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

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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.

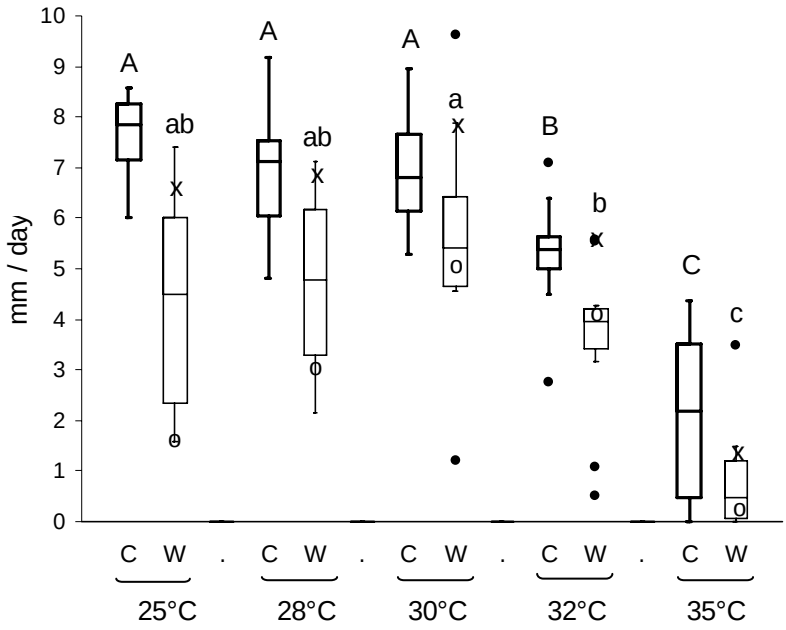


Figure 1: Distribution of the mycelium growth rate at five incubation temperatures.
C: Brazilian cultivars (C), W: wild strains. ●: data outside the distribution, x : ATCC 76739, o : hybrid.
Within a same group of strains, growth rate distributions with a same letter did not differ significantly at $p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

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.

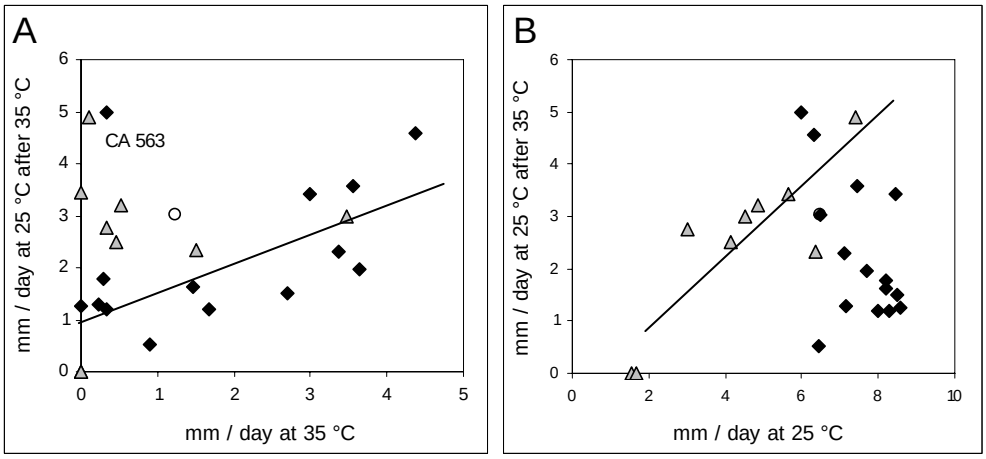


Figure 2: Radial growth rate observed at 25°C after previous 14-day incubation at 35°C compared to (A) radial growth at 35 °C and (B) radial growth at 25°C with no pre-incubation.
♦ Cultivars, ○ ATCC 76739, △ *A. subrufescens*

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).

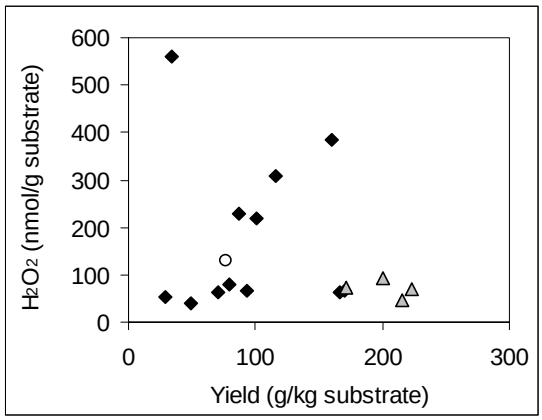


Figure 3: Concentrations in hydrogen peroxide and biomass produced by the Brazilian cultivars and the wild strains.
♦ Cultivars, ○ ATCC 76739, △ *A. subrufescens*

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}).

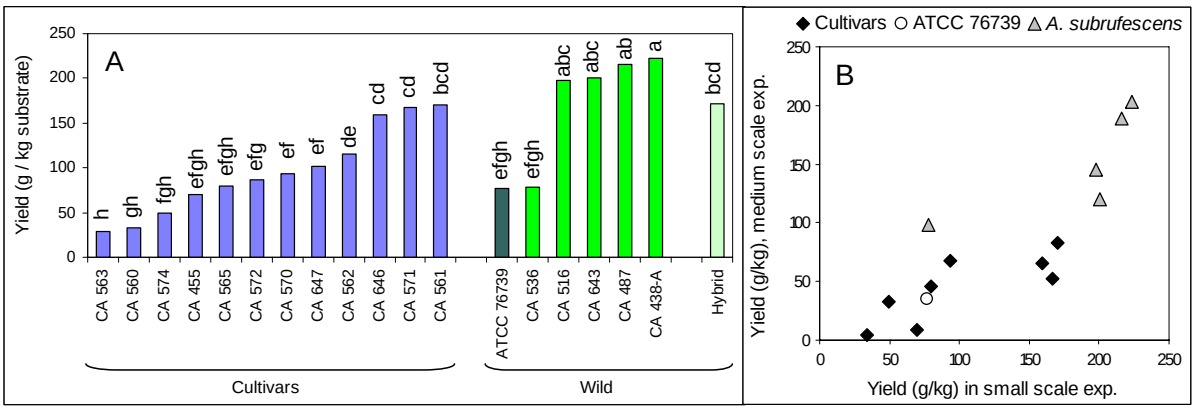


Figure 4: Mushroom yield after a 65-day fruiting period.
A: small scale experiment; B: comparison of the small and medium scale experiment.
Data for medium scale are means of 2 to 4 experiments of 4 replicates

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.

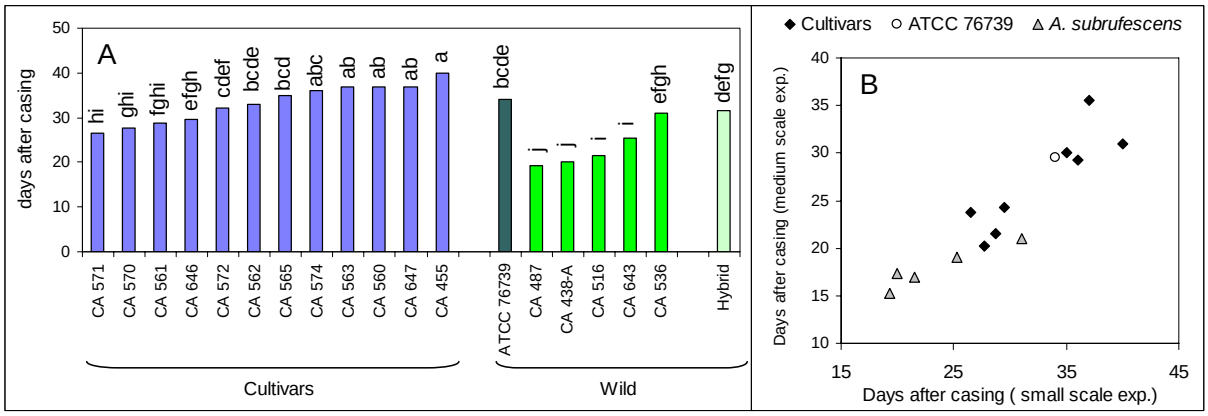
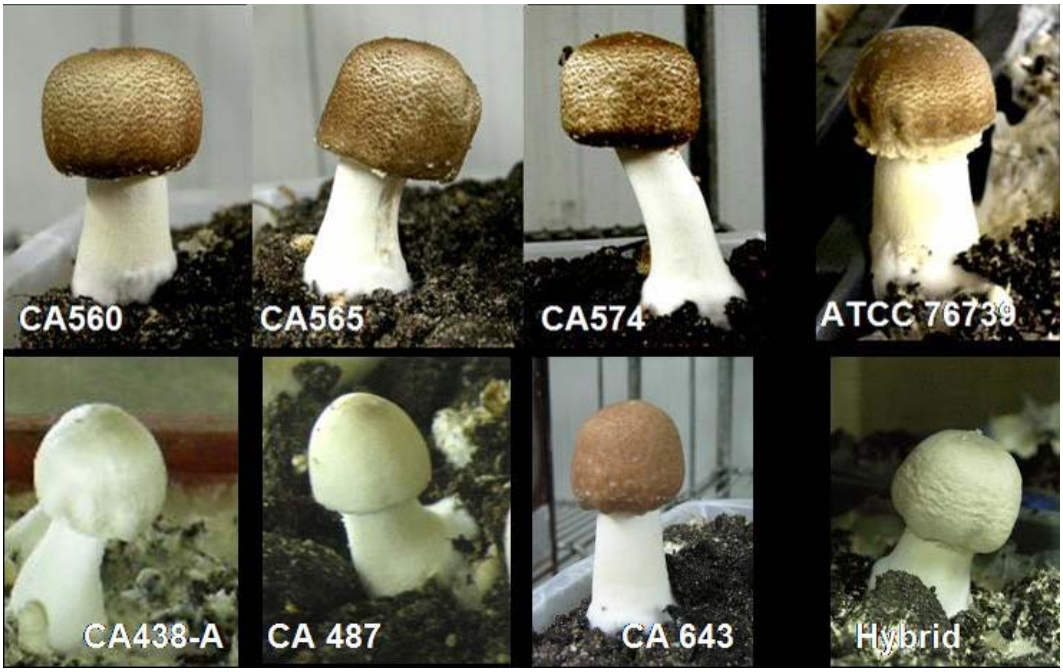


Figure 5: Time to fruiting (in days after casing) of the cultivars and the wild strains: (A) small scale experiments; (B) comparison of small scale and medium scale experiments.

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)

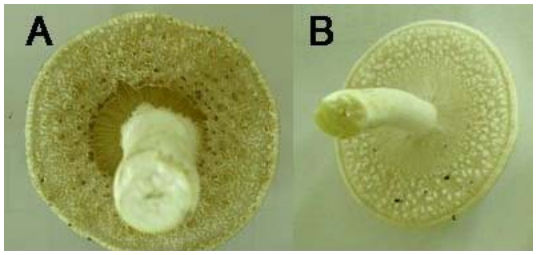


Figure 8: Veil of cultivar (A) and wild strain (B).

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*.

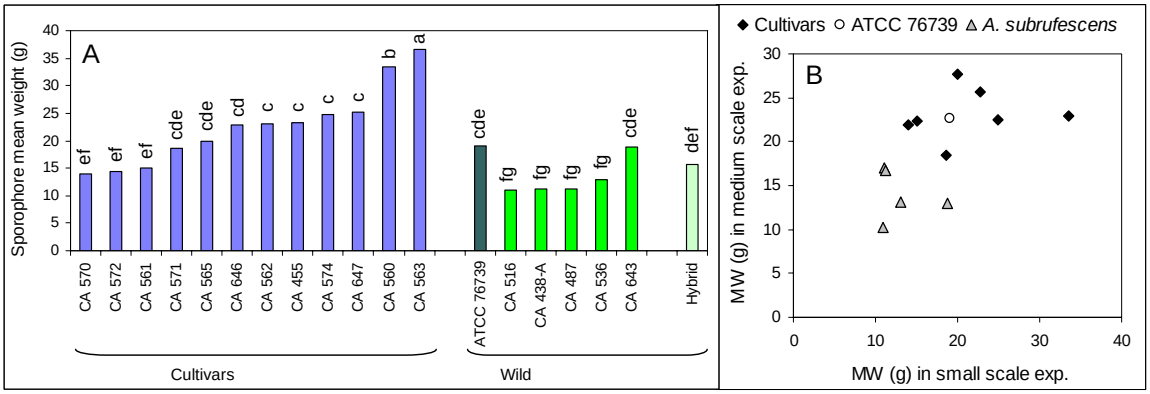


Figure 6: Mean weight of sporophores: (A) produced in small scale experiment and (B).comparison of small scale and medium scale experiments

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.*

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