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Volatile organic compounds (VOCs) produced by biocontrol yeasts

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ABSTRACT

The Volatile organic compounds (VOCs) produced by biocontrol yeast strains which belong to the *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima*, *Aureobasidium pullulans* and *Saccharomyces cerevisiae* species were identified by solid phase microextraction (SPME) coupled with Gas Chromatography-Mass Spectrometry (GC-MS).

Alcohols (ethyl alcohol, 3-methyl-1-butanol and phenylethyl alcohol) and esters (ethyl acetate and isoamyl acetate) were found to be the main VOCs emitted by the yeast strains, which had different production rate over a 16-day period. In addition, the tested yeast strains showed a remarkable ability to consume oxygen and to produce high percentages of carbon dioxide over a 5 days incubation period in a model system.

The yeast strains, which were proven to very efficiently suppress *in vivo* the growth of postharvest fungal by VOCs, also quickly produced high percentages of ethyl acetate and carbon dioxide. .

For all these reasons, we believe that the level of yeast biocontrol efficacy through the production of volatiles could be the result of a synergistic effect between VOCs and carbon dioxide in the packaging environment.

1. Introduction

Postharvest losses of fruit can account for a significant portion of the total production, in particular when manipulation and storage conditions are not properly conducted. The total losses account for 25% of the total production in industrialized countries and for more than 50% in developing countries (Nunes, 2012). In fruit most of such losses are caused by fungal pathogens (Droby et al., 1992), most of which penetrate in the fruit through wounds that inevitably occur during any of the processes of the postharvest system (Barkai-Golan, 2001). The primary measure to control all these diseases is the use of postharvest synthetic fungicides, although toxicological risks, resistant fungal strains and the demanding requirements in sustainable agriculture, integrated crop management and organic production, have resulted in the need of developing alternative methods to control postharvest decays (Droby et al., 2016).

The biological control of the postharvest decay of fruit, vegetables and grains using antagonistic yeasts has been explored as one of several promising alternatives to chemical fungicides, since they possess peculiar features, such as tolerance to multiple stress factors (Liu et al., 2013), simple nutritional requirements, and ability to colonize dry surfaces for long time periods (Spadaro and Droby, 2016). Several mechanisms have been proposed to explain their antagonistic activity, including the competition for nutrients and space, the parasitism of the pathogen, the secretion of antifungal compounds, the formation of biofilms, the ability to secrete killer toxins and to synthesize Volatile Organic Componds (VOCs) (de Lima et al., 2013; Liu et al., 2013; Muccilli and Restuccia, 2015; Parafati et al., 2015, 2016, 2017a,b).

VOCs are produced by yeasts, molds and bacteria during their primary and secondary metabolism (Korpi et al., 2009); they are low molecular weight compounds (< 300 Da), characterized by low polarity and high vapour pressure, whose production is biologically dynamic and strongly influenced by the microbial species and by the growth conditions and phase (Korpi et al., 2009). The production of VOCs is species-specific and acts as a chemical communication signal among cells, as a carbon release mechanism, and as promoter or inhibitor of microbial growth (Kai et al., 2009).

The antifungal activity of the VOCs produced by yeasts was evaluated and proposed as an effective biological control strategy against *Botrytis cinerea*, *Colletotrichum acutatum, Penicillium expansum, P. digitatum* and *P. italicum* (Huang et al., 2011, 2012; Di Francesco et al., 2015). VOCs could be considered as ideal antimicrobials, since the contact between the biocontrol agent and the pathogen or between the biocontrol agent and food is not required to perform their activity. Referring to this peculiarity, Parafati et al. (2017b) proposed the use of hydrogel spheres as a support for VOC-generating biocontrol yeasts and

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opened a new way for the exploitation of bio-emitters in postharvest packaging.

Solid phase microextraction (SPME) is an effective analytical method to identify the VOCs present in the headspace of a microbial culture. SPME has recently become increasingly popular, because the extraction, concentration, and transfer to the gas chromatograph are carried out in a single step and device, considerably simplifying the sample preparation and, thus, improving the sensitivity of the detector with respect to other analytical methods (Zhang and Li, 2010).

The aim of this study is to determine the compositional changes, in terms of carbon dioxide production and VOCs emission in the headspace of a model system, induced by four yeast strains belonging to different species and selected for their proved biocontrol efficacy against postharvest pathogenic molds. The main VOCs produced by the yeast strains under study were identified by SPME and GC-MS and the ability of the evaluated yeasts to act as biological oxygen scavenger/carbon dioxide emitter, under defined growth conditions (model system), was also investigated.

2. Materials and methods

2.1. Yeast cultures

The *Wickerhamomyces anomalus* (BS91), *Metschnikowia pulcherrima* (MPR3), *Aureobasidium pullulans* (PI1) and *Saccharomyces cerevisiae* (BCA61) yeast strains, which are part of the Di3A (Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, Italy) collection, were selected for the study due to their ability to synthetize VOCs with antifungal activity (Parafati et al., 2015, 2017b). The yeast cultures were routinely maintained on Yeast Extract Peptone Dextrose Agar [YPDA; yeast extract, 10 g; peptone, 10 g; dextrose, 20 g; agar, 20 g (Oxoid, Basingstoke, UK) per liter of distilled water] at 4 °C.

2.2. Quantification of oxygen (O_2) and carbon dioxide (CO_2) in a model system

In order to quantify the amount of O_2 and CO_2, respectively consumed or produced by each yeast strain in a model system, 17 mL of molten sterilized YPDA were poured into 50 mL conical tubes and left to set. Separately, yeast cell cultures were prepared by suspending in sterile distilled water 48-h old yeast cultures at a concentration of 10^8 cells/mL. Yeast suspensions (500 µL) were separately added to 500 µL of molten sterile YPDA at double concentration, gently vortexed and poured on the surface of the medium into the 50 mL conical tubes. The tubes were then sealed with 6 turns of Parafilm® and incubated at 25 °C for 5 d. Not inoculated tubes containing only YPDA medium were used as control. The quantification of O_2 and CO_2 in the tube headspace was performed by using a CheckPoint portable gas analyser (PBI Dansensor, Denmark). The measurement was performed on three replicate tubes at the beginning and after a 5-d incubation.

2.3. Identification of volatile organic compounds (VOCs) produced by yeasts

The composition of the VOCs produced by the yeasts was qualitatively determined by headspace (HS)-SPME coupled with gas chromatography-mass spectrometry (GC–MS).

Thirty mL headspace glass vials were sterilized at 121 °C for 15 min, while the screw caps with embedded Mininert Precision Sampling Valves (Restek Corporation, Bellefonte, PA, USA) were sterilized by exposure to UV-C light for 12 h. Then, 17 mL of molten sterilized YPDA were poured into the sterile glass tubes, and inoculated with each yeast strain as described above. Fifteen vials were prepared for each yeast strain (3 replicates × 5 sampling times). The vials were incubated at 25 °C for 16 d and the headspace was collected after 5, 9, 12, 14 and 16 d. The experiment was repeated in triplicate.

At each sampling time, the vials were kept at 55 °C for 1 h to equilibrate the volatiles in the headspace. The needle of a syringe containing 100 µm polydimethylsiloxane SPME fibers (PDMS; Supelco-Sigma-Aldrich, Bellefonte, PA, USA) was inserted into the vials through the port and left in for 30 min at room temperature to collect the headspace. Trapped headspace compounds were thermally desorbed into the GC injection port at 250 °C for 2 min, and separated in a GCMS-QP5050A (Shimadzu Italia S.r.l., Milano, Italy) equipped with a fused-silica capillary SLB-5ms (Supelco-Sigma Aldrich, St. Louis, MO, USA) column, (30 m × 0.25 mm × 0.25 µm). The separation of VOCs was achieved by using the following temperature gradient: 2 min hold at 35 °C, ramp up to 100 °C at 5 °C/min and then to 280 °C at 7 °C/min until the end of the analysis (~35 min).

Helium was used as carrier gas at a flow rate of 8.2 mL/min and a pressure of 32 kPa. A splitless injection mode was used.

Mass Spectrophotometry (MS) parameters were as follows: full scan acquisition mode with scan range of 50–650 amu at a rate of 0.817 scan/s. The ion source temperature was 250 °C with an ionizing energy of 70 eV and a mass transfer line temperature of 300 °C.

The identification of VOCs was carried out by comparing both the retention times (Rt) and mass spectra of the unknown species to the available standards. Moreover, the identification was confirmed using spectra in NIST 05 library and the calculated linear retention indices (LRI).

The analysis of the growth medium not inoculated with yeast antagonists (blank sample) was performed under the same conditions to exclude any interference caused by volatile medium compounds. The measurements were made with three replicates, each replicate representing the analysis of a different vial. GC peak area data were used to estimate the relative abundance (relative peak area, RA) of each volatile compound.

2.4. Statistical analysis

Data were submitted to one-way analysis of variance (ANOVA) and post-hoc comparison of means was performed by the Duncan's test ($p \leq 0.05$) through the statistical package IBM® SPSS® Statistics 13.0 (Armonk, NY, USA).

3. Results and discussion

3.1. Quantification of oxygen (O_2) and carbon dioxide (CO_2) in the model system

Combined O_2/CO_2 measurements, after 5 d of incubation, showed a different decrease of O_2 percentage, from the starting value of 21%, to values ranging from 4.7% to 0.1%, depending on the yeast strain. With respect to the not inoculated control sample, which showed negligible variation in the gas composition, the greatest O_2 reduction was observed for the *W. anomalus* BS91 strain, which consumed 99.9% of O_2 (Fig. 1A), followed by *M. pulcherrima* MPR3, *S. cerevisiae* BCA61 and *A. pullulans* PI1 with, respectively, consumption of 98.7%, 98.1% and 95.3%. With respect to the production of CO_2 (Fig. 1B), the quantitative measurements evidenced significant ($p \leq 0.05$) percent increases for all strains. In particular, the highest percentages were recorded for *M. pulcherrima* MPR3 (52.3%) and *W. anomalus* BS91 (48.4%); lower values were detected for *S. cerevisiae* BCA61 (35.8%) and *A. pullulans* PI1 (32.5%).

It has been generally established that low O_2 and/or high CO_2 partial pressures, such as those adopted in Modified atmosphere packaging (MAP), prolong the shelf-life of fresh or minimally processed fruit and vegetables, by reducing the respiration rate and ethylene biosynthesis. In addition, most of the common spoilage bacteria and fungi require oxygen to grow; therefore, the package atmosphere should contain a low concentration of residual oxygen to increase the shelf life of food (Sandhya, 2010). However, many mold species can

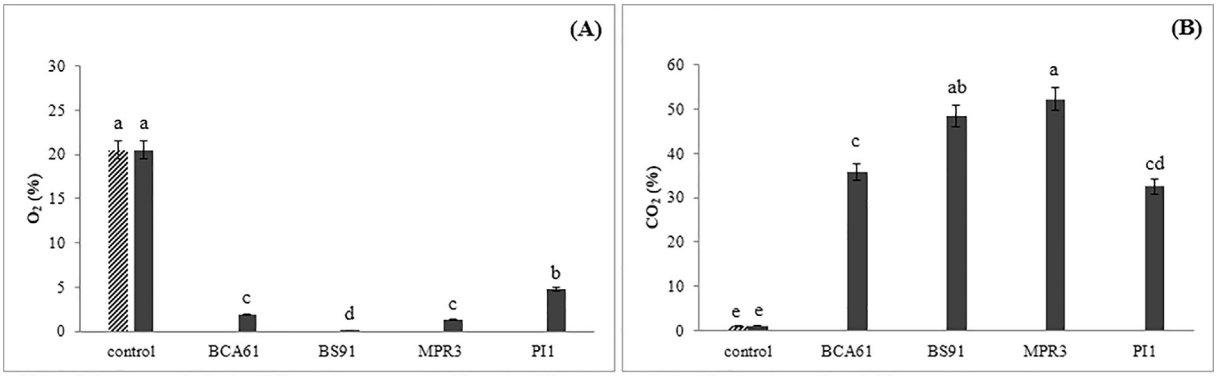

Fig. 1. Percentage of O_2 (A) and CO_2 (B) measured for each yeast strain immediately after the inoculum (t_0) and after 5 d-incubation at 25 °C (t_5). Bars indicate the standard error of the mean (SEM). t_0 t_5

Table 1
Main volatile organic compounds (VOCs) produced by biocontrol yeasts during incubation at 25 °C for 16 d.

Identified compound	Molecular formula	Molecular weight	Retention time (min)	Quantification ion	Linear Retention Index
Ethyl alcohol	C_2H_6O	46	1,75	45; 46	485
Ethyl acetate	$C_4H_8O_2$	88	2,44	43	614
Isoamyl alcohol	$C_5H_{12}O$	88	4509	55;70;87;88	740
Isoamyl acetate	$C_7H_{14}O_2$	130	7,85	43; 70;130	879
Phenethyl alcohol	$C_8H_{10}O$	122	18,52	91; 94;122;65	1128

grow even with very low oxygen levels, suggesting that CO_2 in high concentrations is the key inhibition factor. Ke et al. (1991) studied the effect of high CO_2 levels (20–80%) and found a clear inhibition of the fungal spoilage of strawberries at such CO_2 levels. This also agrees with the results of Agar et al. (1990) who showed that the growth of *B. cinerea* was unaffected by CO_2 treatments up to 20%. With reference to stored grains, Zhai et al. (2015) found a significant correlation ($p < 0.05$) between changes in CO_2 concentration and the growth of mycotoxigenic fungi such as *Aspergillus flavus*, *Penicillium* sp. and *A. ochraceus* in addition to the production of mycotoxins.

The use of microorganisms as oxygen scavengers has been investigated in various studies: Altieri et al. (2004) proposed a method to produce an oxygen scavenging film by embedding *Kocuria varians* and *Pichia subpelliculosa* as active ingredients; Anthierens et al. (2011) described a model system based on an endospore forming *Bacillus amyloliquefaciens* as an active oxygen scavenging ingredient for oxygen sensitive foods. In the present study, biocontrol yeast strains in addition to their ability to scavenge oxygen and to prevent oxygen to be available for other aerobic microorganisms, released high amounts of carbon dioxide as a metabolic reaction end-product inside the vial, thus creating a biological MAP. Even though such biocontrol mechanism has not been yet considered against postharvest fungal pathogens, the depletion of oxygen and, especially, the increase in the carbon dioxide concentration produced by the metabolic activity of the evaluated yeasts may make a significant contribution to effectively inhibit mold growth in packaged fruit (Grzegorczyk et al., 2017; Parafati et al., 2015; 2017b).

MAP gas mixtures must be specifically formulated for each packaged product: O_2 and CO_2 concentrations are generally in the 1–21% and 1–20% ranges (reviewed in Sandhya, 2010). The almost total consumption of O_2 and the substantial production of CO_2 (up to 52.3%) detected for the evaluated yeast strains may be tailored for each specific stored product by fine tuning the yeast inoculum levels and available carbon source in the nutrient media.

3.2. Identification of VOCs by gas chromatography-mass spectrometry (GC-MS)

In recent years, it has become more and more evident that microbial volatiles can play important roles in long-distance interactions within soil microbial communities, as infochemical molecules affecting the population dynamics and gene expression in target microorganisms and as competition tools (Morath et al., 2012; Garbeva et al., 2014; Schmidt et al., 2015).

In the present study the VOCs produced by four yeast strains (BCA61, BS91, MPR3, PI1), which are effective against some fruit postharvest pathogens and belong to four different species, were identified by SPME-GC-MS. Alcohols (ethyl alcohol, 3-methyl-1-butanol and phenylethyl alcohol) and esters (ethyl acetate and isoamyl acetate) (Table 1) were found to be the main VOCs produced. All the identified compounds were produced by all the evaluated biocontrol yeast strains.

The VOCs emitted by each tested yeast strain were reported in terms of percentage (%) of the total peak areas calculated at each sampling time (5, 9, 12, 14 and 16 d), proving that their production rate as a function of the incubation time was different for each strain (Fig. 2a-d).

In particular, for *S. cerevisiae* BCA 61, ethyl alcohol was the main produced compound after 5 d (+88.9%), then decreasing to approximate +50% for longer incubation time. The second most abundant compound was ethyl acetate, with a 33.53% increase after 9 d (Fig. 2A), which remained essentially unchanged until the end of the incubation period. .

The *W. anomalus* BS91 strain had a considerable production of ethyl alcohol until day 9, slightly decreasing thereafter; on the contrary the production of ethyl acetate rapidly increased already after 5 d of incubation (+63.18%), and reached its peak after 12 d (+78.63%) then remaining stable until the end of the considered period (16 d) (Fig. 2B).

M. pulcherrima MPR3 strain showed high production of ethyl alcohol after 5 d (+56.00%), then gradually decreasing to +38.27% up to 16 d. Conversely, the maximum increase of ethyl acetate was observed after 9 d (+62.95%); the amount of these VOC remained then stable until the end of the incubation period (Fig. 2C).

Also for *A. pullulans* PI1 ethyl alcohol and ethyl acetate were the VOCs produced in the highest concentration. In particular the first

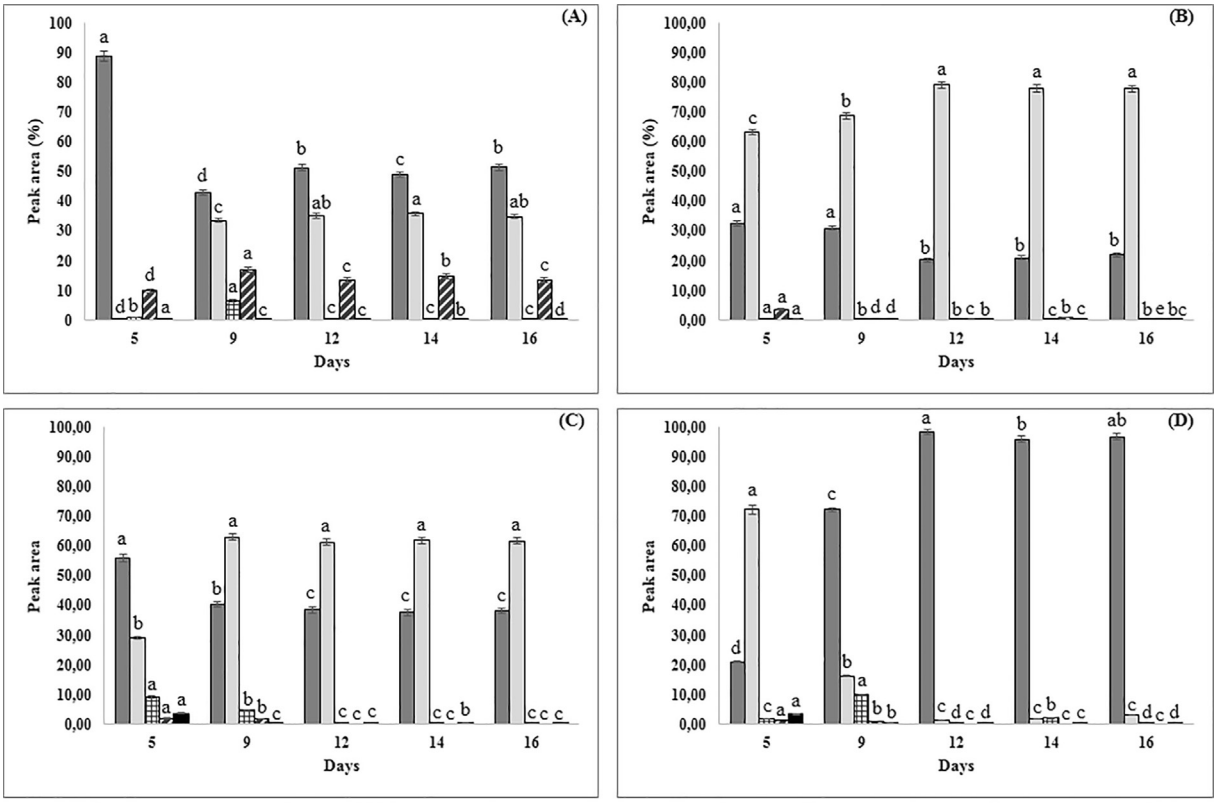

Fig. 2. Peak areas, expressed in terms of percentage (%) of the total peak areas calculated at each sampling time (5, 9, 12, 14 and 16 d) for the strains *S. cerevisiae* BCA61 (A), *W. anomalus* BS91 (B), *M. pulcherrima* MPR3 (C) and *A. pullulans* PI1 (D). Bars indicate the standard error of the mean (SEM).

compound was produced at the highest level after 5 d, then slowly stabilizing to an approximate increase of +38% until the 16th d; conversely, ethyl acetate reached a maximum increase of +62.95% after 9 d of incubation, thereafter remaining unchanged until the end of the experiment (Fig. 2D).

With regard to the minority compounds, the maximum increase of 3-methyl-1-butanol was observed after 9 d for *S. cerevisiae* BCA 61 and *A. pullulans* PI1, with +6.53 and + 9.88%, respectively, and already after 5 d for *M. pulcherrima* MPR3 (+9.23%); no measurable amounts of 3-methyl-1-butanol were produced by *W. anomalus* BS91 (< 1%).

Except for *S. cerevisiae* BCA 61, which produced moderate amounts of isoamyl acetate throughout the incubation period, the remaining yeast strains produced low percentages of such VOC.

Finally, phenylethyl alcohol was noticeably produced only after 5 d of incubation by *A. pullulans* PI1 and *M. pulcherrima* MPR3, with increases of +3.55% and +3.67%, respectively.

The exploitation of yeast strains as VOCs producers in the flavour industry has been already proposed in the past decades. Buzzini et al. (2003) first identified VOCs produced by 98 tropical ascomycetous yeast strains (representative of 40 species) to focus their ecological and biotechnological roles; the VOCs produced were found to be alcohols (amyl alcohol and isoamyl alcohol), aldehydes (2-methyl-2-hexenal and 2-isopropyl-5-methyl-2-hexenal) and esters (ethyl isobutyrate, isobutyl acetate, isoamyl acetate, 2-methylbutyl acetate, ethyl isovalerate, isoamyl propionate and phenylmethyl acetate). Differences in the VOC profiles were used to cluster yeast strains into 25 VOC phenotypes and the different frequency of VOC phenotypes in three specific habitats was correlated to the divergent environmental conditions, possibly affecting the selection of specific yeasts. More recently, the profile and efficacy of the volatiles produced by *C. intermedia* to control Botrytis fruit rot of strawberry have been determined (Huang et al., 2011); among 49 volatiles identified (esters, alcohols, alkenes, alkanes, alkynes, organic acids, ketones, and aldehydes), two compounds, 1,3,5,7-cyclooctatetraene and 3-methyl-1-butanol, were the most abundant.

The same authors evaluated the efficacy of VOCs, including 2-ethyl-1-hexanol, produced by a strain of *S. pararoseus* in suppressing *B. cinerea* on strawberries under the air-tight conditions (Huang et al., 2012).

With regards to the *A. pullulans* biocontrol yeast species, Di Francesco et al. (2015) identified the antifungal volatiles emitted by the two strains L1 and L8 by SPME-GC technique; compounds as 2-phenyl, 1-butanol-3-methyl, 1-butanol-2-methyl and 1-propanol-2-methyl belonging to the group of alcohols were mainly produced for both strains, in the first 96 h of growth, with the 2-phenethyl alcohol the most active.

Previous studies carried out on the same yeast strains under study demonstrated that, on the basis of the proposed mechanisms, VOCs play an important role in determining the biocontrol efficacy against different postharvest pathogenic molds (Parafati et al., 2015). In particular, the VOCs produced by *M. pulcherrima* MPR3 and *W. anomalus* BS91 had the best efficacy in controlling gray mold decay of grape berries (Parafati et al., 2015). More recently, the same authors used immobilized VOC-generating yeasts in hydrogel spheres in experimental *in vivo* trials performed on strawberries and mandarin fruits demonstrating the best efficacy for VOCs produced by *W. anomalus* BS91, which totally inhibited gray mold decay on strawberries and significantly reduced green mold infections on mandarin fruits; on the other hand, blue mold decay on mandarin fruits was more effectively managed by *A. pullulans* PI1 VOCs (Parafati et al., 2017b). Since the above-mentioned experiments were carried out *in vivo* over a period of 5 d, by comparing the VOCs produced in the same time interval, it can be supposed that the early production of ethyl acetate, rather than ethanol, may play a key role in reducing disease parameters caused by

B. cinerea and *P. digitatum*. The antifungal activity of ethyl acetate, together with 2 methyl 1 butanol and 3 methyl 1 butanol, was already observed by Fialho et al. (2011) against *Sclerotinia sclerotiorum* in bean seeds.

4. Conclusions

The inhibitory role of the VOCs produced by yeasts against postharvest food pathogens has been investigated, although variable results were found among species and isolating sources. In the present study the VOCs produced by effective biocontrol yeast strains of the *S. cerevisiae*, *W. anomalus*, *M. pulcherrima* and *A. pullulans* species have been identified in a model system to exclude any interference with the pathogen and/or the commodity. Ethanol and ethyl acetate were the main compounds produced by all the studied strains. However, by comparing the VOCs production rate as a function of time with the results of previous *in vivo* biocontrol studies, the key role of ethyl acetate in inhibiting the growth of postharvest fungal pathogens can be hypothesized. In addition, the fact that *W. anomalus* BS91 and *M. pulcherrima* MPR3 strains produced the greatest quantities of CO_2 through their metabolic activities after a 5-day incubation period, could suggest a synergistic effect of VOCs and CO_2 in inhibiting the growth of postharvest pathogenic molds in packaged fresh fruit.

Although this study could be considered as a solid scientific base on the yeast biocontrol mechanism obtained by the emission of VOCs, further work will be carried out to evaluate the role of each of the identified volatile compounds, alone or in combination with increasing percentages of CO_2 in the experimental environment, to demonstrate their potential synergistic effect on the inhibition of fungal pathogenic species. Such approach could be also useful to design new active packaging strategies aimed at improving food shelf life and therefore reducing food losses during storage.

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