USE OF VOLATILE METABOLITE PROFILES TO DISTINGUISH THREE *MONILINIA* SPECIES

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SUMMARY

Analyses of volatile organic compounds (VOCs) produced by three *Monilinia* species (*M. laxa*, *M. fructigena* and *M. fructicola*) grown on potato dextrose agar (PDA) were done by head space solid phase micro extraction combined with gas chromatography/mass spectrometry (HS-SPME-GS/MS). A total of 14 compounds of different chemical structure were identified, the most frequent of which were ethanol, dodecane and alpha-Muurolene. Specific VOCs were identified, which allowed the discrimination of the three *Monilinia* species. If coupled with the use of the electronic nose and upon validation under commercial conditions, the results of this study may have potential applications in postharvest for detecting and identifying diseases of stone and pome fruits, in relatively early stages of their development.

Key words: brown rot, HS-SPME-GC/MS, postharvest, VOCs.

INTRODUCTION

Volatile organic compounds (VOCs) produced by many fungal species have been analyzed using different methods (Quellette et al., 1990; De Lacy Costello et al., 2001; Kushalappa et al., 2002; Vikram et al., 2004a, 2006; Ibrahim et al., 2011; Morath et al., 2012; Strobel, 2014) and their possible biotechnological applications have recently been reviewed (Morath et al., 2012). One of these methods, head space solid phase micro extraction combined with gas chromatography/mass spectrometry (HS-SPME-GS/MS), is very sensitive, simple and rapid and allows VOCs analysis without the use of solvents. Furthermore, it requires a small sample volume and, therefore, has been widely applied to investigate VOCs from many sources (Boyd-Boland et al., 1994; Yang and Peppard, 1994; Chin et al., 1996; Matich et al., 1996; Clark and Bunch, 1997; Elmore et al., 1997; Song et al., 1997; Steffen and Pawliszyn, 1997; Deng et al., 2004; Sousa et al., 2004; Ceballos et al., 2010; Burkhup et al., 2011; Pickl et al., 2011; Giorgi et al., 2012; Kotowska et al., 2012; Xie et al., 2013; Benevides et al., 2014; Durant et al., 2014).

Significant losses have been recorded all over the world in fruits crops due to brown rot caused by *Monilinia* spp. such as *M. laxa* (Aderhold et Ruhland), *M. fructigena* (Aderhold et Ruhland) and *M. fructicola* (Winter) Honey (van Leeuwen and van Kesteren, 1998; Hrustic et al., 2012). The last fungal species is included in the A2 EPPO List of quarantine pest organisms in Europe (EPPO Standards, 2004; Hrustic et al., 2012).

*M. laxa* is an economically important pathogen of stone fruits causing blossom, twig and branch blight and fruit rot (Holb, 2006, 2008; Hrustic et al., 2012). Severe disease outbreaks are commonly registered during blooming periods associated with low temperatures and repeated rainfalls.

*M. fructigena*, one of the main and economically relevant pathogens of apples, pears, and stone fruits in Europe, causes primarily fruit rot before and during storage (Jones and Aldwinckle, 1990; Holb, 2006; Hrustic et al., 2012), and is widespread also in Asia, North America and North Africa (Batra, 1991).

*M. fructicola* is considered a dangerous fungal pathogen of stone fruits. In Italy, it was detected for the first time on stored nectarines (Pellegrino et al., 2012), and very heavy losses (ranging between 30% and 90%) were reported from Canada (Hong et al., 1997; Hrustic et al., 2012). The same pathogen had previously been recorded in Austria, France, Hungary and Switzerland (Bosshard et al., 2006; Pintércz and Palkovics, 2006).

There is no doubt that further spread of these phytopathogenic ascomycetes in Europe would lead to increased control costs, which would be even higher should resistance to some fungicides appear (van Leeuwen et al., 2001).

Reducing *Monilinia*-induced losses is not an easy task since there are several limiting factors, one of the most important being the non-availability of an early detection system for the presence of these fungal pathogens. Furthermore, modern detection methods like ELISA and PCR, are not suitable in storage, regardless of their sensitivity threshold. On the contrary, VOCs analyses are appropriate to detect presence of fungal pathogen in infected fruits and vegetables in storage facilities (Kallio et
al., 1990; Quellette et al., 1990; De Lacy Costello et al., 2001; Kushalappa et al., 2002; Vikram et al., 2006; Ibrahim et al., 2011).

MATERIALS AND METHODS

Fungal isolates. The plant pathogenic fungi tested, derived from single-spore colonies, were stored as pure cultures in the mycotheca of the School of Agricultural, Forestry, Food, and Environmental Sciences (SAFE), at the University of Basilicata (Potenza, Italy). Fungal species were grown and maintained on potato dextrose agar (PDA, Oxoid, UK) at 22-24°C and 4°C, respectively. Ten isolates of each Monilinia species were used, all of them in the anamorphic phase of the corresponding Monilia species.

Identification of fungal species. All Monilinia species were first identified morphologically followed by molecular confirmation by PCR. Total nucleic acids were extracted from single Monilinia pure cultures with a commercial kit (DNeasy Plant mini kit, Qiagen, USA) according to the manufacturer's instructions. DNA was amplified using the universal primer pair ITS4/ITS5 (White et al., 1990), the amplicons were directly sequenced (by BMR Genomics, Padua - Italy) and the resulting sequences were compared with those available in GenBank using the BLAST software (Altschul et al., 1997). One sequence for each species was deposited in GenBank under the following accession Nos.: HF678387 (M. larsa), HF678388 (M. fructicola) and HF678389 (M. fructigena).

Volatile compounds accumulation and GC/MS analysis. PDA discs 0.5 cm in diameter were axenically cut from a 10-day-old colony of each Monilinia species grown at 22-24°C, placed into a 20 mm mouthed glass vials used for GC/MS analysis and incubated at 36°C for 20 min. VOCs of each Monilinia isolate were determined with a 100-µm PDMS-SPME fiber (57300-U, Supelco, Italy) and analyzed using an HP 6890 Plus gas chromatograph equipped with a Phenomenex Zebron ZB-5 MS capillary column (30-m x 0.256-mm i.d x 0.25-µm FT) (Agilent, Italy). An HP 5973 mass selective detector was used with helium at 0.8 ml/min as the carrier gas. A splitless injector was maintained at 250°C and the detector at 230°C. The oven was held at 40°C for 20 min, then warmed at 8°C/min until 250°C was reached, and held for 10 min at this temperature. Tentative identification of aroma components (qualitative analysis) of each Monilinia species was based on mass spectra and Wiley 6 and NIST 11 L library comparison. The sensitivity threshold of the system was set to 12, in a scale between 0-25, in order to eliminate baseline peaks. The peak was considered as identified when the experimental spectrum matched that in the library with a > 80% score. Negative controls were sterile 0.5 cm PDA discs.

Principal Component Analysis (PCoA) based on fungal metabolites. VOCs data were processed as haploid binary data and diversity was assessed using the expected heterozygosity index (HE) calculated as HE = [1 – (p²+q²)], where p is the frequency of the “present” compound and q = 1-p the frequency of the “absent” compound (Weir, 1996). VOCs profiles were analyzed with Nei’s distance (Nei and Li, 1979) to generate a distance matrix with all pairwise comparisons used as input for Principal Coordinates Analysis (PCoA) into the Genalex v. 6.5 program (Peakall and Smouse, 2006, 2012). PCoA was performed on all 14 VOCs identified in the tested Monilinia species. The coordinates (1 and 2) were used to reveal the compound associations to each other and to the fungal species.

RESULTS AND DISCUSSION

Volatile compounds produced on PDA by 10 isolates of each of the three Monilinia species, determined by HS-SPME-GS/MS, are listed in Table 1. All isolates of each species showed an identical VOCs profile whereas no VOCs were identified at any retention time in the negative controls (Table 1). The headspace gas anaylses of PDA discs with the considered Monilinia spp. yielded a total of 14 different VOCs. Among them, ethanol, dodecane and alpha-Muurolene predominated (Table 1). Five VOCs were detected for M. larsa: ethanol; heptane, 2,4-dimethyl-octane, 4-methyl-dodecane and eicosane. Among them, heptane, 2,4-dimethyl-octane and eicosane were specific to M. larsa (Table 1).

HS-SPME-GS/MS analysis of M. fructigena detected the following six VOCs: ethanol; dodecane; alpha-Muurolene; 4-methyl-octane; 3,7-dimethyl-decane and hexadecane (Table 1). The last three compounds were only detected in M. fructigena. VOCs profile of M. fructicola included six metabolites: 2,4,6-trimethyl-octane; heptadecane; copaene; alpha-Muurolene; caryophyllene and alloaromadendrene. 2,4,6-trimethyl-octane; heptadecane; copaene; caryophyllene and alloaromadendrene were specific and found only in M. fructicola. Of the three analyzed species, M. fructicola seemed to have the highest number of specific VOCs (five), followed by M. fructigena (three) and M. larsa (two).

Data relative to VOCs produced by plant pathogenic fungi are increasing every year (Kallio et al., 1990; Quellette et al., 1990; De Lacy Costello et al., 2001; Kushalappa et al., 2002; Vikram et al., 2006; Ibrahim et al., 2011; Morath et al., 2012; Strobel, 2014). Nevertheless, this is the first preliminary study which provides detailed information on VOCs of M. larsa, M. fructigena and M. fructicola. In fact, the few papers that presented data on VOCs produced by Monilinia spp. and compared them with those found in other fungal pathogens (Botrytis cinerea, Mucorpiriformis and Penicillium expansum) did not specify which species was investigated (Vikram et al., 2004a,
2004b). In particular Vikram et al. (2004a) identified in apples of cvs. Cortland and Empire inoculated with a non specified Monilinia species, six and five VOCs, respectively, from a total of 34 (cv. Cortland) and 36 (cv. Empire) compounds detected. Furthermore, 18 (cv. Cortland) and 25 (cv. Empire) of these VOCs were also revealed in the non wounded (N-control) and 21 (cv. Cortland) and 27 (cv. Empire) in wounded control (W-control). Thus, the results of our study differ from those by Vikram et al. (2004a, 2004b) as they refer specifically to each of three Monilinia species investigated and provide a clear-cut identification of VOCs profiles for each of them.

VOCs detection using highly sensitive sensors may permit the early identification of various fungal pathogens, e.g. Monilinia, Fusarium, Botrytis, Phytophthora etc., in storage facilities, thus allowing for prompt actions to be taken to reduce economical losses. The knowledge acquirements achieved with the present study are initial data on VOCs profiles detected within the genus Monilinia. As a future development, it would be interesting to see if there is any difference between VOCs of Monilinia isolates belonging to the same species but collected from different sources and geographical areas. These data should also be compared with those collected in vivo from infected fruits since volatile metabolite profiles could be different. Following appropriate validation tests, the results of

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Detected compound [a] and the retention time (min) [b]</th>
<th>Monilinia laxa</th>
<th>Monilinia fructigena</th>
<th>Monilinia fructicola</th>
<th>Negative Control (PDA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>+</td>
<td>1.576</td>
<td>+</td>
<td>1.518</td>
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<tr>
<td>Heptane, 2,4-dimethyl-</td>
<td>+</td>
<td>5.048</td>
<td>−</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>Octane, 4-dimethyl-</td>
<td>+</td>
<td>5.925</td>
<td>−</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>Octane, 4-methyl-</td>
<td>−</td>
<td>+</td>
<td>5.925</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>Octane, 2,4,6-trimethyl-</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>Dodecane</td>
<td>+</td>
<td>9.917</td>
<td>+</td>
<td>9.917</td>
<td>−</td>
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<td>Decane, 3,7-dimethyl-</td>
<td>−</td>
<td>+</td>
<td>10.805</td>
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<td>Eicosane</td>
<td>+</td>
<td>14.335</td>
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<td>−</td>
<td>0</td>
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<tr>
<td>Heptadecane</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>20.611</td>
<td>−</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>−</td>
<td>+</td>
<td>14.474</td>
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<tr>
<td>alpha-Muurolene</td>
<td>−</td>
<td>+</td>
<td>17.854</td>
<td>+</td>
<td>17.830</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>16.596</td>
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<td>Alloaromadendrene</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>17.265</td>
</tr>
<tr>
<td>Total metabolites / fungal species</td>
<td>5</td>
<td>6</td>
<td>6</td>
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<td></td>
</tr>
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</table>

**Table 1.** Volatile organic compounds and their retention time determined by HS-SPME-GC/MS in three Monilinia species grown on PDA medium.

Presence (+) /Absence (−) of the compound.
this investigation combined with the use of the electronic nose (Marsili, 1999) could have potential applications for controlling postharvest diseases at a relatively early stage (Magan and Evans, 2000; Morath et al., 2012).

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REFERENCES


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