Outcrossing *via* the Buller Phenomenon in a Substrate Simultaneously Inoculated with Spores and Mycelium of *Agaricus bisporus* Creates Variability for Agronomic Traits.

Philippe Callac, Micheline Imbernon, Jean-Michel Savoie
INRA, UR1264, Mycologie et Sécurité des Aliments, BP 81, F-33883 Villenave d'Ornon, France. E-mail: savoie@bordeaux.inra.fr, callac@bordeaux.inra.fr

Abstract

A novel outcrossing method proceeding through the Buller phenomenon was evaluated for its efficiency in creating a useful variability in agronomics traits and being considered of value in the early generations of selection. Hybrids were obtained by outcrossing via the Buller phenomenon of S608-2 homokaryon (white hybrid) with spores of a single spore print of a C9 sporocarp (brown strain). Forty fruiting bodies resulting from the Buller phenomenon were collected. Spawn was obtained from each collected hybrid and culture trials were performed under conventional conditions or with artificial contamination by the pathogen *Verticillium fungicola*. Forty-two percent of the hybrids came from the non-cultivatable *mat-x* homokaryon of C9. This group of hybrids was not significantly different to the group inheriting the *mat-1* homokaryon for the yield, mean mass and cap colour, but it was significantly less susceptible to the pathogen. The dispersion of the data for the susceptibility to the pathogen was compared with those observed in a sample of wild strains and in a sample of hybrids obtained by conventional crossings. The variability created by outcrossing for this trait is significant and could be used to select interesting strains.

Key Words: *Agaricus bisporus*; Outcrossing; Buller phenomenon; Agronomic traits; *Verticillium fungicola*

Introduction

In *Agaricus bisporus* var. *bisporus* most of the basidiospores are heterokaryotic (predominant pseudohomothallic life cycle). Heterothallic basidiospores are homokaryotic (n) and generally give rise to self-sterile homokaryotic mycelia. Plasmogamy between two sexually compatible homokaryons restores a fertile heterokaryon. Pseudohomothallic basidiospores are heterokaryons (n + n) and contain two non-sister post-meiotic nuclei with different mating type alleles. Mycelia growing from these spores are sexually fertile. In wild strains of *A. bisporus* var. *bisporus*, only 1.3% of the basidia, on average, are tetrasporic (Callac *et al*. 1996) and produce homokaryotic basidiospores. From this, the outcrossing classical method (confrontation of homokaryons *in vitro)* is hampered by the difficulty to recover homokaryotic strains. However, it was recently shown that numerous hybrid sporocarps are easily produced *in vivo* by simultaneously inoculating a standard compost of culture with a homokaryon from one parent and the spores from a second parent (Callac *et al.* 2006). When a homokaryon is cultivated on compost like a commercial heterokaryotic cultivar (Figure 1), it cannot form sporocarps by itself and requires genetic information from the spores of a second parent to do so; on the other hand, mycelia derived from the heterokaryotic spores of the second parent can fructify by themselves. In this case, it was indirectly shown that crosses occurred between the heterokarotic spores (or mycelia issued from them) and the inoculated homokaryon (Buller phenomenon). Outcrossing *via* the Buller phenomenon in a substrate simultaneously inoculated with spores and mycelium of *A. bisporus* is a promising outcrossing method that could be used in commercial breeding programs, and in genetic studies.

Homokaryons recovered from some *A. bisporus* strains always have the same mating type allele, whether they are obtained from spores or from protoplasts, suggesting that they carry a recessive deleterious or lethal allele at a locus linked to *MAT*. This characteristic was used to determine whether outcrossing proceeds through the Buller phenomenon or through heterothallism (Callac *et al.* 2006). Nuclei that cannot support independent homokaryotic growth could easily survive in a heterokaryon as long as their partner carried an allele that complemented their lethal or deleterious allele(s). With outcrossing occurring mainly via the Buller phenomenon, lethal or deleterious alleles can be transmitted through multiple outcrossed generations. But this process allows also preserving and transmitting putative beneficial alleles from these nuclei.

One objective in the present study was to determine if the novel outcrossing method proceeding through the Buller phenomenon could create a useful variability in agronomics traits for being considered of value in the early generations of selection. The second objective was to show that interesting phenotypes can be recovered from nuclei that cannot support independent growth thanks to *in situ* outcrossing via the Buller phenomenon.

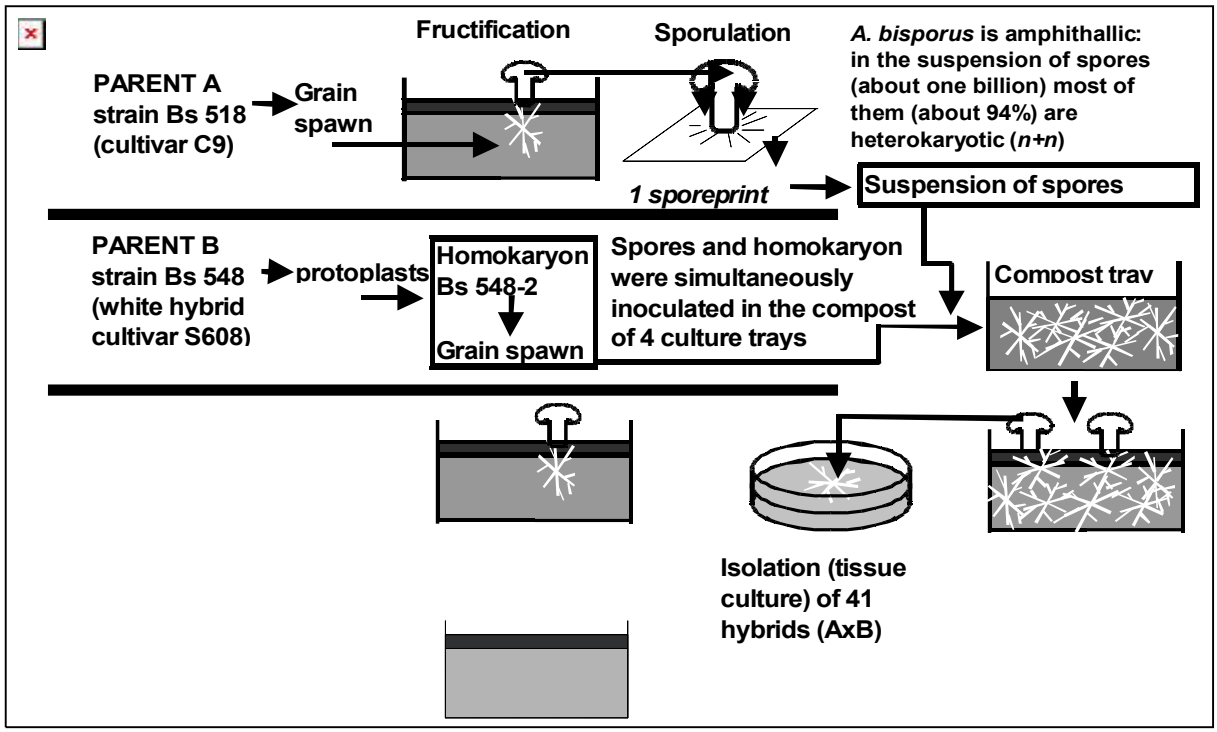

Figure 1. Schematic representation of the experimental design including *in vivo* outcrossing, collection of the hybrids, and cultural assays for determination of their agronomical traits.

Materials and Methods

Parental strains and their mating type genotypes

Homokaryotic mycelium (S608-2 = Bs548-2) from S608 was confronted with spores from C9 (Bs518), (Figure 1). Both of the parental strains, S608 and C9, are bisporic cultivars, but they produce sporocarps with white and brown caps, respectively. S608 (Somycel,

Langeais, France) is a classic "white hybrid" that is genetically very similar to cultivar U1 (Mushroom Research Unit, Horst, The Netherlands). Homokaryon S608-2 was derived from S608 by protoplasting and is genetically very similar to the U1-2 homokaryon and carries the *Mat-2* (mating type) allele. C9 (Le Lion, Saumur, France), the heterokaryotic parent of the spores, is a well-known traditional "brown" cultivar. All the homokaryons derived from C9 carry the *Mat-1* allele, consequently the mating type genotype of C9 is partially undetermined (*Mat-1/x*).

Forty-five hybrids (H) obtained by conventional crossing between homokaryons similar to U1-7 carrying the *Mat-7* allele and homokaryons of *A. bisporus* var *burnettii* (Callac *et al.* 1997) were used as control of the level of variability for the susceptibility to *V. fungicola*. Another sample of 33 wild strains from various geographical origins (collection CGAB, INEA, France) was also used.

Outcrossing to produce hybrids

Hybrids were obtained by outcrossing via the Buller phenomenon of Bs608-2 homocaryon with spores of a single sporeprint of a C9 sporocarp as described in Callac *et al.* (2006). Forty-one fruiting bodies were collected before caps opened and transfers were made from each fruiting body in Petri dishes on compost agar medium (Figure 1). The outcrossing origin of each isolate was checked with molecular markers (Callac *et al*. 2006). Outcrossing heterokaryons resulting from the Buller phenomenon were distinguished by the alleles inherited from the C9 parent at the PR6 locus. *PR6* is a molecular marker derived from restriction fragment length polymorphism marker *P1N150* and is tightly linked to *MAT* on chromosome 1 (Callac *et al.* 1997; Kerrigan *et al.* 1993). In C9, the allele *PR6-1* is linked to *Mat-x*, and *PR6-2* is linked to *Mat-1*. The genotype of S608-2 is *PR6-1*.

Evaluation of the hybrids in cultures

Rye grain spawn cultures of each hybrid were prepared under sterile conditions like for commercial spawn (Elliot, 1995). *A. bisporus* hybrids were cultivated on commercial compost spawned at the rate of 0.8% in 0.1 m² crates containing 8 kg of compost. Three trays were spawned with each hybrid. The incubation was performed at 24 °C for 13 d before a conventional casing layer was overlaid on the compost. Nine days after casing, the trays were separated in two groups, each placed in separated rooms, both with the temperature regulated at 16 °C and air humidity at 89%. A conidial suspension of *V. fungicola* var. *fungicola* was sprayed on the top of the casing layer of one group of trays 11 days after casing at a rate of 10^6 conidia m^{-2}. Mushrooms were harvested for 4 weeks and the weights of healthy or affected mushrooms were recorded for each tray (Juarez *et al*. 2002). One tray of each hybrid was used in this group. The second group containing two trays per hybrid was cultivated under standard conditions without inoculation of pathogen (Figure 1). Mushrooms were harvested for 4 weeks, their weight and number were recorded and cap colour was measured on five sporocarps from the first flush with a Minolta chromameter (CR221, 3 mm), immediately after harvest. The yield was expressed as kg of fresh mushroom per m² of compost and the mean weight of mushrooms was calculated (= weight / number, as g / mushroom) (Rodier *et al.* 2000).

In another trial, wild strains were cultivated as above but only with inoculation of the pathogen and one tray per strain. The 15 hybrids H were cultivated in a third trial with inoculation of the pathogen and 3 trays per hybrid.

Statistical analyses

Descriptive statistics, analyses of variance and mean comparisons were performed with SYSTAT software (SPSS Inc.)

Results and Discussion

Ten of the 41 hybrids were not used in data analyses because of no or abnormal fruiting during the cultivation test in presence of *V. fungicola*. In the resulting sample of 31 hybrids, 13 had the *PR6-1/1* genotype and resulted from crossings of the nucleus of S608-2 and the nucleus of C9 carrying *Mat-x* that can not be obtained as homocaryon (Callac *et al.* 2006). The 18 others had the *PR6-1/2* genotype and resulted from crossings of the nucleus of S608-2 and the nucleus of C9 carrying *Mat-1*. Despite this bimodal distribution of the hybrids, the distribution of the points on Figures 2 and 3 indicated that there was no observable discrimination between the groups for yield, mean mass and cap colour. For the susceptibility to the pathogen there was a trend of lower values of percentiles of diseased mushroom in the groups with PR6-1/1 genotype, even if some highly susceptible hybrids were found in this group.

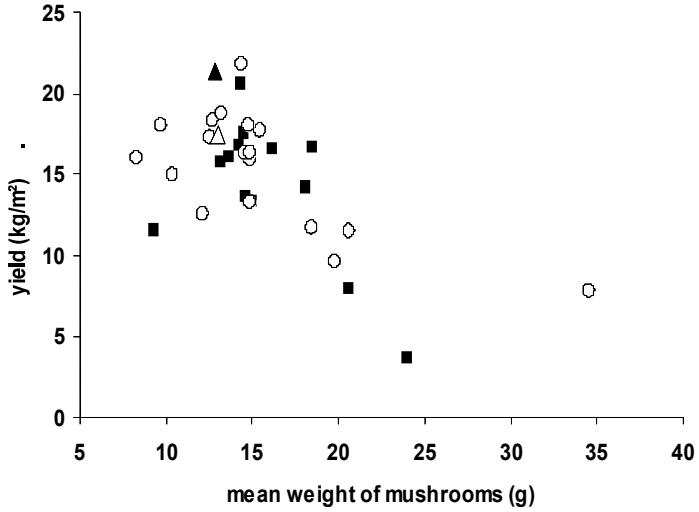

Figure 2. Distribution of the *A. bisporus* hybrids resulting from outcrossing for their yield and calibre estimated as the mean weight.
Squares = genotype PR6-1/1, rings = genotype PR6-1/2, white triangle = parent A C9, black triangle = parent B S608.

This distribution is shown by the descriptive statistics (Table 1). The median is the value above which half of the data fall. Medians and means are close in the two genotypes for the agronomical traits except for susceptibility. 75 % of the data of *PR6-1/1* genotype are lower than the median value of *PR6-1/2* genotype and 9/13 (69 %) are lower than the lower hinge. None of the means between the two genotypes are significantly different except for susceptibility where the difference is significant at $P<0.05$. These data indicate a link between the genotype and the susceptibility to *V. fungicola,* with some possible recombination. In the present case, the level of susceptibility transmitted by the non cultivable *Mat-x* nuclei of the parent C9 is favourable.

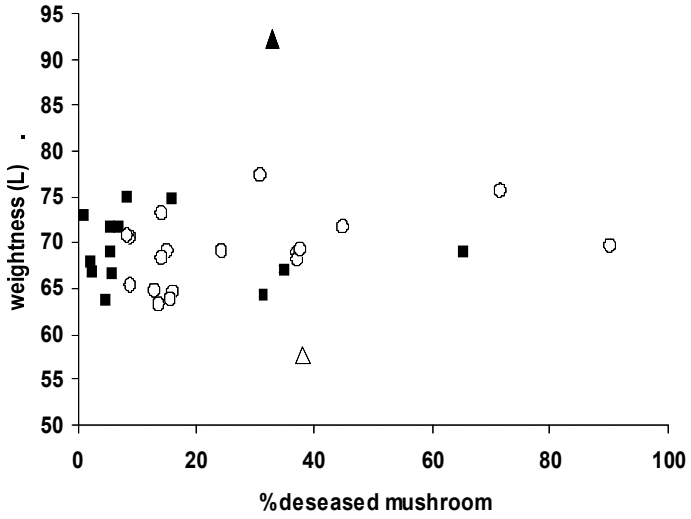

Figure 3. Distribution of the *A. bisporus* hybrids resulting from outcrossing for their cap color and susceptibility to *V. fungicola*.
Squares = genotype PR6-1/1, rings = genotype PR6-1/2, white triangle = parent A C9, black triangle = parent B S608.

Table 1. Descriptive statistics of the agronomical traits measured on the group of hybrids having a different genotype at the marker PR6.

Genotype	Variable	Stem and Leaf Plots				
		Lower hinge	Median	Upper hinge	Mean	Standard error
PR6-1/1	% diseased	4.3	5.5	15.8	14.4	5.2
PR6-1/2		13.7	16.0	37.4	28.0	5.3
PR6-1/1	Yield (kg/m²)	13.4	15.9	16.8	14.4	1.2
PR6-1/2		12.5	16.1	17.2	15.3	0.8
PR6-1/1	Mean weight (g)	14.2	14.5	18.0	15.8	1.0
PR6-1/2		12.6	14.7	15.5	15.4	1.4
PR6-1/1	L (%)	66.8	69.1	71.7	69.4	1.0
PR6-1/2		65.2	69.0	70.6	68.9	0.9

One of the interesting features of the proposed new outcrossing method for hybridisation and selection is that production of numerous homokaryons is not necessary. Nuclei that cannot support independent homokaryotic growth could easily survive in a heterokaryon as long as their partner carried an allele that complemented their lethal or deleterious allele(s). However, they can bear other interesting alleles that could not be selected and used in conventional breeding methods.

The dispersion of the data for the susceptibility to the pathogen was compared with those observed in a sample of wild strains and in a sample of hybrids obtained by conventional crossings. Only the Kurtosis coefficient of the outcrossing sample is considered as significant because Kurtosis/ Standard error Kurtosis is higher than 2 (Table 2). This indicates that the variable has longer tails than those for a normal distribution, due to the existence of a significant difference between the two genotypes. The coefficient of variation of the outcrossing sample is also the higher. The high level of variability in the hybrids

obtained by outcrossing is the result of the genetic variability of the spores in the sporeprint from the parent C9.

Table 2. Comparison of the variability in susceptibility to *V. fungicola* in three groups pf *A. bisporus* strains having three origins: hybrids from outcrossing, wild strains, hybrids obtained by conventional method.

	Origin of the *A. bisporus* strains		
	Outcrossing	Wild strains	Hybrids H
No. of cases	31	33	45
Median	14.3	21.0	30.7
Mean	22.3	28.9	40.3
95 % CI upper	30.3	38.0	46.5
95 % CI lower	14.3	19.8	34.0
Coefficient of variation	0.98	0.89	0.53
Kurtosis	2.4	- 0.18	- 0.52
SE Kurtosis	0.82	0.80	0.68

These data stress that the variability created by outcrossing through the Buller phenomenon by simultaneously inoculating spores and the grain spawn of a homokaryon into a culture substrate, is significant for some traits and could be used to select interesting strains in the early generations of selection.

Acknowledgments

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