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FULL RESEARCH PAPER

Verticillium disease of *Agaricus bisporus*: variations in host contribution to total fungal DNA in relation to symptom heterogeneity

Michèle L. Largeteau · Catherine Regnault-Roger · Jean-Michel Savoie

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Abstract The pathogenic fungus *Verticillium fungicola*, responsible for dry bubble disease of the common mushroom *Agaricus bisporus*, causes various symptoms on its host, bubbles (undifferentiated spherical masses), bent and/or split stipes (blowout) and spotty caps. Host DNA quantification by real-time PCR was used to observed relationships between the type of symptom and the relative amount of *A. bisporus* and *V. fungicola* in diseased mushrooms. *Verticillium fungicola* is involved in bubble formation but does not appear to regulate its growth. Quantifications in bubbles and stipe-bubbles (morphology between bubble and sporophore with stipe blowout) showed that the pathogen has no effect on the growth of undifferentiated host hyphae but prevents morphological differentiation if not initiated and stops it when initiated hyphae are affected. Mushrooms with stipe blowout exhibiting both mature and abortive lamellae reveal that *V. fungicola* has a restricted area of action in host tissues. Despite their visual aspect, healthy looking parts of mushrooms showing spots or stipe blowout were actually contaminated. Discolouration and symptom development are two distinct events. The colour of the tissues was correlated to the percentage of *A. bisporus* DNA, suggesting that discolouration is not an efficient defensive mechanism, and occurs at the time *V. fungicola* developed enough to induce tissues necrosis.

Keywords Mushroom · Discolouration · Dry bubble · Host DNA detection · *Verticillium fungicola* · Real-time PCR

M. L. Largeteau (✉) · J.-M. Savoie
INRA (Institut National de la Recherche Agronomique), UPR1264 MYCSA,
33883 Villenave d'Ornon, France
e-mail: largeteau@bordeaux.inra.fr

C. Regnault-Roger
UPPA (Université Pau et Pays de l'Adour),
UFR Sciences et Techniques,
64012 Pau Universite Cedex, France

Introduction

Dry bubble disease of the cultivated button mushroom, *Agaricus bisporus*, caused by *Verticillium fungicola* is distributed worldwide and responsible for large losses to the mushroom industry. The fungal pathogen affects the morphogenesis of fruiting bodies in its fungal host. Infection is manifested in three types of symptoms, (i) an undifferentiated, non-necrotic spherical mass (dry bubble) in place of the sporophore, (ii) mushrooms with a bent and/or split stipe (blowout) and (iii) superficial cinnamon-brown lesions of the mushroom cap (spotty cap) (van de Geijn 1982; North and Wuest 1993; Rinker and Wuest 1994). The recognition system between the

A. bisporus fruiting body and *V. fungicola* was demonstrated (Bernardo et al. 2004). Some information on the physical and chemical mechanisms involved are available, and the coexistence of host and pathogen hyphae in diseased tissues was visualised by microscopy (North and Wuest 1993; Dragt et al. 1995 and 1996; Calonje et al. 1997), but the ratio of host to pathogen is not documented. In contrast to sporophores which vary little in size, initiate and reach maturity quite simultaneously during each peak of production for a single cultivar, bubbles show great variations in size and no synchronisation in development. Sometimes, diseased mushrooms of morphology between bubble and stipe blowout exhibiting abortive lamellae are observed. Sporophores with stipe blowout can show both healthy-looking and diseased parts, mature and abortive lamellae. Tissue discolouration is currently observed but it does not affect some diseased mushrooms. These observations raise questions about the place of each symptom in the determination of the disease severity assessed during pathological tests (Largeteau et al. 2004) and their links with the time and level of infection, in order to progress in the control of this disease.

Our aim was to explain the morphological heterogeneity observed among diseased mushrooms especially bubbles heterogeneity, to detect if tissue discolouration of the diseased part of mushrooms with stipe blowout or spotty cap reflects a defensive mechanism, and to analyse if healthy looking tissues of mushrooms showing spots or stipe blowout remain uninfected. To this end, we used real-time PCR assay to detect a putative relationship between the amount of *A. bisporus* in diseased mushrooms and the type of symptom, taking into account the stage at which morphogenesis was affected.

Materials and methods

Fungal material and sampling

The commercial hybrid 2100 (Amycel, France) of *A. bisporus* was cultivated at INRA (Institut National de la Recherche Agronomique) facilities and contaminated with *V. fungicola* var. *fungicola* (isolate VCTC) as described by Savoie and Largeteau (2004). In short, a conidial suspension was sprayed at the surface of the casing soil covering the substrate. Inoculation was performed before fruiting initiation. Ten small primordia (P, 2 mm high, undifferentiated tissues), six young bubbles (YB, 0.5–0.8 cm high), 27 bubbles (B, spherical masses, Fig. 1), three mushrooms called stipe-bubbles (SB) based on their morphology between bubble and stipe blowout (Fig. 2), three mushrooms with stipe blowout (BO, Fig. 3), and three spotty caps (SC), were collected at random 13–42 days after contamination of the casing soil. The weight of the bubbles was recorded. Diseased mushrooms were washed with sterile water, peeled, and cut down the middle. Tissue colours were visually assessed. Three classes, white (W), light brown (LBr) and brown (Br) were defined for bubbles (Fig. 1). Cap and stipe tissues of mushrooms showing complete or partial morphological differentiation (spotty mushrooms or stipe-bubble, respectively) were separated. Diseased tissues (discoloured or not) and healthy looking tissues of mushrooms with stipe blowout were separated. Tissues of healthy sporophores (SP) from an uncontaminated culture carried out in another climatic room, on the same substrate and under the same environmental conditions, were used as the uninfected control. After colour measurement, tissues were frozen and lyophilised. Mycelium of *V. fungicola* was cultivated for 21 days in Cristomalt® (Dif.Al, Seysses, France) liquid medium. The fungal biomass was recovered by filtration, the inoculum plugs were removed, and the mycelium was washed with sterile water and lyophilised.

Colour measurement

The colour was measured on fresh tissues of each class (W, LBr and Br) using the parameter *L* (0 for black to 100 for white) given by a Minolta chromameter® CR221 (Minolta Camera Co., Japan) (Sapers et al. 1994; Moquet et al. 1997; Soler-Rivas et al. 2000). Data were reported as means of three measurements per sample.

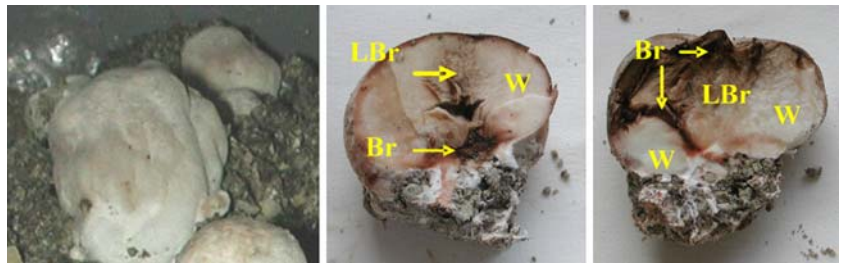

Fig. 1 Bubbles showing heterogeneous discolouration. W = white, LBr = light brown, Br = brown tissues

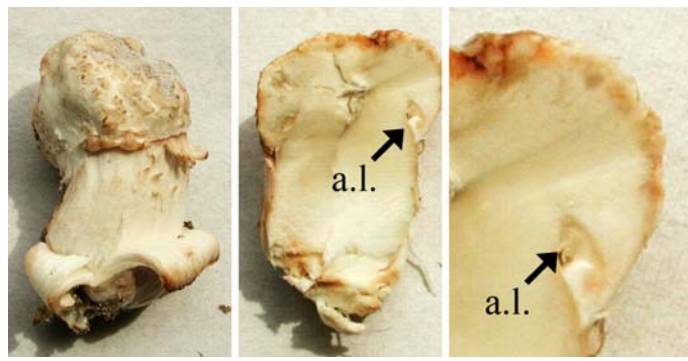

Fig. 2 General aspect and longitudinal cut of a stipe-bubble. a.l. = aborted lamellae

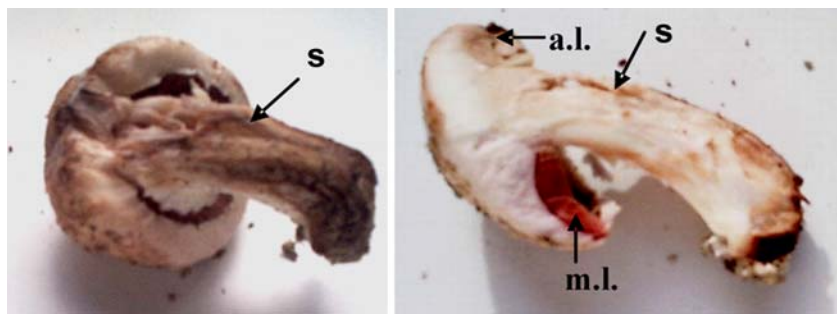

Fig. 3 Mushroom with stipe blowout showing mature (m.l.) and aborted lamellae (a.l.). s = split

Real-time PCR

Because some tissue samples were in too small quantity to allow reliable quantification of the extracted DNA using a spectrophotometer, we used real-time PCR absolute quantification to measure both total DNA and *A. bisporus* DNA quantities in all samples through calibration with standard curves obtained with DNA pools quantified by absorbance. DNA was extracted from lyophilised samples with the Amersham™ Nucleon™ Phytopure™ RPN 8510 Kit (Amersham Int. plc, England) according to the manufacturer's procedure. The DNA samples P and YB represented pools of the ten harvested primordia and the six young bubbles, respectively.

Primers were designed using the programme available at the website: www.genome.wi.mit.edu/cgi-bin/primer/primer_3 www.cgi. To this end, the ITS1–5.8S-ITS2 region of *A. bisporus* (consensus sequence) and VCTC (identical to that of isolate VV1, Genbank accession number AF324879, according to Collopy et al. 2001) were aligned using the ClustalW software (www.Infobiogen.fr). The primer pair 5.8S1F (5′-CAACGGATCT CTTGGCTCT-3′) and 5.8S2R (5′-CGCAAGA TGCGTTCAAAGAT-3′), designed in the 5.8S gene which showed almost similar sequences in the two fungi was used to quantify total DNA. It amplified a 106 bp region. The primer pair AB1F (5′-GTCTTTACATGGGCTATGCC-3′) and AB3R (5′-GATAAGTTATCACACTTGTG

GC-3′) designed in ITS1and ITS2 regions specific to *A. bisporus*, amplified a 331 bp product. We failed to find in these regions an efficient primer pair specific of *V. fungicola* that could be used for quantification by real-time PCR. The primer set VFF6F (5′-GTGAACATACCAATCGTTG-3′) and VFF8R (5′-CGGATTCAGAAGATACT GGT-3′) amplified a 130 bp sequence of the ITS1 region of *V. fungicola*, but it also amplified *A. bisporus* DNA, with a far lower efficiency. Primers VFF6F and VFF8R were, however, used to detect the presence of *V. fungicola* in samples (but without quantification), because the melt peaks for the *V. fungicola* product (Tm = 94.12 ± 0.03°C) and for the *A. bisporus* product (Tm = 88.39 ± 0.03°C) were different. Real-time PCR amplifications were performed with the LightCycler 2.0 Instrument (Roche Diagnostics, Germany). Amplification mixture consisted of 4 µl of FastStart DNA Master^{PLUS} SYBR Green I MIX (Roche Diagnostics, Germany), 500 nM of both forward and reverse primer and 5 µl of DNA in a final volume of 20 µl. Cycling conditions were 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, 56°C for 20 s and 72°C for 15 s for primers 5.8S1F/5.8S2R ; 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, 58°C for 15 s and 72°C for 20 s for primers AB1F/AB3R. Data were acquired at 72°C. Melt curve analysis was performed at the end of each PCR run to test for the presence of a unique PCR reaction product. A melt curve profile was obtained by heating the mixture to 95°C, cooling to 65°C (20 s), and slowly heating to 98°C at 0.1°C s^{-1} with continuous measurement of fluorescence at 520 nm. To check for identical detection efficiency of DNA in healthy and diseased tissues, standard curves were constructed using ten-fold serial dilutions of DNA of healthy fruiting bodies (pool A) and of diseased mushrooms (pool B) that spanned six DNA quantities per reaction (500, 50, 5, 0.5, 0.05 and 0.005 ng). DNA concentration in the pools was assessed by measurement of the absorbance at 260 nm (BioPhotometer, Eppendorf, Hambourg). Standard curves plotted the $\log_{10}$ of DNA quantity on the *X*-axis and the PCR cycle at which the reaction crossed a threshold value of fluorescence (C_t, calculated by the LightCycler software 4.0) on the *Y*-axis. Sample DNA was diluted to provide approximately 10 ng per reaction. Total and *A. bisporus* DNA quantities in samples were calculated by the LightCycler software 4.0 programmed for absolute quantification with reference to the standard curve (pool A) obtained in the same run. The ratio of the DNA quantity detected with primers AB1F/AB3R to the total DNA quantity detected with primers 5.8S1F/5.8S2R was calculated. DNA of *A. bisporus* was expressed as the quantity (ng) per 100 ng of total DNA. Data reported in the Tables 1–4 were means of four PCR replicates.

Statistical analyses

Values recorded for the *L* parameter and DNA quantities were submitted to ANOVA followed by the Student-Newman-Keuls test to detect significant differences, and correlation coefficients between both parameters were determined using the SAS system (SAS Institute Inc., Cary, NC, USA)

Results

Sensitivity and specificity of the method used to quantify *A. bisporus* DNA in the samples

The primer set 5.8S1F/5.8S2R (total DNA) generated standard curves of identical efficiency with DNA of pool A and pool B (Fig. 4a). The melt curves obtained for all the amplicons showed a single peak, and the same melting point (Tm = 81.72 ± 0.04°C). The standard curve generated with the primer set AB1F/AB3R (*A. bisporus* DNA) and DNA of pool A and pool B showed the same efficiency, (Fig. 4b). A single melting peak (Tm = 83.62 ± 0.03°C) was observed for the amplicons obtained with each pool of DNA. No amplification was obtained with DNA of *V. fungicola*. For all the standard curves, the correlation between the C_t value and the $\log_{10}$ of the target DNA quantity was high ($R^2 \geq 0.994$). These results showed that the selected primer sets were accurate over DNA quantities ranging from 5 pg to 500 ng. The C_t values obtained for samples DNA with primers 5.8S1F/5.8S2R ranged from 0.91 to 1.31, corresponding to 20.4–8.2 ng of total DNA.

Table 1 Colour measurements and quantities of *A. bisporus* DNA detected in primordia, young bubbles and bubbles of homogeneous colour

Sample	*L* value [a]	*A. bisporus* DNA (ng per 100 ng total DNA)
SP[b]	90.0 a	99.9 a
P	W [c]	85.0 f
YB	W	89.1 e
B30	90.0 a	97.1 bc
B69	90.0 a	91.1 e
B70	90.0 a	97.9 b
B71	90.0 a	93.5 d
B75	90.0 a	94.8 cd
B91	90.0 a	95.6 bc
B95	90.0 a	96.8 bc
B96	90.0 a	94.9 cd
B65	83.0 ab	75.9 g
B92	79.3 b	88.9 e
B74	71.9 c	96.7 bc
B66	66.0 cd	69.0 h
B54	65.9 cd	38.2 l
B53	65.1 d	51.0 k
B32	64.1 d	64.5 i
B51	60.1 d	27.4 m
B52	58.9 d	56.4 j
B31	52.7 e	22.6 n

Within a column, values followed by different letters differ significantly at $P < 0.05$ (Student-Newman-Keul's test)

[a] Colour parameter (0 for black to 100 for white) given by the chromameter

[b] SP = healthy sporophores used as reference for colour and *A. bisporus* DNA quantity

[c] Visual colour (white)

The primer set VFF6F/VFF8R detected *V. fungicola* (presence of the specific melt peak) in all the tissue samples of diseased mushrooms, including healthy looking tissues. The *L* value of 90.0 measured on tissues of uninfected sporophores (SP) was considered as the reference for the colour of healthy tissues.

Discolouration and amount of *A. bisporus* DNA in primordia and young bubbles

The primordia and young bubbles, too small for colour measurement, were visually white. The quantity of *A. bisporus* DNA in a pool of 10 primordia (85 ng per 100 ng total DNA) was significantly lower than the 89.1 ng per 100 ng total DNA detected in a pool of 6 young bubbles (Table 1).

Discolouration and amount of *A. bisporus* DNA in bubbles of homogeneous colour

All bubbles showed a similar external aspect. The visual colour of tissues recorded after a longitudinal cut revealed that white and discoloured bubbles were collected the same days. Eighteen from the 27 sampled bubbles showed tissues of homogeneous colour, which varied from white ($L = 90.0$) to brown ($L = 52.7$), (Table 1). Eight bubbles gave a homogeneous *L* value of 90.0, identical to the tissue colour of uninfected sporophores (SP) used as the control. The others showed significant discolouration. All bubbles contained significantly less *A. bisporus* DNA than the healthy control SP, but high quantities (>90 ng per 100 ng) were detected for samples showing white tissues ($L = 90.0$). Except B69, these white bubbles contained significantly more *A. bisporus* DNA than young bubbles. Highly variable quantities of *A. bisporus* DNA, ranging from 96.7–22.6 ng per 100 ng total DNA, were found among discoloured tissues (Table 1). The classification of the bubbles for *L* value and DNA quantity showed some differences but both variables were significantly correlated at $P < 0.01$ ($r = 0.882$; df = 16). Neither discolouration *vs* bubble weight nor DNA percentages versus bubble weight were significantly correlated ($P = 0.05$), (data not shown).

Discolouration and amount of *A. bisporus* DNA in bubbles of heterogeneous colour

Nine bubbles exhibited tissues heterogeneous in discolouration (Table 2). Based on the *L* values, none of the tissues of class W were as white as those of healthy mushrooms. Dramatic discolouration giving *L* values below 40 was observed in class Br. Within a bubble, the *L* values measured on tissues initially assigned to different classes of colour by visual assessment differed significantly ($P < 0.05$), (Table 2). The location of the most discoloured tissues varied depending on the bubble (Fig. 2). Significant variations in quantity of *A. bisporus* DNA were observed within each class of tissue colour. The quantity decreased significantly with the increase in discolouration (Table 2). Considering all the tissues samples, *L*

Table 2 Colour measurements and quantities of *A. bisporus* DNA in bubbles with tissues of heterogeneous discolouration

Sample	W tissues		LBr tissues		Br tissues	
	L value[a]	*A. bisporus* DNA (ng per 100 ng total DNA)	*L* value	*A. bisporus* DNA (ng per 100 ng total DNA)	*L* value	*A. bisporus* DNA (ng per 100 ng total DNA)
SP [b]	90.0 a	99.9 a				
B29	87.5 ab A	81.8 d A	– [c]	–	51.2 b B	69.1 a B
B50	85.4 ab A	96.8 b A	71.0 ab B	43.6 d B	–	–
B36	84.3 ab A	78.2 d A	–	–	59.5 a B	42.9 b B
B7	83.0 b A	88.8 c A	63.3 ab B	64.9 c B	38.1 c C	20.2 c C
B90	81.3 b A	80.6 d A	–	–	44.1 c B	46.4 b B
B35	–	–	79.6 a A	73.4 b A	66.4 a B	62.8 a B
B33	–	–	79.2 a A	89.4 a A	61.9 a B	65.9 a B
B5	–	–	73.6 ab A	91.0 a A	64.0 a B	25.3 c B
B8	–	–	60.5 b A	6.4 e A	38.5 c B	12.2 d B

Within a column, values followed by different lower case letters differ significantly at $P < 0.05$; within a line, values followed by different upper case letters (normal for *L*, bold for DNA) differ significantly at $P < 0.05$ (Student-Newman-Keul's test)

[a] Colour parameter (0 for black to 100 for white) given by the chromameter

[b] SP = healthy sporophores used as reference for colour and *A. bisporus* DNA quantity

[c] absent

values and DNA quantities were significantly correlated at $P < 0.01$ ($r = 0.762$; df = 17).

Discolouration and amount of *A. bisporus* DNA in stipe bubbles and spotty mushrooms

The quantity of *A. bisporus* DNA in cap or stipe tissues of stipe-bubbles differed significantly from that detected in the healthy sporophore. It showed a tendency for being linked to discolouration as observed with bubbles (Table 3).

The spotty mushroom SC77 developed a very thin layer of light brown tissues under the spot, not abundant enough for colour measurement and sampling. The necrosis on SC59 and SC68 only affected the pilei-pellis of the cap; the whole tissues remained white, with $L = 90.0$ (Table 3). Quantities of *A. bisporus* DNA detected in cap tissues were significantly lower than those found in stipe tissues. The latter were similar to those observed in white bubbles (Tables 1 and 3).

Discolouration and amount of *A. bisporus* DNA in mushrooms with stipe blowout

White tissues of mushrooms with stipe blowout, including those of the non-discoloured diseased part of BO27, contained high quantities of *A. bisporus* DNA (Table 4) similar to those detected in white bubbles. The quantities assessed in the healthy part of the three mushrooms differed significantly from those of the healthy control SP. BO34 and BO56 exhibited well developed, mature lamellae on the healthy looking side of the mushroom, and white abortive lamellae on the diseased side (Fig. 3). Significantly lower quantities of *A. bisporus* DNA were found in brown tissues of the diseased part of these mushrooms (Table 4). Similar ranges of host DNA quantities were detected in bubbles, stipe-bubbles and diseased tissues of mushrooms with stipe blowout (Tables 1, 3 and 4).

Discussion

Primers VFF6F/VFF8R detected *V. fungicola* in all diseased mushrooms, including healthy looking tissues. We failed to find an efficient primer pair specific to *V. fungicola* that could be used for quantification by real-time PCR. Consequently, we quantified the host and total fungal DNA. No competitor or pathogen other than *V. fungicola* was observed, and only dry bubble symptoms were present on the cultivation trays. Consequently we consider that variations in the host

Table 3 Colour measurements and quantities of *A. bisporus* DNA in stipe-bubbles and spotty mushrooms

Sample	Cap		Stipe	
	L value[a]	*A. bisporus* DNA (ng per 100 ng total DNA)	*L* value	*A. bisporus* DNA (ng per 100 ng total DNA)
SP[b]	90.0 a *a*	99.9 a *a*	90.0 a *a*	99.9 a *a*
Stipe-bubble				
SB67	90.0 a	83.2 c B	90.0 a	91.0 b A
SB79	68.0 b	92.7 b A	68.0 b	52.3 c B
SB80	71.0 b	62.3 d B	89.0 a	92.6 b A
Spotty mushroom				
SC59	90.0 *a*	78.1 *c B*	90.0 *a*	94.8 *b A*
SC68	90.0 *a*	91.6 *b B*	90.0 *a*	99.5 *a A*
SC77	90.0 *a*	82.4 *c B*	90.0 *a*	90.7 *b A*

Within a column, for each type of diseased mushroom, values followed by different lower case letters differ significantly at $P < 0.05$; within a line, values followed by different upper case letters differ significantly at $P < 0.05$ (Student-Newman-Keul's test)

[a] Colour parameter (0 for black to 100 for white) given by the chromameter

[b] SP = healthy sporophores used as reference for colour and *A. bisporus* DNA quantity in the study of stipe-bubbles and spotty mushrooms

Table 4 Colour measurements and quantities of *A. bisporus* DNA in mushrooms with stipe blowout

Sample	Healthy part		Diseased part	
	L value[a]	*A. bisporus* DNA (ng per 100 ng total DNA)	*L* value	*A. bisporus* DNA (ng per 100 ng total DNA)
SP[b]	90.0	99.9 a		
BO27	90.0	94.1 c A	90.0	96.3 a B
BO34	90.0	96.0 b A	LBr[c]	22.3 c B
BO56	90.0	94.0 c A	LBr	69.5 b B

Within a column, values followed by different lower case letters differ significantly at $P < 0.05$; within a line, values followed by different upper case letters differ significantly at $P < 0.05$ (Student-Newman-Keul's test)

[a] Colour parameter (0 for black to 100 for white) given by the chromameter

[b] SP = healthy sporophores used as reference for colour and *A. bisporus* DNA percentage

[c] LBr = light brown, not enough discoloured tissues for colour measurement

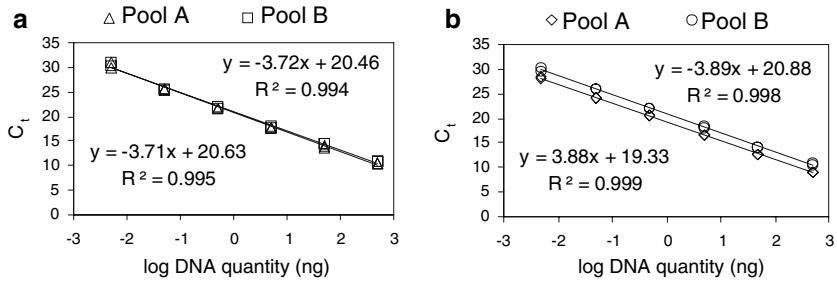

Fig. 4 Standard curves showing identical amplification efficiency with DNA from healthy (pool A) and diseased (pool B) mushrooms. a = Primers 5.8S1F/5.8S2R (total DNA); b = primers AB1F/AB3R (*A. bisporus* DNA)

DNA / total fungal DNA were related to the occurrence of infection by *V. fungicola*.

Hyphal discolouration is commonly observed on mushroom surfaces as a consequence of superficial pathogen attack or senescence after harvest (Burton et al. 1995). In the present study we measured discolouration inside mushrooms affected by a fungal endopathogen and we

observed a negative correlation between the intensity of tissue discolouration and the level of contribution of host DNA to the total fungal DNA. Necrosis of *A. bisporus* hyphae occurred only when *V. fungicola* mycelia represented a significant part of the fungal biomass in the affected mushrooms, and this necrosis resulted in host hyphal death. The literature reports that interactions of *A. bisporus* with another pathogen, *Pseudomonas tolaasii*, the causal agent of brown blotch disease, resulted in localised discolouration of the sporophore. The bacterium produces a toxin (tolaasin), which breaks down the cell membrane of the host (Rainey et al. 1991; Hutchinson and Johnstone 1993). This alteration is followed by the induction of a tyrosinase-specific mRNA, and the activation of a latent tyrosinase (Soler-Rivas et al. 1997 and 2001) involved in tissue discolouration. The activation of a latent tyrosinase also occurred after infection of *A. bisporus* by *V. fungicola* (Thapa and Jandaik 1989; Soler-Rivas et al. 1997 and 2000), and even though the pathogen grows predominantly intercellularly, it is able to penetrate *A. bisporus* fruiting body cell walls (Dragt et al. 1996; Calonje et al. 1997). Based on these reports and our data, we postulate that *V. fungicola* would not affect cell walls of aggregated *A. bisporus* hyphae of white tissues but could have penetrated those of discoloured tissues, when becoming dominant after reaction with *A. bisporus*. The lowest amounts with *A. bisporus* DNA found in discoloured tissues strengthen the idea that browning does not appear to be an efficient defensive system against *V. fungicola* but indicates tissue alteration. Otherwise, several bubbles and a stipe-bubble showed exclusively white tissues, and a mushroom with stipe blowout exhibited white diseased tissues, proving that tissue discolouration and development of the symptoms are two distinct events.

The development stage of *A. bisporus* at the time of infection by *V. fungicola* and the amount of inoculum are currently suspected to determine the type of symptoms. Because *V. fungicola* is unable to attack vegetative mycelium (Calonje et al. 2000), the quantity of A. *bisporus* DNA detected in primordia (85 ng) suggests that a high level of infection at the very early stage of fruiting body development is needed to induce bubble formation. The typical bubble is a mass of mycelium growing out of the cultivation substrate and showing none of the differentiated tissues found in sporophores. These anamorphous masses contain mycelium from both *A. bisporus* and *V. fungicola*. North and Wuest (1993) concluded from inoculation of conidial suspensions of *V. fungicola* at the surface of fruiting bodies at various stages of development that bubbles develop only when primordia (very small spherical mass of undifferentiated tissues) were inoculated. Considering that the host to pathogen ratio depends both on the quantity of pathogen at infection time and on its spread during bubble growth, the increase in the amount of host DNA from primordium stage to white bubble showed that *A. bisporus* mycelium spread faster than *V. fungicola* mycelium during bubble growth. The pathogen is involved in bubble formation, it prevents tissues differentiation, but it does not appear to influence the growth rate of undifferentiated hyphae as no correlation was found between bubble weights and quantities of host DNA.

The literature describes spotty cap and stipe blowout as the result of infection of mature or immature sporophores, generally by conidia produced by bubbles grown on the same crop. The detection of lower amounts of *A. bisporus* DNA in mushrooms with spotty cap or stipe blowout than in tissues of the healthy control showed that even though symptoms were localised, infection affected the whole mushroom including healthy looking tissues. Stipe-bubbles which show an hymenial cavity without gills, or with sterile gills, would result from the infection of primordia at the earliest stage of differentiation, at the time a very small empty cavity was visible on a longitudinal cut. These observations mean that V. *fungicola* stops morphological differentiation when initiated but, as observed for bubbles, has no effect on the growth of undifferentiated host hyphae. When infection occurs at a later stage of differentiation the effect of *V. fungicola* on host morphogenesis is more complex. The coexistence of mature and abortive lamellae on mushrooms with stipe blowout, and the correlation between their development and the quantity of host DNA

indicate that *V. fungicola* has a restricted area of action in host tissues.

In conclusion, neither the quantity of host DNA nor the intensity of tissue discolouration was related to the type of symptom and the growth of diseased mushrooms. This work was carried out with a single host/pathogen pair, and it would be interesting to look at the behaviour of other combinations, as great variations in susceptibility levels of host strains and in the aggressiveness of *V. fungicola* isolates exist (Largeteau et al. 2005). Our inoculation conditions were close to natural contamination, in contrast to those used by North and Wuest (1993), and our observations stress that the dependence of symptom type on the morphological stage of the mushroom at infection time observed by these authors would similarly exist in natural conditions of infection. The relationship between the mushroom stage and the symptom is strengthened and completed by our analyses of *A. bisporus* DNA quantities in stipe-bubbles and mushrooms with stipe blowout. The absence of regulation of host hyphal growth as a consequence of morphogenesis disruption and tissue discolouration could explain the heterogeneity observed in bubble size and the various amounts of host DNA among mushrooms showing similar symptoms. The absence of synchronisation in bubble production raises questions about the level of deregulation induced by *V. fungicola*.

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