid-state ^{13}C NMR and antioxidant activities showed that the wild strains as well as the cultivars proved a valuable source of functional food to prevent cardiovascular diseases, cancers and diabetes. Using cultivation substrate produced for *A. bisporus* and modifying cultivation conditions allowed fruiting of *A. subrufescens*, then increase in strain yield and optimization of agronomic traits of interest. Wild material with good antioxidant activity and high productivity was identified. The evaluation of intercontinental hybrids showed the possibility to transfer traits of interest to offspring. *A. subrufescens* could be proposed to French mushroom growers as an alternative to *Agaricus bisporus* during the summer season.

Keywords: Antioxidant, biodiversity, cultivation, quality.