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Fusarium temperatum and *Fusarium subglutinans* isolated from maize in Argentina

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ABSTRACT

Fusarium temperatum and *Fusarium subglutinans* isolated from the Northwest region (NOA region) of Argentina were characterized using a polyphasic approach based on morphological, biological and molecular markers. Some interfertility between the species was observed. The phylogenetic analysis showed that the two species represented two clades strongly supported by bootstrap values. The toxigenic profile of the strains was also determined. *F. temperatum* strains were fusaproliferin and beauvericin producers, and only some strains were fumonisin B_1 producers. All *F. subglutinans* strains produced fusaproliferin but none produced beauvericin, indicating a potential toxicological risk from maize harvested in the NOA region of Argentina. This study provides new information about *F. temperatum* isolated from maize in Argentina.

1. Introduction

Maize (*Zea mays* L.) is, after wheat, the second most important cereal crop in human and animal diets worldwide (FAO, 2011). In Argentina maize is cultivated in the main maize growing region (Buenos Aires, Santa Fe and Cordoba provinces) and in the NOA region (SIIA, 2012). In the latter, there are also native maize races, which are cultivated in small farms and are used for domestic consumption (ILSI, 2006).

Several maize diseases are caused by *Fusarium* species, leading to significant yield losses and potential risk of mycotoxin contamination. *Fusarium subglutinans,* a member of the *Fusarium fujikuroi* species complex (FFSC), is a globally distributed pathogen causing stalk and ear rot of maize (Leslie and Summerell, 2006). Several studies in Argentina showed that the most prevalent species isolated from this cereal were *Fusarium verticillioides, Fusarium proliferatum* and *F. subglutinans* (Chulze et al., 2000; Reynoso, 2002; Torres et al., 2001) depending on geographical and climatic conditions. *F. subglutinans* was the predominant species in cold and temperate zones such as the NOA region which has an average annual temperature ranging from 18 to 24 °C (SIGA INTA, 2014; Torres et al., 2001).

Species within the FFSC are able to produce a wide range of mycotoxins such as fumonisins, and other toxins such as fusaproliferin, beauvericin and moniliformin (Jestoi, 2008). *F. subglutinans* has been reported to produce moniliformin, fusaproliferin and beauvericin (Logrieco et al., 1996, 1998; Marasas et al., 1986; Moretti et al., 1995), although no fumonisin production was observed (Proctor et al., 2004).

In the FFSC, morphological, biological and molecular phylogenetic studies have revealed that this complex includes 50 phylogenetically distinct species that comprise three biogeographically structured clades. This complex also includes 13 biological species (Aoki et al., 2014; Geiser et al., 2013).

Steenkamp et al. (2002) found two major groups in populations of *F. subglutinans* isolated from maize. The groups, called group 1 and group 2, showed some interfertility between the strains under laboratory conditions (Desjardins et al., 2000; Srobarova et al., 2002). The phylogenetic concordance analysis indicated that these two groups were reproductively isolated, representing cryptic species (Steenkamp et al., 2002). Population studies on *F. subglutinans* isolated from maize from various regions of the world have shown that the strains belonging to both groups were taxonomically divergent (Moretti et al., 2008; O'Donnell et al., 2000; Steenkamp, et al., 1999; Viljoen et al., 1997). Scauflaire et al. (2011) using a polyphasic approach, described a new species within the FFSC naming it *Fusarium temperatum* corresponding to the one previously classified as *F. subglutinans* group 1.

F. temperatum was recently reported from maize in Belgium (Scauflaire et al., 2012) and from sorghum in Serbia (Lević et al., 2013), and later this species was reported from maize in Spain and China too, causing seedling malformation and maize stem rot (Pintos et al., 2013; Wang et al., 2013). The ability of *F. temperatum* to produce diverse mycotoxins such as moniliformin, beauvericin, enniatins and fumonisin B_1 has also been observed (Scauflaire et al., 2012; Wang et al., 2013).

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The aims of this study were to characterize *F. temperatum* and *F. subglutinans* isolates collected from maize harvested in the NOA region of Argentina, using a polyphasic approach based on: identification of the isolates by morphological markers; identification at the biological species level by sexual crosses with tester strains; determination of the mating type and female fertility of the isolates; assessment of the cross-fertility between *F. temperatum* and *F. subglutinans;* molecular identification by sequencing of translation elongation factor 1α (EF-1α); determination of the toxigenic profile of the isolated strains; and determination of the phylogenetic relationships among the Argentinean, Belgian and Chinese *F. temperatum* isolates from maize based on EF-1α and β-tubulin combined genes, and determination of the phylogenetic relationships among our isolates with FFSC strains by EF-1α, β-tubulin and *RPB2* combined genes.

Our working hypothesis was that some of the strains previously reported as *F. subglutinans* isolated from maize from the NOA region of Argentina were *F. temperatum* and could be misidentified as *F. subglutinans,* and some interfertility among these strains could occur.

2. Materials and methods

2.1. Strain isolation and identification

F. temperatum and *F. subglutinans* were isolated from native and commercial maize harvested in the NOA region of Argentina. Native maize was collected from a region located at an elevation ranging from 1260 to 3300 m.a.s.l., with an annual mean temperature of 16 °C and an annual relative humidity of 53% (region 1); commercial maize was collected from one region located at an elevation of 450 m.a.s.l., with an annual mean temperature of 21 °C and an annual relative humidity of 71% (region 2) (SIGA INTA, 2014).

Morphological identification was done from monosporic cultures plated on potato dextrose agar (PDA), carnation leaf agar (CLA) and Spezieller Nahrstoffarmer agar (SNA) and incubated 10–14 days at 25 °C under cycles of 12 h white light–12 h black light. Morphology was observed on PDA, and conidiogenous cells, conidial characteristics and sporodochia were observed on CLA and SNA. Monosporic cultures were cryopreserved in sterile 15% glycerol (Leslie and Summerell, 2006). Strains were maintained in the culture collection at the Department of Microbiology and Immunology, UNRC (National University of Rio Cuarto), as RCFS and RCFT, corresponding to *F. subglutinans* and *F. temperatum* respectively.

2.2. Identification of the isolates at the biological species level

2.2.1. Mating type specific PCR and crossing procedures

Crosses to determine biological species were done on carrot agar as described in Leslie and Summerell (2006). Tester strains used were *F. subglutinans* KSU 0990 (MATE1-1) and *F. subglutinans* KSU 2192 (MATE1-2) from Kansas State University, (Kansas, U.S.), and *F. temperatum* ITEM 16196 (MAT1-1) and *F. temperatum* ITEM 16190 (MAT1-2) from the Institute of Sciences of Food Production (Bari, Italy).

Prior to making the crosses, mating type (MAT1-1 or MAT1-2) was determined by PCR as described by Steenkamp et al. (2000) in order to reduce the number of crosses. Crosses were done in triplicate and fertility was confirmed by observation of a cirrhus on the top of perithecia and by microscopic observation of mature asci with ascospores, within 4–5 weeks of incubation. Female fertility was determined as described above, but using the field isolates as female parents and tester strains as male parents.

2.2.2. Recombinant progeny from the interfertile crosses

Isolates that produced fertile crosses with tester strains of *F. temperatum* and *F. subglutinans* were evaluated to determine the presence of recombinant progeny. Interfertile isolates and tester strains were marked with different types of nitrate nonutilizing (*nit*) mutations. *Nit* mutants were obtained as fast-growing sectors on minimal medium amended with 2% chlorate. The *nit* phenotypes were determined on basal medium amended with different nitrogen sources. Sexual crosses were performed between *nit* complementary mutants of field isolates and tester strains on carrot agar as described in Leslie and Summerell (2006) with tester strains as female parents and the field isolates as male parents. Crosses were tested in triplicate and fertility was confirmed by observation of cirrhus on the top of perithecia. Cirrhi were carefully removed with a sterile needle and were placed in a tube containing 4.5 ml of sterile 2.5% Tween 60 solution. The tube was mixed for 5 to 10 s with a Vortex, after which 300 μl was spread on MMTS medium (minimal medium is amended with 0.05% (vol/vol) tergitol type NP-10 and 2% (wt/vol) L-sorbose instead of 3% sucrose). After 5 to 7 days of incubation, characteristics of the colonies were observed. Each cross was made in triplicate and from each replication three perithecia were randomly selected for the account. Colonies growing thin, with little or no aerial mycelium were considered mutants; in contrast, dense button-like colonies with cottony white aerial mycelium were considered wild type colonies. Prototrophic wild types were assumed to be the result of sexual recombination (Bowden and Leslie, 1999).

2.3. Identification of the isolates at the phylogenetic species level

2.3.1. DNA isolation, PCR amplification and sequencing

The strains were grown in 50 ml of complete medium (CM) (Leslie and Summerell, 2006) and incubated on an orbital shaker (150 r.p.m.) for 3 days at 25 °C. Fresh mycelia were collected by vacuum filtration using a Millipore system and stored at −20 °C. Frozen mycelia were ground to a powder under liquid nitrogen with a mortar. The ground mycelia were transferred to a 1.5 ml microcentrifuge tube. Fungal DNA was extracted by using the cetyl-trimethylammonium bromide (CTAB) method (Leslie and Summerell, 2006). DNA was quantified in a 0.8% agarose gel with ethidium bromide, and diluted to achieve a concentration of 1–10 ng/μl. Amplification of the translation elongation factor 1α (EF-1α) gene was carried out with PCR primers EF1 and EF2 using the amplification conditions of O'Donnell et al. (1998). Amplification of β-tubulin was carried out with PCR primers T1 and T2 using the amplification conditions of O'Donnell and Cigelnik (1997). Amplification of RNA polymerase II beta subunit (*RPB2*) was carried out with PCR primer pairs 5f2–7cf and 7cf–11ar using the amplification conditions of O'Donnell et al. (2007). PCR products were purified with the DNA Wizard-Clean up purification kit (Promega, Madison, WI., USA) according to the manufacturer's instructions and sequenced in both directions in a ABI Prism 3100 (Applied Biosystem, USA) sequencer. Sequences were edited with BioEdit Sequence Alignment Editor Version 7.1.3.0 (Hall, 1999) and compared with FUSARIUM-ID (Geiser et al., 2004) and GenBank data bases for identification of the field isolates.

2.3.2. Phylogenetic analysis

Sequences were aligned using the software ClustalX2 (Larkin et al., 2007). Phylogenetic analyses were performed using the combined sequences of EF-1α and β-tubulin, and based on *RPB2* sequence. Maximum parsimony analyses were made with PAUP*4.0 (Swofford, 1998) with 1000 bootstrap replications to test clade support. Consistency index (CI) and retention index (RI) were calculated. *F. proliferatum* NRRL 22944 was used as outgroup. Sequences included in the analysis were obtained from GenBank and they are listed in Table 1.

2.4. Mycotoxin profile of the isolates

F. temperatum and *F. subglutinans* isolates were cultured on 50 g of yellow maize kernels sterilized by gamma irradiation in a ^{60}Co source (National Commission of Atomic Energy, Buenos Aires, Argentina), with a dose of 1200 kRad (Chulze et al., 1999). Grains were adjusted to 40% moisture in 500-ml Erlenmeyer flasks and inoculated with 2 ml