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Microbiological properties of casing

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ABSTRACT: The importance of casing bacteria for the pattern of mushroom fruitbody initiation has been studied. Bacterial populations of different casing materials, such as coir and peats, were found to vary greatly. Of the casing materials tested only activated charcoal and lignite retained an ability to stimulate mushroom primordia formation when sterilized. Isolated casing bacteria (mainly *Pseudomonas putida,* also an *Alcaligenes* sp.) were assessed for their effect on mushroom primordia using an agar plate bioassay and microcosms containing cased mushroom spawn. Specific bacterial isolates, identified by ribotyping, were inoculated into microcosms to determine the effect of bacterial dose and timing onprimordium formation. The stability and reproducibility of the bacterial effect, after storage by seven methods over 1 year, on initiation is reported. The bacterial effect diminished if isolates were regularly sub-cultured on agar. A molecular marking system *(luxAB* and tetracycline antibiotic resistance reporter genes) was used to monitor bacteria inoculated into non-sterile casing.

1 INTRODUCTION

Mushroom production depends on covering colonized compost with a casing layer. The casing layer is a major source of variation in yield, quality and uniformity of commercial cropping (Noble and Gaze, 1995). The role of the casing layer is not precisely defined but it must have particular physical, chemical (including nutritional) and microbiological properties (reviewed by Visscher,1988; Rainey, 1989 and Colauto,1998). Air and water holding capacities are important physical parameters; salinity, osmotic potential and pH are chemical parameters which are known to affect mycelial growth (Noble *et al,* 1999). A bacterial casing microflora is essential for commercial fruiting and the species *Pseudomonas putida* has been identified as playing a stimulatory role (Hayes cited in Wood, 1976). The casing layer is a major source of variation in, yield, quality and uniformity of cropping.

We have sought to gain greater understanding, as have many researchers before us, of the key properties of the casing layer and their interrelationships. In particular we have investigated the importance of specific bacteria, of defined population size, in timing and abundance of initiation. Our targets have been threefold:

- stimulation and control of fruitbody initiation to give better control over quality and numbers of mushrooms,
- alternatively, a similar end may be attained by suppression of initiation and mycelial growth,
- to use small scale testing of ARP and other wild strain germplasm to find strains with different initiation patterns, eg. strains producing less initials but with a higher percentage developing into fruit-bodies or strains which do not require casing.

2 METHODS

2.1 *Bacterial populations in casing and ribotyping*

Total bacteria and Pseudomonas spp. communities in the casing layer were quantified using Nutrient Agar and Pseudomonas Isolation Agar (PIA; Difco) respectively. Key tests used to distinguish *P. putida* isolates from all other *Pseudomonas* spp. were: their fluorescence under UV light on Pseudomonas Agar F (PAF,Merck) inability to hydrolyse gelatin and testing positive for arginine dehydrolase. The isolated *P.putida* strains were tested for their effects on mushroom mycelial growth and initiation using a Petri-dish bioassay *withAgaricus bitorquis* W19 on compost extract malt agar (Rainey et al.,1990; Fig.l). Selected *P.putida* strains were further characterised by ribotyping. Riboprint analysis was performed using a Qualicon RiboPrinter™ (Wilmington, Delaware, USA). Fully automated ribotyping of single bacterial isolates was by a five stage process following the manufacturers instructions:

- DNA preparation and restriction enzyme digestion *(EcoRl),*
- Separation of DNA fragments by gel electrophoresis, linked directly to membrane,
- Hybridisation of the rRNA "universal" probe for detection,
- Extraction and visualisation of the pattern (RiboPrint),
- Characterisation and where possible identification by computerised comparison with a RiboPrinter database.

Eight mushroom primordia-stimulatory bacteria were tested for host pathogenicity and natural antibiotic resistance to kanamycin, streptomycin and tetracycline. To enable these bacteria to be identified and monitored in non-sterile casing materials Tn5 minitransposon promotorless *luxAB* (bioluminescent) and tetracycline antibiotic resistance reporter genes were randomly inserted in the bacterial chromosome (Colauto, 1998).

2.2 *Inoculation ofcasing -with bacteria*

A microcosm was used to study the effect of bacteria added to casing on fruitbody initiation. Each microcosm consisted of a Kilner jar (500ml; Fisher Scientific) which contained^, *bisporus* A12 spawn (30g; Somycel) cased with peat/chalk casing (60g, 2cm deep, ca. 30% m/c, surface area 80 cm^2).Sterile distilled water (15ml) was added to the casing when the microcosms were set up, before incubation at 25°C for 3-4d. At this stage a small container with soda lime (Fisher, self-indicating, 1-2.5mm granules) was placed on the surface of the casing, to keep CO_2 levels below 1200ppm. to prevent excessive *Agaricus* mycelium growth on the casing surface thus inhibiting initiation. The microcosm was then moved to a growth cabinet (Fisons) and incubated at 16-18°C for 21 d. Soda lime was checked and replaced when necessary. Primordia >lmm. diameter were counted.

Microcosms were used to investigate the effect on primordia formation of adding *P.putida* n!2 to casing. The casing, of 70% moisture content, was sterilized by gamma-irradiation (Technical Service Consultants Ltd., Heywood,UK).

2.3 *Alternative casing materials*

The effects of alternative casing materials on *A.bisporus* A12 primordia formation were determined using the microcosms previously described. Peat and coir based casing (70% moisture content) were sterilized by gamma irradiation; charcoals, lignite, used horticultural rockwool and vermiculite were sterilized by autoclaving twice for one hour at 121 °C.

2.4 *Stability of bacterial isolates*

To assess the stability of bacterial stimulatory activity, *P.putida* n!2 was grown in Nutrient Broth. Bacteria were harvested by centrifugation, re-suspended in sterile distilled water to ca. $Ix1O^9$ cfu ml^{-1} and stored using seven different methods:

on PAF agar at 20°C, sub-cultured every two weeks; on PAF agar slopes, in sterile distilled water or sterile casing all maintained at 5°C; freeze dried in glycerol (10% v/v); frozen in 10% v/v glycerol at -20°C or stored in liquid N. After 1 month or 1 year bacteria were retrieved from

their storage media by isolation of single colonies on PAF agar. Isolated bacteria were then assessed for their continued ability to stimulate primordia formation using the *A.bitorquis* W19 agar plate bioassay. *P.putida* n!2 strains, retrieved from storage, were also grown in liquid culture, cells harvested and suspended in sterile distilled water to ca. $7x10^9$ cfu ml^{-1}. Bacterial suspensions (1ml) were then inoculated onto sterile casing in microcosms. Five replicate microcosms were set up for each bacterial storage treatment.

3 RESULTS AND DISCUSSION

3.1 *Bacterial populations in casing materials*

Bacteria from seven different materials were enumerated on a range of selective media at casing, pinning and end of cropping (Colauto,1998; Colauto and Eira,1998). Bacterial populations varied greatly from dry coir (predominantly *Corynbacteriaceae* and *Bacillus* spp.) to wet black peat (mainly *Pseudomonas* spp. 40% of which were *P.putida).* Bacterial populations on different types of casing materials are shown in Fig.2. Storage of casing materials caused reductions in total bacteria populations, particularly *Pseudomonas* spp., these populations recovered after the stored casing material was watered (Colauto and Eira,1998).

3.2 *Alternative casing materials*

In experiments to determine the effect of alternative casing materials on *A.bisporus* A12 primordia formation in a microcosm only two of the non-sterile materials tested, animal charcoal and vermiculite, failed to support formation of primordia (Table 1). Only activated charcoal and lignite allowed primordia formation in axenic conditions. Microcosms with brown milled Irish peat casing formed more primordia than commercial ready-mixed peat (80% black + 20% brown peat + sugar beet lime (SBL)), which in turn was more productive than wet black peat + SBL. All the materials listed in Table! produced primordia and normal mushrooms in tray culture (except charcoal, which has not been tested).

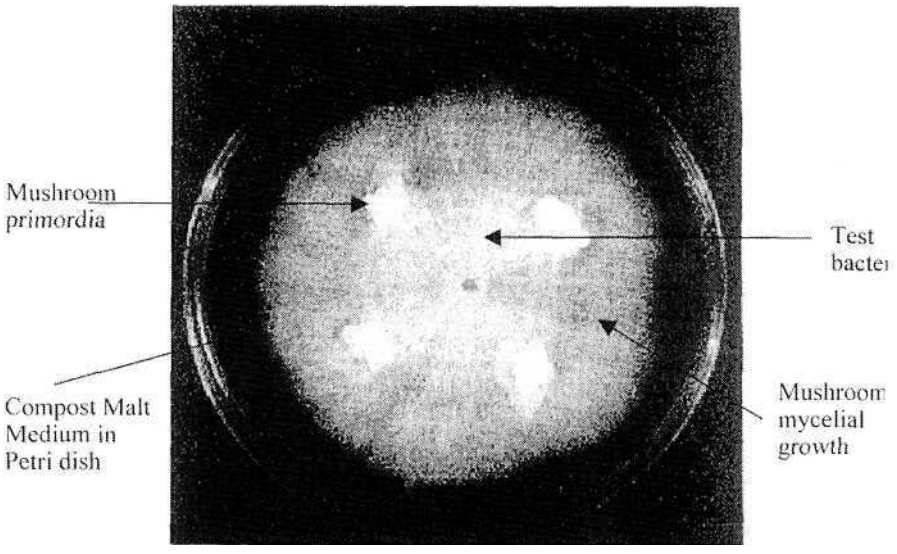
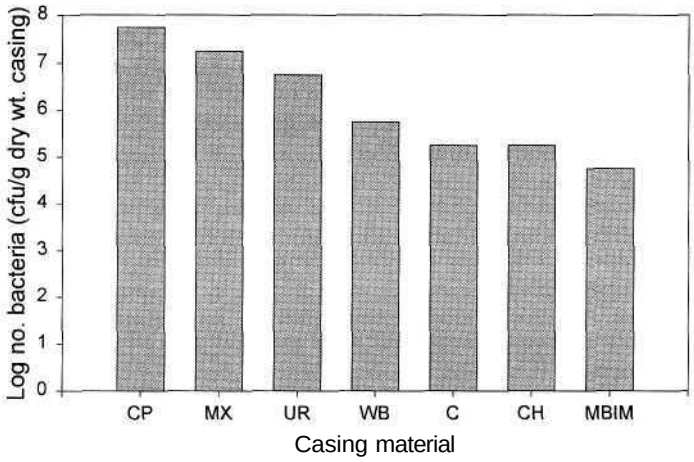


Fig.l *Agaricus bitorquis* W19 bioassay (Rainey *et al* 1990). The petri dish containing 25 ml CMM was inoculated centrally with a 5mm plug of W19. After incubating for 1 week at 30°C, four bacterial streaks were inoculated onto the surface of the agar (at right angles). Primordia formed where W19 extended over the bacterial colonies.



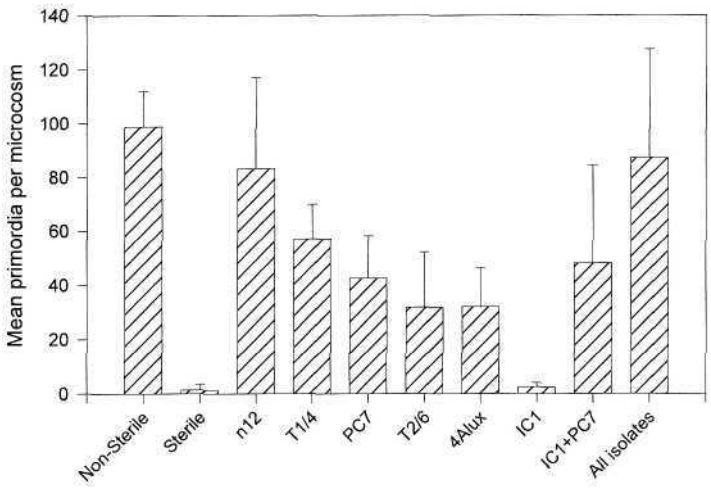
CP Peat + CaCOs .
MX Brown peat (80%), black peat (20%) + CaCOs .
UP Used Rockwool.
WB Wet black peat.
C Coir (coconut fibre).
CH Chalk
MBIM Milled brown Irish peat.

Fig.2 Bacterial populations on different types of casing material (from Colauto, 1998)

Table 1. Effect of alternative casing materials on *A. bisporus* A12 primordia formation in a microcosm

Casing material		Details	Number of Primordia[1] Non-sterile	Sterile
Charcoal				
	Activated	8-20 mesh; Sigma	40-160	70-170
	Animal	Granular; Fisher	0	0
	Lumpwood		5-10	0
Lignite				
	"Brown coal"	Particulate	ND[2]	35
Peat[3]				
	Black		<150	0
	Brown		100-250	0
	Commercial	Ready-mix (80%black+20%brown).	40-170	0
Coir[3]				
	Filipino	Growing medium.	210	0
	Indian B2	Growing medium.	110	0
	Indian B4	Growing medium.	70	0
	Sri Lanka	Carpet factory waste.	10	0
Rockwool		Water absorbent. used in tomato crop	>1	0
Vermiculite		Growing medium, medium mesh.	0	0

[1] per microcosm, minimum of 5 replicates per treatment
[3] ND, No data,always contaminated with *Trichoderma* sp.
- mixed 4:1(w/w) with SBL.



NS Non-sterile control. Treatment (bacterial isolates)
S Sterile control.
n12 from commercial peat/chalk casing.
T1/4 from brown peat.
PC7 from filipino coir.
T2/6 from used rockwool.
4Alux marked isolate from brown peat.
IC1 *Alcallgenes* sp.

Fig. 3. Effect of 'stimulatory' bacterial inocula on production of *A. bisporus* A12 primordia in sterile brown peat/chalk casing

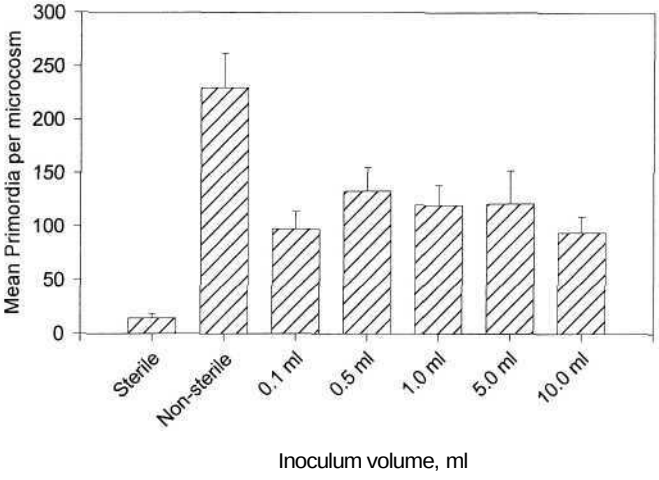


Fig. 4 Effect of *P.putida* n!2 inoculum level on *A. bisporus* A12 primordia formation. The bacterial stock solution contained 8 x 10^8 cfu ml"[1]

3.3 *Ribotyping*

Automated ribotyping of putative *P.putida* isolates proved an effective but expensive means of confirming strain identity. Eleven *P.putida* strains isolated from casing materials, plus two

"type" strains, *P.putida* (biovar A) ATCC12633 isolated from soil in the USA and *P.putida* ("arvilla") PaW8, were successfully typed (Fermor *et al*, unpublished results). All isolates were confirmed as authentic strains *of P.putida.*

Three strains isolated from black peat, brown and used rockwool respectively, had identical RiboPrint patterns. Isolate T6/6, from a brown-peat / pasteurised compost / chalk casing mix, was authenticated as a *P.putida* strain, both by standard bacteriological tests and ribotyping. However, this strain was a non-stimulator and had a RiboPrint pattern easily distinguished from primordia stimulating isolates.

3.4 *Inoculation of casing with bacteria*

Fig.3 shows the effect on *A. bisporus* primordia production of adding individual and mixed populations of primordia-stimulatory bacteria to sterile peat/chalk casing. Only two treatments, a mixture of six bacteria *or P.putida* n!2 alone, lead to primordia numbers equivalent to those found with non-sterile casing. *Alcaligenes* sp. (IC1), originally isolated from "sterile", irradiated peat gave a positive stimulatory response in *iheA.bitorquis* W19 bioassay, but little response in the microcosm experiments.

After 42 days (approximating to end of crop in a commercial production cycle) bacterial populations in each microcosm ranged from $1x10^7$ to $1x10^8$ cfu g^{-1} of dry casing; *Alcaligenes* sp. (IC1) was an exception ($1x10^5$ cfu g^{-1}).

3.5 *Timing and dosage experiments*

P.putida n!2 a known stimulating bacterial strain, was added to sterile peat/chalk casing over a dosage range of $8x10^7$ to $8x10^9$ cfu per microcosm. No significant dosage effect was found (Fig.4).

However, it was found that when *P.putida* n!2 was inoculated into sterile casing at the time when casing was applied, more primordia formed than when an inoculum was added 4 or 7 days after casing was applied (Fig.5). Primordia were first seen in these microcosm systems 14 days after casing.

3.6 *Tracking of marked bacteria*

When genetically marked populations *of P.putida* were introduced into non-sterile peat/chalk

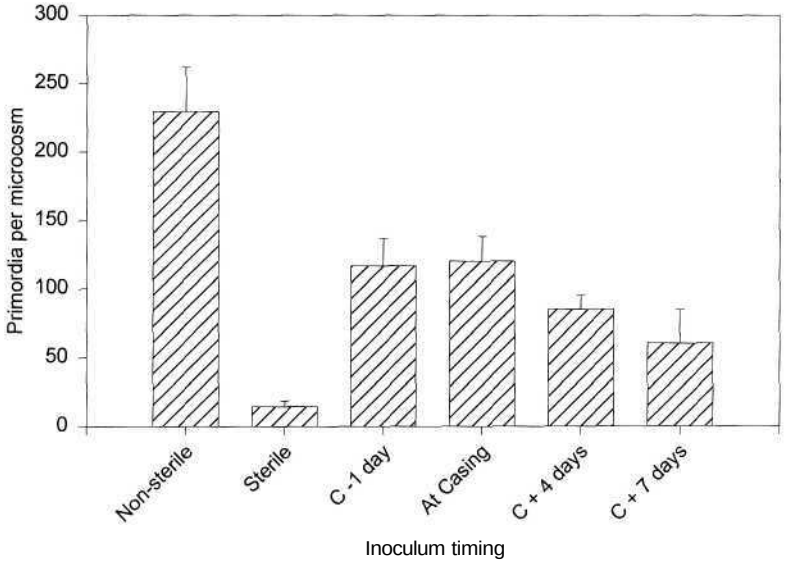


Fig.5 Effect of timing *of P.putida* inoculation on *A. bisporus* A12 primordia formation

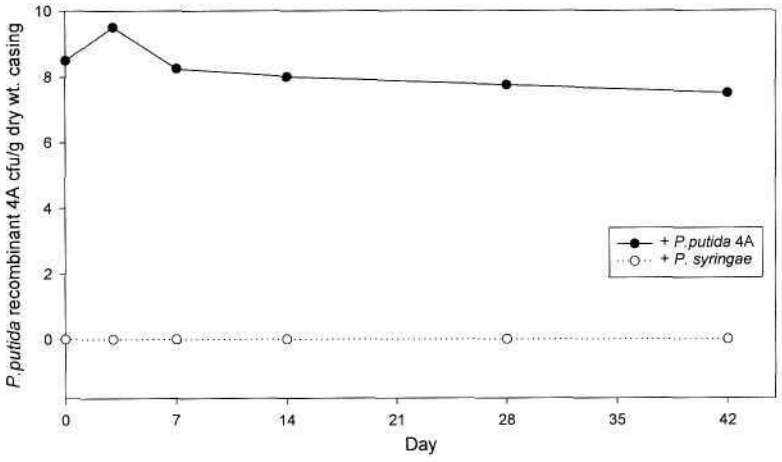


Fig. 6. Survival of marked *P.putida* in non-sterile casing monitored using Nutrient Agar selective media containing tetracycline (Colauto, 1998)

Table 2. Effect of storage on ability of *Pseudomonas putida* n!2, added to sterile peat/chalk casing, to stimulate formation *ofAgaricus bisporus* A12 primordia in jar microcosms.

Bacterial storage	% primordia formed '		
	P. putidaW2 storage time		
	Freshly isolated	1 month	1 year
Non-sterile casing[1] (no added bacteria).	100 (+12)	100 (+11)	100 (+20)
Sterile casing.	1 (+1)	5 (+5)	7 (+12)
Sterile casing + *P.putidanl2*	85 (+33)		-
Regular sub-culture on agar ,20°C.		52 (+17)	55 (+12)
Refrigerated, 5°C			
- agar slope		104 (+39)	49 (+24)
- casing mix		83 (+23)	88 (+17)
-distilled water		84 (+34)	98(+29)
Freezer, -20°C		100 (+11)	68 (+15)
Liquid N		75 (+24)	68(+13)
Freeze dried		82 (+28)	56(+32)

'Number of primordia counted on non-sterile casing per microcosm was in range 40-120.
[2]Each result is the average of 5 replicate microcosms.
[3]The percentage of primordia formed is expressed as percentage of average primordia formed in non-sterile casing control microcosms assayed at the same time.

casings, in small-scale mushroom house cropping trials, the introduced *P. putida* showed a remarkable capacity to survive in the presence of large mixed bacterial populations (Fig.6). Because of high humidities (>90%RH) maintained in the cropping house, they also spread rapidly to non-inoculated control plots.

3.7 *Stability ofbacterial isolates*

After storage for 1 month all except the regularly sub-cultured isolate stimulated primordia

formation in the Petri dish bioassay. Only *P.putida* n!2 maintained by regular sub-culturing on PAF agar incubated at 20°C had a significantly decreased ability to stimulate primordia formation in microcosms (Table 2) after storage for 1 month. After storage for 1 year all isolates had retained some stimulatory activity. However, only those isolates stored in sterile peat casing mix at 5°C showed unimpaired stimulatory activity when compared to that of the freshly isolated strain.

Bacteria may only be of indirect importance in fruitbody initiation. Morphogenetic cell death plays a key role at different stages during the formation of mycelial strands and formation of hyphal aggregates which differentiate to form fruitbody initials (Umar and Van Griensven, 1998). We are currently studying the effect of adding "stimulatory" bacteria to casing, on mushroom mycelial cord and hyphal aggregate formation together with calcium oxalate crystal presence, using the microcosm system. Unravelling the complexities of casing remains an elusive target, which now requires the use molecular biology techniques to provide unambiguous answers. Work presented here has added a few more pieces to the jigsaw, it cannot show the whole picture.

4 ACKNOWLEDGEMENTS

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