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

**Régulo Carlos Llarena-Hernández · Michèle L. Largeteau · Anne-Marie Farnet · Marie Foulongne-Oriol · Nathalie Ferrer · Catherine Regnault-Roger · Jean-Michel Savoie**

**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. *Braquiaría* 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).

**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 $\mu l^{-1}$. 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

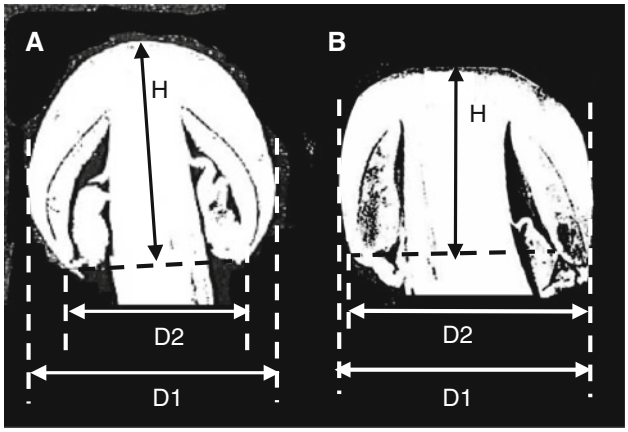


**Fig. 1** Parameters used to analyse sporophore morphology. Examples of morphology: ovoid cap (**a**) and cylindrical cap (**b**). H = cap height, D1 = widest cap diameter, D2 = diameter at the cap margin

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:

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

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,

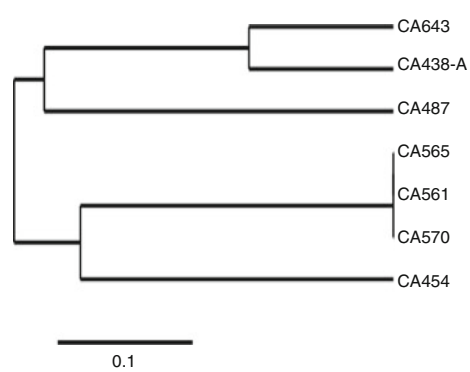


**Fig. 2** Dendrogram of genetic distances between the seven strains, based on the UPGMA analysis resulting from SSR fingerprints

**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*.

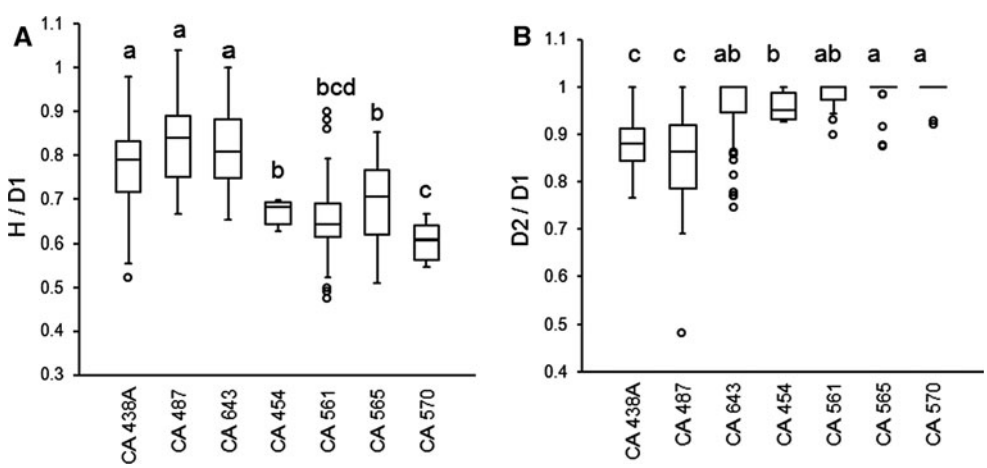


**Fig. 3** Representation of the mushroom cap morphology. **a** Distribution of the ratios cap height (H)/widest cap diameter (D1). **b** Distribution of the ratios D1 (widest cap diameter)/D2 (diameter at the cap margin)

**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

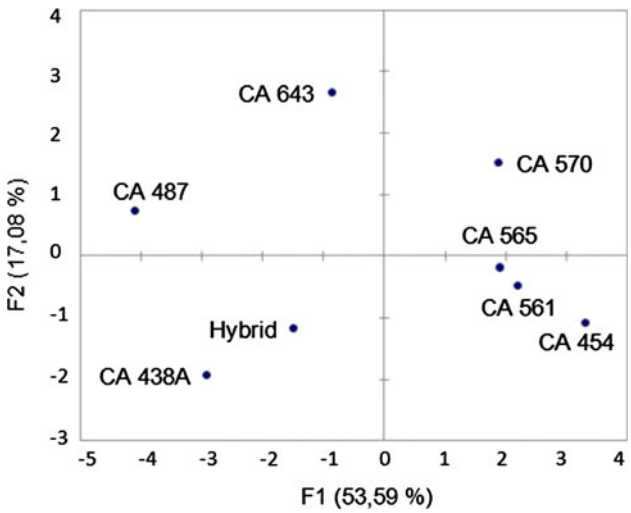


**Fig. 4** Principal component analysis of the phenotypic variation among the eight strains, namely the Brazilian cultivars (CA561, CA565, CA570), CA454, the wild *A. subrufescens* (CA438-A, CA487, CA643) and the hybrid

## 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.

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