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In vivo investigation of the volatile metabolome of antiphytopathogenic yeast strains active against *Penicillium digitatum* using comprehensive two-dimensional gas chromatography and multivariate data analysis

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ABSTRACT

Penicillium digitatum is the responsible for green mold decay on citrus fruits, which contributes to economic losses in agriculture industry during storage, transportation and in the market. Biocontrol agents may be a viable and low-cost alternative in the control of different plant pathogens: metabolites from some yeast strains can prevent infection, decreasing host tissue colonization and reducing pathogen survival and sporulation with varying degrees of efficiency. Eight *S. cerevisae* strains and one *Candida stellimalicola* strain all were evaluated *in vitro* as biological control agents against *P. digitatum*. After *in vitro* tests the headspace of yeast strains were extracted *in vivo* using an optimized HS-SPME + GC × GC–MS method. The correlation between antiphytopathogenic activity with the volatile metabolite profiles was assessed using principal component analysis. Nine compounds were associated with the volatile metabolome of strains with higher antifungal action: ethyl propionate, 3-methyl-1-butanol, 2-methyl-1-butanol, ethyl butyrate, 3-methylbutyl acetate, 3-methylbutyl propionate, n-pentyl butyrate, phenylethyl alcohol and 2-phenethyl acetate. This study allowed for the first time a comprehensive overview of the effect of individual volatile metabolites from yeast strains on *P. digitatum* infection.

1. Introduction

Postharvest diseases due to fungal infection contribute to economic losses in agriculture industry during storage, transportation or in the market [1]. Citrus fruits may be damaged during harvest, handling, transport, storage and marketing, providing entry points to different pathogens, specially *Penicillium digitatum* and *P. italicum*, causal agents of green and blue mold, respectively [2]. *P. digitatum*, the main pathogen found in postharvest citrus fruits, is responsible for 90% of fruit losses caused by diseases occurring during the storage period, leading to serious damages in commercialization [3]. Synthetic fungicides such as Thiabendazole and Imazalil have been widely applied to control this pathogen, but the repeated use of these compounds could induce the resistance by *P. digitatum*. Furthermore, the application of high concentrations of these fungicides to control strains increases the risk of toxic residues in the products. Therefore, using these products is extremely harmful to health, since they are consumed in a short period of time after harvest [3–5].

Due to the impact of chemical control strategies on human health, as well as environmental concerns and the risk of developping resistant pathogen strains, the interest for alternative methods such as biopesticides [6] is rising. Some microorganisms can act as antagonists to phytopathogens acting directly on cell life cycle by mechanisms that have not yet been studied, but this action may derive from competition for nutrients, production of hydrolytic enzymes and antibiotic compounds, especially microbial volatile organic compounds (MVOC) [7]. Use of MVOC as antiphytopathogen agents is extremely convenient because it does not require direct contact with the product, minimizing handling; also, the residual effect is limited. Despite of their ecological role and potential phytopathogen control, a relatively small number of MVOC blends with aniphytopathogen action were investigated [8].

Some yeast species can act as biocontrol agents, preventing infection, decreasing host tissue colonization and reducing pathogen survival and sporulation with varying degrees of efficiency [9]. They have been applied as a potential biocontrol agent against known postharvest fungal pathogens such as *Geotrichum citri-aurantii* [8], *Fusarium oxysporum* [10], *Colletotrichum musae* [11], *Phyllosticta citricarpa* [12], *Penicillium digitatum and P. italicum* [13,14]. Nevertheless, the composition of volatile metabolite blend generated by this antiphytopathogen agents is poorly explored and more studies are necessary in order to

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assess in depth their action.

The analytical protocols to study MVOC should combine steps extraction and pre-concentration of analites, their separation and pre-concentration and evaluation of the collected data. As for the first step, Headspace Solid Phase Microextraction (HS-SPME) is becoming prevalent, if not almost universal, for these samples. HS-SPME is capable of fulfill many of the criteria for ideal sample preparation in metabolomic investigations considering the ease of use, small sample volume requirements, no solvent consumption, easy automation and, in particular, the short sample preparation time and availability of various fiber coatings [15,16] capable to isolate a broad range of metabolites in a single procedure. *In vivo* SPME easily combines sampling, metabolite extraction and metabolism quenching in one step with reduced loss, discrimination or degradation of metabolites. Therefore, it has been successfully applied for targeted and untargeted metabolomics analysis of various biosystems [17].

Combined to HS-SPME, GC–MS is a well-established analytical technique for studies involving volatile metabolites because it provides more selective and higher resolution separations. However, in highly complex matrices, coelution and presence of analytes over a wide concentration range may cause resolution limitations [18]. Hence, multidimensional separation techniques such as Comprehensive Two-Dimensional Gas Chromatography (GC × GC) have been successfully employed to explore different microbiomes [19]. GC × GC provides superior selectivity and higher resolution resulting in a higher peak capacity, particularly for blends containing analytes with high diversity of structures and functionality. Additionally, GC × GC also increases detectability and sensitivity, which facilitates reliable identification of metabolites based on the retention indexes and spectra [18,20].

In this study, different potentially active strains of yeasts *Saccharomyces cerevisiae* and *Candida stellimalicola* were tested as antagonists to control *P. digitatum,* causal agent of green mold disease. In parallel experiments, the MVOC produced by these strains was assessed *in vivo* using a method combining HS-SPME and GC × GC–MS, which was previously optimized using factorial design. The correlation between antifungal activity of the MVOC of each strain and their corresponding GC × GC–MS chromatograms was studied applying Principal Component Analysis (PCA) to these data sets.

2. Materials and methods

2.1. Chemicals and materials

Potato-dextrose-agar (PDA) culture media was used for the metabolic profiling assays (BD Bioscience, Franklin Lakes, NJ, USA) and was manipulated according to supplier's instructions. For the determination of Linear Temperature Programmed Retention Indices (LTPRI) of the peaks on the GC × GC–MS chromatograms, a mix of C8–C20 n-alkanes (Sigma–Aldrich, St. Louis, MO, USA) was used to spike the PDMS fibers before the extraction of selected samples. SPME fibers coated with 85 μm polyacrilate (PA), 100 μm polydimethylsiloxane (PDMS), 65 μm divinylbenzene on PDMS (PDMS/DVB) and 50 + 30 μm DVB and Carboxen on PDMS (DVB/CAR/PDMS) (Supelco, Bellefonte, CT, USA) were employed.

2.2. Isolation of active yeast strains

Among the samples used, six industrial yeast strains of *Saccharomyces cerevisiae* (ACB-CR1, ACB-KD1, ACB-CAT1, ACB-BG1, ACB-K1 and ACB-PE2) were isolated from the fermentation process for ethanol production, characterized by electrophoretic karyotyping [7]; three other yeast strains were isolated from citrus leaves (*Candida stellimalicola* - ACBL-08) and citrus flowers (*S. cerevisiae*, ACBL-14 and ACBL-52). All the strains here evaluated are deposited at the "Sylvio Moreira" Citrus Research Center, Campinas Agronomical Institute (Cordeirópolis – São Paulo, Brazil). A highly virulent strain of *Pennicilium digitatum* was isolated from decayed oranges and is also stored in the same facility.

2.3. Evaluation of antagonism from induced by MVOC

To evaluate the effect of volatile compounds produced by each yeast isolate over *P. digitatum*, the yeast and this pathogen were simultaneously cultivated on PDA culture media over split Petri dished, which prevented non-volatile compounds produced by the yeast to get contact with the fungus. A *P. digitatum* culture disk (5 mm in diameter) was placed on PDA medium on one side of the split plate; on the other side, 250 μL of a suspension containing (approximately 10^8 CFU ml^{-1}) of a yeast strain were spread in the medium. Control experiments were performed using similar Petri dishes innoculated with the pathogen but in absence of yeast. The innoculated plates were wrapped with Parafilm® and kept on a BOD oven at 26 °C, using a 12 h clear/12 dark photoperiod. The diameter of the mycelial spots was determined after seven days incubation. The inhibition power of each strain was defined by the ratio between the diameters of the *P. digitatum* spots on dishes innoculated and non-innoculated with the yeast (control) and expressed as %. The values obtained were compared using a Tukey test with 95% confidence level. All the essays were performed in quadruplicate and the average was used for the calculation of percentage inhibition.

2.4. GC × GC and in vivo HS-SPME Isolation of MVOC

2.4.1. GC × GC

Chromatographic analyses were performed on a GC × GC–QMS prototype based on a QP2010 + GC (Shimadzu Corp., Tokyo, Japan) fitted with a lab-made miniaturized sealed two-stage cryogenic modulator previously described [21]. All the chromatographic parameters were optimized for these samples, including the modulation conditions. The column set consisted of (26 m × 0.25 mm × 0.25 μm) HP-5MS column (Agilent, Santa Clara, CA, USA) connected to a (100 cm × 0.1 mm × 0.1 μm) DB-Wax column (Agilent) through a Sil-Tite zero-volume inert FS union (SGE Analytical, Ringwood, VC, Australia). Hydrogen was used as a carrier gas at constant flow rate of 0.7 mL.min^{-1}. The injector and transfer lines were both kept at 250 °C, and the MS ionization source was maintained at 250 °C. The mass spectrometer was operated with a scan range of 40 to 350 *m/z*, resulting in an acquisition rate of 25 spectra s^{-1}. The column oven temperature was programmed as follows: 45 °C for 2 min, increased from 45 to 130 °C at the rate of 3 °C/min, increased to 240 °C at 20 °C/min and kept at 240 °C for 2 min. Data acquisition was performed in the GCMS Solution software package (Shimadzu), and the data were further processed using the GCImage GC × GC software package (Zoex, Houston, TX, USA). The modulation period for all analyses was 6.0 s.

2.4.2. HS-SPME: optimization and application

A suspension containing 1.0 × 10^8 CFU/mL of *Saccharomyces cerevisiae* (ACBL14) was obtained after the spores of this solution were identified and counted in a Neubauer chamber under an optical microscope. Aliquotes of 150 μL of suspension (containing *ca.* 1.5 × 10^7 CFU) were transferred to 50 mL polypropylene tubes containing PDA culture medium, which were sealed with screw caps fitted with polytetrafluoroethylene/silicon septa (Sigma–Aldrich) and incubated for seven days as aboved described before extractions. To optimize the HS-SPME operational parameters, a 2^4 factorial design (four variables at two levels) experiment was performed. The assessed variables were the nature of the SPME fiber (PA and DVB/CAR/PDMS), extraction time (30 and 60 min), volume of culture media (10 and 25 mL) and desorption temperature (250 and 270 °C). The total area of detected peaks on the chromatograms were used as the response. After optimization, the significative factors were evaluated individually. After optimization, triplicate analysis of the MVOC of the assessed strains were performed; each strain was analysed in triplicate.