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Influence of Sporophore Development, Damage, Storage, and Tissue Specificity on the Enzymic Formation of Volatiles in Mushrooms (*Agaricus bisporus*)

Emilie Combet,[#,†,‡] Janey Henderson,[#,§] Daniel C. Eastwood,[†] and Kerry S. Burton*,[†]

[†]Warwick HRI, University of Warwick, Wellesbourne, U.K. CV35 9EF, [‡]Medical Sciences, Faculty of Medicine, the University of Glasgow, G11 6NT, U.K., [#]Faculty of Health and Life Sciences, Coventry University, Coventry, CV1 5FB, U.K., and [§]School of Science and Technology, Teesside University, Middlesbrough, TS1 3BA, U.K.

The enzymic oxidation of the polyunsaturated fatty acid−linoleic acid leads, in fungi, to the formation of a unique class of nonconjugated hydroperoxides, which are cleaved to form eight-carbon volatiles characteristic of mushroom and fungal flavor. However, the enzymes involved in this biosynthetic pathway, the bioavailability of the fatty acid substrate, and the occurrence of the reaction products (hydroperoxides and eight-carbon volatiles) are not fully understood. This study investigated the lipids, fatty acids, and hydroperoxide levels, as well as eight-carbon volatile variations in the fungal model *Agaricus bisporus*, according to four parameters: sporophore development, postharvest storage, tissue type, and damage. Eight-carbon volatiles were measured using solid phase microextraction and gas chromatography−mass spectrometry. Tissue disruption had a major impact on the volatile profile, both qualitatively and quantitatively; 3-octanone was identified as the main eight-carbon volatile in whole and sliced sporophore, an observation overlooked in previous studies due to the use of tissue disruption and solvent extraction for analysis. Fatty acid oxidation and eight-carbon volatile emissions decreased with sporophore development and storage, and differed according to tissue type. The release of 1-octen-3-ol and 3-octanone by incubation of sporophore tissue homogenate with free linoleic acid was inhibited by acetylsalicylic acid, providing evidence for the involvement of a heme-dioxygenase in eight-carbon volatile production.

KEYWORDS: *Agaricus bisporus*; fungi; 1-octen-3-ol; linoleic acid; eight-carbon volatile; gas chromatography−mass spectrometry

INTRODUCTION

The breakdown of polyunsaturated fatty acids in plants and fungi gives rise to a wide range of volatile short-chain aliphatic compounds, understood to be functionally active (*1*). In fungi, eight-carbon volatiles are the main volatiles produced from this breakdown and are involved in repelling pests and pathogens (*2*−*4*) and controlling sporulation (*5*−*7*) as well as contributing to the mushroom aroma (*4*).

A pioneering set of studies unraveled linoleic acid as the substrate of the reaction leading to 1-octen-3-ol synthesis, the involvement of atmospheric oxygen in the peroxidation reaction taking place as well as a nonconjugated hydroperoxide, 10-HPOD, as the intermediate of the reaction (*8*−*10*). The authors concluded that 1-octen-3-ol is enzymically formed from the oxidative cleavage of linoleic by a lipoxygenase-type (LOX) enzyme and a hydroperoxide lyase. However, the enzymic system has not yet been fully characterized. By convention, LOX targets a *cis*−*cis* pentadiene motif in linoleic acid and produces, after radical movement and double-bond rearrangement, either a conjugated 9-HPOD or a conjugated 13-HPOD, which is cleaved by hydroperoxide lyase into either nine- or six-carbon volatiles, respectively. Thus, LOX is theoretically unable to produce nonconjugated hydroperoxides (such as 10-HPOD) from linoleic acid. However, *A. bisporus* and fungi in general produce eight-carbon volatiles, suggesting a novel, fungal-specific, enzymatic system, different from the well described plant lipoxygenase pathway (*11*), via a nonconjugated 10-HPOD intermediate (*12*, *13*). Heme-dioxygenases, such as the *Gaeumannomyces graminis* linoleate diol synthase (LDS), are able to produce conjugated hydroperoxide and would therefore be a possible candidate to carry out peroxidation of linoleic acid in fungi (*12*, *14*).

Eight-carbon volatiles are abundantly produced by *A. bisporus* mushrooms and account for 44.3% to 97.6% of the total volatile fraction, depending on the extraction method used (distillation, solvent extractions, and Tenax traps) (*4*, *15*−*17*). Relationships between linoleic acid levels and 1-octen-3-ol production were studied by Cruz et al., establishing that the correlations between substrates and products varies according to strains and developmental stages (*17*). A large number of similar studies, based on

*Corresponding author. Phone: +44 (0)2476 575137. Fax: +44 (0) 2476 574500. E-mail: kerry.burton@warwick.ac.uk.

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the use of sample disruption and solvent extractions to study volatiles, often focused on 1-octen-3-ol production only, rather than the whole eight-carbon volatile spectrum (*9*, *13*).

This study aimed to clarify the eight-carbon volatile biosynthesis pathway in fungi, via measurement of substrate, intermediates and products of the reaction, according to fungal development, harvest, and damage. By investigating eight-carbon volatile levels in whole, sliced, or homogenized mushrooms, using solid-phase microextraction and gas chromatography−mass spectrometry, the study takes into account the effect of experimental damage on volatile production, without interference of a solvent extraction procedure requiring further sample disruption. Meanwhile, hydroperoxides and fatty acids were measured during development and between tissue type to investigate the relationships between substrate, intermediate compounds, and products of the reaction. The enzymic system present in *A. bisporus* is further investigated by monitoring the volatile profile of homogenized samples and acetylsalicylic acid, a known heme-dioxygenase-specific inhibitor (*18*).

MATERIALS AND METHODS

Reference Standards and Solvents. All reference standards (1-octen-3-ol, 3-octanone, 3-octanol, 1-octen-3-one, 1-octene, and octenal, 99% purity) were purchased from Sigma-Aldrich, Poole, U.K. All solvents were HPLC grade, purchased from Sigma-Aldrich.

***A. bisporus* Sporophore Sampling and Treatments.** *A. bisporus* strain A15 was supplied by Sylvan, U.K. Sporophore tissues were derived from strain A15 mushrooms grown on composted wheat straw according to a standard commercial procedure at the University of Warwick BioConversion Unit. Sporophores were harvested from a first flush crop of *A. bisporus* from the pin developmental stage at stage 1, to veil break at stage 4, to open cap mushroom at stage 7, according to the Hammond and Nichols classification, and transferred to the laboratory with minimum delay, avoiding bruising (*19*). Alternatively, stage 2 sporophores were stored at 18 °C and 90−95% relative humidity in a controlled environment room for 1, 2, or 3 days to monitor the effect of postharvest development on volatile production. When applicable, fresh sporophores were sliced with a sharp scalpel blade, from cap to stipe, in four slices of equal thickness and immediately placed in a gastight chamber for equilibration of the headspace volatiles, unless specified otherwise. Stage 3 sporophores were dissected into stipe, skin, cap, and gill tissues prior to homogenization. When applicable, whole sporophores were homogenized in MES buffer at pH 7.0 in a 2:1 ratio (v/w) of buffer volume to sporophore weight; dissected sporophore tissues (stage 3) were homogenized in a 4:1 ratio (Ultraturax homogenizer, 1 min).

Total Lipid Extraction. Lipids were extracted from sporophores using a modified version of the method of Blight and Dyer (*20*). Whole sporophores and dissected tissues (cap, stipe, and gills) were homogenized in ice-cold 0.1 M Tris buffer (pH 7.5) in a 1:2 w/v ratio, while the skin tissue was homogenized at a 1:4 w/v ratio (Ultraturax homogenizer, 1 min). Acetic acid (0.15 M, 1 mL) and chloroform/methanol (1:2 v/v, 7.5 mL) were added to the sporophore homogenate; the sample was mixed by inversion (ten times) prior to chloroform (2.25 mL) and H_2O (2.25 mL) addition. The samples were centrifuged at 900*g* for 1 min, and the lower chloroform layer containing the lipids was transferred to a clean tube. Lipid extracts were stored at −20 °C under nitrogen atmosphere.

Hydroperoxide Analysis. Aliquots of total (fresh) lipid extract (1 mL) were dispensed in two amber vials and allowed to dry under a stream of nitrogen at room temperature. Samples were resuspended in HPLC grade methanol, either in 100 μL without triphenylphosphine (TPP) or 90 μL supplemented with 10 μL of TPP (25 mM in methanol). Samples were incubated on ice in the dark for 30 min, followed by the addition of 900 μL of working FOX2 reagent. The FOX2 reagent was prepared as previously described by Nouroozzadeh et al. (*21*), by dissolving xylenol orange and ammonium ferrous sulfate in 250 mM H_2SO_4 to final concentrations of 1 mM and 2.5 mM, respectively, and diluting this stock (1:10) in HPLC grade methanol containing 4.4 mM BHT (butylated hydroxytoluene). Samples were incubated for 30 min at room temperature in the dark and absorbance measured spectrophotometrically at 560 nm. Hydroperoxide concentrations were determined by subtracting the absorbance of the sample treated with TPP from the absorbance of the untreated sample using the extinction coefficient for linoleate hydroperoxide, $\varepsilon = 6.0 \times 10^4\ M^{-1}\ cm^{-1}$ (*22*).

Fatty Acid Methylation. Total lipid extract (1 mL) was mixed with 50 nmol of heptadecanoic acid (17:0, internal standard) in hexane. The mixture was dried under a stream of nitrogen at 70 °C. Dried lipids were methylated with 2 mL of freshly prepared methanolic sulfuric acid; the reaction was left to reflux at 60 °C for 2 h. The reaction was cooled before the addition of hexane (1.5 mL) and H_2O (2 mL). The sample was inverted repeatedly to partition the fatty acid methyl esters, prior to centrifugation at 900*g* for 5 min. An aliquot of the hexane phase (1 mL) was dried under a stream of nitrogen at room temperature for 20 min and reconstituted in 100 μL of chilled HPLC-grade hexane.

Chromatographic Conditions for Fatty Acids Analysis. The gas-chromatographic analysis was performed on a 6890 GC system (Agilent Technologies, U.S.A.) equipped with a 5973 Network MSD mass spectrometer. The GC-MS was equipped with a standard split/splitless inlet and predrilled PTFE septum (Supelco, U.K.). All injections were performed in splitless mode with an injection volume of 2 μL and the inlet port maintained at 220 °C. Fatty acids were separated on a 30 m × 0.25 mm 19091N-133 Innowax column (Agilent Technologies, U.K.) maintained at 40 °C for 1 min, with a temperature increment to 190 °C at a rate of 10 °C/min, and then to 240 at 15 °C/min. Helium was used as the carrier gas at a velocity of 47 cm/s. The internal standard (IS) heptadecanoic acid (17:0) was used for quantification. Fatty acids were detected by mass spectrometry in full scan mode with an ionization energy of 70 eV.

Solid Phase Microextraction (SPME) Analysis of Volatile Compounds. Volatile analysis was performed on whole, sliced, and homogenized sporophores, as well as homogenized dissected tissues. Sporophore homogenates were filtered on a double layer of Miracloth (Amersham Bioscience, U.K.) and 7.65 mL of the filtered homogenate was transferred to a 20 mL headspace vial (Supelco, U.K.) equipped with magnetic stirrer. Samples were left to equilibrate (25 °C) for 5 min. Linoleic acid and the internal standard 1-hepten-3-ol (2 mM and 1 μg/mL, respective final concentrations) were added to homogenates before capping.

Whole and sliced sporophore samples were placed in gastight jars, and the volatile compounds were left to equilibrate in the headspace for 5 min, at a constant temperature of 25 °C. After 5 min of equilibration, the headspace volatiles were extracted by solid phase microextraction for 5 min. All experiments were performed in triplicate. A 50/30 μm DVB/Carboxen/PDMS fiber (Supelco, U.K.) was conditioned at 270 °C for at least 3 h, before exposure to the headspace volatiles for 5 min. The SPME fiber was withdrawn from the sampling vial and directly inserted in the gas chromatographer inlet (230 °C, 1 min, splitless injection), where the analytes were thermally desorbed.

Chromatographic Conditions for Volatiles Analysis. The gas-chromatographic analysis was performed on a 6890 GC system (Agilent Technologies, U.S.A.) equipped with a 5973 Network MSD mass spectrometer. The GC-MS was equipped with a specific narrow-bore inlet (Supelco, U.K.) and a predrilled PTFE septum (Supelco, U.K.). The column was maintained at 40 °C for 1 min before ramping the oven temperature at a rate of 10 °C/min to 90 °C, then 6 °C/min to 160 °C, and finally maintaining it at 160 °C for 3 min. Helium was used as the carrier gas at a velocity of 36 cm/s. Volatiles were detected by mass spectrometry in full scan mode with an ionization energy of 70 eV.

Volatiles Identification and Quantitation. Volatile compounds were identified by mass spectra library search (Chemstation, Agilent), based on the National Institute of Standards and Technology (NIST) database. Volatile compound identities were also confirmed by comparing their mass spectra and retention times with those obtained for their respective standards. Volatile levels from whole and sliced samples were measured in standardized gastight jars and the headspace volume recorded. As sample matrix influences the partitioning of volatiles to the headspace, no internal standard was added to either whole or sliced mushroom samples: volatiles levels were expressed according to their

Table 1. Total Fatty Acid Levels, Fatty Acid Distribution (% of Total Fatty Acids), Unsaturated/Saturated Fatty Acid Ratios, Hydroperoxide Levels, and Ratios to Fatty Acid Content According to Stages of Sporophore Development, Postharvest Storage (0,1, 2, and 3 Days after Harvest), and Tissue Types[a]

		total FA (μg/g fwt)	14:0 (% FA)	15:0 (% FA)	16:0 (% FA)	16:1 (% FA)	18:0 (% FA)	18:2 (% FA)	19:0 (% FA)	20:0 (% FA)	21:0 (% FA)	22:0 (% FA)	unsaturated/ saturated	HPOD (nmols/g fwt)	FAOR (% total FA)
stages of development	1	359.9 a	0.31 a	0.53 a	16.54 a	0.98 a	10.53 a	64.22 a	0.22 a	3.41 a	0.61 a	2.50 a	1.88 a	4.96 a	0.95 ac
	2	291.9 a	0.59 a	0.86 a,b	17.77 a	1.20 a	5.01 a	52.82 a	0.15 a	7.25 b	1.37 ab	6.46 b	1.38 a	6.39 a	1.38 a
	3	728.3 ab	0.58 a	0.80 a,b	12.31 ab	1.20 a	8.80 a	61.87 a	1.59 b	5.68 b	1.90 ab	3.98 ab	1.78 a	9.39 a	0.44 ac
	4	488.6 ab	0.41 a	0.72 a,b	14.62 ab	0.92 a	8.00 a	58.47 a	3.30 c	4.92 b	2.17 b	3.94 ab	1.55 a	9.29 a	0.61 ac
	5	887.1 b	0.41 a	0.76 a,b	10.49 b	1.44 a	10.52 a	67.06 a	0.78 b	4.50 b	1.01 ab	2.68 a	2.20 a	7.13 a	0.30 ac
	6	586.1 ab	0.56 a	1.18 b	12.66 ab	1.17 a	8.31 a	58.88 a	1.62 bc	4.83 b	1.96 ab	7.57 b	1.54 a	5.86 a	0.32 ac
	7	804.3 b	0.40 a	1.21 b	9.90 b	1.07 a	7.93 a	70.53 a	0.67 b	4.01 b	1.32 ab	3.47 ab	2.57 a	5.49 a	0.20 bc
number of days postharvest	H0	291.9 a	0.60 a	1.00 a	17.77 a	1.20 a	5.01 a	52.82 a	0.15 a	7.25 b	1.37 a	6.46 a	1.38 a	6.39 a	1.38 a
	H1	469.3 ab	0.70 a	0.90 a	11.43 a	0.95 a	10.61 a	59.66 a	1.98 b	7.55 b	2.91 a	3.66 ab	1.58 a	5.96 a	0.38 a
	H2	740.4 ab	0.20 a	0.50 a	10.84 a	0.73 a	9.20 a	60.34 a	1.27 ab	5.32 ab	3.02 a	4.60 ab	1.68 a	8.18 a	0.43 a
	H3	834.8 b	0.70 a	0.80 a	9.99 a	0.95 a	8.54 a	69.97 a	0.36 ab	3.39 a	1.85 a	2.39 b	2.47 a	7.18 a	0.25 a
tissues	cap	408.9 b	0.68 b	0.87 b	11.06 a	1.47 a	8.70 a	68.23 ab	0.85 a	3.32 a	0.92 a	1.63 a	2.34 a	5.70 a	0.49 b
	gills	789.7 ab	0.19 c	0.47 a	11.77 a	1.00 a	7.28 a	76.81 b	0.87 a	2.08 a	0.78 a	3.53 a	3.42 a	27.71 b	0.97 b
	stipe	640.2 b	0.67 b	0.76 b	13.48 a	0.45 a	12.44 a	54.82 a	1.16 a	6.58 a	2.82 a	2.58 a	1.32 a	8.97 a	0.40 b
	skin	2093.7 a	0.34 a	0.50 a	11.90 a	1.42 a	6.24 a	69.08 ab	0.44 a	4.03 a	2.39 a	3.04 a	2.36 a	6.49 a	0.11 a

[a] Statistical significance ($p < 0.05$) is displayed using letter combinations. Only values followed by different letters within each column are significantly different.

total ion current total peak area in arbitrary units per gram fresh weight per milliliter of headspace (AU/g/mL). Meanwhile, 1-hepten-3-ol was added as an internal standard (final concentration 1 μg/mL) to homogenized samples; no further calibration or recovery experiment was carried out, and volatile levels were expressed in ng of 1-hepten-3-ol equivalent per gram fresh weight per milliliter headspace (ng/ g fwt /mL).

Inhibition of Volatile Production. The heme-dioxygenase inhibitor acetylsalicylic acid (250 μM) was added to the chilled MES buffer prior to the homogenization of whole stage 2 sporophores. After filtration on a double layer of Miracloth (Amersham Bioscience, U.K.), 7.65 mL of the filtered homogenate was transferred to a 20 mL headspace vial (Supelco, U.K.) equipped with a magnetic stirrer. Samples were left to equilibrate (25 °C) for 5 min. Linoleic acid and the internal standard 1-hepten-3-ol (2 mM and 1 μg/mL, respective final concentrations) were added to homogenates before capping. Volatile analysis by SPME was carried out as described above. The effect of the inhibitor was tested alongside a water control, and all experiments were performed in triplicate.

Statistical Analysis. Results are presented as the average of at least three independent experiments. Statistical analysis of all data was performed on $\log_{10}$ transformed data due to a mean-variance relationship, by ANOVA or one-tailed paired Student's *t*-test. The least significant difference (LSD) was used for comparison of means between treatments. Mean values were associated with letters as an indication of significance ($p < 0.05$), with different letters displaying statistically significant differences for each individual volatile series between treatments.

RESULTS

Fatty Acids and Hydroperoxides in *A. bisporus*. Total fatty acid measurements varied according to stages of development, with levels in the late stages 5 and 7 significantly higher than total fatty acid levels for immature stages 1 and 2 ($p < 0.05$, **Table 1**). Postharvest development was accompanied by an increase in total fatty acids levels between harvest (H0) and storage day 3 (H3) ($p < 0.05$, **Table 1**). Meanwhile, individual sporophore tissues varied in their total fatty acid levels, with the skin the richest ($p < 0.05$, **Table 1**). Individual fatty acids, expressed as a percentage of total fatty acids, showed little variation according to sporophore development, postharvest storage, or tissue type (**Table 1**). Fatty acids identified in *A. bisporus* were linoleic acid (18:2), stearic acid (18:0), and palmitic acid (16:0), while the monounsaturated fatty acids oleic (18:1) and palmitoleic acids (16:1) were rarely detected, along with low amounts of unusual odd-numbered fatty acids, such as pentadecanoic (15:0), nonadecanoic (19:0), and heneicosanoic acids (21:0). The three main fatty acids 18:2, 18:0, and 16:0 were ubiquitous to all developmental stages and tissue types. Regardless of sporophore development, storage, or tissue origin, 18:2 was the most abundant fatty acid (53 to 76% of the total fatty acids), with the highest level measured in the gills. The unsaturated/saturated fatty acid ratio remained unaffected by natural development, postharvest development, or tissue type (**Table 1**).

Fatty acid oxidation was expressed as the quantity of fatty acid hydroperoxide molecules either relative to the sample fresh weight (HPOD) or relative to the total amount of fatty acids of the sample (the fatty acid oxidation rate or FAOR). During the two developmental trends, the HPOD levels showed no significant variation. The FAOR decreased during sporophore development between stage 2 and stage 7 ($p < 0.05$), but there was no trend for postharvest storage (**Table 1**). HPOD levels were significantly higher in the gill tissue than in other tissues, with 27.71 nmol of HPOD/g tissue, approximately 3-fold the amount measured in stipe tissue and more than 5-fold the amount measured in cap tissues ($p < 0.05$). The FAOR was significantly lower in the skin (0.11%) compared to that in any other tissue ($p < 0.05$, **Table 1**).

Eight-Carbon Volatiles in *A. bisporus* Whole Sporophores. Three eight-carbon volatiles (1-octen-3-ol, 3-octanone, and 1-octen-3-one) were detected by SPME-GCMS in the headspace of whole sporophore samples. The most abundant volatile was 3-octanone, which decreased between stages 1 and 4 (696 and 18 AU/g/mL, respectively), with levels remaining stable for the later stages of development ($p < 0.05$, **Figure 1A**). 1-Octen-3-ol levels initially showed a decrease between stages 1 and 2, followed by an increase between stages 2 and 3, and then a decrease between stages 3 and 4 (136, 9, 67, and 0.5 AU/g/mL of headspace, respectively) ($p < 0.05$, **Figure 1A**). It was not possible to monitor the evolution of volatile compounds in stored (postharvest day 1 to 3) intact whole sporophores, as the volatile concentrations were below detection levels after the first day of storage.

Eight-Carbon Volatiles in *A. bisporus* Sliced Sporophores. 1-Octen-3-ol, 3-octanone, 1-octen-3-one, 3-octanol, and 1-octene were detected in the headspace of sliced sporophore samples; 3-octanone was the most abundant volatile (**Figure 1B**).

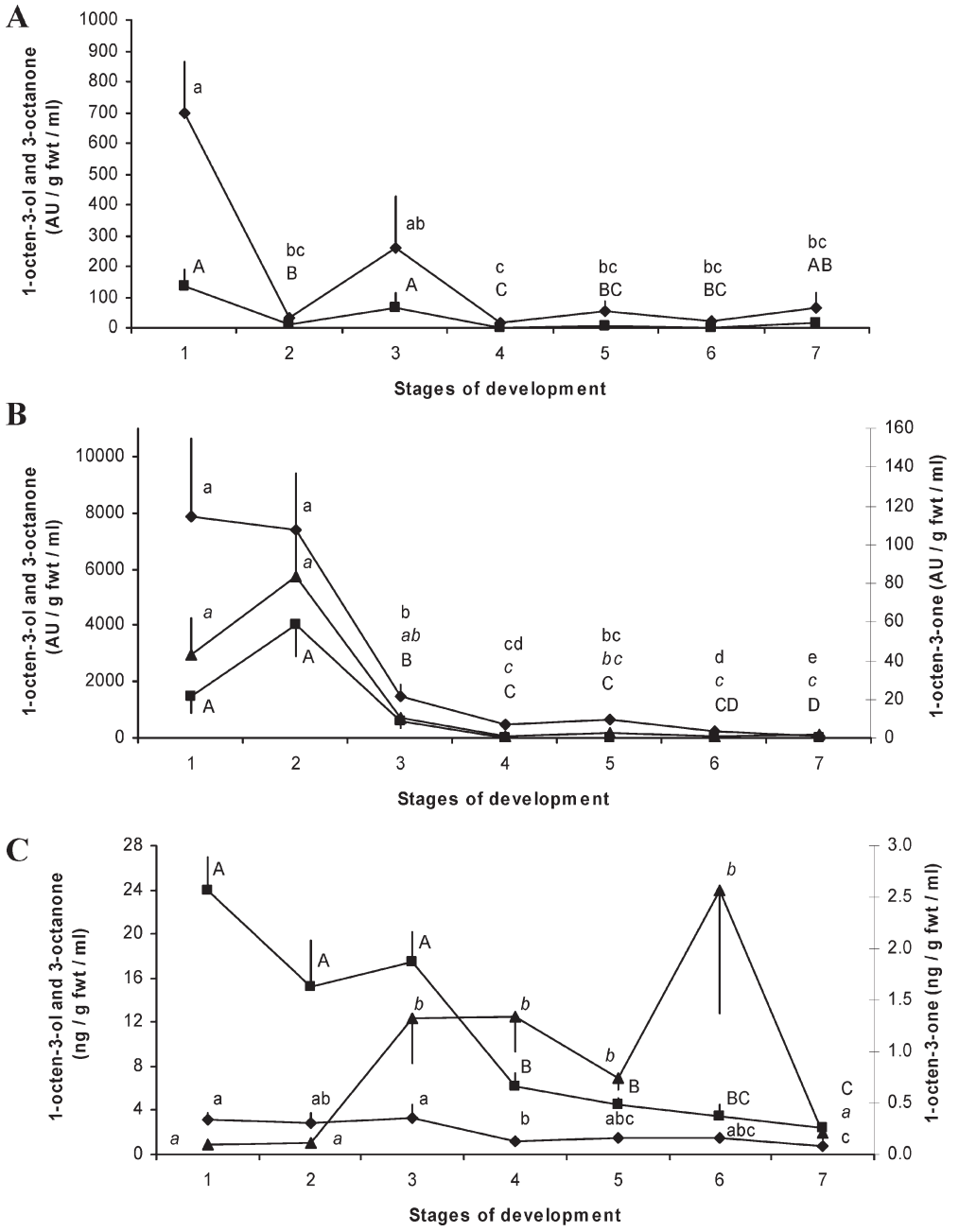

Figure 1. Evolution of the eight-carbon volatiles 3-octanone (◆),1-octen-3-ol (■), and 1-octen-3-one (▲) from whole (**A**), sliced (**B**), and homogenized (**C**) sporophores according to stages of development (results expressed as mean ± SE). Only values with different letters show significant differences within each volatile series ($p < 0.05$); statistical differences of 1-octen-3-ol are shown with upper case letters to avoid confusion with italic letters of 1-octen-3-one or lower case letters for 3-octanone.

Levels of 1-octen-3-ol and 3-octanone were approximately 10 times higher in sliced sporophores than in whole sporophores (**Figure 1A** and **B**). Levels of the three eight-carbon volatiles decreased in the headspace of sliced sporophore samples during natural development. Between stages 1 and 7, 3-octanone levels decreased from 7891 to 62 AU/g/mL, 1-octen-3-ol levels decreased from 1500 to 4 AU/g/mL, and 1-octen-3-one levels decreased from 42 to 2 AU/g/mL ($p < 0.05$, **Figure 1B**). During sporophore development, 3-octanone levels were 3- to 33-fold higher than 1-octen-3-ol levels in sliced samples and 4 to 38-fold higher than 1-octen-3-ol levels in whole samples, with a peak in the 3-octanone/1-octen-3-ol ratio at stage 4 in both whole and sliced sporophores ($p > 0.05$) (**Table 2**). The evolution of the headspace levels of both 3-octanone and 1-octen-3-ol were monitored with sliced sporophores (stages 2 and 3) over a 40 min time period. After 40 min, volatiles were undetectable in the headspace of stage 2 sporophores, while they decreased from 1538 to 310 AU/g for 3-octanone and 3296 to 3 AU/g for 1-octen-3-ol in the headspace of stage 3 sporophores (**Table 3**).

The three eight-carbon volatiles were also detected in the headspace of sliced sporophores during postharvest storage. Levels of 3-octanone, 1-octen-3-ol, and 1-octen-3-one fell significantly during the 3 day postharvest period ($p < 0.05$, **Figure 2A**). During postharvest storage of sliced sporophores, 3-octanone/1-octen-3-ol ratios varied between 1.5 and 28.9, with a minimum at day 1 postharvest and a maximum at day 3 postharvest (**Table 2**).

Comparisons of the volatile and fatty acid hydroperoxide data for sliced sporophore revealed a strong positive correlations between the fatty acid oxidation rate (FAOR) and

Table 2. 3-Octanone/1-Octen-3-ol Ratios for Whole, Sliced, and Homogenized Sporophores, According to Development and Postharvest Storage[a]

		whole sporophores	sliced sporophores	homogenized sporophores
stages of development	1	5.72 a	5.47 ab	0.12 a
	2	3.82 a	17.88 bc	0.16 a
	3	4.18 a	2.78 a	0.14 a
	4	38.52 c	32.74 c	0.20 ab
	5	15.30 b	29.48 c	0.33 cd
	6	10.38 ab	30.56 c	0.41 d
	7	4.60 a	17.24 bc	0.22 bc
postharvest storage	H0	n.d.	17.88 ab	0.16 a
	H1	n.d.	1.53 a	0.4 b
	H2	n.d.	10.43 ab	0.4 b
	H3	n.d.	28.94 b	0.5 b

[a] Statistical significance ($p < 0.05$) is displayed using letter combinations. Only values followed by different letters within each column are significantly different.

Table 3. 3-Octanone and 1-Octen-3-ol Concentration in the Headspace of Stage 2 and 3 Sporophores after Slicing over a 40 min Time Period[a]

	stage 2		stage 3	
time (min)	3-octanone	1-octen-3-ol	3-octanone	1-octen-3-ol
0	2003.46	185.20	0	2003.46
20	71.47	n.d.	20	71.47
40	n.d.	n.d.	40	n.d.

[a] Volatile concentrations are given in arbitrary unit (AU) per gram of fresh tissue weight per mL of headspace immediately (t0), 20 min (t20), and 40 min (t40) after slicing.

3-octanone levels ($r^2 = 0.58$, $p < 0.01$) and 1-octen-3-ol levels ($r^2 = 0.52$, $p < 0.05$) during natural and postharvest development (**Figure 3A**).

Eight-Carbon Volatiles in *A. bisporus* Homogenized Sporophores and Tissues. Analysis of eight-carbon volatiles from homogenized whole sporophores and dissected tissues was supplemented with linoleic acid to provide a nonlimiting environment (fatty acid substrate/oxygen) to study the enzymatic reaction leading to 1-octen-3-ol and 3-octanone formation. The five eight-carbon volatiles, 1-octen-3-ol, 3-octanone, 1-octen-3-one, 3-octanol, and 1-octene, were detected in the headspace of all homogenized samples, while five more were more seldom detected (1-octanol, 1,3-octadiene, 3,5-octadien-2-ol, octenal, and 2-octen-1-ol). In contrast to whole and sliced sporophores, 1-octen-3-ol was the main volatile in homogenized samples, and the 3-octanone/1-octen-3-ol ratios increased between stage 1 to stage 6 from 0.12 to 0.41 and from 0.16 to 0.5 during postharvest storage ($p < 0.05$) (**Table 2**).

Significant decreases ($p < 0.05$) in the levels of both 3-octanone (3.1 to 0.68 ng per gram of fresh weight per milliliter of headspace (ng/g/mL)) and 1-octen-3-ol (23.9 to 2.33 ng/g/mL) were observed between stages 1 and 7 (**Figure 1C**). The volatile levels decreased most between stages 3 and 4 for both 3-octanone and 1-octen-3-ol (3.4 to 1.2 and 17.4 to 6.2 ng/g/mL, respectively) ($p < 0.05$, **Figure 1C**). There was a strong linear relationship between 3-octanone and 1-octen-3-ol concentrations in the headspace of sporophore homogenates during natural development ($r^2 = 0.85$, $p < 0.01$), while 1-octen-3-one levels did not correlate with 1-octen-3-ol and 3-octanone levels. 1-Octen-3-one levels were significantly higher in stages 3, 4, 5, and 6 (between 0.7 and 2.5 ng/g/mL) than stages 1, 2, and 7 (between 0.09 to 0.2 ng/g/mL) ($p < 0.05$, **Figure 1C**).

1-Octen-3-ol levels postharvest decreased from 15.3 to 3.4 ng/g/mL from freshly harvested to 3-day stored sporophores ($p < 0.05$, **Figure 2B**). 3-Octanone levels did not change significantly during postharvest storage. 1-Octen-3-one levels measured from sporophore homogenates did increase as a result of sporophore postharvest storage with the highest levels one day after harvest (2.7 ng/g/mL) ($p < 0.05$, **Figure 2B**).

In homogenates, 1-octen-3-ol levels significantly correlated to FAOR ($r^2 = 0.54$, $p < 0.01$) and during natural and postharvest development, as observed for sliced sporophores (**Figure 3B**).

Volatile analysis in tissue homogenates revealed a specific volatile profile for each tissue (**Figure 4**). The skin tissue produced the highest concentration of 3-octanone in its headspace (2.9 ng/g/mL), while the gill tissue displayed the lowest (0.3 ng/g/mL). 1-Octen-3-ol levels were the highest in the headspace of stipe and cap tissue homogenate (6.1 and 3.4 ng/g/mL, respectively) and the lowest in the skin (0.1 ng/g/mL). 1-Octen-3-ol and 3-octanone concentrations in the headspace of cap and stipe tissues were not significantly different ($p < 0.05$, **Figure 4**). 1-Octen-3-one was detected in cap and stipe tissues only and at similar levels ($p < 0.05$, **Figure 4**).

Incubation of Sporophore Homogenates with Acetylsalicylic Acid. Sporophores were homogenized with and without the dioxygenase inhibitor acetylsalicylic acid in order to investigate the enzyme system producing eight-carbon volatiles in *A. bisporus*. Acetylsalicylic acid (250 μM) had a significant effect on volatile production, reducing the release of both 1-octen-3-ol and 3-octanone by 36 and 67%, respectively ($p < 0.05$, **Figure 5**). However, acetylsalicylic acid addition had no significant effect on 1-octen-3-one release ($p < 0.05$, **Figure 5**).

DISCUSSION

In this study, we analyzed the effect of sporophore development, postharvest storage, damage, and tissue variation on the fatty acids, hydroperoxides, and eight-carbon volatile profile of *A. bisporus* in order to gain new insights into the regulation of this important pathway. The volatile analysis was conducted using SPME-GC-MS, a novel technique enabling measurement of headspace volatiles without further sample disruption. Volatile partitioning between the headspace and sample can be heavily influenced by the sample matrix (*23*, *24*). For this reason, an internal standard was not added to sliced and whole samples; as a consequence, volatile levels were expressed in arbitrary units per gram mushroom per milliliter headspace, according to peak areas. Meanwhile, volatiles in homogenized samples were quantified as 1-hepten-3-ol equivalent. Further calibrations and recovery experiments would be required to draw conclusions based on quantified levels, and this is a limitation to the present study. However, the data presented here provides comparative assessment of levels within an experimental data set from which conclusions may be drawn. A major consideration when analyzing headspace volatiles is the degree of cellular disruption, as this

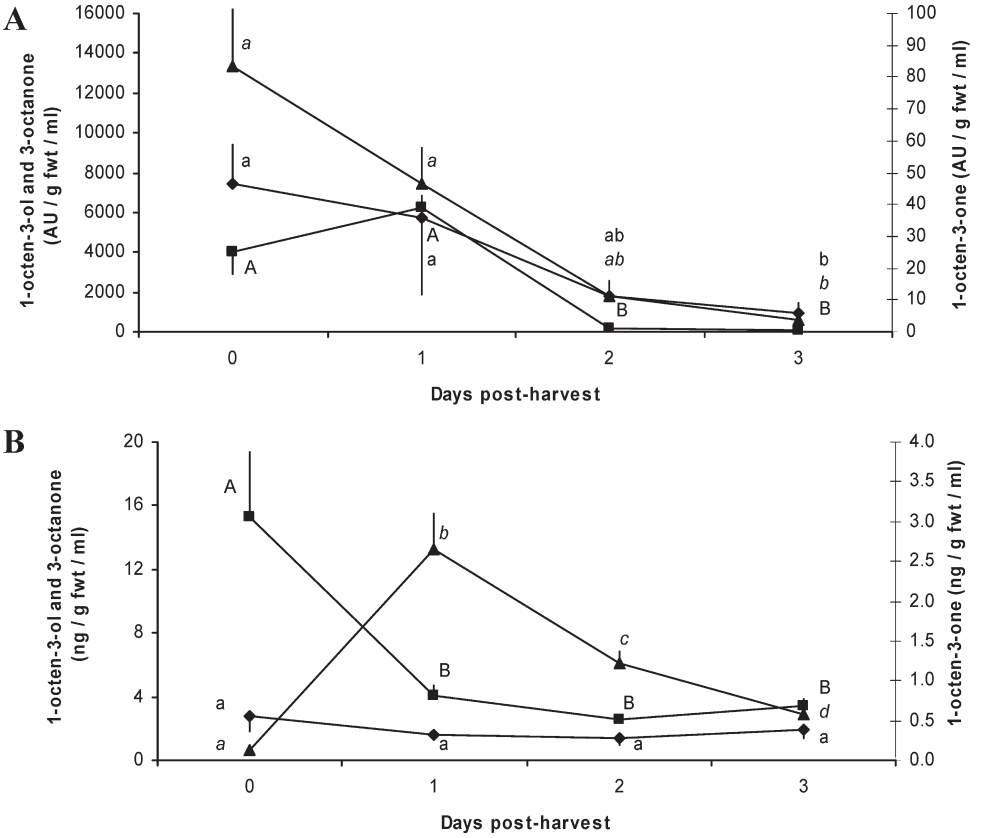

Figure 2. Evolution of the eight-carbon volatiles 3-octanone (◆),1-octen-3-ol (■), and 1-octen-3-one (▲) from sliced (**A**) and homogenized (**B**) sporophores according to stages of postharvest storage (0 to 3 days) (results expressed as mean ± SE). Only values with different letters show significant differences within each volatile series ($p < 0.05$); statistical differences of 1-octen-3-ol are shown with upper case letters to avoid confusion with italic letters of 1-octen-3-one or lower case letters for 3-octanone.

alone will induce the release of free fatty acid from membrane lipids and lipid globules, as well as breaking down the cellular compartments, which keep enzymes apart from substrates (*25*), and trigger nonphysiological volatile formation, interfering with the measure of physiologically produced volatiles. Previous studies have focused on fungal volatile profiling using tissue disruption and solvent extraction techniques (*4*, *17*, *26*) and have identified 1-octen-3-ol as the main eight-carbon volatile in *A. bisporus*. The range of volatiles reported in our study is in good agreement with previously published work (*4*, *17*); however, the use of the nondestructive SPME-GC-MS analysis identified the ketone 3-octanone as the most-abundant eight-carbon volatile in nonhomogenized samples, while 1-octen-3-ol is the main eight-carbon volatile in homogenized *A. bisporus* sporophores. This novel finding bears significant implication for the understanding of the biochemical pathway leading to the formation of eight-carbon volatiles in fungi as, until now, previous theories were based on data acquired using more damaged samples (*4*, *16*, *17*). 1-Octen-3-ol may only be produced due to cellular damage, and the small amount detected from whole sporophores may be the result of damage caused by the separation from mycelial cells upon harvest. Tissue damage had a major effect on volatile formation: the partial wounding treatment of slicing resulted in 10 times more volatiles being produced than from whole sporophores, indicating that the enzymic machinery was not operating maximally in whole mushrooms, possibly due to substrate limitation, e.g., oxygen, or substrates in different intracellular locations. Both 1-octen-3-ol and 3-octanone increased by approximately similar levels from whole sporophore to sliced sporophore, suggesting that the productions of these volatiles share a common synthesis and/or regulatory mechanism. Damage also increased the range of volatiles detected. Metal ions (possibly associated with the use of a scalpel blade) can catalyze linoleic acid autoxidation as well as the decomposition of linoleic acid hydroperoxides (*27*). According to Badings, 1-octen-3-ol could be formed via nonenzymatic formation and breakdown of the hydroperoxide 10-HPOD (*28*). However, this would lead to the concomitant formation of 2-octen-1-ol, which was not observed in the headspace of sliced sporophores. We therefore deduced that eight-carbon volatile formation via autoxidative mechanisms was likely to be negligible. The rapid decline in volatile levels measured continuously for 40 min after slicing (**Table 3**) indicated that the reaction substrate(s) available, fatty acid and/or molecular oxygen, may rapidly become expended or that compartmentalization reoccurs after wounding, preventing the access of enzyme to the substrate.

Both eight-carbon volatile synthesis and fatty acid oxidation rate (FAOR) decreased during sporophore development, while total fatty acid levels increased. The proportion of linoleic acid does not change significantly during natural or postharvest development and cannot account for the decrease in volatile production during development. The regulation of eight-carbon synthesis is therefore not dependent on the levels of total fatty acid substrate present. The correlation between eight-carbon volatiles and hydroperoxides indicate that early reactions, including either free fatty acid release from lipids or formation of hydroperoxides by dioxygenases, are likely to be the steps limiting the reaction. This is consistent with findings by Wurzenberger and Grosch, showing that the mushroom hydroperoxide lyase fraction maintains its activity to cleave HPOD even after 5 h of storage at 0 °C, while the lipoxygenase-containing fraction lost its activity to convert linoleic acid to

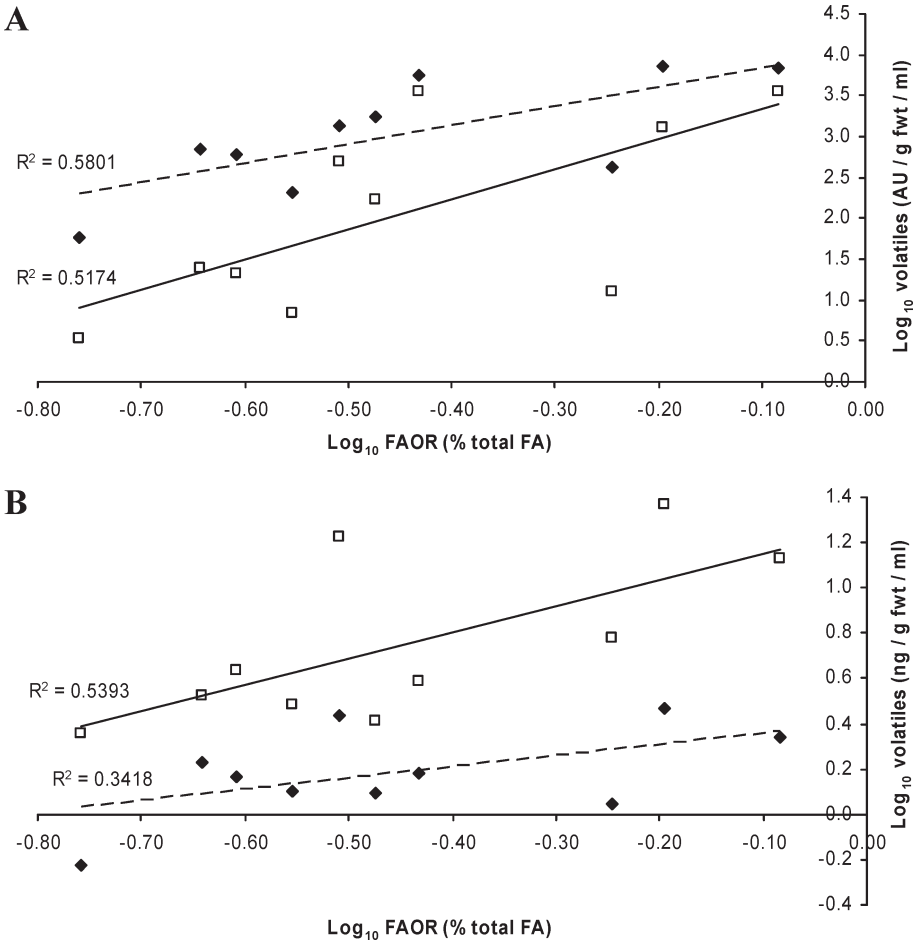

Figure 3. Correlation between $\log_{10}$ corrected fatty acid oxidation rate (FAOR, in % total fatty acids) and 1-octen-3-ol (□) and 3-octanone (◆) levels (in ng or AU/ng fwt/mL) in sliced (**A**) and homogenized samples (**B**). Results expressed as mean ± SE.

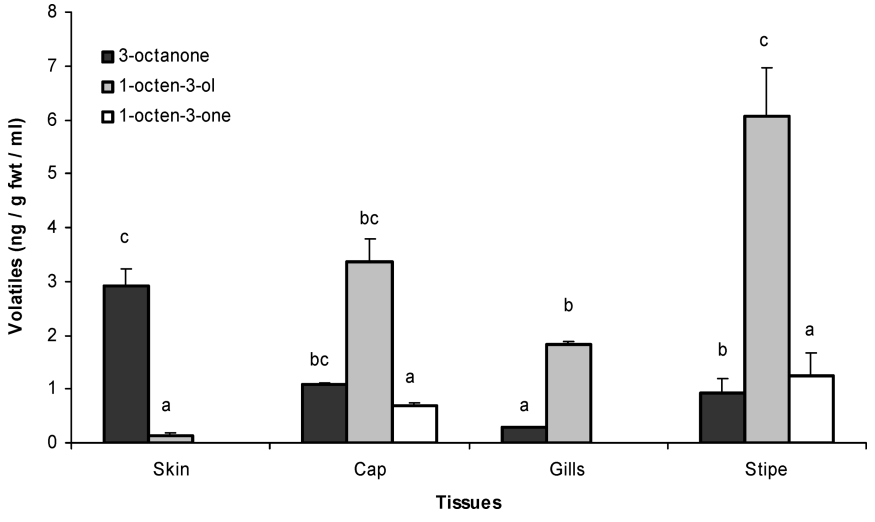

Figure 4. 8-Carbon volatile levels in the headspace of homogenized sporophore (stage 3) tissues: 3-octanone (black), 1-octen-3-ol (gray), and 1-octen-3-one (white). Only values with different letters show significant differences between tissues for each individual volatile ($p < 0.05$). Results expressed as mean ± SE.

the 10-HPOD (*9*). It also appears that there is a switch toward proportionately more 3-octanone production at the time of spore formation. The 3-octanone to 1-octen-3-ol ratio showed an approximate 10-fold significant increase between stages 3 and 4 in whole and sliced sporophores during natural development. Interestingly, a recent study by Nemcovic et al. reported the potential of eight-carbon volatiles, especially 3-octanone, in inducing conidiation in *Trichoderma* spp. both inter- and intraspecifically, while 1-octen-3-ol has been linked to inhibition of sporulation in *Penicillium* (*5*, *7*). This highlights that, while substrate availability and enzyme regulation may vary with sporophore development and influence the amount of hydroperoxides and volatiles released, the mechanisms in place to modulate the eight-carbon volatile profile are also affected by development, with potentially crucial outcomes for the organism's life cycle.

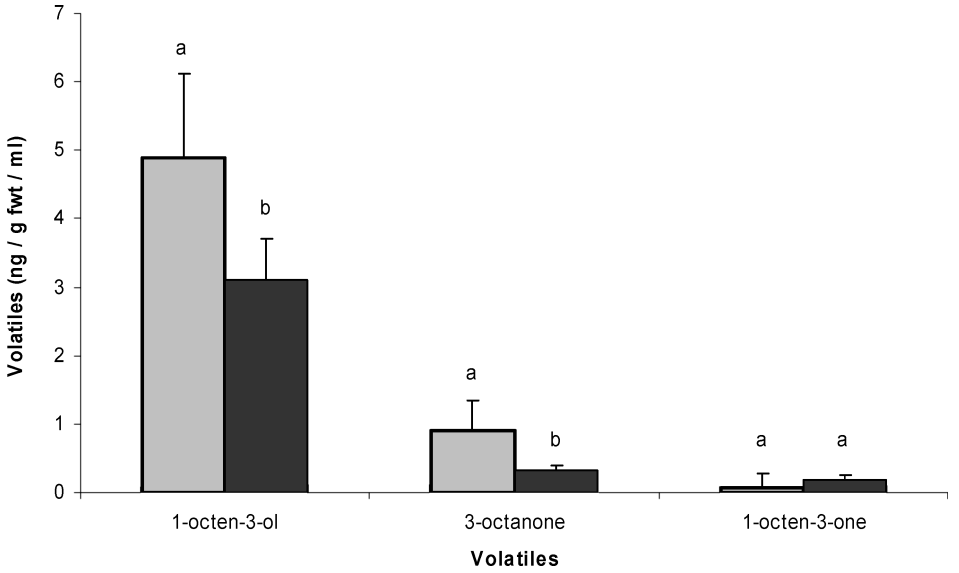

Figure 5. Effect of acetylsalicylic acid on the production of eight-carbon volatiles in homogenized sporophore: water control (gray) or acetylsalicylic acid (black). Significant differences in volatile levels between the control and acetylsalicylic acid treatment are shown by different letters ($p < 0.05$). Results expressed as mean ± SE.

Sporophore homogenization with added linoleic acid and oxygen (by aeration) enabled us to study the volatile production abilities of sporophore and tissues without the constraints of intracellular partitioning and substrate limitations, providing new insight into the regulation of the enzyme system itself. Different volatiles were detected compared with those of whole and sliced sporophores, indicating further chemical and biochemical conversions. The principal volatile produced in homogenized fresh and postharvest samples was 1-octen-3-ol, in agreement with previous studies in *A. bisporus* (*4*, *29*). Meanwhile, the link between 1-octen-3-ol formation and tissue disruption, enabled by the use of SPME, is consistent with previous findings reporting a large 40-fold increase in the 1-octen-3-ol released upon homogenization of the mushroom *Tricholoma inamoenum* (*30*). The levels of 1-octen-3-ol from homogenates declined during development and postharvest storage, and positively correlated with 3-octanone levels. Correlations were also identified between the FAOR (hydroperoxide as a percentage of fatty acids), 3-octanone, and 1-octen-3-ol released by sliced sporophores, both for natural and postharvest developments. This provides good evidence that 3-octanone and 1-octen-3-ol originate via a hydroperoxide intermediate, possibly common to both volatiles. Meanwhile, only 1-octen-3-ol levels significantly correlated with FAOR in homogenized samples ($p < 0.01$). 1-Octen-3-one levels did not correlate with 1-octen-3-ol levels in sliced or homogenized sporophores and showed peaks during development and storage. Moreover, while acetylsalicylic acid inhibited both 1-octen-3-ol and 3-octanone release, it did not influence 1-octen-3-one emissions. These two observations possibly indicate that 1-octen-3-one may have a distinct synthesis pathway from that of 1-octen-3-ol and 3-octanone.

Large quantitative differences were detected in the volatile levels between homogenates of different tissues, with findings consistent with previous observations (*16*). The skin tissue produced largely 3-octanone, while the cap, stipe, and gills produced mainly 1-octen-3-ol, and only the cap and gills released detectable quantities of 1-octen-3-one. Moreover, significant differences in HPOD levels were also detected. These marked differences observed for tissue homogenates may either indicate different enzymes present between tissue or tissue-specific regulations of a shared pathway. The physiological relevance of these findings is unclear and requires better characterization of the enzyme system(s) present in each tissue; however, as the tissues fulfill very different biological functions, the volatile profile may be a reflection of this.

However, the formation of eight-carbon volatiles other than 1-octen-3-ol (such as 3-octanone and 1-octen-3-one) is poorly documented, with no enzymatic system characterized so far (*26*). Previous reports suggested the involvement of a lipoxygenase and a hydroperoxide lyase, consistent with the plant biochemical model, converting linoleic acid to 1-octen-3-ol via a 10-HPOD (*9*, *13*). However, the presence of a nonheme-dioxygenase (lipoxygenase) would not be compatible with the proposed intermediate, a conjugated 10-HPOD: by definition, lipoxygenase only produces nonconjugated hydroperoxides from polyunsaturated fatty acid, yielding either a 13-HPOD or a 9-HPOD from linoleic acid (*12*). By incubating the sporophore homogenate with acetylsalicylic acid, a known inhibitor of heme-dioxygenase, we were able to test whether a lipoxygenase might be involved in the formation of eight-carbon volatiles in fungal homogenates. Acetylsalicylic acid acetylates a serine residue near the heme-dioxygenase active site, preventing the binding of fatty acids. This inhibition is irreversible and specific for heme-dioxygenases (*18*). Acetylsalicylic acid has no effect on lipoxygenase activity. Sample homogenization in presence of the inhibitor led to a significantly decreased production of 1-octen-3-ol and 3-octanone, providing evidence of a likely heme-dioxygenase activity. The incomplete inhibition could be due to a level of activity prior to inhibition or the presence of a lipoxygenase-dependent additional pathway. Reductase activity has been reported in *A. bisporus* by Chen and Wu, converting 1-octen-3-one to 1-octen-3-ol and 3-octanone in a pH-dependent manner (*26*). Similarly, conversion of 1-octen-3-one to 3-octanone was attributed to an enone reductase in *Saccharomyces cerevisiae* (*31*). It is not clear to what extent these pathways contribute to the eight-carbon volatile equilibrium, in either intact or disrupted tissues. According to our results, 3-octanone is the main volatile in whole and sliced sporophores, yet we cannot confirm whether 3-octanone is the product of 1-octen-3-ol conversion, or its precursor. Sporophore homogenization may either alleviate a physical constraint otherwise limiting conversion of 3-octanone to 1-octen-3-ol or enable the formation of high, saturating amounts of 1-octen-3-ol, which cannot be

effectively converted to 3-octanone. Further studies are required in order to elucidate the secondary pathways involved in volatile production in *A. bisporus*.

ABBREVIATIONS USED

10-ODA, 10-oxodecanoic acid; HPOD, hydroperoxide; GC-MS, gas chromatography−mass spectrometry; BHT, butylated hydroxytoluene; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; SPME, solid-phase microextraction; AU, arbitrary units; FAOR, fatty acid oxidation ratio; fwt, fresh weight.

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