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# Quorum sensing as a method for improving sclerotiorin production in *Penicillium sclerotiorum*

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ABSTRACT

Quorum sensing has been extensively studied in the bacterial kingdom but little is known about it in filamentous fungi. γ-Butyrolactones have been established as quorum sensing molecules in Gram-negative bacteria (as acyl-homoserine lactones) and Gram-positive bacteria (as A-factor) and they are present in many filamentous fungi (e.g. as butyrolactone I in *Aspergillus terreus*). This study investigates possible role of multicolic acid (and related derivates) as quorum sensing molecule(s) in *P. sclerotiorum* and its effect on the production of secondary metabolite sclerotiorin. Exogenous addition of an ethyl acetate extract of supernatants from *P. sclerotiorum* IMI 104602 (Strain M) at 48 h of growth resulted in maximum sclerotiorin yield of 8.5 mg $g^{-1}$ in Strain M at 168 h post-inoculation, a 1.8-fold increase as compared to the control. Addition of spent medium containing γ-butyrolactone molecules from this strain to *P. sclerotiorum* IMI 040574 (Strain S) resulted in 6.4-fold increase in sclerotiorin yield at 168 h post-inoculation without causing a significant change in the biomass production ($p > 0.05$) or carbohydrate consumption rate ($p > 0.05$). The results presented here suggest that multicolic acid (and related derivatives) function as quorum sensing molecules in the filamentous fungus *P. sclerotiorum*.



## 1. Introduction

Butyrolactones have been extensively studied in recent years due to their association with quorum sensing. Quorum sensing is a phenomenon in which low molecular weight signals produced by micro-organisms are perceived either at intraspecies or interspecies level evoking a response from the microbial population (Fuqua and Greenberg, 1998). Some of these responses are the production of secondary metabolites (carbapenem by *Erwinia carotovora* (McGowan et al., 1995)), and streptomycin by *Streptomyces griseus* (Recio et al., 2004), pathogenesis—biofilm formation by *Pseudomonas aeruginosa* (de Kievit, 2009) and morphological differentiation (*Streptomyces griseus*, Ochi, 1987). In Gram-negative bacteria acyl-homoserine lactones have been identified as signalling molecules whereas in Gram-positive bacteria these signals encompass modified or unmodified oligopeptides (Miller and Bassler, 2001). In actinomycetes γ-butyrolactones such as the A-factor (2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone, Miyake et al., 1990) and virginiae butanolides (Yamada et al., 1987) have been reported as signalling molecules regulating the production of streptomycin and virginiamycin, respectively. Signalling molecules have also been identified in some fungi for example farnesol and tyrosol in *Candida albicans* regulating the morphological switch between the yeast and the mycelial form (Hornby et al., 2001). Although very little is known about quorum sensing in filamentous fungi, recent advances have identified Butyrolactone I as a quorum sensing molecule in *Aspergillus terreus* enhancing lovastatin production (Raina, 2008) and oxylipins as quorum sensing molecules regulating sporulation in *Aspergillus nidulans* (Sorrentino, 2009). Oxylipins have also been suggested as quorum sensing molecules regulating morphological transitions in *Aspergillus flavus* (Horowitz Brown et al., 2008). There have been reports of similar lactone molecules being present in other filamentous fungal strains but their role in cell–cell signalling has not been established (Schimmel et al., 1998).

*Penicillium sclerotiorum* also known as *P. multicolor*, is a soil microorganism which produces a number of commercially important secondary metabolites such as azaphilones and polyketides (Turner and Aldridge, 1983). Multicolanic, multicolosic and multicolic acids were isolated by Gudgeon et al. from *P. multicolor* (*sclerotiorum*) IMI 104602 (Gudgeon et al., 1979). These γ-butyrolactone-containing compounds are synthesised by oxidative cleavage of an aromatic precursor 6-pentylresorcylate (Holker et al., 1987) and classified as hexaketides because of their polyketide origin (Turner and Aldridge, 1983). Sclerotiorin, a secondary metabolite synthesised by *P. sclerotiorum* is a yellow, chlorine containing pigment possessing phospholipase A2 inhibitor activity (Michael et al., 2003). Sclerotiorin and other azaphilones synthe-

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sised by *P. sclerotiorum* have shown human endothelin (ET) receptor binding activity. They exhibit physiological activities resulting in bronchoconstriction, chronotropic and positive ionotropic effects and renal effects (Pairet et al., 1995). Sclerotiorin has also been correlated with induction of morphological changes in filamentous fungi. This is thought to be a result of its reactivity with methylamine (Natsume et al., 1988). In addition, sclerotiorin also possesses cholesterol ester transfer protein (CETP) inhibitory activity. CETP is a lipophilic thermo-stable glycoprotein responsible for exchange and transfer of cholesterol esters and triacylglycerol among plasma lipoproteins (Tabata et al., 1999). CETP inhibitory activity together with efficacy against Methicillin resistant *Staphylococcus aureus* (MRSA) provided by isochromophilone IX (a sclerotiorin derivative) makes sclerotiorin an important candidate for medicinal research (Michael et al., 2003). This study investigated the role of γ-butyrolactone-containing compounds (Multicolic acid and related derivatives) as quorum sensing molecules in *P. sclerotiorum* and their effects on sclerotiorin production.

## 2. Materials and methods

### 2.1. Strains and chemicals

*P. sclerotiorum* IMI 104602 (Strain M) and IMI 040574 (Strain S) were obtained from CABI Biosciences UK Centre, Surrey, United Kingdom. All chemicals used in this study were obtained from Sigma–Aldrich Company Limited, Dorset, United Kingdom. Analytical grade reagents were used for quantitative and qualitative assays and HPLC grade reagents were used for high performance liquid chromatography assays.

### 2.2. Media and growth conditions

#### 2.2.1. P. sclerotiorum IMI 104602 (Strain M)

A stock culture of *P. sclerotiorum* Strain M was maintained on modified Czapek Dox agar slopes (Oxoid). The slopes were grown at 28 °C for 8 days for generation of spores and subsequently stored at 4 °C. A spore suspension containing $10^7$ spores/ml was prepared in 20% (v/v) glycerol solution and maintained at −80 °C.

For production of multicolic acid (and related derivatives) Strain M was grown in Raulin Thom medium with the following composition: sucrose (30 g $l^{-1}$), ammonium tartrate (2.6 g $l^{-1}$), $NH_4H_2PO_4$ (0.4 g $l^{-1}$), $(NH_4)_2SO_4$ (0.16 g $l^{-1}$), tartaric acid (2.6 g $l^{-1}$), $MgCO_3$ (0.28 g $l^{-1}$), $K_2CO_3$ (0.4 g $l^{-1}$), $ZnSO_4{\cdot}7H_2O$ (0.07 g $l^{-1}$), $CuSO_4{\cdot}5H_2O$ (0.005 g $l^{-1}$), and $FeSO_4{\cdot}7H_2O$ (0.06 g $l^{-1}$). The pH of the medium was adjusted to 3.5 before sterilisation. One hundred millilitres of the sterile medium was inoculated with 1 ml of spore suspension ($10^7$ spores/ml). The cultures were subsequently incubated in an orbital shaker at 26 °C set at a speed of 150 rpm with 2 cm throw for 8 days to allow production of multicolic acid.

For production of sclerotiorin, Strain M was grown in potato dextrose broth (PDB). One millilitre of spore suspension was used to inoculate 100 ml of PDB which was then incubated at 26 °C and a speed of 150 rpm with 2 cm throw for 9 days. Samples were collected at 24 h intervals and analysed for sclerotiorin production.

#### 2.2.2. P. sclerotiorum IMI 040574 (Strain S)

*P. sclerotiorum* Strain S was maintained on modified malt extract agar slopes. The inoculated slopes were incubated for 8 days at 28 °C for generation of spores. A spore suspension containing $10^7$ spores/ml was prepared in 20% (v/v) glycerol solution and maintained at −80 °C. Sterile potato dextrose broth was inoculated with *P. sclerotiorum* Strain S spores ($1 \times 10^7$ $ml^{-1}$) and incubated at 27 °C at a speed of 120 rpm with 2 cm throw for 9 days in an orbital shaker for production of sclerotiorin.

**Table 1**
HPLC gradient profile for analysis of multicolic acid and related derivatives.

| Stage | Time (min) | Mobile phase A (1% formic acid in acetonitrile) | Mobile phase B (water) |
|---|---|---|---|
| 1 | 0 | 10% | 90% |
| 2 | 1 | 10% | 90% |
| 3 | 14 | 100% | 0% |
| 4 | 19 | 100% | 0% |
| 5 | 20 | 10% | 90% |
| 6 | 30 | 10% | 90% |

### 2.3. Addition of potential inducer(s) to sclerotiorin producing cultures

#### 2.3.1. Crude extract containing butyrolactone molecules

An ethyl acetate extract containing multicolic acid and related derivatives was prepared from *P. sclerotiorum* Strain M as described by Gudgeon et al. (1979). The extract was filter sterilised using a 0.2-μm cellulose acetate membrane filter. The LC–MS analysis of the extract suggested the presence of multicolic acid and related derivatives in the extract. This γ-butyrolactone-containing extract (0.1%, v/v) was added to the test cultures of *P. sclerotiorum* Strain M at 48 h post-spore inoculation.

#### 2.3.2. Spent medium (containing multicolic acid)

*P. sclerotiorum* Strain M was grown in Raulin–Thom medium as described earlier for 8 days. The culture broth was filtered using a Whatman filter paper (No. 1). The cell-free broth was then filter sterilised using a 0.2-μm cellulose acetate membrane filter and added to the test cultures in volumes of 0.5, 1.0, 1.5, 2.0 and 5.0% (v/v). The spent medium was added to *P. sclerotiorum* Strain S cultures at 48 h post-spore inoculation.

#### 2.3.3. Addition of 4-hydroxybutyric acid lactone

To investigate the effect of purified γ-butyrolactone, 4-hydroxybutyric acid lactone was added to the growing cultures of Strain S at 48 h post-spore inoculation at a final concentration of 1.625 mM. The concentration of 4-hydroxybutyric acid lactone was chosen based on preliminary studies conducted for optimal sclerotiorin production (data not shown).

### 2.4. Quantification of sclerotiorin

Quantification of sclerotiorin was carried out using an HPLC method as described by Weng et al. (2004). Freeze dried mycelia were extracted with HPLC grade methanol (1 ml) and analysed using a Dionex, Acclaim® 120 reverse phase column. The mobile phase was acetonitrile and water (65:35, v/v). The peaks were detected by spectrophotometry at 370 nm. A standard curve was prepared using a sclerotiorin HPLC standard (Wako Chemicals, Germany).

### 2.5. HPLC–MS for identification of multicolic and related derivatives

The ethyl acetate extract (0.2 g) from *Penicillium sclerotiorum* Strain M shaken flask fermentation was dissolved in methanol (2 ml) for introduction into the HPLC interface of the mass spectrometer. HPLC was performed using a Dionex HPLC system (Chromelon) and a Symmetry Shield RP18 (150 mm × 4.6 mm, 5.0 μm), (Waters, USA). The mobile phase composition and gradient profile is given in Table 1. The flow-rate was set at 1 ml $min^{-1}$. These conditions were held for 5 min followed by returning to the initial conditions over 5 min. In-line MS was performed using a Surveyor MSQ Plus Mass Spectrometer system with a Z-Spray electrospray