



Obtaining new hybrid strains of *Agaricus bisporus* by crossing homokaryons from wild Mexican and commercial strains

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Abstract

Breeding *A. bisporus* is complicated due to its two pseudohomothallic and heterothallic life cycles resulting from the production of both heterokaryotic and homokaryotic basidiospores, respectively. This makes it difficult to retrieve homokaryotic offspring required for outcrossing. Mexico has a long history of using wild edible mushrooms, and mushroom cultivation, especially *A. bisporus*, and these practices have steadily increased in recent decades. However, the production of *A. bisporus* still depends on foreign cultivars. Wild collections of *A. bisporus* were recently reported from Mexico. This study aimed to obtain new hybrid strains of *A. bisporus* by crossing homokaryons from these wild strains and the homokaryon U1-7 from the commercial strain U1. Single spore isolates (SSIs) were obtained from spore prints directly from cultivated sporocarps. Putative homokaryotic SSIs were first selected based on culture morphology and confirmed using a cleaved amplified polymorphic sequence (CAPS) marker in the gene *mip*. Selected homokaryotic SSIs and the homokaryotic strain U1-7 were crossed. Six putative hybrid strains were obtained and tested for fruiting. Their primordia appeared after 40 to 55 days. The variability was recorded for unit weight (5.3 to 44.6 g), pileus diameter (3.3 to 8.5 cm) and pileus firmness (5.01 to 8.77 N). These are the first hybrid strains of *A. bisporus* obtained by crossing wild strains from Mexico.

Key words – button mushroom – CAPS marker – outcrossing – native germplasm – sporocarp morphology

Introduction

Agaricus bisporus (Lange) Imbach was first cultivated in France three and a half centuries ago (Savoie & Mata 2016). It is probably the most famous edible mushroom worldwide, and is known by various common names, such as the button mushroom, chestnut mushroom, portobello mushroom and champignon, depending on its color and maturity (De Mattos-Shipley et al. 2016). Due to its unique taste and medicinal properties, *A. bisporus* is currently one of the most cultivated species worldwide (Beelman et al. 2003, Royse & Sánchez 2017). Its Latin name is derived from a notable characteristic of the species; in general, the sporocarps predominantly produce bisporic basidia. Exceptions are found with the two varieties: *A. bisporus* var. *eurotetrasporus* Callac & Guinberteau and *A. bisporus* var. *burnettii* Kerrigan & Callac, in which the majority of basidia are

tetrasporic. These varieties differ in their life cycle, spore size and average spore number per basidium (Callac et al. 2003). Three different life cycles have been observed for *A. bisporus* species: homothallism (= primary homothallism) in *A. bisporus* var. *eurotetrasporus*; amphithallism with predominant pseudohomothallism (= secondary homothallism) in the traditionally cultivated variety, *A. bisporus* var. *bisporus*; and amphithallism with predominant heterothallism in *A. bisporus* var. *burnettii* (Callac et al. 2003).

Mexico has a long history of using wild edible mushrooms as food and as medicine (Perez-Moreno et al. 2009, López-García et al. 2020). Mushroom cultivation was introduced to Mexico in the 1930s, increasing notably during the last two decades of the 1900s (Martinez Carrera & Lopez Martinez de Alva 2010). By 2014, the national production of *A. bisporus* was estimated at 59,349 tons, representing 93.7% of the total national edible mushroom production (Martinez-Carrera et al. 2016), however, *A. bisporus* production in Mexico depends on foreign cultivars. It is known that commercial strains derived from a limited number of genotypes (Savoie et al. 2013), and most of the white-capped cultivars are identical or highly similar to the first hybrids obtained by crossing the white and off-white traditional cultivars (Foulongne-Oriol et al. 2011, Fritsche 1983, Xu et al. 1997). Such a limited degree of genetic variability significantly slowed the progress of genetic improvement in this very commercially important species (Yadav et al. 2003). There is a growing demand for excellent commercial strains of edible cultivated species due to the rapid expansion of the international market share for edible mushrooms (Nazrul & YinBing 2011).

Recently, *A. bisporus* has been reported from Mexico, and these wild collections are genetically different from all of the commercial strains (Mata et al. 2016). According to that study, the strains found showed variations between 44% and 82.5% (average = 64.3%) of typical bisporic basidia. Such percentages suggest that the production rates of heterokaryotic ($n+n$) and homokaryotic (n) spores should be relatively balanced. This discovery makes it possible and desirable to utilize wild Mexican strains to obtain new hybrid strains for several reasons: to develop new high yielding strains, to enrich regional germplasm resources, and to select strains able to adapt to local environments.

Intra-specific hybridization has been a very effective approach of exploiting heterosis in various field crops and mushroom species; for example, with success in the button mushroom (Yadav et al. 2003). The major difficulty in cross-breeding of this species is their amphithallic predominantly pseudohomothallic life cycle with generally a relatively low rate of homokaryotic spores. Studying wide SSI offspring, Royse & May (1982) and Summerbell et al. (1989) using allozyme or RFLP markers, respectively, found that 3% of SSIs were homokaryotic” but from the count of basidia of Callac et al. (1996) this rate is certainly much more variable and especially higher in the strain from Mexico (Mata et al. 2016). In addition, like other *Agaricus* species, *A. bisporus* lacks morphological characters like clamp connections that characterize heterokaryosis in species such as *Pleurotus pulmonarius* (Fr.) Quél. (Salmones et al. 2020). Therefore, traditionally the following criteria have been used alone or in combination for identification of homokaryons in *A. bisporus*: mycelium morphology, slow mycelial growth rate, non-fruiting, source of spores (Kamal et al. 2019).

Additionally, the development of molecular markers such as allozymes, restriction fragment length polymorphisms (RFLP), internal transcribed spacers (ITS), amplified fragment length polymorphisms (AFLP) and cleaved amplified polymorphic sequences (CAPS) has facilitated the rapid and reliable assessment of genetic diversity, homokaryon isolation and confirmation in mushroom breeding programs (Callac et al. 1993, 1996, Chen et al. 2016, De Brito et al. 2016, Foulongne-Oriol et al. 2011, Horgen et al. 1991, Kerrigan et al. 1992, 1994, Ling et al. 2019, Royse & May 1982, Thongklang et al. 2014, Xu et al. 1997). CAPS codominant markers are easily developed from heteromorphisms confirmed in both strands of DNA sequences. These heteromorphisms generally reflect allelic polymorphisms, such as single-nucleotide polymorphisms (SNPs) or length polymorphisms such as insertion or deletion (Ling et al. 2019).

The objectives of the present study were, first, to select homokaryotic single spore isolates (SSIs) from the offspring of wild Mexican strains of *A. bisporus* and, second, to obtain new hybrid

strains by crossing wild Mexican homokaryotic strains and commercial homokaryotic stock. The new hybrid strains were all tested for fruiting.

Materials & Methods

Parental strains

To get the sporocarps, we used IE 623 and IE 746 as parental strains – field isolates from the state of Tlaxcala, Mexico where the climate is semi-dry temperate (Mata & Rodríguez Estrada 2005) – both of which had been deposited in the Fungus Strain Collection of the Institute of Ecology, A.C. in Xalapa, Mexico. Offspring were obtained from the cultivated sporocarps of IE 623 and IE 746, respectively. Strain BS 26-7 is from the CGAB (Collection of the Germplasm of *Agaricus* at Bordeaux), now located at CIRM-CF Marseille, France (accession 2982; https://www6.inrae.fr/cirm_eng/Filamentous-Fungi/Strains-catalogue). It is a homokaryon obtained using the protoplast method from a strain considered to be a clone of the hybrid cultivar HorstU1 (Kerrigan et al. 1994). This strain is also called U 1-7 in Kerrigan et al (1994) and deposited at ATCC (96326) as a reference strain bearing the mating type allele *Mat-2*.

Single spore isolates

For sporocarp production, the mycelia of IE 623 and IE 746 strains were cultivated in an environmentally controlled culture room following the method described in Salmones et al. (2018). Single spore isolates (SSIs) were obtained from spore prints directly from cultivated sporocarps following the method described by Thongklang et al. (2014). After spore germination, the early-appearing larger mycelia were removed and late-appearing smaller mycelia from germinating basidiospores were isolated, transferred individually to compost extract medium (aqueous extract of pasteurized commercial mushroom compost plus 1% glucose and 2% agar), and incubated at 25°C in the dark for further observation.

Screening of putative homokaryons

Putative homokaryotic SSIs were first selected based on culture morphology. The SSIs that produced mycelial characters similar to those of the parental heterokaryotic strains were excluded. The SSIs with mycelial characters different from those of the parental heterokaryotic strains or slow growing isolates were selected for further morphological observation and molecular confirmation.

CAPS marker development

A CAPS marker based on the allelic polymorphism present in the sequences of the mitochondrial intermediate peptidase gene (MIP) was used. This marker has been used to study the offspring of *A. sinodeliciosus* and was found to be sufficient alone to identify homokaryons in this species due to (1) the gene MIP is link to the MAT locus and to a centromere and (2) non-sister meiotic nuclei are preferentially paired in the same spore (Ling et al. 2019). In the present study, loci showing restriction fragment length polymorphism between alleles within the parental strains of IE 623 and IE 746 were included for analyzing the SSIs. Co-dominant alleles and their constituent DNA fragment sizes are given in Table 1.

DNA extraction and polymerase chain reaction (PCR) amplifications were run following Chen et al. (2019). Cleavage reaction procedures followed the protocol recommended by the manufacturer. After digestion with the appropriate restriction endonuclease, DNA fragments were separated by electrophoresis in a 2% agarose gel stained with GelRed, running at 90 V for 60 min.

Four PCR products of presumed homokaryons from the offspring of each parental strain were randomly selected for sequencing. PCR products were purified and sequenced at Macrogen Inc., Korea.

Cross-fertility test of single spore isolates

Crosses among selected putative homokaryotic SSIs and with the homokaryotic strain U1-7 were performed by placing two mycelial plugs (2 mm cubes of inoculum) about 10 mm apart in compost extract medium. The plates were incubated for 3–4 weeks at 25°C in the dark. The compatible mating interaction zones (fluffy mycelium) were isolated to subculture. For each pairing, a second subculture was made from a mycelium originating from the confluent zone. These subcultures were used for fruiting tests. All hybrid strains were preserved and deposited in the Fungus Strain Collection of the Institute of Ecology, A.C. in Xalapa (Mexico).

Fruiting test

Spawn were prepared with hydrated sorghum grains with 0.5% (dry weight) of a 1:1 ratio of calcium carbonate to calcium sulfate added. Seeds were autoclaved at 121°C for 90 min in polyethylene bags. Sterilized grains were inoculated with a plug of pre-cultured dikaryotic strains and incubated at 25°C in darkness until the grains were completely covered by mycelia.

Plastic boxes were filled with 4 kg of commercial compost (for *A. bisporus* cultivation) and spawned at 5%. The compost was based on wheat straw (65%), chicken manure (25%), sugar cane bagasse (9%) and urea (1%), with pH 8.1 and moisture 66%. Five samples were prepared for each dikaryotic strain. After 20 days of incubation at 25 ± 1°C, boxes of spawn run compost were cased with a 4 cm casing layer (peat moss and limestone). Seven days after casing, the surface of the casing soil was ruffled deeply. Irrigation and ventilation conditions in the growing room were set to stimulate the formation of pinheads and the development of the sporocarps.

Agaricus bisporus mushrooms were harvested when their veils were intact. Earliness was estimated as the number of days between casing and primordium initiation. Mushroom unit weight was recorded as the range in the weight of the carpophores. Pileus diameter (cm) was measured manually. Firmness of pileus was determined with a GY-4 digital fruit penetrometer. The force applied during the test was recorded as the measurement of firmness (N). Tests were performed using eight replicates for each strain.

Results

Homokaryon selection

In total, 58 and 120 single spore isolates were obtained from the parental strains IE 623 and IE 746, respectively. Thirteen and 20 presumed homokaryons from IE 623 and IE 746 were first selected according to their gross morphology; i.e. slow growth rate and different mycelial morphologies compared to the parental lines (Fig. 1). They were further screened with the CAPS marker.

CAPS marker

Six double peaks were observed in electropherograms of both strand sequences of the MIP gene from parental strains IE 623 and IE 746. These double peaks reflect allelic single-nucleotide polymorphisms (SNPs). One of the SNPs was chosen to develop the CAPS marker. Phenotypes and genotypes at locus MIP:100 were determined using restriction endonuclease HpyCH4III (Table 1). The same SNP [aagaRctgt] was found in both parental strains IE623 and IE 746. Codominant alleles and their constituent DNA fragment sizes are given in Table 1. Finally, 10 and 14 homokaryotic SSIs were identified from parental strains IE 623 and IE 746, respectively.

Cross-fertility test

Based on the growth rate, ten homokaryons from parental strains IE 623 and IE 746 were selected for the cross-fertility test. Twenty-eight different paired combinations of homokaryons were tested. Among the paired combinations, six pairs were observed to react positively (i.e., fluffy mycelium). Cubes of inoculum (1 mm³) were removed from the confluent zone and subcultured on

the compost extract medium. The subcultures had a denser and fluffier mycelium compared with the parental homokaryons from wild Mexican strains (Fig. 2).

Fruiting test

All the cultivated putative hybrid strains fruited. This confirms that they were heterokaryotic and, therefore, that hybridization succeeded. In addition, this confirms the sexual intercompatibility between the cultivars and the wild population from Mexico.

The mycelia took 20 ± 1 days of incubation to colonize of the compost substrate, while the colonization of the casing layer was more variable, taking from 9 to 12 days depending on the strain. Primordium initiation, or earliness, was observed between 40 (IE 746-22 \times IE 623-30) and 49 (BS 26-7 \times IE 623-12 and BS 26-7 \times IE 623-30) days from the spawning day, and the mushrooms were picked between 46 (IE 746-22 \times IE 623-30) and 55 (BS 26-7 \times IE 623-12, BS 26-7 \times IE 623-30 and IE 746-22 \times IE 623-30) days, respectively. The size and weight of the fruiting bodies varied among the strains (Fig. 3, Table 2).

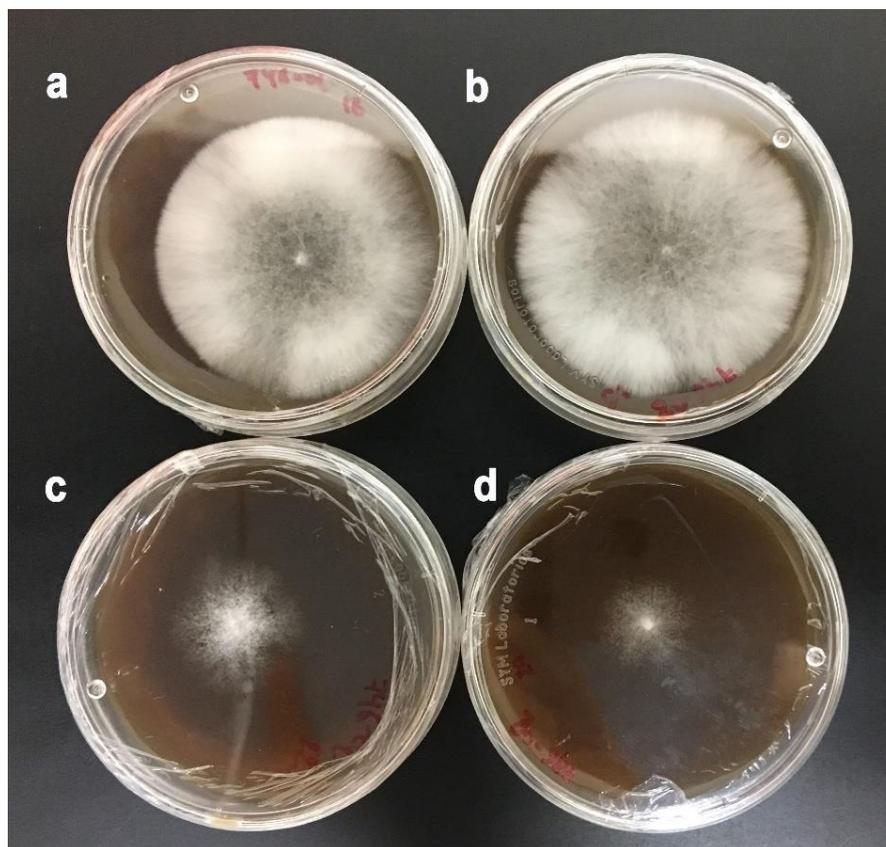


Fig. 1 – Differences in mycelial growth between homokaryons and heterokaryons. a, b Fast-growing discarded SSIs. c, d Putative homokaryotic SSIs. All cultures shown after 7 days of incubation.

Table 1 CAPS marker and expected genotypes in the offspring of IE 623 and IE 746

Sequenced DNA fragment Forward primer (5'→3') Reverse primer (5'→3')	Locus ^a Endonuclease Recognition site ^b	Phenotype (DNA fragment in bp)	Genotype	GenBank accession number
MIP	MIP:100	403	1 (or 1/1) ^c	MW881227
91BF:ACAGGCGCAGATGATTATGC	HpyCH4III	303 + 100	2 (or 2/2) ^c	MW881226
91BR:GGTTCAGGTGGGCAATAAA	aagaACTGTtag	403 + 303 + 100	1/2	

^a The locus is designated by the name of the sequenced DNA fragment followed by its position on the 5'→3' amplified sequence

^b Nucleotides of the recognition site are in capital letters with the polymorphic position in bold type
^c Because *MIP:100* is tightly linked to the *MAT* locus and to a centromere and because non-sister post-meiotic nuclei are preferentially paired in the same spore, it is unlikely to find heterokaryotic SSIs that should be homozygous at the *MIP:100* locus

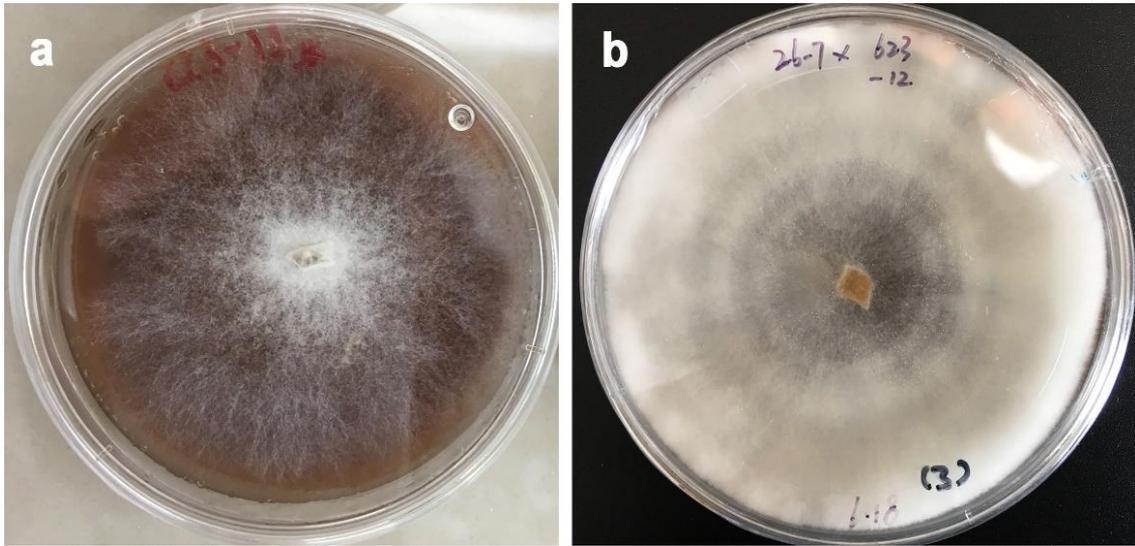


Fig. 2 – Difference between homokaryotic and heterokaryotic mycelia of *A. bisporus*. a homokaryotic mycelia of SSI IE623-12. b heterokaryotic mycelia of subculture from the confluent zone of BS 26-7 × IE 623-12. The visible concentric growth zones are typical of the heterokaryons in *A. bisporus*.

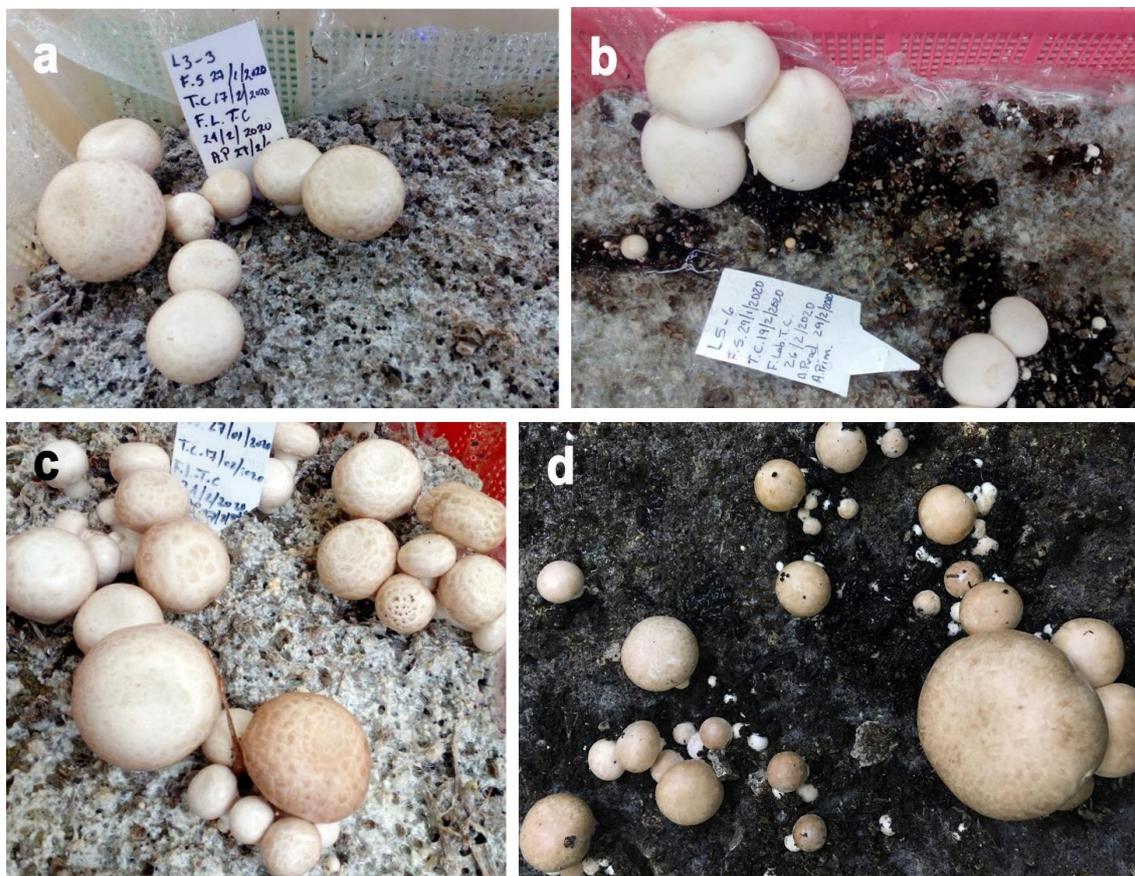


Fig. 3 – Sporocarps from the different strains evaluated. a IE 746-22 × IE 623-30. b IE 746-63 × IE 623-12. c IE 746-110 × IE 623-30, d BS 26-7 × IE 746-22.

Table 2 Phenotypic characteristics of the cultivated hybrids

Strain	Earliness (days)	First flush (days)	Mushroom unit wg (g)	Pileus unit diameter (cm)	Pileus firmness (N)
BS 26-7 × IE 623-12	45–49	51–55	11.4–25.2	3.9–7.8	6.28–8.69
BS 26-7 × IE 623-30	47–49	53–55	17.8–28	3.6–6.6	6.92–8.77
BS 26-7 × IE 746-22	42–45	51–53	10.5–24.6	4.2–5.1	7.08–8.10
IE 746-110 × IE 623-30	44–46	51–53	13.8–40.9	3.9–5.6	5.01–8.65
IE 746-63 × IE 623-12	43–46	51–54	17.6–31.1	4.0–8.5	6.03–6.90
IE 746-22 × IE 623-30	40–48	46–55	5.3–44.6	3.3–7.6	5.89–7.08

Discussion

The use of wild germplasm and molecular genetic approaches in breeding studies have contributed to successful outcomes in mushroom cultivation, especially in species with a tetrapolar heterothallic sexuality pattern since the presence of clamp connections facilitates the identification of homokaryons.

Breeding *A. bisporus* var. *bisporus* is complicated because of its pseudohomothallic life cycle. In our study, six hybrid strains were obtained from the homokaryons of wild Mexican strains and a homokaryotic stock of the commercial strain U1. The morphological characteristics of the mycelia and the CAPS marker used by Ling et al. (2019) were rapid and efficient at identifying and selecting homokaryons for crossing. These are the first hybrid strains of *A. bisporus* obtained by crossing wild strains from Mexico.

The putative hybrid strains fruited. This confirmed their heterokaryosis status knowing that haploid fruiting is only reported in the homothallic variety *eurotetrasporus*. In addition, these fruiting hybrids confirmed that the biological concept of *A. bisporus*, which includes a population of this species in Mexico. However, obtention of second generation hybrids would definitely confirm the interfertility between the wild population from Mexico and the cultivars likely of European origin. Second generation hybrids would be also useful to study the segregation of the characters and better evaluate the potential interest of the strains from Mexico.

The ability to rapidly colonize the substrate, the formation of primordia and development of the sporocarps are also priorities in the selection of commercial germplasm. In our study, the hybrids colonized the substrate within 19–21 days of incubation, whereas primordium initiation or earliness was observed at 40 to 49 days and the first flush was harvested between 46 and 55 days. These values are greater than those reported by Pardo et al. (2004), who cultivated commercial strains of *A. bisporus* on a different casing mixture and required 14 days for the colonization period and 38–42.1 days to obtain the first crop. However, the values are similar to those reported by Kumar & Suman (2014) and Sharma et al. (2016) who characterized *A. bisporus* strains from different resources and reported 33–40 to 34–59 days for the first harvest, respectively. The strains we obtained fall within a satisfactory timeframe for commercial cultivation.

Additionally, mushrooms should be of good quality (size, weight, color and firmness) to be well accepted in the market. We identified some variability in pileus diameter (range: 3.3 to 8.5 cm) of different strains. Interestingly, Braaksma et al. (1999) reported the size of the pileus in strain U1, one of the parental strains used in this study, to be 2–5 cm. Therefore, it is likely that the Mexican parental stains contributed to the increased pileus diameter in the hybrids obtained. Indeed, Salmones et al. (2018) reported that in the two Mexican parental strains maximum pileus diameter was 15 cm, though this was with the caps fully expanded. It is possible to use these parental homokaryons to increase pileus diameter to meet the market demand in Mexico.

The obtained hybrid fruiting bodies also varied in cap color. The commercial strain is a classic white button mushroom, while the IE 623 and IE 746 strains have a white stipe but a brownish pileus. None of the hybrid strains were white. Only strain BS 26-7 × IE 623-30 is lighter in color but is not completely white. The other hybrid strains are more similar to their Mexican parents.

Firmness is an important commercial parameter for increasing shelf life. Ni et al. (2021) analyzed commercial strains of *A. bisporus* from a market in Hangzhou, China, and observed a firmness of 11 N in the uncoated control mushroom, which is higher than our results. In contrast, Zivanovic et al. (2000) reported an initial firmness of 2.6 N in an off-white F-140 hybrid. The firmness observed in our study (from 5.01 to 8.77) could be a characteristic of commercial interest for subsequent genetic crosses. Other parameters, such as tolerance to disease and yield, could be evaluated in future studies.

In conclusion, the cultivated hybrids had some interesting characteristics, such as variations in diameter, weight, and intermedium firmness, compared to previous studies. Although the strains were not evaluated for their yield, the phenotypic characteristics observed in the mushrooms harvested are promising and allow us to propose that the method used for the generation of new hybrids should be tested on a larger scale, including a greater number of cultivars with different characteristics of interest to the *Agaricus* market.

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