CONTRACT REPORT M 15

Evaluation of *A. bisporus/A. bitorquis* *hybrids*

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Application

This project sought to confirm that hybrid strains had been obtained from crossing *Agaricus bisporus* with *Agaricus bitorquis* and to evaluate the behaviour - growth and possible fruiting of the hybrid strains.

The project confirmed on the basis of molecular DNA markers and conventional genetic markers that two strains, coded *Abb1* and *Abb2* had hybrid characteristics.

This project has been shown for the first time that it is possible to create stable *A. bisporus/A. bitorquis* hybrids. Whilst these two strains *Abb1* and *Abb2* are not directly commercially useful they suggest a methodology for the creation of a whole range of *A. bisporus/A. bitorquis* hybrids. The analysis of *Abb1* and *Abb2* shows that they are more like *A. bisporus* than *A. bitorquis*. It is probable, therefore, that only a small amount of the DNA from *A. bitorquis* is present in the hybrid. Making and testing a lot more hybrids could yield strains of value to growers with the desired combinations of characteristics of both species, for example incorporating the post-harvest properties of *A. bitorquis* into *A. bisporus*.

Summary

Making hybrids can be a very effective way of producing novel and useful variation. A major step in the improvement of spawns of *Agaricus bisporus* has been the development of hybrid strains produced by crossing the 'smooth white' and 'rough' traditional strains. Hybrids from such crosses now dominate the spawn market.

These hybrids were made by crossing strains within *A. bisporus*; is it possible to make more distant crosses, to make hybrids between different species? A second *Agaricus* species, *A. bitorquis*, with spawns such as K26 and K32, has been grown commercially but its success has been limited. It requires higher temperatures for cropping, has a longer flushing pattern and produces lower yields than *A. bisporus*. However, it has post-harvest characteristics in respect of colour, bruising resistance and texture which are potentially advantageous. In addition, it does not appear to be susceptible to the viruses which attack *A. bisporus*. Hybrids between *A. bisporus* and *A. bitorquis* might show useful diversity that could be exploited by the spawn companies and the industry.

Interspecies hybrids are rare in fungi but two possible *A. bisporus/A. bitorquis* hybrids have been produced. The first step in the production of a hybrid is the establishment of a hybrid mycelium. This involves growing together mycelial colonies of specific strains of the two species which carry independent, distinct, and recognisable genetic markers and then selecting hybrid mycelium which appears to contain both markers. Two types of markers (i) resistance and (ii) auxotrophic have been used to create these hybrids. With resistance, mutant strains which are able to grow in the presence of a chemical eg a fungicide which prevents the growth of normal strains are used. (These mutants are comparable to strains of a mushroom pathogen which become resistant to the control chemicals used). With auxotrophic markers

the mutants cannot grow unless specific amino-acids are provided in the growth medium whereas 'normal' strains grow quite happily in their absence.

Two mycelial hybrids which were stable in repeated subculturing were produced using these methods. The hybrids and their component strains were tested for growth rate at different temperatures. *A. bitorquis* grows best at 30°C whereas *A. bisporus* grows best at 25°C. Hybrids *Abb1* and *Abb2* differed from their component strains but in general were more like *A. bisporus* than *A. bitorquis* in response to temperature.

Fruiting trials

The two potential hybrids, called *Abb1* and *Abb2* have been tested for fruiting ability in trials on the HRI Mushroom Unit. Hybrid *Abb1*, which was produced by combining auxotrophic markers proved to be non-fertile, it did not produce any mushrooms, whereas *Abb2*, produced by combining resistance markers was fertile. Single spores were collected from *Abb2* mushrooms; these mushrooms had the characteristics of *A. bisporus.* Colonies from these spores were screened for resistance and both markers used in the cross were shown to be present; one from *A. bisporus*, the other from *A. bitorquis.*

Molecular markers

Molecular markers were used to evaluate *Abb1* and *Abb2*. These markers are more reliable than auxotrophic and resistance markers where the presence or absence of growth is the criterion used and may not be clear-cut. The DNA is randomly sampled using a technique which gives rise to a strain specific pattern of separate bands, and analogous to a supermarket bar code. Each band represents an individual fragment of DNA and fragments were identified which were unique to each of the strains involved in the production of a hybrid. If these fragments, and/or novel fragments not present in the strains involved in the cross, appear in the banding, bar code, pattern of the possible hybrid then hybridity is confirmed (Figure 1). Using these techniques *Abb1* was confirmed as a hybrid. The results for *Abb2* were less clear but this strain is probably also a hybrid.

4. SCIENCE SECTION

4.1 INTRODUCTION

New and improved mushroom spawns can be produced in a variety of ways (Elliott, 1985). One possible approach is to try and develop hybrids between different mushroom species. Two species of *Agaricus*, *A. bisporus* and *A. bitorquis* are cultivated, and more recently *Agaricus arvensis* has been grown commercially on a small scale. Mushroom production is dominated by *Agaricus bisporus*, which accounts for approximately 40% of the total world production. *A. bisporus* is predominantly a crop of the temperate regions of the world but it has been also successfully introduced into sub-tropical and tropical countries like Mexico, India and Taiwan.

The second species which has been cultivated, *A. bitorquis*, was first described in 1968 from Central Africa. As with *A. bisporus*, strains of *A. bitorquis* produce edible, relatively dense and durable white mushrooms with good yields. However, *A. bitorquis* differs from *A. bisporus* in a range of properties. It will fruit over a wider range of temperatures (15-25°C) and at higher CO_2 (0.1-0.2%) concentrations.

In the 1970's, the use of *A. bitorquis* increased because of virus outbreaks in *A. bisporus* crops. Since *A. bitorquis* was proven to be resistant to the known viruses of *A. bisporus*, different strains of *A. bitorquis* were grown successfully throughout Europe. *A. bitorquis* was first introduced to the market by commercial spawn makers such as Somycel in 1973 under the name of *"Psalliota edulis"*. After this, other strains of *A. bitorquis* such as *P. rodmanii* (444) from spawn maker Le Miz; and Horst B30, K26, K32 and K46 from the Dutch Mushroom Experimental Station, Horst, Netherlands were released for commercial cultivation (Fritsche, 1977). However, the success of these strains of *A. bitorquis* has been limited due to the problems of sensitivity to growing conditions and occasional off-flavours. In addition, the cropping cycle (flushing pattern) of these strains is irregular and the time between flushes is generally longer than in *A.bisporus*.

Within the group homobasidiomycetidae (of which *A. bisporus* and *A. bitorquis* are a part) hyphal fusion and genetic exchange is an essential part of the sexual cycle within a species. Hyphal fusion *between* species leading to the production of stable hybrids is not known in this group of fungi. Genetic complementation between mutant strains from each species suggests itself as a possible way of generating interspecific hybrids in the genus *Agaricus*. In this study we have looked at hyphal fusion between defined mutant strains of *A. bisporus* and *A.bitorquis* and evaluated the degree of "hybridity" of any putative hybrids produced.

4.2 MATERIALS AND METHODS

4.2.1 Strains

Mutant strains (carrying auxotrophic or resistance markers) were recovered from liquid nitrogen storage in the HRI culture collection. These strains are shown below:

Species	Strain code	Genotype/phenotype
A. bisporus	W9-ad.his	Auxotroph which requires exogenous supply of adenine and histidine for growth
A. bisporus	NI-ura	Auxotroph which requires exogenous supply of uracil for growth
A. bisporus	C54-8	Resistance mutant which can grow in the presence of the fungicide carboxin; an SDH mutant
A. bitorquis	W2-G	Autotroph which requires exogenous supply of methionine and threonine for growth
A. bitorquis	W2-H	Auxotroph which requires exogenous supply of nicotinic acid for growth
A. bitorquis	JA-2	Resistant mutant which grows in the presence of the antimetabolite para-fluorophenylalanine

4.2.2 Hyphal fusion/crossing

Auxotrophic and resistance markers for each species were combined in pairs eg W9-ad.his crossed with W2.G, W9-ad-his crossed with W2-H, C54-8 crossed with JA-2. These crosses were carried out in three ways to promote the possibility of interspecific hyphal fusion.

(i) Mycelial macerates were mixed together and plated on selective and non-selective media.

(ii) Hyphal plugs were plated out 1 cm apart on non-selective medium and transfers made from the junction zone between mycelia to selective media.

(iii) Hyphal plugs were placed on top of each other with mycelia in direct contact on selective medium.

Any putative hybrids which were able to grow on selective media were transferred to fresh selective media.

4.2.3 Growth rate tests

The growth of the mycelium of any putative hybrids and their component strains was compared at 20°, 25° and 30°C. Growth was determined by measuring colony diameter twice at right angles across the colony. For each test mycelium 5 replicate plates were measured at 4 day intervals up to 20 days.

4.2.4 Fruiting trials

A replicated fruiting trial was carried out. Spawn of putative hybrids was prepared and used to spawn compost at 2% by weight using a mini-culture system based on 10½" plastic pots which can hold 2.5 kg of spawned compost. Fruiting trials were conducted following standard HRI procedures for *A. bisporus*.

Spore progeny

Spores were collected from any sporophores (mushrooms) produced, germinated using the double plate technique and tested for the segregation of markers present in the parental strains. (Elliott, 1978).

4.2.5 Molecular markers

DNA was extracted from mycelia of putative hybrids and parental strains and RAPD markers generated using the commercially available Operon 10-mer kit (18 primers). Banding patterns were compared for all primers.

4.3 RESULTS AND DISCUSSION

Two putative hybrid stable mycelia were recovered from all crosses. Stability was confirmed by growth on both selective and non-selective media and transfers between them. These putative hybrids were given the designations *Abb1* and *Abb2*. *Abb1* was produced by crossing the *A. bisporus* auxotrophic homokaryon W9-ad.his (requirement for adenine and histidine) with the *A. bitorquis* homokaryon W2-H (requirement for nicotine acid). *Abb2* was produced by the cross between *A. bisporus* C54-8 (resistant to the fungicide carboxin) and *A. bitorquis* JA-2 (resistant to the antimetabolite para-fluorophenylalanine). The hybrids differed in growth and general colony morphology from the parent strains on non-restrictive medium. The growth morphology of *Abb1* was more distinct from its parents than that of *Abb2*. Fig. 2 shows the growth of *Abb1* on both restrictive and non-restrictive media.

The established optimum for vegetative growth of the mycelium of *A. bisporus* is 25°C and that for *A. bitorquis* is 30°C. The results of the comparison of growth rate are shown in Figure 3. The growth profile of *Abb1* is similar to that of the W9.ad.his. parental strain. The growth profile of *Abb2* differs from that of both its parental strains, JA-2 and C54-8. Neither isolate of *A. bitorquis* grew best at 30°C but nevertheless they made more growth at this temperature than the *A. bisporus* strains.

The ability of *Abb1* and *Abb2* to grow on restrictive media linked to their distinctive colony morphologies and their growth responses to temperature are all characteristics suggesting hybridity.

In the replicated (x 2) fruiting trial, putative hybrid strain *Abb1* proved to be non-fertile; no mushrooms were produced. Putative hybrid *Abb2* proved fertile and yield figures are given in Table 2 in comparison with a commercial strain coded C43 which was used as a control.

Table 2 - Cropping data for putative hybrid *Abb2*

Trial 1		
Strain:	Wt of mushrooms/replicate	No. of mushrooms/replicate
*Abb2**	464 g	43
C43**	583 g	47
Trial 2		
Strain:	Wt of mushrooms/replicate	No. of mushrooms/replicate
*Abb2**	511 g	26
C43**	557 g	40

* mean of 7 replicates
** mean of 8 replicates

Spore prints were collected from *Abb2* mushrooms and a sample of one hundred and fifteen single spore isolates recovered. These isolates were grown on media containing either one or other of the antimeabolites/fungicides used or both. Three isolates showed a phenotype suggesting the presence of a gene for resistance to para-fluorophenylalanine but with sensitivity to carboxin, fpa^r, $carb^s$, one isolate with sensitivity to both compounds, fpa^s, $carb^s$ and one isolate showing resistance to carboxin and sensitivity to para-fluorophenylalanine, fpa^s, $carb^r$. These data though limited suggest that both resistances are segregating through meiosis.

The molecular analysis of strain *Abb1* and its component strains found eight markers from seven primers which were informative. Two markers from primer 1 designated RAPD1 and RAPD2 indicate the integration of genetic material from the parental strains into the hybrid (Figure 4). Six RAPD markers designated RAPD 3-8 show that the putative hybrid *Abb1* differs from its component strains by the presence of non-parental DNA bands. This data is summarised in Table 3. Thirteen other RAPD markers were potentially informative but were

faint and their reproducibility needs confirming.

Table 3 - RAPD markers which might become informative, if they are shown to be consistent and reproducible in difference PCR reactions

Primer	Components		Hybrid *Abb1*	
	A W9 ad.his.	**B W2-H**		
P2	0	0	1	RAPD.9
	0	0	1	RAPD.10
	0	0	1	RAPD.11
P3	0	0	1	RAPD.12
P8	0	0	1	RAPD.13
	0	0	1	RAPD.14
P11	0	1	1	RAPD.15
	0	1	1	RAPD.16
	1	0	1	RAPD.18
	1	0	1	RAPD.19
P14	0	0	1	RAPD.20
P16	0	0	1	RAPD.21

The molecular analysis of *Abb2* and its component strains showed that of the 18 primers tested 17 gave identical banding patterns to those of the carboxin resistant parent C54-8. Three RAPD markers from primer 20 suggest the integration of genetic material from the parent strains into the hybrid (Table 4).

Table 4 - Informative RAPD markers for the hybrid *Abb2*

Primer	Components		Hybrid *Abb2*	
	A JA2	**B C54 carb.**		
P20	1	0	1	RAPD.22
	0	1	1	RAPD.23
	0	1	1	RAPD.24

4.4 CONCLUSIONS

Two putative *A. bisporus/A. bitorquis* hybrids have been evaluated using growth rate tests, growth response tests, fruiting trials, segregation analysis and molecular analysis using RAPD markers.

These tests provide strong evidence that the two strains, *Abb1* and *Abb2* are *bona fide* hybrids. The non-fertile putative hybrid *Abb1*, on the basis of the molecular analysis is a hybrid. For the fertile putative hybrid *Abb2* the molecular evidence is less clear cut but both resistance markers segregated in the progeny of this cross. A working hypothesis, which merits further study, is that only a relatively small amount of the genome of *A. bitorquis* has been integrated into the hybrids, specifically that part of the DNA carrying the resistance gene or the auxotrophic gene.

A more extended study should be carried to determine the mechanism of hybridity and the amount of 'foreign' DNA that can participate in a viable hybrid.

4.5 REFERENCES

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4.6 GLOSSARY

SDH	Succinate dehydrogenase
Selective medium	Growth medium which is selective for the growth of a hybrid. Component strains are unable to grow on this medium.
fpa^{s}, *fpa*^{r}	Sensitive or resistant to para-fluorophenylalanine respectively
carb^{s}, *carb*^{r}	Sensitive or resistant to carboxin respectively