International Journal of Food Microbiology 145 (2011) 414–419

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

AFLP variability, toxin production, and pathogenicity of *Alternaria* species from Argentinean tomato fruits and puree

Stefania Somma [a,*], Graciela Pose [b], Alejandro Pardo [b], Giuseppina Mulè [a], Virginia Fernandez Pinto [c], Antonio Moretti [a], Antonio Francesco Logrieco [a]

[a] Institute of Sciences of Food Production, Research National Council (ISPA-CNR), Via G. Amendola 122/O, 70126, Bari, Italy
[b] Departamento de Ciencia y Tecnologia, Universidad Nacional de Quilmes/CONICET, Buenos Aires, Argentina
[c] Departamento de Quimica Organica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 24 August 2010
Received in revised form 9 December 2010
Accepted 4 January 2011

Keywords:
Alternaria alternata/tenuissima
A. arborescens
Tomato black mould
Tenuazonic acid
Monomethyl ether
Alternariol

ABSTRACT

Large amounts of tomato fruits and derived products are produced in Argentina and may be contaminated by *Alternaria* toxins. Limited information is available on the genetic variability, toxigenicity, and pathogenicity of *Alternaria* strains occurring on tomato. We analyzed 65 *Alternaria* strains isolated in Argentina from tomato fruits affected by black mould and from tomato puree, using amplified fragment length polymorphisms (AFLPs) technique. AFLP analysis resolved the set of strains in 3 main clusters (DICE similarity values of 58 and 60%) corresponding to *A. alternata/tenuissima* (44 strains), *A. arborescens* (15 strains) and to an unknown group (6 strains). Most of the representative strains, belonging to each AFLP cluster, when cultured on rice, produced tenuazonic acid (up to 46,760 mg/kg), alternariol monomethyl ether (AME, up to 1860 mg/kg), and alternariol (up to 70 mg/kg). The toxin profile related to the strains was not related to any AFLP cluster, except for AME which was produced at lower level by *A. arborescens.* Most of strains were pathogenic on two types of commonly cultivated tomato fruits. These findings provide new information on the variability within the *Alternaria* species complex associated with tomato disease.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Alternaria species occur in different habitats worldwide, causing infections to a wide range of plants and agricultural commodities, due to their pathogenic and saprophytic nature (Thomma, 2003). Diseases caused by *Alternaria* species can affect several important crops such as cereals, oil crops, vegetables, and different fruit plants (Logrieco et al., 2009; Thomma, 2003). Among these, a well-known *Alternaria* plant disease is the black mould of tomato, characterized by black lesions on green and ripe fruits (Pearson and Hall, 1975). Tomato fruits are easily infected because of their thin skin and weak tissues that allow a rapid penetration and growth of the different *Alternaria* infecting species (Pitt and Hocking, 1997). However, some species are also involved in post-harvest infection before fruit processing (Rotem, 1994; Wilson and Wisniewski, 1994). Therefore, the risk of contamination of industrialized products derived from pre and post-harvest infected tomato fruits, such as tomato sauce, juice, pulp, or puree, is very high (Ostry, 2008). Several of these species of *Alternaria* produce toxic metabolites which can be harmful for human health (Logrieco et al., 2009).

Among the *Alternaria* mycotoxins, tenuazonic acid (TA), alternariol (AOH), and alternariol monomethyl ether (AME) are the most occurring on plants. TA is a metabolite characterized by toxicity towards animals (Ostry, 2008), antibacterial activity (Gallardo et al., 2004) and it is considered as a possible causal factor of Onyalai, a human haematological disorder (Steyn and Rabie, 1976). TA has been shown also to inhibit of protein production and cell proliferation in three mammalian cell lines (Zhou and Qiang, 2008). AOH and AME, two toxins frequently found in combination, were reported to be mutagenic (Schrader et al., 2001), and Lehmann et al. (2006) reported also an estrogenic activity and genotoxic effects of AOH in mammalian cells.

Large volumes of tomato fruits and derived products are produced in Argentina (680,000 tonnes of tomato fruits produced on 18,000 hectares in 2008; FAOSTAT source). Most of the crop is used in processed products, where toxin contamination may result from preformed *Alternaria* toxins in tomato fruits. Recently Terminiello et al. (2006) reported contamination by TA, AOH, AME in Argentinean tomato puree that could pose serious risks for human health related to the consumption of these products. *Alternaria alternata* and *A. tenuissima* were considered the dominant species on tomato fruits analyzed, but only a morphological identification was provided (Terminiello et al., 2006).

The identification of species in *Alternaria* (Nees:Fr.) genus has been mainly based on morphological traits such as conidial shape and the type of catenulation, even though these traits can have a great intraspecific variability. Molecular tools have been recently used with

* Corresponding author. Tel.: +39 0805929326.
E-mail address: stefania.somma@ispa.cnr.it (S. Somma).

0168-1605/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2011.01.006

increasing efficiency for *Alternaria* species identification (Logrieco et al., 2009; Peever et al., 2004). Amplified fragment length polymorphisms (AFLPs) represent a powerful, highly reproducible, PCR-based DNA-fingerprinting technique (Vos et al., 1995). Because a large number of polymorphic loci can be investigated in a single experiment, the AFLP technique has become one of the major methods used for investigation of genetic diversity of fungal species (Fahleson et al., 2003, Leissner et al., 1997; Majer et al., 1996).

The objectives of this work were (a) to investigate by AFLP the species composition and genetic variability of *Alternaria* species occurring on tomato fruits affected by black mould and tomato puree in Argentina, (b) to evaluate their mycotoxin production, and (c) to test their pathogenicity on tomato fruits.

2. Materials and methods

2.1. Fungal strains

Among the 65 strains studied here, a set of 57 strains of *Alternaria* species was isolated from 38 tomato fruits of two different types affected by black mould (28 strains from “Redondo” type and 29 strains from “Perita” type, belonging to Platense, Petitt, Rhodas, and Superman varieties). The tomatoes were collected in 2004/2005 years from 8 fields in Buenos Aires province. A further 8 strains were isolated from 30 samples of Argentinean commercial tomato puree. Infected tissue portions of each tomato fruit and 0.2 ml of each tomato puree sample were inoculated on Dichloran Chloramphenicol Malt Extract Agar (DCMA) (Pitt and Hocking, 1997). Petri dishes were incubated for 7 days at room temperature under fluorescent lamps, with a 12-h/12-h day/night photoperiod. The plates were observed under the stereomicroscope and single-conidial cultures, representing each suspected *Alternaria* colony, were obtained on Water–Agar (WA) and plated on Potato–Carrot–Agar (PCA). Plates were stored at 25 °C under cool-white fluorescent lamps with an alternating 8/16 light/dark cycle (Simmons and Roberts, 1993) to induce sporulation. The strains were stored and deposited in the Institute of Sciences of Food Production (ISPA) Collection, Italy (ITEM accession: http://www.ispa.cnr.it/Collection/).

Four strains used in this work as reference strains were *A. alternata*, EGS 34.016; *A. tenuissima*, EGS 34.015; *A. arborescens*, EGS 39.128; and the *A. infectoria* ex type, EGS 27.193 (kindly provided by Dr. E. G. Simmons, Mycological Services, Crawfordsville, IN).

2.2. DNA extraction

Each strain was grown on PDA and inoculated in 100 ml of Wickerman's medium (glucose 4%, malt extract 0.3%, yeast extract 0.3% and peptone 0.5%). The shaken cultures (120 rpm), incubated at 25 °C, were filtered after 2 days and lyophilized. Genomic DNA was isolated from powdered lyophilized mycelia by using the E.Z.N.A. (EaZy Nucleic Acid Isolation) Fungal DNA Miniprep Kit (Omega Bio Teck Inc., USA), according to the manufacture's protocol. Quality and quantity of DNA were checked by comparison with standard DNA markers (1 kb DNA Ladder, ready-to-use, with fragments ranging from 250 bp to 10,000 bp, Fermentas GmbH, St. Leon-Rot, Germany) on 0.8% agarose gel.

2.3. AFLP analysis

AFLPs were performed by using the Applied Biosystem AFLP Microbial Fingerprinting Kit (Perkin Elmer Corporation, USA). *Eco*RI and *Mse*I restriction enzymes were used for DNA digestions, followed by ligation of *Eco*RI and *Mse*I adapters to the DNA fragments and subsequent preselective amplification. *Eco*RI fluorescent dye-labeled primers were used in three different primer combinations (*Eco*RI-AC FAM/*Mse*I-CC, *Eco*RI-AT NED/*Mse*I-CG, *Eco*RI-G JOE/*Mse*I-T), performed in Gene Amp PCR System 9700 (Applied Biosystems). The samples, combined with deionized formamide and a size standard (GeneScan-500 ROX), were denatured and loaded for electrophoresis on ABI Prims 310 Genetic Analyzer (Applied Biosystems) for 26 min at 15 kV. The peaks on electrophorogram were scored as present (1) or absent (0); the size of the fragments was calculated by the ABI Prism GeneScan Analysis Software (Applied Biosystems) comparing their migration to the standard. For each primer combination a binary matrix of bands with present and absent fragments for each strain was generated by ABI Genotyper 2.5.2 Software (Applied Biosystems). A combined matrix derived by the three primer combinations was analyzed by NTSYS version 2.0.2. software by UPGMA (Unweighted Paired Group Method of Arithmetic Averages) cluster analysis, to generate a similarity tree with the DICE similarity coefficient.

2.4. Mycotoxin production and analysis

Fungal cultures were grown on 12.5 g of autoclaved polished rice with 40% moisture in flasks of 250 ml. Each strain of *Alternaria* was inoculated with three agar plugs (1 × 1 cm) of 1-week-old cultures. The flasks were incubated in the dark at 25 °C for 21 days (Li et al., 2001).

The method for the detection of *Alternaria* toxins in rice was described by Li et al. (2001). The culture material was homogenized with 30 ml of methanol and filtered through a Whatman filter paper (n. 1). Briefly, the filtrate was clarified with 60 ml of 20% ammonium sulphate and divided into two parts. One part (40 ml) was extracted three times with 10 ml of chloroform. The organic phases were combined, evaporated to dryness, and dissolved in 4 ml of methanol for AOH and AME analysis by high-performance liquid chromatography (HPLC). Another part (20 ml) was adjusted to pH 2 with 6 N HCl and extracted twice for TA with 15 ml of chloroform. TA was then partitioned into 10 ml of 5% sodium bicarbonate, acidified to pH 2 again, and extracted twice with 10 ml of chloroform. The chloroform extracts were combined, washed with 7.5 ml of water, and evaporates to dryness. The residue was made up to 4 ml of methanol and analyzed for TA by HPLC.

The HPLC system consisted of Shimadzu LC-CA liquid chromatography (Shimadzu, Kyoto, Japan) equipped with a Rheodyne sample valve fitted with a 20 µl loop and a Shimadzu SPD-M10Avp UV photodiode array detector. The analytical column was Jupiter 4.6× 250mm 5 µ C18 (Phenomenex, USA). Standards of TA, AME and AOH were purchased from SIGMA Chemical Company (St. Louis, MO, USA). The mobile phase was methanol/water (80:20) containing 300 mg ZnSO4.H2O/l, for AOH and AME, and methanol/water (85:15) containing 300 mg of ZnSO4.H2O/l for TA. A flow rate of 0.4 ml/min was used. The wavelength for recording chromatograms was 258 nm for AME and AOH, and 280 nm for TA. A calibration curve was constructed for quantification purposes using the toxins standards and correlating peak-area versus concentration. The spectra were acquired in the range of 200–300 nm. Reference spectra were acquired during the elution of associated standards and used for peak identification by comparison after spectra normalization. The detection limits were 11 µg/kg for TA, 2 µg/kg for AME, and 5 µg/kg for AOH, respectively. The analyses were performed twice, with similar results.

2.5. Pathogenicity

A set of 23 representative strains, belonging to all the three AFLP clusters previously characterized (Fig. 1), was tested for pathogenicity on tomato fruits. In particular, 18 strains of the *A. tenuissima*/*alternata* AFLP cluster, 4 strains of the *A. arborescens* AFLP cluster and one of the 6 unknown strains, were tested. The pathogenicity and host specificity for each strain was determined on two types of fruit (Redondo var. Bonanza and Perita var. Roma) just after picking, by means of the toothpick-inoculation technique (Serdani et al., 2002). Halved toothpicks

Download English Version:

https://daneshyari.com/en/article/4368043

Download Persian Version:

https://daneshyari.com/article/4368043

Daneshyari.com