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Volatile C8 compounds and pseudomonads influence primordium formation of *Agaricus bisporus*

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Abstract: Primordium formation of *Agaricus bisporus* depends on the presence of a 'casing layer' containing stimulatory bacteria, and on sufficient air exchange. The influence of specific pseudomonad populations and volatile organic compounds (VOCs) on primordium formation of *A. bisporus* was studied in microcosm cultures. VOCs produced by *A. bisporus* mycelium were predominantly C8 compounds, some of which could inhibit primordium formation, with 1-octen-3-ol being most inhibitory. A VOC produced by the rye grain substrate on which *A. bisporus* was grown, 2-ethyl-1-hexanol, also inhibited primordium formation. 2-Ethyl-1-hexanol and 1-octen-3-ol were metabolized by pseudomonad populations and adsorbed by activated charcoal, with both modes of removal enabling primordium formation in the casing. Removal of VOCs by ventilation also enabled primordium formation to occur under axenic conditions. The presence of 2-ethyl-1-hexanol and 1-octen-3-ol in the microcosms resulted in higher total bacterial and pseudomonad populations in the casing. The stimulatory effects of the casing and its microbiota, and air exchange on primordium formation of *A. bisporus* are due, at least partly, to the removal of inhibitory C8 compounds produced by the mycelium and its substrate. Monitoring and

controlling the levels of these inhibitory VOCs in mushroom culture should enable primordium formation of *A. bisporus* to be more efficiently and precisely controlled.

Key words: casing, 2-ethyl-1-hexanol, mushroom, 1-octen-3-ol, *Pseudomonas putida*

INTRODUCTION

Fructification in the button mushroom (*Agaricus bisporus*) begins with the aggregation of mycelium into thick strands, followed by the formation of primordia, some of which develop further into sporophores. This is a highly inefficient process, with generally less than 7 % of initiated primordia developing further into sporophores (Flegg 1979; Noble et al 2003). Primordium formation is stimulated by a reduction in temperature, and an increase in ventilation, which is considered to have an effect through reduced CO_2 concentration (Tschierpe & Sinden 1964; Flegg & Wood 1985). *A. bisporus* also has a requirement for a 'casing layer' (casing) which has imprecisely defined chemical and microbiological properties that stimulate primordium formation (Wood 1976; Flegg 1979). The presence of stimulatory bacteria in the casing, of which *Pseudomonas putida* is regarded as most significant (Hayes et al 1969; Rainey et al 1990), is usually necessary for primordium formation to occur. Activated charcoal and other adsorbent materials can obviate the requirement for stimulatory pseudomonads (Eger 1961; Long & Jacobs 1974; Noble et al 2003), possibly by adsorbing compounds that are inhibitory to primordium formation. Attempts to isolate putative inhibitors to primordium formation from activated charcoal casing have been confounded by the large number of compounds adsorbed and identified (Grove 1981). However, several volatile C8 compounds were identified that were characteristic of *A. bisporus* and of sufficiently low molecular weight to be readily adsorbed by activated charcoal. Of these, 1-octen-3-ol was found to inhibit primordium formation *in vitro* on malt agar without inhibiting mycelial growth (Wood & Blight 1982).

Since the function of the casing and mechanism of primordium formation are not fully understood, fructification in button mushroom culture has relied on the empirical selection of casing materials (Noble et al 2003; Beyer 2004) and by reducing temperature and CO_2 concentration at an appropriate stage (Flegg & Wood 1985). Attempts to reduce the

stimulation of excess numbers of primordia and improve the efficiency of fructification, by manipulation of temperature or ventilation, or selection of less conducive casing materials, have resulted in excessive vegetative mycelial growth in the casing ('stroma') and/or reduced sporophore production (Flegg 1979; Flegg & Wood 1985).

This work aimed to improve the understanding of the mechanism of primordium formation of *A. bisporus in vivo* by: (i) determining the stimulatory effect of isolates of *P. putida* and related *Pseudomonas* species defined by their 16S rRNA sequences (Elomari et al 1996; Anzai et al 2000); (ii) measuring the effect of the casing microbiota on the concentration of specific VOCs produced by mushroom mycelium and its substrate; and (iii) determining the effect of these VOCs on primordium formation and on the populations of bacteria in the casing.

MATERIALS AND METHODS

Axenic microcosm tests.—A 500 mL glass microcosm culture system (Noble et al 2003) was used to examine growth in axenic casing materials. The microcosms were filled with a 10 mm layer (30 g) of sterile rye grain 'spawn', which served as a nutritional substrate, colonized with the *A. bisporus* strain A15 (Sylvan Spawn, Peterborough, UK), except where stated. This substrate was covered with 60 g (about 17 mm layer) of casing, consisting of a mixture of black peat and $CaCO_3$ (4:1 v/v) or activated charcoal (granular, 4–8, Sigma Aldrich, Poole, Dorset, UK). Casing material was autoclaved at 121 C for 2 h for axenic treatments. Temperatures (25 C for 7 d followed by 16 C), CO_2 concentrations (>0.5%v/v followed by 0.08–0.12%v/v) and casing matric potential (−1 to −2 kPa) in the microcosms were maintained as described in Noble et al (2003). Microcosms were arranged in an incubator in a randomized block design with each incubator shelf containing a complete replicate block of each set of treatments. The numbers of primordia > 1 mm diameter were recorded 28 d after the microcosms were filled, i.e. 12 to 16 d after the first primordia had reach this size. Bacterial numbers in the casing were recorded before and after mushroom culture as described below.

Bacterial isolates and populations in casing materials. —Seven isolates of *P. putida* or closely related species were obtained from casing materials and other growing media (Fermor *et al*. 2000): *P. veronii* (MAR2 and MAR12), *P. poae* (n12,), *P. putida* (T2/6 and T1/4) and an unnamed species *P.* PsI (NSC4 and NSC6). Three further isolates of *P. putida* (4Alux, Paw8 and Paw340) were obtained from the Warwick HRI culture collection. The taxonomy of these isolates was determined by comparison of the 16 S rRNA gene sequences with sequences that have been obtained from related *Pseudomonas* isolates in the EMBL database, using the program FASTA (Genetics Computer Group, University of Wisconsin) (TABLE I).

The *Pseudomonas* isolates were grown in nutrient broth (LB broth (Miller) Merck, Darmstadt, Germany). The bacteria were harvested by centrifugation, re-suspended in sterile distilled water to 10^9 cfu mL^{-1}. Bacterial suspensions (1 mL) were then inoculated on to axenic peat + $CaCO_3$ casing in *A. bisporus* microcosm cultures. Axenic and non-axenic casing were used as controls. There were three replicate microcosms of each *Pseudomonas* isolate and axenic or non-axenic culture treatment. The experiment was repeated four times.

Bacterial populations in the casing in microcosms were determined by preparing suspensions of 1 g casing in 9 mL of sterile Ringers solution (Fisher Scientific, Loughborough, UK). Serial dilutions of the suspension were then plated on nutrient agar (CM309, Oxoid, Basingstoke, UK) and pseudomonas isolation agar (PIA) (Difco Laboratories, Detroit, USA) and then incubated at 25 C for 48 h to determine the total bacterial populations as colony forming units per g casing (cfu g^{-1}), and to estimate the proportion which were pseudomonads.

Analysis of volatile organic compounds in microcosms.—Two methods involving gas chromatography-mass spectrometry (GC-MS) were used for analyzing the VOCs produced by *A. bisporus* mycelium in the casing and in the headspace of axenic and non-axenic microcosms. A solvent extraction method involved washing of casing in diethyl ether followed by low temperature analysis of the solvent. Casing (5 g) was put in the porous thimble of a Soxhalet apparatus and VOCs were extracted in 45 mL of diethyl ether for one

hour. The resulting samples (1 μL) were then injected on to a GC column (SPB1, 30 × 0.25 mm, 0.25 μm film thickness, Supelco, Bellefonte, PA, USA) of a Shimadzu QP 5000 GC-MS system (Shimadzu UK, Milton Keynes). The flow rate of the eluting gas, helium, was 1.1 mL min^{-1}. The temperature ramp of the GC oven was set to: rate: temperature (C): time (s) (0:50:5, 20:150:0 and 4:250:5).

Although unsuitable for analysis of high moisture content casing, a thermal desorption method was used for analyzing VOCs in the headspace of microcosms, due to higher resolution than the solvent extraction method. VOCs from the microcosm headspace were firstly pre-concentrated on to adsorbents (Carbotrap 20/40 mesh and Tenax TA 60/80, Supelco, 0.3 g) contained within 6 mL porcelain crucibles placed on the surface of the casing. The concentrated VOCs were then thermally desorbed from 220 mg samples of the adsorbents into a GC-MS system for identification and quantification. A Hewlett Packard (hp, Stockport, Cheshire, UK) GC-MS system consisting of a 5890 II Series gas chromatograph and a 5972A mass selective detector (MSD II) was used for analysis. Chromatographic retention time and mass spectral matching were used to confirm VOC identity. A 25 m fused silica (cross linked methyl siloxane) hp-1 column with an internal diameter of 0.2 mm and a 0.34 μm film with a 1 m, 'Q plot', deactivated fused silica guard column (internal diameter 0.53 mm), containing a porous polymer of divinylbenzene (Supelco) was used. The flow rate of the eluting gas, helium, was 0.75 mL min^{-1}. An Optic temperature programmable injector (Ai Cambridge, Pampisford, Cambridge, UK) was used to desorb headspace samples from the adsorbents and was initially set to 30 C and then heated at 16 C s^{-1} to 250 C. An electronic pressure controller was used to offset peak pressure broadening with increasing GC column temperature. The GC oven conditions were an initial temperature of 40 C, then increased to 220 C at 15 C min^{-1} and remaining at 220 C for 1 min. The GC-MS interface was at 280 C. The mass spectrometer scanned from 35 to 250 mass units every 0.2 s to give responses in the ng range.

VOCs detected by the mass spectrometers were identified using a probability based matching algorithm and a NIST mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD, USA). Compounds were declared unknown if their matching probability was less than 80 (100 being a perfect match).

Samples of adsorbent or casing were analyzed using the solvent extraction and thermal desorption methods respectively, before use for *A. bisporus* culture to test for background VOCs. VOC levels obtained showed evidence of a mean-variance relationship. These concentrations were subjected to a logarithmic transformation prior to analysis.

Primordium formation and production of VOCs by A. bisporus *strains.* —Microcosms were prepared using axenic and non-axenic peat + $CaCO_3$ casing as previously described. The *A. bisporus* strains A15 and 5776 (Le Lion, Varrains, France) were selected on the basis of known difference in primordium formation in culture. Microcosms without *A. bisporus* inoculum were prepared to test for background VOCs in the culture. Crucibles containing the Carbotrap or Tenax adsorbents were placed in each microcosm after 7 d, at the time of transfer from 25 C to 16 C. The adsorbents were analyzed by the thermal desorption method after a further 21 d, at the same of recording numbers of primordia and casing bacteria. Eight replicate microcosms were prepared for each *A. bisporus* inoculum, casing sterility and adsorbent treatments, giving a total of 96 microcosms.

Effect of C8 compounds on primordium formation and casing bacteria. —The effect of introducing specific VOCs identified from the above experiment into the atmosphere of microcosms was examined. Microcosms were prepared using axenic activated charcoal and non-axenic peat + $CaCO_3$ casing as previously described. The following VOCs (10 µL) were placed in 20 ml glass beakers in microcosms, after 7 and 17 d: 2-ethyl-1-hexanol, octane, 1-octanol, 3-octanone, *trans*-3-octene and 1-octen-3-ol. 2-Ethyl-1-hexanol was also used at 1 µL per beaker. Beakers with water were used as a control. Samples of the casing materials were analyzed by the solvent extraction method. Three replicate microcosms of each VOC and casing material treatment were prepared, giving a total of 48 microcosms.

Effect of VOCs on primordium formation and casing bacteria in ventilated flasks. — Composted substrate (Noble et al 1998) (100 g) was filled into 350 mL glass jars. After autoclaving (axenic jars only), the substrate was inoculated at 2% w/w with rye grain spawn of the *A. bisporus* strain Sylvan A15. The jars were incubated under sterile conditions for 14 d at 25 C. The colonized substrate was then covered with 80 g of the above axenic or non-axenic peat + $CaCO_3$ casing and the jars placed in 10 L 'Quickfit' multiadapter flasks (Fisher Scientific, Loughborough, UK) with 5 jars per flask. Sterile water to a depth of 30 mm in the base on the flasks maintained a relative humidity > 90%. The flasks were then sealed with bacterial air vents and incubated at 25 C for about 8 d until mycelium became visible at the surface of the casing, which was then watered with 10 mL sterile water on each jar. The flasks were then transferred to a room at 18 C and connected to a humidified, filtered air flow of 15 L h^{-1} to maintain a CO_2 concentration of 0.7–0.11% v/v.

The air flow to the multiadapter flasks was passed through sealed 300 mL flasks containing 3–5 mL of ethanol, 2-ethyl-1-hexanol, octane, 3-octanone, 1-octen-3-ol or *cis*-3-octen-1-ol. The level of VOCs was maintained by replenishment at 4–5 d intervals and by placing the 300 mL flasks in ice to reduce volatilization. Axenic and non-axenic cultures aerated without VOCs were used as controls. Two replicate multiadapter flasks aerated with each VOC, and four replicate axenic and non-axenic controls were prepared. The numbers of primordia, sporophores (developmental stage 4, Hammond & Nichols 1976), total bacteria and pseudomonads in the casing were recorded 20 d after the casing was applied as previously described. The supply of VOCs to flasks was then removed and the flasks aerated with fresh air only. The number of primordia and sporophores was then recorded after a further 10 d.

Bacterial and primordial populations showed evidence of a mean-variance relationship. These populations were respectively subjected to a logarithmic or square root transformation prior to analysis. All differences in the results section were significant at $P < 0.05$, or if stated, $P < 0.01$ or 0.001.

RESULTS

Effect of Pseudomonas *isolates on primordium formation.*—The *Pseudomonas* isolates added to axenic casing were found to differ in terms of growth in the casing and in stimulating primordium initiation (FIG.1). Isolates n12 and NSC4 stimulated the formation of more primordia than other isolates whereas the *P. veronii* isolates MAR2 and MAR12 stimulated the formation of the fewest primordia, although had the highest pseudomonad populations in the casing at the end of the experiment (3.5×10^8 and 3.7×10^8 cfu g^{-1} respectively compared with $1.4 \times 10^8 - 2.6 \times 10^8$ cfu g^{-1} for the other isolates). None of the *Pseudomonas* isolates added to axenic casing stimulated the formation of as many primordia as non-axenic casing (FIG. 1) ($P<0.001$). In non-axenic casing during *A. bisporus* culture, there were slight increases in the populations of pseudomonads, from 6.0×10^5 to 7.6×10^5 cfu g^{-1}, and in total bacteria, from 5.1×10^6 to 6.0×10^6 cfu g^{-1}. However, the final population of pseudomonads in non-axenic casing was significantly ($P<0.001$) smaller than the final populations in the axenic casing inoculated with *Pseudomonas* isolates.

Primordium formation and production of VOCs by A. bisporus *strains.*—Significant formation of primordia only occurred in the non-axenic microcosms, with the *A. bisporus* strain A15 forming more primordia than the strain 5776 (TABLE II) ($P<0.01$). By the time of primordia assessment, one or two primordia had developed further into immature sporophores (stages 1 and 2, Hammond & Nichols 1976) in some of the microcosms. However, the formation and development of primordia and sporophores was slower in the microcosms than in the larger scale flask culture due to the lower temperature (16 C compared with 18C) (Flegg 1979; Noble et al 2003).

In the presence of *A. bisporus* inoculum, the pseudomonad population of non-axenic casing increased from 7.5×10^5 to 8.1×10^5 cfu g^{-1}, but declined to 1.2×10^4 cfu g^{-1} without *A. bisporus* inoculum. The total bacterial population of the casing declined from 1.9×10^7 cfu g^{-1} to 6.8×10^6 and 2.5×10^5 cfu g^{-1} in the presence and absence of *A. bisporus* inoculum

respectively. Final populations of total bacteria and pseudomonads in the casing of non-axenic microcosms were unaffected by the *A. bisporus* strain and adsorbent treatments.

No VOCs were detected in the adsorbents before use. Over 30 VOCs were identified in the chromatograms of the adsorbent traps from microcosms, with concentrations of most VOCs significantly higher in the Tenax than in the Carbotrap. A number of compounds with molecular weights exceeding 150 were detected in trace amounts (e.g. 3,7-dimethyl-1,6-octadien-3-ol); these are excluded from TABLES II and III. Several VOCs were detected in both the microcosms with and without *A. bisporus* inoculum, with 2-ethyl-1-hexanol, benzyl alcohol and benzone compounds being most abundant (TABLE III). For all of these VOCs, the concentrations were highest in the axenic, uninoculated microcosms and were reduced by the presence of the microbiota in the non-axenic microcosms, and the *A. bisporus* inoculum. The exception was 1-bromo-heptane, which was greater in the microcosms containing *A. bisporus* ($P<0.001$). 1-Bromo-octane was only detected in microcosms inoculated with *A. bisporus* (TABLE II). These halogenated VOCs may be *A. bisporus* metabolites of 1-bromo-decane, which was most abundant in the axenic microcosms ($P<0.001$). VOCs that were present only in the microcosms inoculated with *A. bisporus* were exclusively 8-carbon compounds (TABLE II). With the exception of 1-octanol, the concentrations were higher in the axenic microcosms than in the non-axenic microcosms.

Effect of 8-carbon compounds on primordium formation and casing bacteria.—The 8-carbon compounds in the microcosms did not affect mycelial growth in the casing. The numbers of primordia that formed on axenic activated charcoal and non-axenic peat + $CaCO_3$ casing in the presence of different C8 compounds is shown in FIG. 2. The axenic charcoal casing resulted in more primordia than the non-axenic peat + $CaCO_3$ casing ($P<0.01$). The presence of 2-ethyl-1-hexanol in the microcosms suppressed primordium formation. In the microcosms containing peat + $CaCO_3$ casing, 1 μL of 2-ethyl-1-hexanol was sufficient to cause a suppressive effect; in the microcosms containing axenic activated charcoal, 10 μL were required. The other C8 compounds did not significantly affect primordium formation in the microcosms.

No VOCs were detected in the casing materials before use. At the end of the experiment, no C8 compounds were detected using the solvent method of extraction in any of the microcosms containing peat + $CaCO_3$ casing or in the control microcosms containing axenic activated charcoal (FIG. 2). At the end of the experiment, 2-ethyl-1-hexanol, 1-octen-3-ol, 3-octanone and *trans*-3-octene were detected in the activated charcoal casing at similar concentrations.

The total bacterial population of the control treatment casing did not change significantly during the culture period (2.5×10^7 and 2.3×10^7 cfu g^{-1} at the beginning and end respectively), whereas the pseudomonad population increased from 7.5×10^5 cfu g^{-1} to 1.1×10^6 cfu g^{-1}. Final total bacterial numbers in the peat + $CaCO_3$ casing were higher in the presence of 1-octen-3-ol and 2-ethyl-1-hexanol than in the control microcosms and in the presence of the other C8 compounds (FIG. 3). The pseudomonad population was increased by the presence of 2-ethyl-1-hexanol in the microcosms ($P<0.01$) (FIG. 3).

Effect of VOCs on primordium formation and casing bacteria in ventilated flasks. —The presence of ethanol or C8 compounds into ventilated flask culture reduced mycelial growth in the casing. Primordia and sporophores were produced in both axenic and non-axenic ventilated flask culture, although the numbers of sporophores were greater in non-axenic conditions (TABLE IV). The presence of ethanol or 1-octen-3-ol almost completely inhibited primordium formation, which was also suppressed by 2-ethyl-1-hexanol and *cis*-3-octen-1-ol. Octane suppressed the numbers of primordia but not of sporophores and 3-octanone had no significant effect (TABLE IV). Following the removal of the VOCs from the air stream into the flasks, primordium formation occurred within 7 d and at least one sporophore per jar was produced in all the treatments.

The pseudomonad population of the casing in the control treatment remained stable during the *A. bisporus* culture period although the total numbers of bacteria declined (TABLE IV). 2-Ethyl-1-hexanol and 1-octen-3-ol increased the total bacterial population of the casing

during the culture period ($P<0.001$) and all the VOCs, except 3-octanone, increased the population of pseudomonads in the casing.

DISCUSSION

This work has confirmed earlier research (Grove 1981; Combet et al 2006) that VOCs produced by *A. bisporus* mycelium are predominantly C8 compounds. The *in vivo* results from microcosm cultures support the hypothesis based on *in vitro* agar plate cultures (Wood & Blight 1982), that some of these C8 compounds can inhibit primordium formation, with 1-octen-3-ol being most inhibitory. The results have also shown that a VOC produced by the rye grain substrate on which *A. bisporus* is grown, 2-ethyl-1-hexanol, can inhibit primordial formation. 2-Ethyl-1-hexanol is a metabolite known to be produced by *Acremonium obclavatum* and *Aspergillus versicolor* (Enzeonu et al 1994; Pasanen et al 1997) although its source in the substrate in these experiments was not established. 2-Ethyl-1-hexanol and 1-octen-3-ol were metabolized by the casing microbiota and adsorbed by activated charcoal casing, with both modes of removal enabling primordium formation, as previously suggested by Eger (1972) and Long & Jacobs (1974). 2-Ethyl-1-hexanol was inhibitory to primordium formation at lower concentrations than C8 compounds produced by *A. bisporus* and at concentrations below which a negative effect on mycelial growth was observed. The inhibitory effect was less with activated charcoal casing than with peat-based casing, probably due to the greater adsorption capacity of activated charcoal compared with peat. Wood & Blight (1982) noted that 1-octen-3-ol inhibited fruitbody formation but not mycelial growth on agar culture. Mau et al. (1992) identified a compound 10-oxo-*trans*-8-decenoic acid (ODA) from *A. bisporus* sporophores that stimulated mycelial growth and stipe elongation of *A. bisporus*. Supplementation of the casing with ODA resulted in an increase in the number of sporophores produced and they suggested that ODA may be involved in the initiation of fruiting, although this was not examined.

The presence of 2-ethyl-1-hexanol and 1-octen-3-ol resulted in higher total bacterial and pseudomonad populations in the casing. These bacteria were therefore able to utilize and metabolize the inhibitory VOCs, as previously suggested by Hayes et al (1969) and Eger

(1972). It is well established that pseudomonads are able to utilize C8 compounds as substrates (Chakrabarty et al 1973). In these experiments, the population of pseudomonads in the casing tended to increase during *A. bisporus* culture, whereas the total bacterial population remained stable or slightly declined. The increasing dominance of pseudomanads in the casing bacterial population during *A. bisporus* culture was also observed by Hayes & Nair (1974) and indicates the significance of pseudomonads in metabolizing VOCs produced by *A. bisporus* mycelium. However, none of the *Pseudomonas* isolates tested were able stimulate primordia formation in axenic casing to the same extent as a naturally occurring microbiota in non-axenic casing, in spite of the inoculated casing having a significantly higher pseudomonad population. This confirms earlier work by Eger (1972) and indicates that either a mixed pseudomonad or bacterial population is more stimulatory to primordium formation than individual pseudomonads. An isolate of *P. poae* stimulated the formation of more primordia than several *P. putida* isolates and two isolates of *P. veronii* had little or no stimulatory effect on primordium formation. This confirms previous work which has identified *P. putida* and closely related species as being stimulatory to primordium formation, with several other *Pseudomonas* species, including *P. aeruginosa, P. agarici, P. fluorescens, P. reactans* and *P. tolaasii*, being non-stimulatory (Hayes et al 1969; Rainey et al 1990; Fermor et al 2000). Significant differences in the stimulatory behaviour of several *P. putida* isolates were observed, in agreement with Fermor et al (2000) but not Rainey et al (1990). Further work is needed to test if the VOC metabolizing capability of individual *Pseudomonas* isolates, or of mixed bacterial populations, relates to their stimulatory effect on primordium formation.

Removal of inhibitory VOCs by ventilation enabled primordium formation to occur on peat-based casing under axenic conditions. This has not been previously observed, probably due to the enclosure and inadequate ventilation of axenic culture systems that have been used before (Long & Jacobs 1974; Yeo 1980; Noble et al 2003). The requirement for sufficient air exchange for primordium formation of *A. bisporus* has been attributed solely to a reduction in CO_2 concentration (Tschierpe & Sinden 1964; Flegg & Wood 1985). In the present

microcosm experiments, a reduction in CO_2 concentration using soda lime adsorbent did not overcome the inhibitory effect of the VOCs. Experimentation is needed to separate the independent effects of inhibitory VOCs and CO_2 on primordium formation.

This work has shown that the stimulatory effects of the casing and its microbiota, and air exchange on primordium formation of *A. bisporus* are due, at least partly, to the removal of inhibitory C8 compounds produced by the mycelium and its substrate. Mushroom strains differ in their production of C8 compounds which influence their primordium formation. Monitoring and controlling the levels of these inhibitory VOCs in mushroom culture should enable primordium formation of *A. bisporus* to be more efficiently and precisely controlled. This could be achieved by intermittent adsorbent trapping and subsequent analysis of VOCs from culture room air (Pfeil & Mumma 1992), and adjustment of room ventilation accordingly. The influence of different mushroom strains and substrates on the production of C8 compounds should also be investigated further.

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FIGURE CAPTIONS

FIG. 1. Numbers of primordia > 1 mm diam (square root transformed values) the formed on non-axenic peat + $CaCO_3$ casing (control, □) and on axenic casing inoculated with different *Pseudomonas* isolates (■). No primordia formed on uninoculated sterile casing. Length of vertical bar indicates least significant difference between treatments ($P = 0.05$, 121 df).

FIG. 2. Effect of different C8 compounds (10 µL unless stated) in the atmosphere of microcosms on the numbers of primordia > 1 mm diam (square root transformed values) that formed on non-axenic peat + $CaCO_3$ casing (□) and axenic charcoal casing (■). Length of vertical bar indicates least significant difference between treatments ($P = 0.05$, 32 df). Figures

above bars are the concentrations of the respective C8 compound detected in the casing at the end of the experiment, where measured (μg g^{-1}).

FIG. 3. Effect of different C8 compounds (10 μL unless stated) in the atmosphere of microcosms on the populations of total bacteria (□) and pseudomonads (■) in non-axenic peat + $CaCO_3$ casing. Length of vertical bar indicates least significant difference between treatments (P = 0.05, 32 df).

FOOTNOTE

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TABLE I. Similarity of the 16S rRNA gene sequence of *Pseudomonas* isolates to the best matches in the EMBL database using the program FASTA.

Pseudomonas isolate	Species name with greatest homology and EMBL accession no.		Sequence Length	Similarity (%)
n12	*P. poae*	DSM14936T	1529	100.0
NSC4	*P.* PsI	AF105387	1407	98.6
NSC6	*P.* PsI	AF105387	1407	97.7
4Alux	*P. putida*	D85994	1259	96.5
T1/4	*P. putida*	D85994	1259	100.0
T2/6	*P. putida*	D85994	1259	98.7
Paw8	*P. putida*	KT2440	302614	99.0
Paw340	*P. putida*	KT2440	302614	99.0
MAR2	*P. veronii*	CFML 92-134	1430	97.6
MAR12	*P. veronii*	CFML 92-104	1430	97.6

TABLE II. Volatile organic compounds (VOCs) detected only in axenic and non-axenic microcosms containing *Agaricus bisporus* inoculum, and numbers of primordia > 1 mm diam. Values for primordia and VOC concentrations in adsorbents are respectively square root and logarithmic transformed means of microcosms containing Carbotrap and Tenax adsorbents, with back-transformations shown in parentheses.

Sterility	Axenic	Non-axenic	Axenic	Non-axenic	LSD(*P*=0.05)
A. bisporus strain	A15	A15	5776	5776	69 df
primordia per microcosm	0.68 (0.1)	7.06 (49.4)	0.61 (0.0)	2.1 (4.0)	0.42
VOC (μg g^{-1})					
1-bromo-octane	1.19 (3.29)	–0.10 (0.89)	1.83 (6.22)	0.55 (1.72)	0.88
1,6-octadien-3-ol	1.98 (7.21)	–2.63 (0.06)	1.83 (6.24)	–1.17 (0.30)	1.06
octane	–0.13 (0.87)	–3.41 (0.02)	0.00 (0.99)	–0.90 (0.40)	1.40
1-octanol	–3.19 (0.03)	–2.86 (0.05)	–2.81 (0.05)	–3.30 (0.03)	0.73
3-octanone	3.15 (23.21)	–2.39 (0.08)	3.49 (32.91)	2.17 (8.71)	0.94
1-octen-3-ol	2.13 (8.38)	–1.80 (0.16)	2.19 (8.93)	1.30 (3.65)	1.65

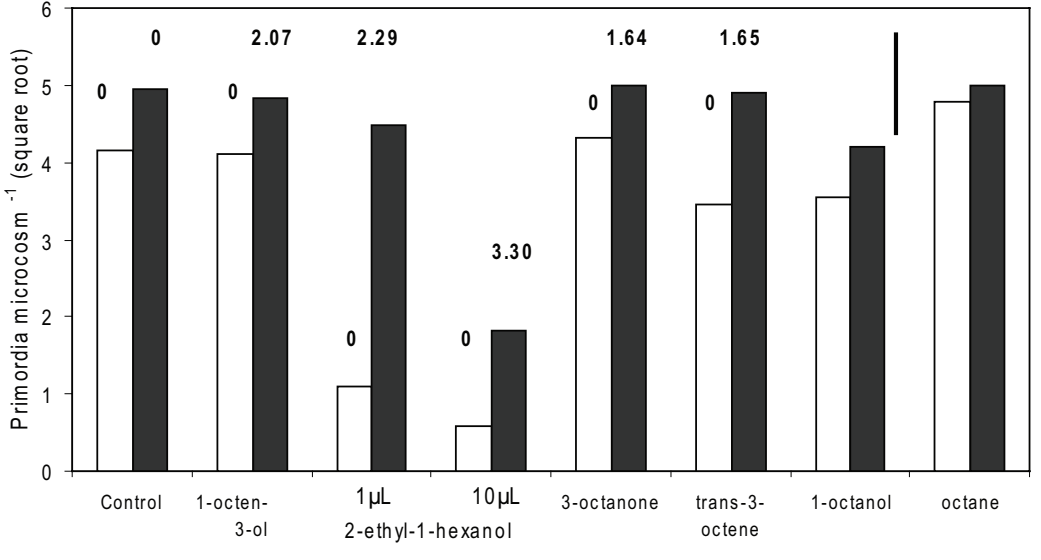
TABLE III. Volatile organic compounds (VOCs) detected in the highest concentrations in axenic and non-axenic microcosms, both with and without *Agaricus bisporus*. Values are logarithmic transformations of mean VOC concentrations in Carbotrap and Tenax adsorbents and of *A. bisporus* strains A15 and 5776, with back-transformations shown in parentheses.

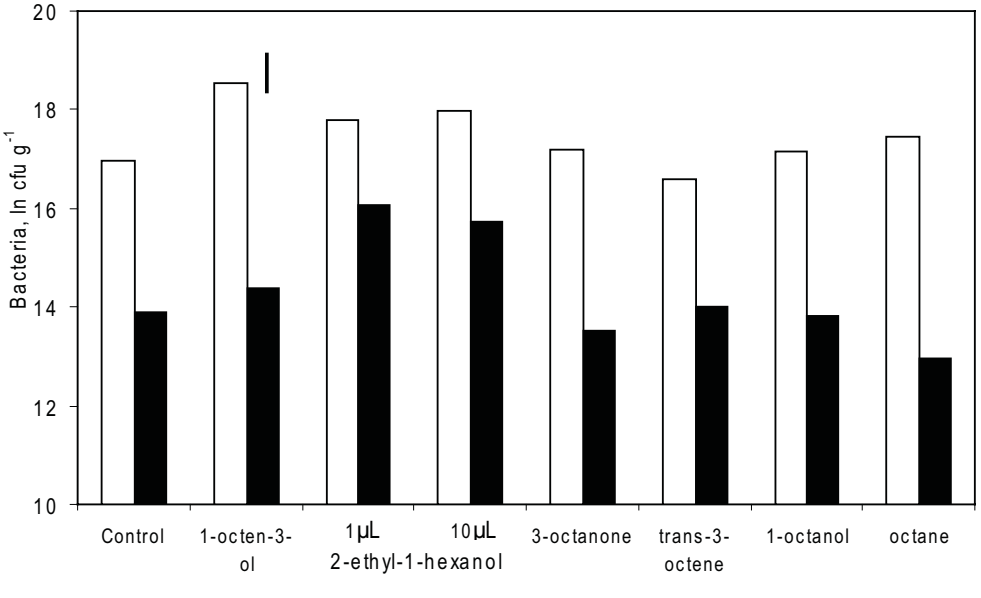
Sterility	Axenic	Non-axenic	Axenic	Non-axenic	LSD(P=0.05)
Agaricus. bisporus	absent	absent	present	present	69 df
VOC (μg g^{-1})					
Benzoic acid	0.39 (1.47)	–4.61 (0.00)	–1.36 (0.25)	–2.05 (0.13)	1.02
Benzone compounds	(5.72) (305.40)	2.15 (8.60)	3.54 (34.53)	2.49 (12.01)	1.35
Benzyl alcohol	5.89 (359.76)	4.96 (142.02)	1.49 (4.66)	1.08 (2.54)	0.71
1-Bromo decane	1.96 (7.10)	–0.50 (0.60)	1.39 (4.01)	0.56 (1.76)	0.45
1-Bromo heptane	–3.39 (0.024)	–4.61 (0.00)	1.44 (6.03)	1.40 (4.04)	0.62
Diethyl ester 2-butanedioic acid	2.16 (8.63)	0.08 (1.07)	1.28 (3.72)	0.33 (1.39)	0.54
2-Ethyl-1-hexanol	6.72 (830.0)	5.32 (204.5)	3.99 (68.9)	3.17 (24.0)	0.49
d-limonene	3.60 (36.59)	–2.82 (0.05)	2.25 (11.10)	1.90 (6.74)	0.67
Mequinol	–0.29 (0.74)	–4.61 (0.00)	–1.24 (0.34)	–1.87 (0.22)	0.59
Naphthalene compounds	3.81 (45.33)	1.40 (4.05)	2.62 (13.70)	1.62 (5.05)	1.06
2-Pentyl furan	4.16 (64.18)	–1.89 (0.14)	1.48 (4.73)	–0.89 (0.68)	0.67

TABLE IV. Effect of volatile organic compounds on the numbers of mushroom primordia > 1 mm diam and sporophores formed, and on the casing bacterial populations in axenic and non-axenic jar cultures contained within ventilated flasks. Primordia numbers are square root transformed values, with back-transformations shown in parentheses.

Volatile organic compound	Numbers per jar			Bacteria (ln cfu g^{-1} casing)	
	Primordia		Sporophores	Total	Pseudomonads
Control (start, axenic)	–	–	–	0	0
Control (start, non-axenic)	–	–	–	16.9	13.3
Control (axenic)	4.7	(22.0)	0.7	0	0
Control (non-axenic)	5.6	(31.4)	1.8	15.6	13.5
Ethanol	0.0	(0.0)	0.0	16.7	14.0
2-Ethyl-1-hexanol	2.1	(4.4)	0.3	18.3	14.1
Octane	4.1	(16.2)	2.1	17.3	14.2
1-Octen-3-ol	1.4	(1.9)	0.0	17.9	14.1
cis-3-Octen-1-ol	3.3	(11.0)	1.1	17.3	14.9
3-Octanone	4.5	(20.0)	1.8	16.1	13.3
LSD (P = 0.05) 12 df	1.2		0.6	0.7	0.5

Primordia microcosm-1 (square root)
0
1
2
3
4
5
6
7
8
9
P. poae
P. Psl
P. putida
P. veronii
n12
NSC4
NSC6
4Alux
T1/4
T2/6
Paw8
Paw340
Mar2
Mar12


Primordia microcosm -1 (square root)
6
5
4
3
2
1
0
0
0
2.07
0
2.29
0
0
3.30
1.64
0
0
Control
1-octen-3-ol
1µL
10µL
2-ethyl-1-hexanol
3-octanone
trans-3-octene
1-octanol
octane


20
18
16
14
12
10
Bacteria, ln cfu g-1
Control
1-octen-3-ol
1µL 2-ethyl-1-hexanol
10µL
3-octanone
trans-3-octene
1-octanol
octane