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ORIGINAL ARTICLE

Quorum sensing activity in *Ophiostoma ulmi*: effects of fusel oils and branched chain amino acids on yeast-mycelial dimorphism

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Ophiostoma ulmi, fungal dimorphism, inoculum size, aromatic alcohols, branched-chain amino acids, quorum sensing activity.

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Abstract

Aims: For *Ophiostoma* (*Ceratocystis*) *ulmi*, the ability to undergo morphological change is a crucial factor for its virulence. To gain an understanding of quorum-sensing activity in *O. ulmi* as it relates to yeast-mycelium dimorphism control, this study examines the effects of branched-chain amino acids as well as their fusel alcohols and fusel acids as quorum sensing molecules.
Methods and Results: In a defined medium containing glucose, proline and salts, *O. ulmi* grew as yeasts when the culture was inoculated with a high density of spores (2×10^7 CFU ml^{-1}) and as mycelia when inoculated with a low spore density (4×10^5 CFU ml^{-1}). The cultures displaying yeast morphology secreted a quorum-sensing factor that shifted the morphology from mycelia to yeast. This quorum-sensing molecule was lipophilic and extractable by organic solvents from the spent medium. Using GC/MS analysis, it was determined that the major compound in the extract was 2-methyl-1-butanol. A similar effect was observed when the branched-chain amino acids (fusel alcohol precursors) were used as the nitrogen source. E, E-farnesol had no effect on the morphology of *O. ulmi*.
Conclusions: Addition of the branched-chain amino acids or one of the compounds detected in the spent medium, 2-methyl-1-butanol or 4-hydroxyphenylacetic acid, or methylvaleric acid, decreased germ tube formation by more than 50%, thus demonstrating a quorum sensing molecule behaviour in *O. ulmi* cultures.
Significance and impact of the study: This study presents advances in the investigation of dimorphism in *O. ulmi*, complementing the existing scientific basis, for studying, understanding and controlling this phenomenon.

Introduction

The Dutch elm disease caused by the fungus *Ophiostoma ulmi* (Buisman), Nannf. and its aggressive variant *Ophiostoma novo-ulmi* (Brasier) (Brasier 1996), has destroyed the majority of American elms and continues to kill trees each year (Anon 2011a). The fungus is transported to the elm trees by bark beetles that deposit their eggs in weak, dying or dead trees thereby disseminating the disease (Agrios 2005). The *O. ulmi* spores are accumulated in the xylem vessels where they reproduce by budding and are dispersed throughout the tree by translocation. *Ophiostoma ulmi* can also grow as mycelia that have an invasive capacity in which they can penetrate the xylem vessels and then continue growing radially (Anon 2011b). Dutch elm disease has been classified as one of the biggest epidemic forestry diseases on a worldwide level (Anon 2009).

For *O. ulmi*, Kulkarni and Nickerson (1981) found that in a defined glucose-salts medium, the nitrogen source was essential to control the yeast-mycelium dimorphism. The amino acid L-proline allowed the formation of yeasts, whereas L-arginine, L-asparagine and ammonium chloride promoted mycelial growth. However, there was also an inoculum size effect; yeasts were only formed in the proline-containing medium when the initial concentration of spores (inoculum) was $\geq 10^6$ colony forming units per ml (CFU ml^{-1}). Hornby *et al.* (2004) determined that the effect of inoculum size reported by Kulkarni and Nickerson (1981) was mediated by a quorum sensing molecule (QSM) secreted by the fungus, which induced a change in the type of growth from mycelia to yeast, suggesting the existence of at least one signal transduction pathway dependent on cell concentration as a mechanism of regulation for dimorphic growth.

The study of *O. ulmi* dimorphism at a molecular level has not been developed. The few studies available are based on the use of compounds that inhibit protein signalling. For example, inhibition of the *O. ulmi* lipoxygenase enzyme favoured the growth of yeast cells (Jensen *et al.* 1992). Interestingly, lipoxygenase inhibitors are naturally produced by plants and tend to accumulate in the xylem (Nadal *et al.* 2008). Similarly, calmodulin is a small acidic protein present in all eukaryotic cells that is a receptor for intracellular Ca^{2+}. The interaction between Ca^{2+} and calmodulin is necessary for hyphal development (Muthukumar and Nickerson 1984) and the use of inhibitors of calmodulin (e.g. calmidazolium, trifluorperazine and the Ca^{2+}ionophore Calcimycin), promotes yeast growth (Muthukumar and Nickerson 1984; Gadd and Brunton 1992).

For this reason, this study examines the existence of quorum-sensing activity mediated by the effects of branched-chain amino acids, as well as fusel alcohols and fusel acids in *Ophiostoma* (*Ceratocystis*) *ulmi* cultures. Each of the branched-chain amino acids, as well as 2-methyl-1-butanol and methyl valerate (the fusel alcohol and fusel acid derived from isoleucine), effectively blocked germ tube formation in *O. ulmi*.

This study presents advances in the investigation of dimorphism in *O. ulmi*, by connecting two separate topics: cell density phenomena and amino acid metabolism via fusel oil production, for studying, understanding and controlling this phenomenon.

Materials and methods

Organism

Ophiostoma ulmi (Buisman), Nannf. also known as *Ceratocystis ulmi* (Buism), C. Moreau was obtained from the National Centre for Agricultural Utilization Research (NRRL 6404) Peoria, Ill. The cultures were kept at 5°C in Petri plates with YPD (yeast extract, peptone, dextrose and agar) culture medium (Kulkarni and Nickerson 1981).

Culture media

The defined culture medium contained per litre of distilled water: 20 g of dextrose, 4 g of KH_2PO_4, 3·2 g Na_2HPO_4 0·5 g $MgSO_4{\cdot}7H_2O$, 20 μg biotin, 200 μg thiamine·HCl, 200 μg pyridoxine·HCl, 1 mg $ZnSO_4{\cdot}7H_2O$, 1 mg $MnCl_2{\cdot}4H_2O$, 1 mg $CuSO_4{\cdot}5H_2O$, 1 mg $FeCl_3$ and 10 mmol l^{-1} nitrogen source. Vitamins were prepared as a 1000X stock solution in 20% ethanol. Trace elements were prepared as a 5000X stock solution in 0·1 mmol l^{-1} HCl. Medium reagents, except the carbon and nitrogen sources, were dissolved in distilled water, deposited into their respective culture bottles and autoclaved at 121°C for 15 min. The nitrogen and carbon (dextrose) sources were autoclaved separately and added under sterile conditions to the culture medium (Hornby *et al.* 2004). Culture media with L-proline and L-arginine as nitrogen sources were designated Glucose–phosphates–proline (GPP) and Glucose–phosphates–arginine (GPR), respectively.

Inoculum preparation

For the preparation of blastospores, 250-ml Erlenmeyer flasks containing 50 ml of defined culture medium with L-proline as the nitrogen source were aseptically inoculated with cell culture stock and incubated on a G52 orbital agitator (New Brunswick Scientific Co., Enfield, CT, USA) at 150 rpm for 5 days at 25°C. The cells were collected aseptically, pelleted by centrifugation at 4750 ***g*** for 5 min and washed three times with equal volumes of 50 mmol l^{-1} phosphate buffer (pH 6·5). The cells were resuspended in 10 ml of the same buffer and stored at 5°C until required (Hornby *et al.* 2004).

Culture condition and growth evaluation

Erlenmeyer flasks of 250 ml and 25 ml, containing 50 ml and 5 ml of defined culture medium, respectively, were inoculated with the NRRL 6404 strain of *O. ulmi* and incubated at 25°C. Aeration was achieved by agitation at 150 rev min^{-1} on an orbital shaker. Cultures were monitored 24 h after inoculation (unless otherwise specified). Culture cell number and morphology were determined using a hemacytometer and phase-contrast microscopy at 640×. Only differentiated cells were quantified and normalized to 100% (yeast/filamentous cells). Cells with buds were counted as yeasts if they had a visible constric-

tion at the bud site and cells forming germ tubes were classified as germinated if the length of the germ tube was greater than half of the diameter of the spore (Hornby *et al.* 2004).

Effect of inoculum size and nitrogen source

Eight Erlenmeyer flasks of 250 ml having 50 ml of defined GPP or GPR culture media were inoculated with two different cell densities (4×10^5 or 2×10^7 CFU ml^{-1}) and agitated at 150 rev min^{-1} at 25°C. After 24 h of growth, three samples per flask were taken and the culture morphology was evaluated by microscopy.

Preparation of spent medium

Spent medium was obtained by inoculating 400 ml of GPP medium in a 2000-ml Fernbach flask with 2×10^7 CFU ml^{-1} of *O. ulmi* (blastospores). The flask was aerated by rotary agitation at 150 rpm on an orbital shaker for 72 h (final pH 5·5 ± 0·2). Cultures were collected by centrifugation (5 min at 7520 ***g***) and filtered and sterilized with 0·22-μm GVWP membrane filters (Millipore, Billerica, MA, USA). The cell-free supernatant was designated as spent medium.

Bioassay with spent medium

Flasks (25 ml) were prepared with 5 ml of medium consisting of 1 ml of 5X GPP fresh medium and different concentrations of sterile spent medium (0, 10, 25, 45, 65 and 75% v/v) made to a total of 5 ml with sterile water. These flasks were inoculated with 4×10^5 spores per ml and aerated by agitation at 150 rev min^{-1} at 25°C and their morphology was evaluated 24 h after inoculation. All experiments were performed in triplicate. A one-way ANOVA was performed.

Extraction of the quorum-sensing molecule with different solvents

Spent medium from *O. ulmi* was extracted with different solvents (ethyl acetate, hexane or diethyl ether). Separation was carried out using a separation funnel and an aqueous: solvent ratio of 5 : 1. The solvent was removed with continuous N_2 flow at room temperature. The residue was resuspended in 50 μl of 100% methanol and bioassayed to measure the QSM activity.

Bioassay of QSM activity

Twenty-five millilitre flasks with 5 ml of GPP culture medium were inoculated with spores at a concentration of 4×10^5 CFU ml^{-1}. A 50 μl volume of the methanol extracts, obtained from the different organic solvents, was added to measure their effect on the morphology of *O. ulmi* cultures. Flasks containing only GPP were used as controls. In addition, 50 μl of 100% methanol was added to flasks having GPP culture medium as a solvent control.

The loss of activity of the spent medium following solvent extraction was evaluated by the addition of 50% (v/v) of the aqueous phase to fresh 2X GPP medium using a low inoculum size. Prior to this addition, the aqueous phase of spent medium that had been extracted with ethyl acetate was extracted again with hexane to remove any remaining ethyl acetate, because trace levels of this solvent have an inhibitory effect on *O. ulmi*. Morphology was evaluated after 24 h of growth and all treatments were carried out in triplicate.

GC/MS Analysis

The spent medium was extracted with ethyl acetate, dried with N_2 at room temperature, resuspended in 100% methanol and analysed by a GC 7890A (Agilent Technologies, Santa Clara, CA, USA) and a MS: 5975 C inert XL EI/CI MSD with a Triple-Axis detector GC/MS. The column used was a 30-m Agilent Technologies HP INNOWAX, 0·25 mm of diameter and 0·25 μmol l^{-1} of flow with an operating range from 40 to 270°C. GC used a 1·5 μl sample, injector and detector temperature of 200 and 235°C, respectively, and a temperature programme of 150°C for 2 min and then 15°C min^{-1} 235°C for 30 min. MS used a 5-min solvent delay and a scan rate of 1·5 scans s^{-1}.

Effect of branched-chain amino acids on *O. ulmi* morphology

Stock solutions (100 mmol l^{-1}) of the amino acids L-isoleucine, L-leucine and L-valine were prepared, autoclaved and added to the culture media at final concentrations of 1 or 10 mmol l^{-1}. The first experiment evaluated the morphology of *O. ulmi* in GPP and GPR enriched with 1 mmol l^{-1} of one of the branched-chain amino acids. For this assay, 25-ml flasks with 5 ml of GPP or GPR were inoculated with two inoculum sizes (4×10^5 or 1×10^7 CFU ml^{-1}) and the culture morphology was examined after 24 h. Treatments were performed in duplicate.

The second experiment evaluated the branched-chain amino acids L-leucine (GPL) and L-isoleucine (GPI) as the sole nitrogen sources at a series of different inoculum sizes (10^4–10^7 CFU ml^{-1}). Morphological observations were carried out after 48 h of growth because the cells did not display a well-defined morphology after 24 h of

growth. All experiments were performed in duplicate using 250-ml flasks with 50 ml of culture.

QSM candidates

Eight compounds were evaluated as possible QSM candidates for the inhibition of germ tube formation in *O. ulmi*. Erlenmeyer flasks (25 ml) with 5 ml of GPR were inoculated with two inoculum sizes: 4×10^5 or 1×10^7 CFU ml^{-1} and the effect of different compounds and concentrations on the morphology of the cultures was analysed. Observations were carried out at 24 h after inoculation, following the previously described methodology. All experiments were performed in duplicate.

Results

Effects of inoculum size and nitrogen source

Table 1 shows the effects of inoculum size and nitrogen source on the formation of germ tubes in cultures of *O. ulmi*. These growth experiments, like all of those described in this study, were conducted at 25°C. Greater than 90% germ tube formation (mycelial growth) was observed in the GPR culture at both low (4×10^5 CFU ml^{-1}) and high (2×10^7 CFU ml^{-1}) inocula, whereas in the GPP culture, germ tube formation was observed with only the low inoculum size (4×10^5 CFU ml^{-1}). The remaining 95% of the high inoculum GPP cells developed as budding yeasts.

Bioassay of *O. ulmi* morphology with spent medium

At a concentration of 4×10^5 CFU ml^{-1} in a series of GPP cultures prepared with increasing concentrations of sterile spent medium, *O. ulmi* exhibited a change in morphology from mycelia to yeasts that was concentration-dependent and statistically significant ($P < 0{\cdot}0001$). The mycelial proportion decreased from 93 to 20% with the addition of up to 75% (v/v) of spent medium (Fig. 1). These effects of spent medium on the growth morphology of *O. ulmi* cultures could be explained by the presence of a compound or group of compounds capable of inducing the yeast-like morphology or blocking the mycelia morphology. The term QSM refers to a cell density–dependent activity with no preconditions on the mode of action of that molecule (Nickerson *et al.* 2006).

Table 1 Effect of inoculum size and nitrogen source on cell morphology in *Ophiostoma ulmi* at 24 h of growth

	Proportion of cells with germ tube formation (%)	
Inoculum size (CFU ml^{-1})	GPP*	GPR†
2×10^7	5 ± 2·5	94 ± 2·0
4×10^5	90 ± 0·5	91 ± 0·8

*GPP, Glucose–phosphates–proline culture medium.
†GPR, Glucose–phosphates–arginine culture medium.

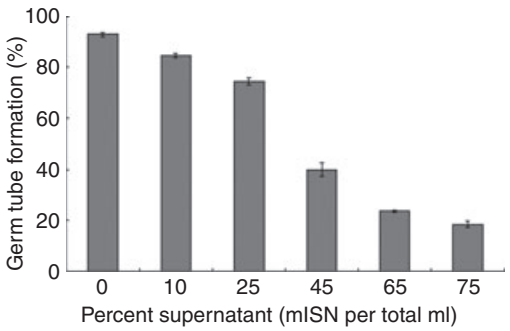

Figure 1 Effect of per cent of supernatant on proportion of cells with germ tube formation in *Ophiostoma ulmi* cultures grown in Glucose–phosphates–proline medium and low inoculum size (4×10^5 CFU ml^{-1}) at 24 h of growth.

A lipophilic QSM

Addition of 50 μl of 1000-fold concentrated spent medium, extracted with ethyl acetate, hexane, or diethyl ether and resuspended in 100% methanol, reduced the germ tube formation from ≥90% (no additions) to 39 ± 1·6, 35 ± 2·2 and 32 ± 2·2% of the spores, respectively. As a solvent only control, the percentage of germinated spores was 89 ± 1·9% with unsupplemented GPP medium and 89 ± 1·3% with the addition of 50 μl of 100% methanol (control). Thus, the QSM activity was extracted into three organic solvents. This view was confirmed by an experiment in which the solvent extracted spent medium was tested to see whether it had any remaining QSM activity. The extracted spent medium was used (50% v/v) in preparing fresh GPP. There was no QSM signalling activity remaining in the spent medium; for all three solvents used the extracted spent medium did not reverse mycelial growth, 90 ± 2% of the spores formed germ tubes. These results prove that the QSM activity of *O. ulmi* is lipophilic. Also, because the extractions with ethyl acetate, hexane and diethyl ether were roughly equivalent, ethyl acetate extraction was used in all subsequent experiments.

GC/MS results

GC/MS analysis of the spent medium extracted with ethyl acetate and resuspended in methanol showed a major peak at 8·79 min which was identified by the library from GC/MS Agilent Technologies as 2-methyl-1-butanol as

well as 8-9 minor peaks between 12 and 21 min (Fig. 2). Of these, only the minor peak at 17·14 min was identified, as 4-hydroxyphenylacetic acid, ethyl ester. The major peak (2-methyl-1-butanol) comprised over 98% of the total (Fig. 2).

Effects of branched-chain amino acids on the morphology of *C. ulmi*

We studied the effect of adding 1 mmol l^{-1} of the branched-chain amino acids (leucine, isoleucine and valine) on the morphology of *O. ulmi* grown in GPP or GPR media (Table 2). Addition of the branched-chain amino acids (1 mmol l^{-1}) showed a significant effect on the morphology of *O. ulmi* cultures grown in GPP (Table 2) at the low inoculum size where they gave a per cent germ tube formation of ca. 25% compared with a control of 90 ± 0·60% (Table 2). In contrast, the percentage of spores that germinated at a high inoculum size (1×10^7 CFU ml^{-1}) was close to 5% with or without the branched-chain amino acids. Thus, these values were not different from the control, demonstrating that the addition of 1 mmol l^{-1} of leucine, isoleucine or valine does not affect the cellular morphology under this condition. In this case, the addition of isoleucine, leucine or valine showed a significant drop in per cent germ tube formation regardless of inoculum size. The control cultures displayed a high percentage of germ tube formation (≥90%), whereas those with added branched-chain amino acids (1 mmol l^{-1}) had only 19–41% (Table 2). L-leucine was the most effective of the three amino acids tested (Table 2).

For Table 2, the *O. ulmi* cells were grown in media containing 10 mmol l^{-1} L-proline or L-arginine, supplemented with 1 mmol l^{-1} of the branched-chain amino acids. Figure 3 graphs the effects of inoculum size on the morphology of *O. ulmi* cultures grown in defined media containing 10 mmol l^{-1} of the branched-chain amino acids (L-isoleucine or L-leucine) as the sole nitrogen sources. Consistently, a lower per cent germ tube formation was obtained with the higher inoculum size for both GPI and GPL (Fig. 3). In both cultures, the germ tube formation was reduced from 65 to 67% at a cell density of 1×10^4 CFU ml^{-1} to only 17 and 8% in the high cell density media containing isoleucine and leucine, respectively, (Fig. 3). These results indicate that inoculum size influences the morphology of *O. ulmi* using branched-chain amino acids as the sole nitrogen source.

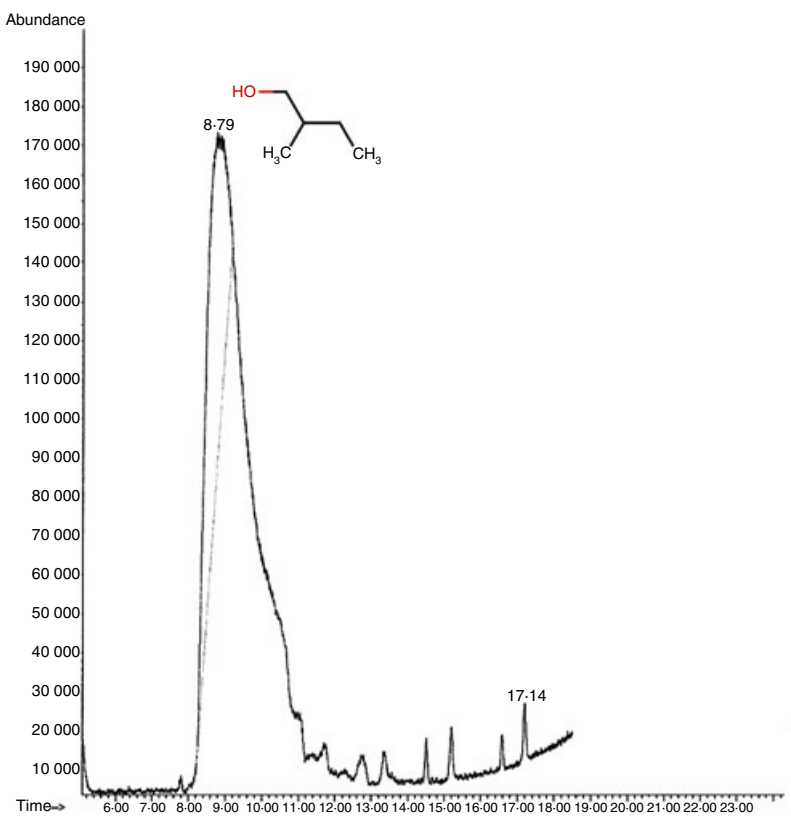

Figure 2 GC/MS analysis of quorum sensing molecules from *Ophiostoma ulmi* using the CI mode for MS. Identified peaks were at 8·79 min (2-methyl-1-butanol) and at 17·14 min (4-hydroxyphenylacetic acid, ethyl ester).

Table 2 Effect of the addition of three branched-chain amino acids (1 mmlo l^{-1}) on proportion of cells with germ tube formation in *Ophiostoma ulmi* cultures grown in GPP and GPR medium at high and low inoculum sizes

	GPP*		GPR†	
Condition	1×10^7 (CFU ml^{-1})	4×10^5 (CFU ml^{-1})	1×10^7 (CFU ml^{-1})	4×10^5 (CFU ml^{-1})
GPP* (Control)	5 ± 0·8	90 ± 0·6	91 ± 0·3	91 ± 1·3
GPP + Isoleucine (1 mmol l^{-1})	5 ± 0·8	25 ± 2·2	24 ± 0·7	41 ± 0·3
GPP + Leucine (1 mmol l^{-1})	3 ± 0·6	23 ± 2·4	19 ± 1·0	26 ± 1·5
GPP + Valine (1 mmol l^{-1})	4 ± 0·3	27 ± 0·7	26 ± 1·4	36 ± 1·4

*GPP, Glucose–phosphates–proline–culture medium.
†GPR, Glucose–phosphates–arginine culture medium.

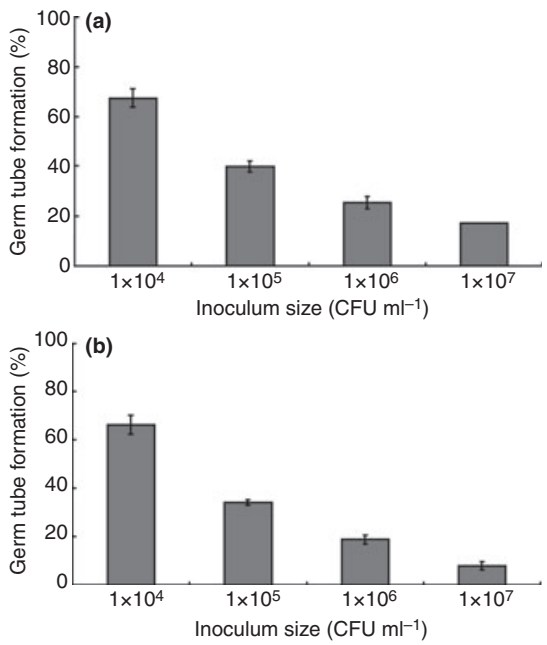

Figure 3 Effect of inoculum size on proportion of cells with germ tube formation in *Ophiostoma ulmi* cultures grown in media containing: (a) glucose–phosphate–isoleucine and (b) glucose–phosphate–leucine at 48 h of growth.

Table 3 Effect of different concentrations of eight quorum sensing molecule candidates on proportion of cells with germ tube formation in *Ophiostoma ulmi* cultures established with high inoculum size (1×10^7 CFU ml^{-1}) and grown in GPR medium at 24 h of culture

	Concentration		
Condition	25 μmol l^{-1}	250 μmol l^{-1}	500 μmol l^{-1}
GPR* + 2-(4-hydroxyphenyl) ethanol	78 ± 0·0	69 ± 0·6	ND
GPR + 3-methylbutanol	74 ± 4·5	68 ± 0·3	ND
GPR + 2-methylpropanol	73 ± 0·2	68 ± 2·0	ND
GPR + 2-methyl-1-butanol	45 ± 0·6	26 ± 0·3	20 ± 4·0
GPR + isovaleric acid	76 ± 4·3	76 ± 0·8	ND
GPR + 2-methylpropanoic acid	72 ± 2·0	76 ± 0·3	ND
GPR + 4-hydroxyphenylacetic acid	40 ± 0·1	38 ± 2·0	18 ± 3·3
GPR + methylvaleric acid	57 ± 4·6	30 ± 0·5	20 ± 3·0
GPR (control)	90 ± 0·5		
GPR + 50 ul methanol (control)	90 ± 0·6		

ND, Not determined.
*GPR, Glucose–phosphates–arginine culture médium.

QSM candidates

Our results with the analysis of the spent medium (2-methyl-1-butanol) as well as the effects of the three branched-chain amino acids (Table 2) led us to select eight compounds (four fusel alcohols and four fusel acids) as QSM candidates. These eight compounds are the alcohol and acid products of the Ehrlich pathway derived from tyrosine and the three branched-chain amino acids. We examined their effects on *O. ulmi* morphology in GPR medium with high (Table 3) and low (Table 4) inoculum sizes.

The aromatic alcohol 2-(4-hydroxyphenyl) ethanol, 3-methylbutanol and 2-methylpropanol, end products in the catabolism of the amino acids tyrosine, leucine and valine, respectively, showed no effect on germ tube formation at either high or low inoculum size with addition of 25 or 250 μmol l^{-1} of the respective alcohols. Spore germination was reduced from 90% (control) to 68–85% (Tables 3 and 4). However, 2-methyl-1-butanol (a fusel alcohol derived from isoleucine) did have a significant effect on the morphology of these cultures; addition of 250 μmol l^{-1} of 2-methyl-1-butanol caused only 26% of the spores to germinate at either high (Table 3) or low (Table 4) inocula.

With respect to the carboxylic acids that were evaluated, neither isovaleric acid (3-methyl butanoic acid) nor isobutyric acid (2-methylpropanoic acid) were able to decrease germ tube formation. Addition of 250 μmol l^{-1} of either of these compounds to *O. ulmi* cultures grown in GPR medium resulted in 75–88% of the spores

Table 4 Effect of different concentrations of eight quorum sensing molecule candidates on proportion of cells with germ tube formation in *Ophiostoma ulmi* cultures established with low inoculum size (4×10^5 CFU ml^{-1}) and grown in GPR medium at 24 h of culture

	Concentration	
Condition	250 μmol l^{-1}	500 μmol l^{-1}
GPR* + 2-(4-hydroxyphenyl) ethanol	75 ± 2·7	ND
GPR + 3-methylbutanol	87 ± 1·7	ND
GPR + 2-methylpropanol	85 ± 2·9	ND
GPR + 2-methyl-1-butanol	26 ± 0·4	20 ± 0·4
GPR + isovaleric acid	81 ± 3·1	ND
GPR + 2-methylpropanoic acid	88 ± 0·9	ND
GPR + 4-hydroxyphenylacetic acid	36 ± 0·3	22 ± 0·2
GPR + methylvaleric acid	36 ± 1·9	23 ± 2·1
GPR (control)	91 ± 0·8	
GPR + 50 μl methanol (control)	90 ± 0·6	

ND, Not determined.
*GPR, Glucose–phosphates–arginine culture medium.

displaying mycelial growth (Tables 3 and 4). On the other hand, methylvaleric acid (2-methylbutanoic acid), derived from isoleucine, at concentrations of 25, 250 and 500 μmol l^{-1} reverted the morphology of *O. ulmi* so that only 57, 30 and 20%, respectively, of the spores formed germ tubes in the high cell density cultures (Table 3). Similar reductions were observed for methylvaleric acid in the low cell density cultures (Table 4).

Discussion

Hornby *et al.* (2004) found that this GPP inoculum size effect, which controls dimorphism in *O. ulmi*, was a manifestation of a quorum-sensing system that is mediated by a molecule secreted extracellularly when the cell concentration is high, and they suggested that quorum sensing was a general phenomenon in dimorphic fungi. By analogy to a study performed in *Candida albicans* by Hornby *et al.* (2001), this compound would be called a QSM and, as expected, the molecule is lipophilic in nature and can be extracted with organic solvents such as ethyl acetate, hexane or diethyl ether. In agreement with Nickerson *et al.* (2006), the QSM from *O. ulmi* is not E,E-farnesol. Separate experiments carried out in our laboratory showed that addition of 10, 25 or 50 μmol l^{-1} of E,E-farnesol did not inhibit germ tube formation in *O. ulmi*.

The present study showed that three molecules, 2-methyl-1-butanol, methylvaleric acid and 4-hydroxyphenylacetic acid, displayed QSM characteristics on the morphology of *O. ulmi*. 2-methyl-1-butanol and methylvaleric acid, respectively, are the fusel alcohol and fusel acid derived from isoleucine (Hazelwood *et al.* 2008). Other branched-chain amino acids like L-leucine and L-valine also generate branched-chain alcohols and collectively these amino acid derived alcohols are called the fusel alcohols or fusel oils (Hazelwood *et al.* 2008). Fusel alcohols are higher order alcohols that are produced during the fermentation process when the growth of the yeasts is limited by the availability of nitrogen. The Ehrlich pathway achieves the extraction of nitrogen from amino acids and results in the reduction or oxidation of the fusel aldehydes, forming the corresponding alcohols or organic acids (Hazelwood *et al.* 2008). At that time, yeasts scavenge any available amino acids, extract the nitrogen by transamination and excrete the remaining carbon skeleton. These fusel alcohols, especially the aromatic alcohols, have been proposed as signalling molecules for the fungi *Saccharomyces cerevisiae* and *C. albicans* (Chen and Fink 2006; Ghosh *et al.* 2008; Hazelwood *et al.* 2008). Several other previous studies demonstrated the effect that fusel alcohols such as 3-methyl-1-butanol (isoamyl alcohol), 2-methyl-1-propanol (isobutyl alcohol), 2-methyl-1-butanol (active amyl alcohol), 2-phenylethanol and 3-(2-hydroxyethyl) indole (tryptophol) (end products of the catabolism of leucine, valine, isoleucine, phenylalanine and tryptophan, respectively) have on the cell morphology of fungi (Martins *et al.* 2007; Dickinson 2008). Isoamyl alcohol and 2-methyl-1-butanol stimulated pseudohyphal or filamentous growth in haploid cells from *S. cerevisiae* (Lorenz *et al.* 2000; Hauser *et al.* 2007), while isoamyl alcohol induced the formation of pseudohyphae in two strains of *C. albicans*, as well as pseudohyphae and extension-type hyphae in *Brettanomyces anomalus*, thus suggesting a common relationship for the development of these two morphologies (Dickinson 1996).

Several microorganisms use extracellular signals to transmit information depending on the population density and environmental conditions (Sprague and Winans 2006) and recent evidence suggests that for the yeast S. *cerevisiae* the production of these self-signalling alcohols is regulated by the nitrogen source (Hogan 2006). For instance, the presence of ammonium restricts the production of aromatic alcohols from the aromatic amino acids by inhibiting the expression of their biosynthetic pathway (Ghosh *et al.* 2008). This nitrogen repression is eliminated when only poor nitrogen sources are available and then the fusel oil pathways are activated. For instance *C. albicans* produced 5–7 times less aromatic alcohols when grown in a defined culture medium containing ammonium as nitrogen source than when grown in the same medium but with proline GPP. The only difference between the two cultures was whether ammonium sulfate or L-proline (both at 10 mmol l^{-1}) was used as the nitrogen source. Production of the aromatic alcohols was also 5–7 times less when the cells were grown in the presence of both proline and ammonium sulfate (10 mmol l^{-1}

each), demonstrating that ammonium sulfate still exerts an inhibitory effect in the presence of L-proline (Ghosh *et al.* 2008). In addition, the production of these alcohols is controlled by cell density. In *S. cerevisiae*, for example, a high cell density stimulates the production of aromatic alcohols by regulating the expression of *ARO9* and *ARO10,* two genes that are key for the biosynthesis of these alcohols. The transcription levels for *ARO9* and *ARO10* in low-density cells (10^5 cells per ml) were very low, even when these cells were cultured in media containing only a small amount of ammonium. However, a high-density culture (5×10^7 cells per ml) in the same medium significantly stimulated their expression (Chen and Fink 2006).

Our study also revealed the effect of branched-chain amino acids on the morphology of *O. ulmi* (Table 2), possibly associated with their acting as precursors for fusel alcohols, fusel acids or other similar secondary metabolites. Furthermore, some amino acids such as valine (Val), isoleucine (Ile) or leucine (Leu) are precursors for other secondary metabolites (Li *et al.* 2009). Likewise, the use of amino acids as a source of nitrogen is influenced by the affinity that different microorganisms have for them. A study carried out in *Geotrichum candidum* revealed that this fungus displayed a clear preference for ammonium over L-leucine as the nitrogen source. Experiments showed that the ammonium was completely depleted at the end of the exponential growth phase (35 h of fermentation), whereas only 5% of the leucine had been utilized. Thereafter, the culture grew slowly until the leucine was completely consumed at 185 h of fermentation (Adour *et al.* 2010).

It is intriguing that the fusel alcohols are reported to cause different and sometimes opposite effects in different fungi as observed by different authors. For instance, they can block germ tube formation in *O. ulmi* (Tables 3 and 4), stimulate germ tube formation in *C. albicans* (Chen and Fink 2006), block germ tube formation in *C. albicans* (Martins *et al.* 2007) and promote pseudohyphal growth in *C. albicans* (Dickinson 1996, 2008) and *S. cerevisiae* (Hauser *et al.* 2007). To help with the interpretation of these reports, we suggest two unifying ideas. First, morphological shifts induced by added amino acids (Table 2) are easier to interpret than shifts induced by alcohols derived from them (Tables 3 and 4). Because the amino acids are not lipid soluble, you do not need to worry about nonspecific membrane effects at high concentrations of the active component. Second, in *S cerevisiae* nitrogen metabolism and the activator protein Gcn4 are regulated by an intricate translation control mechanism, including Gcn2p and eIF2B which are highly conserved in the fungi (Hinnebusch 2005). Gcn4 then activates over 500 target genes (Hinnebusch 2005). Interestingly, yeast eIF2B activity is inhibited by fusel alcohols in a manner dependent on a single amino acid (position 180) in eIF2B (Ashe *et al.* 2001). Thus, the fusel alcohols could easily have different effects on different fungi or even on different strains of the same fungus.

Finally, it is important to mention the possibility that it is not just one single QSM that has regulatory properties, as demonstrated by our results (Tables 3 and 4) with the compounds 2-methyl-1-butanol, methylvaleric acid and 4-hydroxyphenylacetic acid, which all have the same effect on the morphology of *O. ulmi*. For any one species, there may be numerous substances that display QSM characteristics. For example, with *C. albicans* and *Candida dubliniensis*, the addition of different concentrations of isoamyl alcohol, 2-phenylethanol, 1-dodecanol, *E*-nerolidol and *E,E*-farnesol (at concentrations proportional to those found in the culture supernatants from both species) inhibited the transition from yeasts to hyphae by more than 50%. The physiological role of these alcohols was confirmed by comparing the effects of the 96 h supernatant with a synthetic mixture containing these alcohols. The synthetic mixtures induced morphological effects similar the supernatants for both fungal species (Martins *et al.* 2007). This suggestion should be viewed in the context that of the three potential QSMs identified in Tables 3 and 4, only 2-methy-1-butanol would be extracted into ethyl acetate and, to the extent we could determine it, ethylacetate removed all the QSM activity from the cell supernatants. Thus, 2-methyl-1-butanol appears to be the dominant QSM for *O. ulmi*.

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