Analytica Chimica Acta 716 (2012) 155–162

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Capillary and gel electromigration techniques and MALDI-TOF MS – Suitable tools for identification of filamentous fungi

Marie Horká[a,*], Anna Kubesová[a], Jiří Šalplachta[a], Eva Zapletalová[b], Jaroslav Horký[b], Karel Šlais[a]

[a] Institute of Analytical Chemistry of the ASCR, v. v. i., Veveří 97, 602 00 Brno, Czech Republic
[b] State Phytosanitary Administration, Division of Diagnostics, Šlechtitelů 23, 779 00 Olomouc, Czech Republic

ARTICLE INFO

Article history:
Received 29 August 2011
Received in revised form
14 December 2011
Accepted 15 December 2011
Available online 24 December 2011

Keywords:
CIEF and CZE
UV detection
Gel IEF
SDS-PAGE
MALDI-TOF MS
Monilinia spp.

ABSTRACT

Microbial strains are now spreading out of their original geographical areas of incidence and previously adequate morphological identification methods often must be accompanied by a phenotypic characterization for the successful microbial identification. The fungal genus *Monilinia* represents a suitable example. *Monilinia* species represent important fruit pathogens responsible for major losses in fruit production. Four closely related spp. of *Monilinia*: *Monilinia laxa*, *Monilinia fructigena*, *Monilinia fructicola* and *Monilia polystroma* have been yet identified. However, the classical characterization methods are not sufficient for current requirements, especially for phytosanitary purposes.

In this study, rapid and reproducible methods have been developed for the characterization of *Monilinia* spp. based on the utilization of five well-established analytical techniques: CZE, CIEF, gel IEF, SDS-PAGE and MALDI-TOF MS, respectively. The applicability of these techniques for the identification of unknown spores of *Monilinia* spp. collected from infected fruits was also evaluated. It was found that isoelectric points, migration velocities or the protein patterns can be used as the identification markers in the case of cultivated filamentous fungi. Moreover, the results obtained by capillary electromigration techniques are independent on the host origin of the spores. On the other hand, the host origin of the fungi can play an important role in the precise fungi identification by the other techniques.

1. Introduction

Fungi often cause serious crop devastation. The standard method for identifying and classifying filamentous fungi remains in the morphology (fruiting body, spores – shape, color, measurements, etc. or hyphae arrangement) [1], since filamentous fungi have more distinctive morphologies than, e.g., single-cell bacteria and yeasts. Although physiological characteristics have been examined as well (e.g. colony color, growth rates), they are too variable [2].

The genus *Monilinia* is a suitable model example of the filamentous fungi. *Monilinia* species (spp.) are responsible for major losses in fruit production by affecting blossoms and fruits, either on the tree or after a harvest [1,2]. Four closely related spp. of *Monilinia*: *Monilinia laxa* (*M. laxa*), *Monilinia fructigena* (*M. fructigena*), *Monilinia fructicola* (*M. fructicola*) have been identified [3–5] and *Monilia polystroma* (*M. polystroma*) [4–7]. *M. fructicola* is an extremely destructive pathogen which tends to occur more often on peaches and nectarines, while *M. laxa* favors apricots and almonds. *M. fructigena* is more characteristically found on apples and pears. In North America, brown rot of fruit is mainly caused by *M. fructicola* and to lesser extent by *M. laxa*. In Europe, the main causal agents of the disease are *M. fructigena* and *M. laxa* [3–5]. *M. polystroma* is the most similar to *M. fructigena* and it was found only in Japan at first [4]. However, this pathogen has been already identified on apples in Hungary [7] and on apple and peach fruits in Czech Republic [8]. *Monilinia* spp. are traditionally identified by the combination of culture and morphological characteristics. Since these characteristics are overlapping, the identification has to be conducted under standardized conditions. They cannot be in principle distinguished from each other except by laboratory examination [9]. Moreover, classical identification methods (minimum 5–7 days of incubation [9]) are not adequate for the phytosanitary diagnosis, which should be rapid and reliable.

Subsequently, the methods using multiplex polymerase chain reaction (PCR) assays have been developed to detect and identify *Monilinia* spp. [3,4,9–14] in at least of 2 h. The detection based on the universal PCR protocol was developed for *Monilinia* spp. and species-specific primers were designed using the sequence characterized amplified regions (SCAR) markers [10]. As few as 20 spores of *M. fructicola* were detected in air samples by the real-time PCR

* Corresponding author. Tel.: +420 5 32290221; fax: +420 5 41212113.
E-mail address: horka@iach.cz (M. Horká).

0003-2670/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2011.12.032

[9,11,12], but it is still difficult to detect small number of spores resting on the non-infected plant tissue. Probably, the plant tissues contain more PCR inhibitors than the air samples do [12].

Another approach for the fast and reliable fungal identification is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [15–23]. In recent years, MALDI-TOF MS has become a powerful and rapid analytical tool for the classification and identification of whole microorganisms based on species-specific fingerprints [22,24–30]. The observable molecular mass range is usually between 2 and 20 kDa where very few metabolites appear [2,31], which is an advantage because these masses can be easily used as biomarkers. MALDI-TOF MS has important advantages over PCR methods. The technique is rapid and straightforward since it requires the minimum sample handling. Care is required to avoid contaminants and interferences like salts, etc. [32].

In contrast to bacteria, fungal cell walls and spores are mainly composed of different polysaccharides (80–90%), including chitin which maintains the rigidity and the structural stability of the cells, but peptides, proteins, lipids, polyphosphates and inorganic ions are also present [19]. The use of suitable electromigration techniques in both capillary and gel formats for the distinguishing of different fungal species is also very promising. Capillary isoelectric focusing (CIEF) and capillary zone electrophoresis (CZE) of cultivated microorganisms [27–40] or lysates of microorganisms collected from infected fruits [33,41–44] were used for the efficient microbial characterization. The gel electromigration techniques [28,45–50] like sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can be used as stand-alone analytical techniques or as a part of preparative methodology [47], e.g., proteomic microbial identification [28,45,47], two-dimensional gel electrophoresis (2-DE), [46,49] and chip gel electrophoresis [48], respectively. Moreover, the above-mentioned analytical techniques are suitable to associate each other in on-line or off-line combination. In addition, the careful selection of individual detection techniques is necessary for the successful and reliable microbial identification [50,51].

The aim of this study is to develop rapid, effective and reproducible methods for the detection and reliable identification of *Monilinia* spp. For this purpose, five well-established analytical techniques: CZE, CIEF, gel IEF, SDS-PAGE and MALDI-TOF MS, respectively, were used. The techniques were evaluated for identification of filamentous fungi spores. The research was build on our experimental knowledge arising from the CIEF and CZE separation of the hydrophobic conidia from the cultures of different strains of the filamentous fungi, *Aspergillus*, *Fusarium* and *Penicillium* [40] and fungal spores of *M. laxa*, *M. fructigena* and *M. fructicola* preconcentrated and separated by CIEF [52]. The optimized methods were applied for the characterization of spores of *Monilinia* spp. collected from infected fruits and for the preliminary differentiation of four *M. polystroma* strains. The results were compared with those obtained by the conventional microbiological identification procedures.

2. Experimental

2.1. Chemicals

The most chemicals, including wide range molecular weight markers, 6.5–200 kDa, acetonitrile (ACN), trifluoroacetic acid (TFA), formic acid (FA), etc., were purchased from Sigma–Aldrich (Schnelldorf, Germany). Ampholyte high resolution, pH 2–4, and ampholyte pH 3–4.5 and β-mercaptoethanol were from Fluka Chemie (Buchs, Switzerland). Polyethylene glycol, M_r 1000 (PEG 1000), was obtained from Aldrich (Milwaukee, WI, USA). Acrylamide and the solution of synthetic carrier ampholytes, Biolyte, pH 3–10 were obtained from Bio-Rad (Hercules, CA, USA), L-aspartic acid (Asp) from LOBA Chemie (Wien, Austria) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES), *N,N'*-methylenebisacrylamide (Bis), *N,N,N',N'*-tetramethylethylenediamine (TEMED) and bromophenol blue from Merck (Darmstadt, Germany). Glycerol was obtained from Onex (Rožnov pod Radhoštěm, Czech Republic). Tris(hydroxymethyl)aminomethane (Tris), Coomassie Brilant Blue G-250 (CBB), glycine and sodium dodecyl sulfate were obtained from J. T. Baker (Burlington, Canada). 3,5-Dimethoxy-4-hydroxycinnamic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB) and calibration mixture (Protein calibration Mix2) were purchased from LaserBio Labs (Sophia-Antipolis Cedex, France). The specifications [53,54] of the used spacers and simple ampholytes are described in Ref. [55]. The low-molecular p*I* markers [56], p*I* = 2.0, 2.7, 3.3, 4.3 [56], 4.0, 7.1, 9.8 [57], and 5.3, 6.3, 8.0 [58] were synthesized in the Institute of Analytical Chemistry of the ASCR, v. v. i., Brno. All chemicals were of electrophoresis, analytical or MS grade, respectively.

2.2. Plant pathogens

Pure cultures of *M. fructigena*, *M. laxa* and *M. fructicola* were preserved in the collection of the diagnostic laboratory of the State Phytosanitary Administration in Olomouc. The strains of *M. fructigena* and *M. laxa* were isolated from samples originated in the Czech Republic. Strain of *M. fructicola* was taken from the collection (Agroscope, Wädenswil, Switzerland). Strains of *M. polystroma* were from collection FERA, York, UK. Strains of *M. polystroma* 1003843, 1003849 and 1004759, respectively, were taken from the official plant samples originated in the Czech Republic.

2.3. Preparation of microbial samples

All isolates were cultivated on potato dextrose agar medium (HiMedia Laboratories, Mumbai, India) in Petri dishes 90 mm under following laboratory conditions: 22 °C, 10 days, natural rotation of day/night. The colonies of *M. fructicola* cultivated under these conditions were hazel with mostly entire margin and the even surface. Sporulation was abundant in concentric rings. The colony color of *M. fructigena* was creamy to yellow white with mostly entire margin. Sporulation was in concentric rings but more sparsely than *M. fructicola.* Colonies of *M. polystroma* were similar to those of *M. fructigena*, except for intense formation of black stromatal plates after 10–12 days of incubation. The colony color of *M. laxa* was hazel to gray, rosetted with markedly lobel colony margin. Characteristic black arcs were associated with the petals of the rosettes in the colony. Sporulation was sparse. Cultivation was made according to the EPPO diagnostic protocol PM 7/18 (2). For subsequent experiments, spore concentration of all *Monilinia* spp. was adjusted to 1×10^8 cell mL^{-1}.

In the experiments, where the spores of *Monilinia* spp. were taken from the mummified apricot or infected apple surface, the collected spores were not cultivated and were resuspended directly in glass vials with the physiological saline solution (PSS) and their concentration was adjusted to 1×10^8 spores per 1 mL.

2.4. Safety

The potentially pathogenic microorganisms were analyzed by several analytical techniques in this study. Therefore, the adequate safety procedures were necessary to obey. All experiments were performed according to the instructions for the work with infective materials. A special care was taken to avoid contact with these pathogens. The use of the latex gloves and disinfection of