**CAMILA PRIMIERI NICOLLI**

# *Fusarium fujikuroi* SPECIES COMPLEX IN BRAZILIAN RICE

**LAVRAS - MG**
**2018**

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Thesis presented to the Universidade Federal de Lavras, as part of the requirements of the Post Graduate Program in Agronomy/ Phytopathology, area of concentration in Phytopathology, to obtain the title of Doctor.

Prof. Dr. Ludwig H. Pfenning
Advisor

Prof. Dr. Emerson M. Del Ponte
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Thesis presented to the Universidade Federal de Lavras, as part of the requirements of the Graduate Program in Phytopathology, to obtain the title of Doctor.

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**LAVRAS – MG**
**2018**

*To my family Laurindo, Leonida and Karine for the support and affection and my niece for showing me how a small person can change a family.*

*I dedicate*

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## GENERAL ABSTRACT

Members of the *Fusarium fujikuroi* species complex (FFSC) are producers of mycotoxins, mainly fumonisins (FBs). In this work, we studied the FFSC species diversity on rice, the presence of essential fumonisin metabolic pathway genes, fumonisin production capacity *in vitro* and the natural occurrence of mycotoxins in rice. We sequenced 100 isolates for three gene regions (*TEF*, *TUB*, *CAL*). The phylogenetic tree showed that the most frequent species were *F. fujikuroi*, *F. proliferatum* and *F. verticillioides*, then *F. anthophilum*, *F. pseudocircinatum* and *F. sterilihyphosum*. In addition, we found two new phylogenetic lineages, *Fusarium* sp. 1 and *Fusarium* sp. 2. All isolates were analyzed for the presence of the *FUM1*, *FUM8* and *FUM14* genes and for a the subgroup of 61 isolates the fumonisin production capacity *in vitro* was assessed. Almost all isolates were gene positive. The species *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* produced fumonisins B1, B2 and B3 *in vitro*, *F. pseudocircinatum*, *Fusarium* sp. 1 and *Fusarium* sp. 2 produced only FB1, while *F. anthophilum* and *F. sterilihyphosum* did not produce any. The natural occurrence of mycotoxins was analyzed in 93 samples subdivided in rice flour and rice hull. All the mycotoxins analyzed were detected, 87% of rice samples were contaminated with at least one of the mycotoxins and 16 % there is no contamination. Among the mycotoxins analyzed, beauvericin was the most prevalent mycotoxin on Brazilian rice grains. This is the first report on the association of several FFSC members with rice grains in Brazil, the ability of these species to produce fumonisins and the natural contamination of rice grains by mycotoxins. Considering the importance of rice in the Brazilian population, our results contribute with new knowledge for the improvement of models for assessing the risk of mycotoxin production on rice in Brazil. Since this study indicates a potential threat to the quality of Brazilian rice. The thesis is divided into three chapters. The first chapter is the review on bakanae, the complex of *Fusarium fujikuroi* species and mycotoxins. The second chapter on the diversity of FFSC species in rice and the natural occurrence of mycotoxins. And the third chapter on the ability of species present in Brazil to produce fumonisins.



**Key words:** *Oryza sativa*, bakanae disease, *FUM* genes, mycotoxins, grain contamination.

## RESUMO GERAL

Membros do complexo de espécies *Fusarium fujikuroi* (FFSC) são produtores de micotoxinas, principalmente de fumonisinas (FBs). Neste trabalho estudamos: a diversidade de espécies de FFSC em arroz, a presença de genes essenciais da via metabólica de fumonisina, a capacidade de produção de fumonisinas *in vitro* e a ocorrência natural de micotoxinas no arroz. Sequenciamos 100 isolados para três regiões gênicas (*TEF*, *TUB*, *CAL*). A árvore filogenética mostrou que as espécies mais freqüentes foram *F. fujikuroi*, *F. proliferatum* e *F. verticillioides*, depois *F. anthophilum*, *F. pseudocircinatum* e *F. sterilihyphosum*. Além, de duas novas linhagens filogenéticas, *Fusarium* sp. 1 e *Fusarium* sp. 2. Todos os isolados foram analisados quanto à presença dos genes *FUM1*, *FUM8* e *FUM14* e para o subgrupo de 61 isolados foi analisado a capacidade de produção de fumonisinas *in vitro*. Quase todos os isolados foram positivos para os genes. As espécies *F. fujikuroi*, *F. proliferatum* e *F. verticillioides* produziram fumonisinas B1, B2 e B3 *in vitro*, *F. pseudocircinatum*, *Fusarium* sp. 1 e *Fusarium* sp. 2 produziram apenas FB1 e *F. anthophilum* e *F. sterilihyphosum* não produziram nenhuma dessas micotoxinas. A ocorrência natural de micotoxinas foi analisada em 93 amostras subdivididas em farinha de arroz e casca de arroz. Todas as micotoxinas analisadas foram detectadas, 87% das amostras de arroz estavam contaminadas com pelo menos uma das micotoxinas analisadas e 16% não apresentaram nenhuma contaminação. Dentre as micotoxinas analisadas, a beauvericina foi a micotoxina mais prevalente nos grãos de arroz brasileiros. Este é o primeiro relato sobre a associação de vários membros do FFSC com grãos de arroz no Brasil, a capacidade dessas espécies em produzir fumonisinas e a contaminação natural dos grãos de arroz por micotoxinas. Considerando a relevância do arroz na alimentação da população brasileira, nossos resultados contribuem com novos conhecimentos para a melhoria dos modelos de avaliação do risco da produção de arroz com micotoxinas no Brasil. Uma vez que, este estudo indica uma ameaça potencial à qualidade do arroz brasileiro. A tese está dividida em três capítulos. O primeiro capítulo é a revisão sobre bakanae, o complexo de espécies *Fusarium fujikuroi* e as micotoxinas. O segundo capítulo sobre a diversidade de espécies de FFSC no arroz e a ocorrência natural de micotoxinas. E o terceiro capítulo sobre a capacidade das espécies presentes no Brasil de produzir fumonisinas.



**Palavras chaves:** *Oryza sativa*, bakanae, *FUM* genes, mycotoxinas, grãos contaminados.

**SUMMARY**

# FIRST PART

# REVIEW OF BAKANAE DISEASE, *FUSARIUM FUJIKUROI* SPECIES COMPLEX AND MYCOTOXINS

**ABSTRACT**

Rice is basic food for human nutrition and Brazil is among the nine largest rice producers in the world. Bakanae is one of oldest rice diseases and the most common symptom is the elongation of the diseased plant stems. In Brazil, the first report of bakanae disease was in 1967 in São Paulo State. The main causal agent is *Fusarium fujikuroi* that produces gibberellins. However, other members of *F. fujikuroi* species complex (FFSC) can be associated to bakanae disease, such as *F. anthophilum*, *F. proliferatum*, *F. sacchari*, *F. subglutinans* and *F. verticillioides*. FFSC represents a monophyletic group, which species are established based on phylogenetic relationships, and corresponds in large part to the old section Liseola of the genus *Fusarium*, defined taxonomically based on morphological traits. Members of FFSC can produce mycotoxins such as fumonisins, moniliformin, beauvericin, enniatins, fusaric acid, and fusarin C. These mycotoxins have a significant economic impact, reducing the quality of rice grains and are harmful to human and animal health. Fumonisins are the most important mycotoxins that occur in several grains around the world. The production of fumonisin is regulated by 16 genes, which form the genetic cluster called fumonisin biosynthetic gene cluster. Thus, bakanae disease cause several losses in the rice crop worldwide. The most important control method of bakanae disease is using resistant genotypes. However, studies on biology, sexual cycle, and physiology of FFSC species, together with deeper genetic investigations on the whole complex are providing a better understanding of the main mechanism that cause the disease and tools for its management.

**Key words:** *Oryza sativa*, bakanae disease, fumonisins, control methods.

# 1 CHARACTERIZATION OF THE PROBLEM

Rice (*Oryza sativa* L.) is one of the most produced and consumed cereals in the world, being characterized as a staple food for the majority of the world population (FAO, 2018). Due to the importance of rice in the diet, its composition and nutritional characteristics are strictly related to human health. However, even apparently healthy rice grains can be infected by mycotoxigenic *Fusarium* species. In Brazil, *Fusarium asiaticum*, a species belonging to the *Fusarium graminearum* species complex (FGSC), was reported as an important pathogen of rice (GOMES et al., 2015). During the last three years, several species belonging to the *Fusarium fujikuroi* species complex (FFSC) have been isolated from rice grains by our group.

Members of the genus *Fusarium* produce a diverse array of secondary metabolites, including mycotoxins. Trichothecenes are important *Fusarium* mycotoxins produced by species of FGSC isolated from rice also in Brazil (GOMES et al., 2015). The recent finding of species from the FFSC in association with rice grains in southern Brazil is of concern, since members of that complex may also produce mycotoxins, such as fumonisins, moniliformin, beauvericin and enniatins (MUNKVOLD et al., 2017). The production and accumulation of these mycotoxins in rice grains reduce the quality of the production and are harmful to human and animal health, since they are often associated with food poisoning, tumors and other chronic diseases (DESJARDINS et al., 2006).

*Fusarium verticillioides* and *F. proliferatum* are the main producers of fumonisins in the FFSC (MUNKVOLD, 2017). However, fumonisins are produced also by other species of FFSC, such as *F. anthophilum* and *F. fujikuroi* (PROCTOR et al., 2013). In a recent survey of *Fusarium* contaminating rice grains in Brazil, together with strains of *Fusarium graminearum* species complex (FGSC), we identified *F. fujikuroi*, *F. verticillioides*, *F. proliferatum* and *F. anthophilum* (unpublished data from GOMES et al., 2015). These species have different potential for fumonisins production, which cluster has been characterized for several species along the complex showing that multiple horizontal transfers of the cluster from unknown donors to FFSC recipients and cluster duplication and loss were the genetic mechanisms that originated the current genetic traits of FFSC species (PROCTOR et al., 2004, 2013).

This study was designed to (i) identify the members of FFSC isolated from rice grains in Brazil; (ii) identify the *FUM* genes and characterize the gene cluster type from each identified species; (iii) evaluate the ability for mycotoxin production among the species of the FFSC commonly associated with rice grains, in order to elucidate specific differences in the biosynthetic gene cluster that are responsible for intra and inter-specific differences in mycotoxin production and (iv) quantify the mycotoxin levels on naturally infected rice grain.

# 2 STATE OF ART

## 2.1 Brazilian rice production

Rice (*Oryza sativa* L.) stands out among the most important cereal crops of the world, being predominantly grown for domestic consumption (FAO, 2018). Rice is basic food for human nutrition. Brazil is among the nine largest rice producers in the world, producing about 11.3 million tons. The production comes from two production systems: irrigated and highlands. The main rice producing State in Brazil is Rio Grande do Sul (60.4%) followed by Santa Catarina (8.9%), Maranhão (6.4%), Mato Grosso (3.2%) and Tocantins (2.0%) (CONAB, 2018).

## 2.2 Bakanae disease

Bakanae is one of oldest rice diseases in the Orient. Bakanae disease was discovered in 1828 but was named in 1898. The name of disease is derived from "bad" or "foolish" referring to disease-specific stretching symptoms caused by the production of gibberellin after infection by the pathogen (OU, 1987). Bakanae pathogens survive on seeds leading to the development of disease after emergence the rice plants (OU, 1987). The grains are infected in the flowering stage and acquire a reddish color.

The most common symptom is the elongation of the diseased plant stems, which become slender, pale yellowish in color and taller than their undiseased counterparts. Symptoms of bakanae also include leaf narrowing, chlorotic and elongated primary leaf leaves, which appear to be induced by the production of gibberellins by the pathogen. Crown

rot is also seen, resulting in stunted rice plants. Harvest losses caused by bakanae may reach up to 40% (OU, 1987; AMOAH et al., 1995).

Bakanae disease occurs in different countries of Europe, Africa, Asia and the United States (CARTER et al., 2008; WULFF et al., 2010; AMATULLI et al., 2010; MATIC et al., 2017). In Brazil, the first report of bakanae disease was in 1967 in São Paulo State (AMARAL et al., 1970). The disease caused early death of plantlets and death of adult plants. When seedlings die early, the infection occurs in coleoptile causing the abnormal growth that reaches twice the size of healthy seedlings. In adult plants, the infection occurs in the first training and the plant presents gigantism characterized by the elongation and thinning of the stem and leaves (AMARAL et al., 1970). Currently, there is no report of the disease in Brazil.

## 2.3 Etiological agent of bakanae disease

In the sole report of bakanae disease in Brazil in 1967, the causal agent was morphologically identified as "*Fusarium moniliforme"* (AMARAL et al., 1970). *Fusarium fujikuroi* produces gibberellins and is the main pathogen of the bakanae disease of rice around the world (CARTER et al., 2008; MARASAS et al., 2004; WULFF et al., 2010). However, other members of *Fusarium fujikuroi* species complex (FFSC) can be associated with bakanae, such as *F. anthophilum*, *F. proliferatum*, *F. sacchari*, *F. subglutinans* and *F. verticillioides* (ZAINUDIN et al., 2008; CARTER et al., 2008; AMATULLI et al., 2010; HOSSAIN et al., 2016).

In Africa and Asia, Wulff et al. (2010) identified four species of the complex, associated with bakanae disease: *F. fujikuroi*, *F. proliferatum*, *F. verticillioides* and *F. andiyazi*, being *F. fujikuroi* in this study present only in Asia. In Italy, between years 2006 and 2008, *Fusarium* strains were isolated from asymptomatic plants and from bakanae symptoms plants, and identified as *F. fujikuroi* considered the causal agent of the bakanae disease (AMATULLI et al., 2010). Since 2001, increasing economical losses presumably caused by *Fusarium fujikuroi* were recorded in Italy and in 2010, the first report of *Fusarium andiyazi* associated with rice bakanae was published (DAL PRÁ et al., 2010). Moreover, recently, *F. verticillioides*, *F. sacchari*, *F. subglutinans*, *F. proliferatum* and *F. fujikuroi* were isolated

from different fields of rice plants with symptoms of bakanae in Malaysian Peninsula (HSUAN et al., 2011).

## 2.4 *Fusarium fujikuroi* species complex (FFSC)

The *Fusarium fujikuroi* species complex (FFSC) is a monophyletic group, which species are established based on phylogeny relationships, and corresponds in large part to the old section *Liseola* of the genus, taxonomically defined based on morphological traits (O'DONNELL et al., 1998; GEISER et al., 2005). Nowadays, with the new proposed nomenclature "One fungus, one name", this complex was renamed as *Fusarium fujikuroi* species complex (FFSC) (GEISER et al., 2013). This complex was defined through the concept of phylogenetic species (O'DONNELL et al., 1998; KVAS et al., 2009).

The morphogical concept of species within the Liseola Section was firstly established by Wollenweber and Reinking (1935) to group species that produce macroconidia in sporodochia, microconidia in false heads or chains and that do not produce chlamydospores (LESLIE & SUMMERELL, 2006). Using the biological species concept, seventeen genetically isolated biological species were identified in the FFSC by means of laboratory crossings. Thirteen genetically isolated biological species are informally known as mating populations A-M (KVAS et al., 2009). The mating populations of the FFSC are all heterotallic, with one binary mechanism of mating types, genetically defined by one MAT locus and two idiomorphs, *MAT1-1* or *MAT1-2*, and unrelated sequences present at the same locus of the genome (LESLIE & SUMMERELL, 2006). Sexual reproduction only happens between individuals of the same mating population but of different mating types.

A phylogeographic investigation on the species within the FFSC conducted by DNA sequences of four genes, identified three distinct clades of species with putative origins in the American, African and Asian continents, and identifed at least 50 distinct phylogenetic species O'Donnell et al. (1998). The FFSC currently contains 61 formally described species, including 13 species corresponding to mating population and at least 20 species known to produce one or more mycotoxins (KVAS et al., 2009; LAURENCE et al., 2015; HERRON et al. 2015; MOUSSA et al. 2017).

## 2.5 Mycotoxins produced by FFSC

Members of FFSC produce a wide array of mycotoxins such as fumonisins (FBs), moniliformin (MON), beauvericin (BEA) and enniatins (ENNs) families (MARASAS et al., 2004; THRANE et al., 2004; JESTOI et al., 2008). These mycotoxins have a significant economic impact, reducing the quality of rice grains and being harmful to human and animal health. All these mycotoxins are natural secondary metabolites with toxic properties including carcinogenicity, genotoxicity, immunotoxicity, mutagenicity and reproductive and developmental toxicity (BENNETT & KLICH, 2003).

Fumonisins are a family of polyketide-derived molecules structurally similar to the sphingolipid intermediates sphinganine and sphingosine, and capable of disrupting the sphingolipid metabolism in animals by inhibiting the enzyme ceramide synthase (sphinganine N-acyltransferase) (MUNKVOLD et al, 2017). These mycotoxins can cause fatal diseases in animals, e.g. horses and swines, and severe intoxications in humans (DESJARDINS, 2006). Fumonisins were first isolated from *F. moniliforme*, currently known as *F. verticillioides*, and can be classified into four series, fumonisin A, B, C and P (RHEEDER et al., 2002). B-series fumonisins (FBs) are the most abundant, and fumonisin $B_1$ ($FB_1$) is the most commonly found in natural contaminations, besides being the most toxic fumonosin analogue (MARASAS, 2001). A and B fumonisins consist of a linear 20-carbon backbone with functional substitutions, including 3-5 hydroxyls, two methyl groups, one amino group and two tricarboxylic esters. C fumonisins (FCs) have a 19-carbon backbone. These mycotoxins are mainly produced by *F. verticillioides* and *F. proliferatum*, which have worldwide distribution and are usually associated with ear, stalk and seedling rot diseases of maize (MILLER, 2001; MUNKVOLD et al., 2017). Others members of FFSC, such as *F. fujikuroi* and *F. anthophilum*, are also fumonisins producers (PROCTOR et al., 2013).

Moniliformin is a potassium or sodium salt of 1-hydroxycyclobut-1-ene-3, 4 dione. The name of moniliformin is derived from "*F. moniliforme"*, the first fungus identified as producer of this mycotoxin, as in the case of fumonisins (MARASAS et al., 2006). However, based on taxonomic revision, the main species that produce moniliformin are the two FFSC members *F. proliferatum* and *F. nygamai* (DESJARDINS, 2006). The toxicological effects of

moniliformin are inhibition of protein synthesis, cytotoxicity, and chromosome damage. Moniliformin has been reported on maize and rice from different parts of the world (JESTOI et al., 2008).

Beauvericin is a cyclic hexadepsipeptide identified for the first time from *Beauveria bassiana* (HAMILL et al., 1969). This secondary metabolite has been identified in several species of FFSC (MORETTI et al., 1996). Enniatins are structurally closely related to beauvercin. There are seven enniatin analogues produced by *Fusarium*, and the most important are enniatin A, A1, B and B1. Beauvericin and enniatins are cytotoxic in laboratory studies to cell lines of insects and humans (JESTOI et al., 2008). Due to the structural similarity between beauvericin and enniatins, the occurrence of these mycotoxins also has a high correlation. Both types of mycotoxins were reported on maize, wheat and rice in several countries (EFSA, 2014).

## 2.6 *FUM* cluster

The production of fumonisins is regulated by several genes, which form the genetic cluster called fumonisin biosynthetic gene cluster (*FUM* cluster). The *FUM* cluster consists of 16 genes that encode biosynthetic enzymes, transport proteins and a transcription factor (PROCTOR et al., 2003; 2013). The genes of the cluster are *FUM1*, *FUM2*, *FUM3*, *FUM6*, *FUM7*, *FUM8*, *FUM10*, *FUM 11*, *FUM 13*, *FUM 14*, *FUM15*, *FUM16*, *FUM17*, *FUM18*, *FUM19* and *FUM21*. The number, order and orientation of the genes inside the FUM cluster is dependant on the producing species. In *F. verticillioides* and *F. proliferatum* all genes are in the same order and orientation (MORETTI et al., 2013). However, the sequences flanking the cluster differ, indicating that the cluster is in a different genomic location in these two species (PROCTOR et al., 2003; 2008; 2013). The main genes involved in fumonisin biosynthesis are *FUM1*, *FUM8* and *FUM14* (PROCTOR et al., 2008). The *FUM1* gene encodes a polyketide synthase that catalyses synthesis of a linear polyketide that forms the backbone structure of fumonisins. *FUM8* gene encodes a α-oxoamine synthase that defines whether fusaria will produce FBs or FCs by catalysing the condensation of the linear polyketide with alanine, to produce FBs, or with glycine, to produce FCs. *FUM14* catalyzes the esterification of the

CoA-activated tricarboxylic acids to the fumonisin backbone and no fumonisin production occurs after the deletion of this gene (BUTCHKO et al., 2006; PROCTOR et al., 2008).

## 2.7 Control methods of bakanae disease

Bakanae disease caused several losses in the rice crop with a percentage of 30% up to 80% (CARTER et al., 2008). The most important control method on bakanae disease is resistant genotypes (HALIM et al., 2015; FIYAZ et al., 2016; MATIĆ et al., 2016). Since the first report of bakanae disease was observed, great efforts have been made to find resistant rice cultivars. Recent studies have reported thirteen genotypes with different levels of resistance. Currently, by using RNA-seq, the pathways involved in bakanae resistance were found in rice (MATIĆ et al., 2016) and a germoplasm collection of rice was screened for *F. fujikuroi* resistance and new effective sources for resistance against bakanae were identified on rice chromosomes 1 and 4 (VOLANTE et al., 2017). Thus, in the near future new control tools will be available.

Biological control is also a method largely studied to provide tools for developing treated seeds to control bakanae disease that is a seedborne disease. In the last years, some microorganisms have been studied such as as yeasts and bacteria. In particular, *Pichia guilliermonddi* and *Metschnikowia pulcherrima* distributed to seeds both showed significant reduction of the infection rate of *F. fujikuroi* when compared to some commercial biofungicide (MATIĆ et al., 2014). On the other hand, *Bacillus oryzicola* had a biocontrol activity by directly inhibithing *F. fujikuroi* and providing to the rice plants capability to induce a systemic resistance (HOSSAIN et al., 2016). Most recently, the biocontrol activity of a surfactin-producing Bacillus and of the purified surfactin A showed to reduce bakanae disease up to 80% (SARWAR et al., 2018).

Finally, a study aimed to develop RNA interference to reduce fumonisin production by *Fusarium* species in maize plants showed a possible application extended to rice. In this study, *FUM1* and *FUM8* gene segments were cloned. These genes are regulators of fumonisin production and suggested that segments could be expressed in maize for host-induced gene silencing of fumonisin production (JOHNSON et al., 2018).

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**SECOND PART**

# ARTICLE 1 - OCCURENCE OF *FUSARIUM FUJIKUROI* SPECIES COMPLEX AND RELATED MYCOTOXINS ON BRAZILIAN RICE GRAINS

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**ABSTRACT**

Recently, no information on the bakanae disease has been reported in Brazil, although the occurrence on rice of mycotoxins potentially produced by members of the *Fusarium fujikuroi* species complex (FFSC), were reported. A set of 93 rice grains samples, collected from the main rice-growing regions of Brazil, was investigated for the occurrence of species of the *Fusarium fujikuroi* species complex and natural occurrence of *Fusarium* mycotoxins e.g. fumonisins, moniliformin, beauvericin and enniatins. One hundred representative *Fusarium* isolates with characteristics of the FFSC were analyzed using a multi-locus sequence approach. The translation elongation factor 1-α (*TEF*), calmodulin (*CAL*) and β-tubulin (*TUB*) genes were sequenced. A phylogenetic tree obtained by the analyses of three genes sequenced, showed that the main species identified were as *F. fujikuroi*, *F. proliferatum* and *F. verticillioides*, and, at a lesser extent, *F. anthophilum*, *F. pseudocircinatum* and *F. sterilihyphosum*. Moreover, some of the strains sequenced could not be assigned to any known species and therefore were identified as two lineages, defined as *Fusarium* sp. 1 and *Fusarium* sp. 2. All the mycotoxins analysed were detected, although sporadically and at low concentrations in the rice grain samples. This is the first report on the occurrence of several members of FFSC and related mycotoxins on rice. Considering the relevance of rice in the feeding of the Brazilian population, the presence of several species of the FFSC and the natural contamination of related mycotoxins indicate a potential health threat of Brazilian rice. In particular, the high level of beauvericin contamination is worrisome, since previous information on this mycotoxin on rice in Brazil is completely missed. Therefore, in Brazilian rice, more comprehensive mycotoxin monitoring programs may be needed to better evaluate the risk related to *Fusarium* mycotoxin occurrence.

**Key words:** *Oryza sativa*, bakanae disease, molecular phylogeny.

# 1 INTRODUCTION

Rice stands out among the most important cereal crops of the world, being predominantly grown for domestic consumption (FAO, 2018). Rice is a global staple food crop and Brazil the largest producer outside Asia. Rice production in Brazil is concentrated mainly in the southern states of the country, and in 2017 was of 12 million tons (FAO 2018). Among the diseases that affect the rice, bakanae disease has been recorded almost in all countries where paddy rice is grown commercially, especially in Asian countries (Zainudin et al., 2008; Hossain et al., 2016). The typical symptoms of bakanae are slender, chlorotic and elongated primary leaves, that seem to be induced by the production of gibberellins by the pathogen (Ou, 1987; Amoah et al., 1995). According to the literature, bakanae disease is caused mainly by *Fusarium fujikuroi*, which is a member of the so-called *Fusarium fujikuroi* species complex (FFSC), and a well known gibberellin producer (Marasas et al., 2004; Wulff et al., 2010). However, other members of FFSC have been isolated from rice grains in different parts of the world, namely *F. anthophilum*, *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* (Zainudin et al., 2008; Carter et al., 2008; Amatulli et al., 2010; Hossain et al., 2016).

The association of members of the FFSC with the rice crop is not only a concern because of bakanae disease, but also due to the fact that the species that cause the disease can produce several mycotoxins (Marasas et al., 2004; Jestoi et al., 2005). Mycotoxins produced by *Fusarium* species are natural toxic secondary metabolites that cause major concern for public health because of their toxic properties including carcinogenicity, genotoxicity, immunotoxicity, mutagenicity and reproductive and developmental toxicity (Bennett & Klich, 2003). In Brazil, bakanae disease was firstly reported in 1970 and the causal agent identified was *Fusarium moniliforme* (Amaral et al., 1970). After that, no report of this disease in the country has been published. However, other mycotoxigenic *Fusarium* species were reported in rice seeds. Four members of *Fusarium graminearum* specie complex (FGSC) were identified associated with rice seeds in the South of Brazil. Among these species, *F. asiaticum* was the predominant species, followed by *F. graminearum*. Both species can produce nivalenol and deoxynivalenol, respectively, and their acetylated derivatives (Gomes et al.

2015). Beside this latter study, few data have been provided on the association of *Fusarium* and related mycotoxins occurrence on rice crop in Brazil.

Becker-Algeri et al. (2013) reported the occurence of fumonisin B1, a mycotoxin produced by members of FFSC, on grains of commercial rice in the Sao Paulo State. Few years later, Mendes et al. (2015) reported fumonisin B1, on wheat harvested in Southern Brazil. Among the species that are agent causal of bakanae disease, *F. verticillioides*, *F. fujikuroi*, *F. proliferatum* and *F. anthophilum* are fumonisins (FBs), moniliformin (MON), beauvericin (BEA) and enniatins (ENNs) producers and occur in different parts of the world (Marasas et al., 2004; Jestoi et al., 2005).

Since several members of FFSC have been identified on rice in different parts of the world (Carter et al., 2008; Amatulli et al., 2010; Hossain et al., 2016), we suggest that FFSC species could be associated with rice grains in Brazil. All these reports emphasizing the need for further investigations about the diversity of FFSC in Brazilian rice grains samples. In lack of information about the diversity the species and toxigenic potential of tropical *Fusarium fujikuroi* species complex occurring in commercialized rice grains in Brazil, this study was designed (i) to identify the diversity of species of FFSC on rice grains from different producing regions of the country and (ii) to detect the natural occurrence of mycotoxins on rice.

## 2 MATERIAL AND METHODS

### 2.1 Sampling and isolation of fungi

Samples of rice grains were obtained from all production regions in Brazil, in the states Rio Grande do Sul (RS), Santa Catarina (SC), São Paulo (SP), Goiás (GO), Mato Grosso (MT), Tocantins (TO), Maranhão (MA) and Roraima (RR). In total more than six hundred sample were processed and two hundred grains of each sample plated on sterile saline solution soaked blotters (filter papers) in germination boxes (Gerbox) and incubated at room temperature (25±3 °C) for 12 days (Leslie & Summerell, 2006). Colonies with characteristics of the *Fusarium fujikuroi* species complex (FFSC) were isolated in pure culture. Examinations were made using stereo and compound binocular microscopes, and one

strain per grain sample was isolated on MA2%. Preliminary identification of the strains was based on morphological characters of the conidiophores, conidiogenic cells, and presence or absence of polyphialides and conidial chains (Leslie & Summerell, 2006).

**2.2 Phylogenetic analysis**

DNA was extracted and purified from fresh mycelium using the "Wizard® Magnetic DNA Purification System for Food" kit (Promega, USA) according to the manufacturer's protocol. Phylogenetic relationships of strains were investigated by amplifying and sequencing the housekeeping genes translation elongation factor 1-α (*TEF*), calmodulin (*CAL*) and β-tubulin (*TUB*). Before sequencing, PCR products were purified with the enzymatic mixture EXO/FastAP (Exonuclease I, FastAP thermosensitive alcaline phosphatase, Thermo Scientific, Lithuania, Europe). Sequence reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit for both strands, after which they were purified by gel filtration through Sephadex G-50 (5%) (Sigma Aldrich), before they were analyzed on the 3730xl DNA Analyzer (Applied Biosystems – HITACHI). DNA sequences and aligned were determined by BioNumerics Seven software platform (Applied Maths). Phylogenetic analysis by the alignment with Clustal W algorithm (Thompson et al., 1994) in the MEGA 7.0 software (Tamura et al., 2013) and the method used was Maximum Parsimony. Phylogenetic analyses were conducted with three housekeeping genes (*TEF*, *CAL* and *TUB*), individually and combined, the DNA sequences of 100 strains obtained were compared with sequences from strains of reference species in the FFSC already available in GenBank. Clade stability was evaluated using 1000 parsimony bootstrap replicates with 1000 simple addition sequences. *Fusarium oxysporum* and *Fusarium inflexum* were used as outgroups.

**2.3 Identification of mating type**

Both *MAT1-1* and *MAT1-2* idiomorphs were identified by amplifying of a fragment of 200 bp and 250 bp, respectively. The *MAT1-1* fragment was amplified with primer set

fusALPHAfor and fusALPHArev and *MAT1-2* with the fusHMGfor and fusHMGrev primers under conditions as indicated by the authors (Kerényi et al., 2004).

### 2.4 Mycotoxin analysis

A subset of 93 rice samples were investigated for the natural contamination of FBs, MON, BEA and ENNs. The samples were analyzed in subsamples that are rice hulls and flour (Supplementary table 2). *Sample preparation protocol:* 5 g of homogenized sample was performed according to a previous study with slight modifications (Rodríguez-Carrasco et al., 2014). The extract was evaporated to dried out under gentle nitrogen flow at 45°C and the residue reconstituted with a mixture of MeOH:water (70:30, v/v), and filtered (0.22 µm) prior to the UHPLC-Q-Orbitrap HRMS analysis. *Analysis by UHPLC-Q-Orbitrap HRMS:* analysis were performed using an UHPLC instrument (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, Ma, USA) coupled to a Q Exactive Orbitrap mass spectrometer (UHPLC, Thermo Fischer Scientific, Waltham, Ma, USA). The mobile phases were: phase A, water with 0.1% formic acid and 5 mM ammonium formate; and phase B, MeOH with 0.1% formic acid and 5 mM ammonium formate. Detection was performed using a Q-Exactive mass spectrometer. The mass spectrometer was operated in both positive and negative ion mode by setting two scan events (Full ion MS and All ion fragmentation, AIF). Data analysis and processing were evaluated by the Quan/Qual Browser Xcalibur software, v. 3.1.66. (Xcalibur, Thermo Fisher Scientific).

## 3 RESULTS

### 3.1 Sampling and strains collection

We identify 100 strains of FFSC (Suppl. Table 1) and the strains were obtained between the years 2008 - 2016 and isolated from seven states of Brazil: RS (n = 20), SC (n = 40), MT (n = 19), GO (n = 14), TO (n = 2), RR (n = 3) and MA (n = 2) (Table 1). All the strains were characterized morphologically based on the production of color mycelium growth and the presence of microconidia in chains (on monophialides or on polyphialides),

after that the strains were deposited in the Coleção Micológica de Lavras (CML), Department of Plant Pathology, Universidade Federal de Lavras, Brazil. Duplicates were deposited at ITEM - Agro-Food Microbial Culture Collection of Institute of Sciences and of Food Production (ISPA), Puglia, Bari, Italy.

### 3.2 Phylogenetic analysis

The individual dataset of *TEF* were analyzed 649 characters, of which 177 sites were polymorphic, and 136 of which were parsimony-informative (Suppl. Figure 1). The *TEF+CAL+TUB* dataset contained 1093 characters, of which 181 sites were polymorphic, and 153 of which were parsimony informative. Combination of housekeeping genes datasets into a Maximum Parsimony analysis resulted in 81 most parsimonious trees and the consistency index is 0.83, the retention index is 0.99, and the composite index is 0.84 for all sites and 153 parsimony informative sites. According to the combined tree, eight species showed were identified as *Fusarium fujikuroi* (n = 23), *F. proliferatum* (n = 22) and *F. verticillioides* (n = 16), *F. anthophilum* (n = 7), *F. pseudocircinatum* (n = 4) and *F. sterilihyphosum* (n= 2) (Suppl. Table 1; Figure 1) with high support statistic (87-100%). Two lineages were identified as *Fusarium* sp. 1 (n = 8) and *Fusarium* sp. 2 (n = 18) (Suppl. Table 1; Figure 1) with 100% and 97% bootstrap support, respectively. Both, new phylogenetic species were placed in the African clade of the FFSC. Our strains of *Fusarium* sp. 1 from rice grouped together with a strain from Nigeria as well obtained from rice seeds (NRRL 25615), while *Fusarium* sp. 2 formed a sister group of *F. andiyazi* (Figure 1).

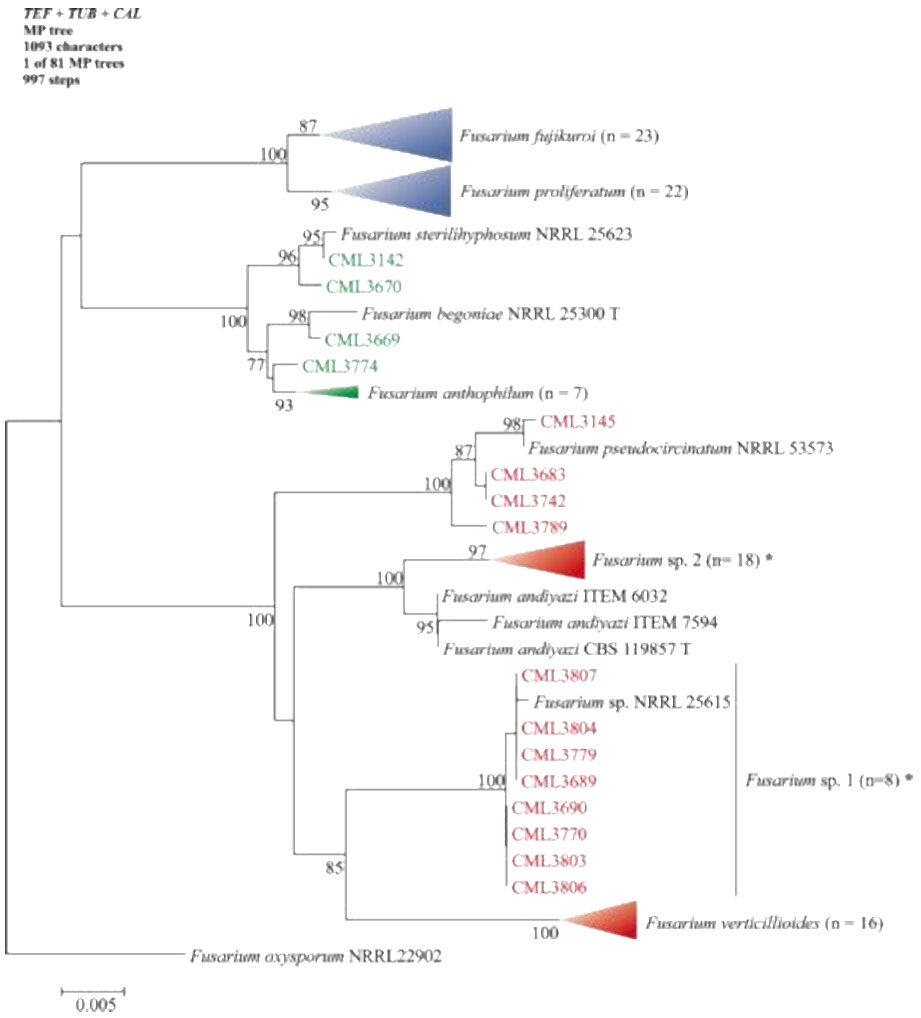


Figure 1 Phylogenetic tree inferred from the combined datasets of *TEF+TUB+CAL* sequences from members of the *Fusarium fujikuroi* species complex analyzed in this study. Sequences generated in the present study are in colorful and the new lineages with arterisks. The evolutionary history was inferred by using the Maximum Parsimony method. Numbers on branches are bootstrap values based on 1000 replicates.

### 3.3 Mating types

The alleles of *MAT1-1* and *MAT1-2* of 100 strains were identified by presence or absence of amplification of fragment. Among of 100 strains, 36 % are *MAT1-1* and 62 % of strains are *MAT1-2* (Suppl. Table 1). The species of FFSC identified have at least one isolate

of each mating type, with the exception of *F. sterilihyphosum*, which was represented by only two strains.

### 3.4 Mycotoxin analysis

The subset of 93 rice samples were analyzed for the natural occurrence of FBs, MON, BEA and ENNs. All the mycotoxins analyzed were detected and 87% (n= 84) of rice samples were contaminated with at least one of the mycotoxin and the 16% there are anyone contamination (n=9). Among the mycotoxins analyzed, beauvericin was the most prevalent on Brazilian rice grains (Suppl. Table 2), which was found in 81 out of 93 rice samples at the concentrations up to 110.4 μg/kg (Table 1). The other mycotoxin on Brazilian rice was moniliformin, which was found in 4 out of 93 rice samples and the concentrations up to 28.1 μg/kg, then fumonisins and ennniatins that were present in maximum 3 out of 93 rice samples and the concentrations up to 4.1 μg/kg and 2.6 μg/kg, respectively (Table 1). In general, all mycotoxins analyzed showed higher levels in the rice flour samples compared to the rice hulls samples. Finally, all the mycotoxins that we analysed were found, however, mycotoxins were found sporadically in the samples and at low concentrations.

Table 1 Incidence, mean and maximum of natural occurrence of mycotoxins of 93 rice samples divided in two subsamples: hull and flour.

| Mycotoxins | Incidence (%) | | Mean (µg/kg) | | Maximum (µg/kg) | |
|---|---|---|---|---|---|---|
| | Hull | Flour | Hull | Flour | Hull | Flour |
| Fumonisin B1 | n.d. | 1 (1.07) | n.d. | 0.044 | n.d. | 4.1 |
| Fumonisin B2 | 3 (3.22) | 1 (1.07) | 0.091 | 0.022 | 3.4 | 2.1 |
| Moniliformin | 1 (1.07) | 4 (4.3) | 0.143 | 0.417 | 13.3 | 28.1 |
| Beauvericin | 61 (65.6) | 81 (87.09) | 6.369 | 20.322 | 110.4 | 810.1 |
| Enniatin A | n.d. | n.d. | n.d. | n.d | n.d. | n.d. |
| Enniatin A1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Enniatin B | 1 (1.07) | n.d. | 0.027 | n.d. | 2.6 | n.d. |
| Enniatin B1 | 2 (2.15) | 2 (2.15) | 0.018 | 0.031 | 1.2 | 2.4 |

Abbreviations: n.d. = not detected.

## 4 DISCUSSION

The isolation of *Fusarium* species from Brazilian rice grains confirm the importance of FFSC members as main contaminants of rice grains in Brazil. Our data showed that several species of FFSC are associated with rice grains and seeds. Beside that, our data showed that the population diversity of FFSC in association with rice in Brazil is wider of what reported worldwide. The main species of FFSC, commonly associated to rice, are *F. fujikuroi*, *F. verticillioides*, *F. proliferatum* and *F. anthophilum*, detected on Brazilian rice grains as well. However, we detected other species never previously reported such as *F. pseudocircinatum* and *F. sterilihyphosum*. These latter two species have been reported as pathogens of mango, in Brazil (Lima et al., 2009), in Mexico (Freeman et al., 2014) and Dominican Republic (García-López et al., 2016). In addition, to the above mentioned we isolated from rice two new lineages that we designated as *Fusarium* sp. 1 and *Fusarium* sp. 2. The *Fusarium* sp. 1 in our phylogenetic analysis have 100% the bootstrap support with strains NRRL 25615 that was originally isolated in Nigeria from rice seeds (Laurence et al., 2015). The other new lineage, *Fusarium* sp. 2 is a sister clade of *F. andiyazi*, originally isolated from sorghum in Africa

(Marasas et al., 2001), and detected from rice seed samples collected in Africa, Asia and California (Wulff et al., 2010).

Species of FFSC isolated from rice in Brazil rice reproduce primarily asexually. The 100 strains analyzed, showed a ratio of *MAT1-1* and *MAT1-2*, the two mating type genes, of 0.58, that is significantly lower than the ratio 1:1, as should be in a population in which sexual reproduction occur. In Brazil, no reports on sexual reproduction of FFSC members isolated from rice are available. On the other hand, no symptoms on rice plants similar to those of bakanae disease. Thus, we suggest that species of FFSC have endophyte lifestyle on rice and crop of rice can be a reservoir of pathogens. However, the monitoring of species of FFSC on rice to needs attention in Brazil, especially since the species that we detected associated to rice produce mycotoxins (Marasas et al., 2004; Proctor et al., 2013).

For the two new lineages that we have identified in this study, no information about the potential of production of mycotoxins is available. However, the species are sister of *F. verticillioides* and *F. andiyazi*, and since both are fumonisin producing species, the possibility that both can be mycotoxins producers is considerable. Moreover, *F. sterilihyphosum* and *F. pseudocircinatum* have been poorly investigated for their capability of producing mycotoxins. Therefore, the contribution of these two species in the contamination of Brazilian rice needs more data. The identification of species from Brazilian rice grains showed a wide diversity of species belonging to FFSC associated with rice. On the other hand, the mycotoxin analysis of the natural samples was performed separately for rice hulls and flour. The data showed that in general there is not imminent risk of contamination on rice grains by micotoxins. Among the species that we found on rice grains, we detected *F. fujikuroi*, *F. verticillioides*, *F. proliferatum* and *F. anthophilum*, all well known mycotoxin producers on several crop products such as maize, wheat, barley, rye, and sorghum kernels (Marasas et al., 2004; Proctor et al., 2004; Proctor et al., 2013).

Thus, the members of FFSC we found on rice grains can contributed with contamination of mycotoxins. Our data from mycotoxin analysis contribute with indication that mycotoxins produced by species of the *Fusarium fujikuroi* complex can occur on rice grains. Beside that, the analysis of natural occurrence of mycotoxins in flour rice in

comparation with hull rice corroborates with affirmation that the dissemination of agent causal is by seeds. Moreover, when the concentration of the pathogen is high, this is reflected by the concentrations of mycotoxin. In Brazil, there is legislation to establishing maximum tolerated levels (MTLs) for a range of mycotoxins in cereals grains and derivatives (ANVISA 2011). For rice, in our country the MTLs were defined for three *Fusarium* mycotoxins: fumonisins, deoxynivalenol and zearalenona (ANVISA 2011). Our data showed that the legislation need take in account the role played by other mycotoxins, such as beauvericin, which occurrence in rice grains was the highest, among the mycotoxin analyzed. Finally, since, no information is available on the potential of production of mycotoxins by the two new lineages detected and by *F. sterilihyphosum* and *F. pseudocircinatum*, more investigations about their ability to produce mycotoxins is needed in order to better evaluate the risk associated to the consumption of rice contaminated by *Fusarium* in Brazil.

## 5 CONCLUSIONS

This is the first report about presence of members of *Fusarium fujikuroi* specie complex on rice grains and the natural contamination of FBs, MON, BEA and ENNs in rice samples. Considering the relevance of rice in the feeding of the Brazilian population, the presence of several species of the FFSC and the natural contamination of mycotoxins, this study indicates the potential health threat of rice due to mycotoxins. The extensive occurrence beauvericin on rice in Brazil is reason of further concern. Therefore, in Brazilian rice, more comprehensive mycotoxin monitoring programs may be needed in rice, due to the emerging *Fusarium* contamination of grains. Moreover, the ability of the news species to produce mycotoxins and the influence of factors as environmental, nutritional or genetic factors on the contamination by mycotoxins on Brazillian rice, requires wider investigations and higher attention.

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Supplementary Table 1 Collection of strains of *Fusarium fujikuroi* of this study and Genbank accession numbers of sequences used to generate the phylogenetic analysis.

| **Code CML[a]** | **Equivalent code[b]** | **Phylogenetic species[c]** | **Geographical origin[d]** | **Year** | **MAT[e]** | **Number Access GenBank[f]** | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | ***TEF*** | ***TUB*** | ***CAL*** |
| 3143 | ITEM 17550 | *F. anthophilum* | Palmares do Sul RS | 2009 | 2 | LS423132 | LS423240 | LS423348 |
| 3144 | ITEM 17551 | *F. anthophilum* | Capivarí do Sul RS | 2012 | 2 | LS423133 | LS423241 | LS423349 |
| 3668 | ITEM 17572 | *F. anthophilum* | Itajaí SC | 2016 | 2 | LS423154 | LS423262 | LS423370 |
| 3669 | ITEM 17573 | *F. anthophilum* | Nova Veneza SC | 2016 | 2 | LS423155 | LS423263 | LS423371 |
| 3684 | ITEM 17585 | *F. anthophilum* | Formoso do Araguaia TO | 2016 | 2 | LS423167 | LS423275 | LS423383 |
| 3774 | ITEM 17599 | *F. anthophilum* | Nova Veneza SC | 2016 | 1 | LS423181 | LS423289 | LS423397 |
| 4003 | ITEM 17651 | *F. anthophilum* | Itajaí SC | 2016 | 2 | LS423233 | LS423341 | LS423449 |
| 3643 | ITEM 17560 | *F. fujikuroi* | Brazabrantes GO | 2015 | 2 | LS423142 | LS423250 | LS423358 |
| 3785 | ITEM 17610 | *F. fujikuroi* | Santo Antônio do Goiás GO | 2015 | 1 | LS423192 | LS423300 | LS423408 |
| 3778 | ITEM 17603 | *F. fujikuroi* | União do Sul MT | 2015 | 2 | LS423185 | LS423293 | LS423401 |
| 3801 | ITEM 17626 | *F. fujikuroi* | Sinop MT | 2015 | 2 | LS423208 | LS423316 | LS423424 |
| 3140 | ITEM 17547 | *F. fujikuroi* | Cachoeirinha RS | 2010 | 2 | LS423129 | LS423237 | LS423345 |
| 3141 | ITEM 17548 | *F. fujikuroi* | Santa Maria RS | 2012 | 2 | LS423130 | LS423238 | LS423346 |
| 3795 | ITEM 17620 | *F. fujikuroi* | Santa Maria RS | 2016 | 2 | LS423202 | LS423310 | LS423418 |
| 3638 | ITEM 17555 | *F. fujikuroi* | Nova Veneza SC | 2016 | 1 | LS423137 | LS423245 | LS423353 |
| 3639 | ITEM 17556 | *F. fujikuroi* | Nova Veneza SC | 2016 | 2 | LS423138 | LS423246 | LS423354 |
| 3640 | ITEM 17557 | *F. fujikuroi* | Nova Veneza SC | 2016 | 1 | LS423139 | LS423247 | LS423355 |

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Supplementary Table 1 Collection of strains of *Fusarium fujikuroi* of this study and Genbank accession numbers of sequences used to generate the phylogenetic analysis.

| **Code CML[a]** | **Equivalent code[b]** | **Phylogenetic species[c]** | **Geographical origin[d]** | **Year** | **MAT[e]** | **Number Access GenBank[f]** | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | ***TEF*** | ***TUB*** | ***CAL*** |
| 3641 | ITEM 17558 | *F. fujikuroi* | Nova Veneza SC | 2016 | 2 | LS423140 | LS423248 | LS423356 |
| 3642 | ITEM 17559 | *F. fujikuroi* | Nova Veneza SC | 2016 | 1 | LS423141 | LS423249 | LS423357 |
| 3691 | ITEM 17590 | *F. fujikuroi* | Nova Veneza SC | 2016 | 2 | LS423172 | LS423280 | LS423388 |
| 3692 | ITEM 17591 | *F. fujikuroi* | Nova Veneza SC | 2016 | 1 | LS423173 | LS423281 | LS423389 |
| 3693 | ITEM 17592 | *F. fujikuroi* | Nova Veneza SC | 2016 | 1 | LS423174 | LS423282 | LS423390 |
| 3784 | ITEM 17609 | *F. fujikuroi* | Garuva SC | 2016 | 2 | LS423191 | LS423299 | LS423407 |
| 3790 | ITEM 17615 | *F. fujikuroi* | Itajaí SC | 2016 | 1 | LS423197 | LS423305 | LS423413 |
| 3791 | ITEM 17616 | *F. fujikuroi* | Jacinto Machado SC | 2016 | 1 | LS423198 | LS423306 | LS423414 |
| 3792 | ITEM 17617 | *F. fujikuroi* | Itajaí SC | 2016 | 1 | LS423199 | LS423307 | LS423415 |
| 3798 | ITEM 17623 | *F. fujikuroi* | Nova Veneza SC | 2016 | 2 | LS423205 | LS423313 | LS423421 |
| 3809 | ITEM 17634 | *F. fujikuroi* | Nova Veneza SC | 2016 | 2 | LS423216 | LS423324 | LS423432 |
| 3826 | ITEM 17650 | *F. fujikuroi* | Jacinto Machado SC | 2016 | 2 | LS423232 | LS423340 | LS423448 |
| 4004 | ITEM 17652 | *F. fujikuroi* | Garuva SC | 2016 | 1 | LS423234 | LS423342 | LS423450 |
| 3687 | ITEM 17588 | *F. proliferatum* | Santo Antônio do Goiás GO | 2015 | 1 | LS423170 | LS423278 | LS423386 |
| 3782 | ITEM 17607 | *F. proliferatum* | Brazabrantes GO | 2015 | 2 | LS423189 | LS423297 | LS423405 |
| 3786 | ITEM 17611 | *F. proliferatum* | Santo Antônio do Goiás GO | 2015 | 2 | LS423193 | LS423301 | LS423409 |
| 3796 | ITEM 17621 | *F. proliferatum* | Brazabrantes GO | 2015 | 2 | LS423203 | LS423311 | LS423419 |
| 3802 | ITEM 17627 | *F. proliferatum* | Brazabrantes GO | 2015 | 2 | LS423209 | LS423317 | LS423425 |
| 3644 | ITEM 17561 | *F. proliferatum* | Sinop MT | 2015 | 2 | LS423143 | LS423251 | LS423359 |

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Supplementary Table 1 Collection of strains of *Fusarium fujikuroi* of this study and Genbank accession numbers of sequences used to generate the phylogenetic analysis.

| **Code CML[a]** | **Equivalent code[b]** | **Phylogenetic species[c]** | **Geographical origin[d]** | **Year** | **MAT[e]** | **Number Access GenBank[f]** | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | ***TEF*** | ***TUB*** | ***CAL*** |
| 3645 | ITEM 17562 | *F. proliferatum* | Sinop MT | 2015 | 2 | LS423144 | LS423252 | LS423360 |
| 3685 | ITEM 17586 | *F. proliferatum* | Sinop MT | 2015 | 1 | LS423168 | LS423276 | LS423384 |
| 3686 | ITEM 17587 | *F. proliferatum* | União do Sul MT | 2015 | 1 | LS423169 | LS423277 | LS423385 |
| 3741 | ITEM 17593 | *F. proliferatum* | União do Sul MT | 2015 | 2 | LS423175 | LS423283 | LS423391 |
| 3772 | ITEM 17597 | *F. proliferatum* | Sinop MT | 2015 | 2 | LS423179 | LS423287 | LS423395 |
| 3781 | ITEM 17606 | *F. proliferatum* | Sinop MT | 2015 | 1 | LS423188 | LS423296 | LS423404 |
| 3794 | ITEM 17619 | *F. proliferatum* | União do Sul MT | 2015 | 2 | LS423201 | LS423309 | LS423417 |
| 3138 | ITEM 17545 | *F. proliferatum* | Palmares do Sul RS | 2009 | 1 | LS423127 | LS423235 | LS423343 |
| 3139 | ITEM 17546 | *F. proliferatum* | Cachoeirinha RS | 2009 | 2 | LS423128 | LS423236 | LS423344 |
| 3647 | ITEM 17564 | *F. proliferatum* | Santa Maria RS | 2016 | 2 | LS423146 | LS423254 | LS423362 |
| 3646 | ITEM 17563 | *F. proliferatum* | Itajaí SC | 2016 | 1 | LS423145 | LS423253 | LS423361 |
| 3688 | ITEM 17589 | *F. proliferatum* | Nova Veneza SC | 2016 | 1 | LS423171 | LS423279 | LS423387 |
| 3775 | ITEM 17600 | *F. proliferatum* | Nova Veneza SC | 2016 | 2 | LS423182 | LS423290 | LS423398 |
| 3813 | ITEM 17638 | *F. proliferatum* | Nova Veneza SC | 2016 | 2 | LS423220 | LS423328 | LS423436 |
| 3815 | ITEM 17640 | *F. proliferatum* | Nova Veneza SC | 2016 | 1 | LS423222 | LS423330 | LS423438 |
| 3821 | ITEM 17645 | *F. proliferatum* | Formoso do Araguaia TO | 2015 | 1 | LS423227 | LS423335 | LS423443 |
| 3789 | ITEM 17614 | *F. pseudocircinatum* | Paraibano MA | 2016 | 2 | LS423196 | LS423304 | LS423412 |
| 3742 | ITEM 17594 | *F. pseudocircinatum* | Boa Vista RR | 2016 | 2 | LS423176 | LS423284 | LS423392 |
| 3145 | ITEM 17552 | *F. pseudocircinatum* | Palmares do Sul RS | 2009 | 2 | LS423134 | LS423242 | LS423350 |

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Supplementary Table 1 Collection of strains of *Fusarium fujikuroi* of this study and Genbank accession numbers of sequences used to generate the phylogenetic analysis.

| **Code CML[a]** | **Equivalent code[b]** | **Phylogenetic species[c]** | **Geographical origin[d]** | **Year** | **MAT[e]** | **Number Access GenBank[f]** | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | ***TEF*** | ***TUB*** | ***CAL*** |
| 3683 | ITEM 17584 | *F. pseudocircinatum* | Nova Veneza SC | 2015 | 1 | LS423166 | LS423274 | LS423382 |
| 3142 | ITEM 17549 | *F. sterilihyphosum* | Pelotas RS | 2012 | 2 | LS423131 | LS423239 | LS423347 |
| 3670 | ITEM 17574 | *F. sterilihyphosum* | São Sepé RS | 2016 | 2 | LS423156 | LS423264 | LS423372 |
| 3676 | ITEM 17576 | *F. verticillioides* | Brazabrantes GO | 2015 | 2 | LS423158 | LS423266 | LS423374 |
| 3800 | ITEM 17625 | *F. verticillioides* | Brazabrantes GO | 2015 | 1 | LS423207 | LS423315 | LS423423 |
| 3808 | ITEM 17633 | *F. verticillioides* | Paraibano MA | 2016 | 1 | LS423215 | LS423323 | LS423431 |
| 3771 | ITEM 17596 | *F. verticillioides* | Boa Vista RR | 2016 | 2 | LS423178 | LS423286 | LS423394 |
| 3773 | ITEM 17598 | *F. verticillioides* | Boa Vista RR | 2016 | 2 | LS423180 | LS423288 | LS423396 |
| 3146 | ITEM 17553 | *F. verticillioides* | Caçapava do Sul RS | 2012 | 2 | LS423135 | LS423243 | LS423351 |
| 3425 | ITEM 17554 | *F. verticillioides* | Pelotas RS | 2012 | 2 | LS423136 | LS423244 | LS423352 |
| 3652 | ITEM 17568 | *F. verticillioides* | Uruguaiana RS | 2015 | 1 | LS423150 | LS423258 | LS423366 |
| 3653 | ITEM 17569 | *F. verticillioides* | Arroio Grande RS | 2015 | 1 | LS423151 | LS423259 | LS423367 |
| 3654 | ITEM 17570 | *F. verticillioides* | Arroio Grande RS | 2015 | 2 | LS423152 | LS423260 | LS423368 |
| 3678 | ITEM 17577 | *F. verticillioides* | Quaraí RS | 2015 | 1 | LS423159 | LS423267 | LS423375 |
| 3679 | ITEM 17578 | *F. verticillioides* | Santa Vitória do Palmar RS | 2015 | 1 | LS423160 | LS423268 | LS423376 |
| 3788 | ITEM 17613 | *F. verticillioides* | Uruguaiana RS | 2015 | 1 | LS423195 | LS423303 | LS423411 |
| 3799 | ITEM 17624 | *F. verticillioides* | Quaraí RS | 2015 | 1 | LS423206 | LS423314 | LS423422 |
| 3655 | ITEM 17571 | *F. verticillioides* | Nova Veneza SC | 2016 | 1 | LS423153 | LS423261 | LS423369 |
| 3675 | ITEM 17575 | *F. verticillioides* | Nova Veneza SC | 2016 | 2 | LS423157 | LS423265 | LS423373 |

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Supplementary Table 1 Collection of strains of *Fusarium fujikuroi* of this study and Genbank accession numbers of sequences used to generate the phylogenetic analysis.

| **Code CML[a]** | **Equivalent code[b]** | **Phylogenetic species[c]** | **Geographical origin[d]** | **Year** | **MAT[e]** | **Number Access GenBank[f]** | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | ***TEF*** | ***TUB*** | ***CAL*** |
| 3689 | ITEM 17581 | *Fusarium* sp. 1 | Sinop MT | 2015 | 2 | LS423163 | LS423271 | LS423379 |
| 3690 | ITEM 17582 | *Fusarium* sp. 1 | Sinop MT | 2015 | 1 | LS423164 | LS423272 | LS423380 |
| 3770 | ITEM 17595 | *Fusarium* sp. 1 | Sinop MT | 2015 | 1 | LS423177 | LS423285 | LS423393 |
| 3779 | ITEM 17604 | *Fusarium* sp. 1 | Sinop MT | 2015 | 1 | LS423186 | LS423294 | LS423402 |
| 3803 | ITEM 17628 | *Fusarium* sp. 1 | Sinop MT | 2015 | 1 | LS423210 | LS423318 | LS423426 |
| 3804 | ITEM 17629 | *Fusarium* sp. 1 | Sinop MT | 2015 | 1 | LS423211 | LS423319 | LS423427 |
| 3806 | ITEM 17631 | *Fusarium* sp. 1 | Sinop MT | 2015 | 1 | LS423213 | LS423321 | LS423429 |
| 3807 | ITEM 17632 | *Fusarium* sp. 1 | Sinop MT | 2015 | 2 | LS423214 | LS423322 | LS423430 |
| 3651 | ITEM 17567 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | 2 | LS423149 | LS423257 | LS423365 |
| 3682 | ITEM 17583 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | 2 | LS423165 | LS423273 | LS423381 |
| 3780 | ITEM 17605 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | 2 | LS423187 | LS423295 | LS423403 |
| 3787 | ITEM 17612 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | 2 | LS423194 | LS423302 | LS423410 |
| 3819 | ITEM 17643 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | 2 | LS423225 | LS423333 | LS423441 |
| 3812 | ITEM 17637 | *Fusarium* sp. 2 | Tangará da Serra MT | 2016 | 2 | LS423219 | LS423327 | LS423435 |
| 3648 | ITEM 17565 | *Fusarium* sp. 2 | Itajaí SC | 2016 | 2 | LS423147 | LS423255 | LS423363 |
| 3649 | ITEM 17566 | *Fusarium* sp. 2 | Itajaí SC | 2016 | 1 | LS423148 | LS423256 | LS423364 |
| 3680 | ITEM 17579 | *Fusarium* sp. 2 | Itajaí SC | 2016 | 2 | LS423161 | LS423269 | LS423377 |
| 3681 | ITEM 17580 | *Fusarium* sp. 2 | Itajaí SC | 2016 | 1 | LS423162 | LS423270 | LS423378 |
| 3793 | ITEM 17618 | *Fusarium* sp. 2 | Itajaí SC | 2016 | 2 | LS423200 | LS423308 | LS423416 |

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Supplementary Table 1 Collection of strains of *Fusarium fujikuroi* of this study and Genbank accession numbers of sequences used to generate the phylogenetic analysis.

| **Code CML[a]** | **Equivalent code[b]** | **Phylogenetic species[c]** | **Geographical origin[d]** | **Year** | **MAT[e]** | **Number Access GenBank[f]** | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | ***TEF*** | ***TUB*** | ***CAL*** |
| 3805 | ITEM 17630 | *Fusarium* sp. 2 | Itajaí SC | 2016 | 2 | LS423212 | LS423320 | LS423428 |
| 3810 | ITEM 17635 | *Fusarium* sp. 2 | Itajaí SC | 2016 | 2 | LS423217 | LS423325 | LS423433 |
| 3814 | ITEM 17639 | *Fusarium* sp. 2 | Itajaí SC | 2016 | 2 | LS423221 | LS423329 | LS423437 |
| 3816 | ITEM 17641 | *Fusarium* sp. 2 | Garuva SC | 2016 | 2 | LS423223 | LS423331 | LS423439 |
| 3818 | ITEM 17642 | *Fusarium* sp. 2 | Itajaí SC | 2016 | 2 | LS423224 | LS423332 | LS423440 |
| 3822 | ITEM 17646 | *Fusarium* sp. 2 | Garuva SC | 2016 | 2 | LS423228 | LS423336 | LS423444 |
| 3824 | ITEM 17648 | *Fusarium* sp. 2 | Garuva SC | 2016 | 2 | LS423230 | LS423338 | LS423446 |
| - | CBS 119857 T; ITEM 6032 | *F. andiyazi* | South Africa | | | KP662901 | KP662894 | KU603953 |
| - | ITEM 7594 | *F. andiyazi* | | | | N.P. | N.P. | N.P. |
| - | NRRL 13602 | *F. anthophilum* | Germany | | | AF160292 | U61541 | AF158345 |
| - | NRRL 25300 T | *F. begoniae* | Germany | | | AF160293 | U61543 | AF158346 |
| - | NRRL 66233 T | *F. coicis* | Australia | | | KP083251 | - | - |
| - | NRRL 13566 | *F. fujikuroi* | Taiwan | | | AF160279 | U34415 | AF158332 |
| - | NRRL 26131 | *F. globosum* | South Africa | | | AF160285 | U61557 | AF158338 |
| - | CBS 409.97 | *F. guttiforme* | Brazil | | | KC514066 | - | - |
| - | NRRL 25059 T | *F. musae* | Honduras | | | FN552086 | FN545368 | FN552064 |
| - | NRRL 22944 | *F. proliferatum* | Germany | | | AF160280 | U34416 | AF158333 |
| - | NRRL 22946 T | *F. pseudocircinatum* | Ghana | | | AF160271 | U34453 | AF158324 |

Continue 6/7

Supplementary Table 1 Collection of strains of *Fusarium fujikuroi* of this study and Genbank accession numbers of sequences used to generate the phylogenetic analysis.

| **Code CML**[a] | **Equivalent code**[b] | **Phylogenetic species**[c] | **Geographical origin**[d] | **Year** | **MAT**[e] | **Number Access GenBank**[f] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | ***TEF*** | ***TUB*** | ***CAL*** |
| | NRRL 53573 | *F. pseudocircinatum* | | | | GU737399 | GU737345 | GU737372 |
| 283 T | - | *F. sterilihyphosum* | Brazil | | | DQ452858 | DQ445780 | - |
| - | NRRL 25623 | *F. sterilihyphosum* | | | | GU737414 | GU737360 | GU737387 |
| 262 T | - | *F. tupiense* | Brazil | | | DQ452859 | DQ445781 | - |
| - | NRRL 22172 | *F. verticillioides* | Germany | | | AF160262 | U34413 | AF158315 |
| - | NRRL 25615 | *Fusarium* sp. | Nigeria | | | AF160304 | AF160348 | AF158357 |
| - | NRRL 20433 | *F. inflexum* | Germany | | | AF008479 | U34490 | AF158366 |
| - | NRRL 22902 | *F. oxysporum* | United States | | | AF160312 | N.P. | AF158365 |

[a,b]Culture collection abbreviations: CML – Coleção Micológica de Lavras, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil; ITEM – Microbial Culture Collection of Institute of Sciences and of Food Production (ISPA), Puglia, Bari, Italy; NRRL – National Center for Agricultural Utilization Research, Peoria, Illinois, USA; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. [c,f]Phylogenetic identification by three housekeeping genes: *TEF* – elongation factor 1-α, *CAL* – calmudolin and *TUB* – β – tubulin; N.P. = not published. [d]Brazilian cities and states: RS – Rio Grande do Sul, SC – Santa Catarina, GO – Goiás, MT – Mato Grosso, RR – Roraima, TO – Tocantins, MA – Maranhão. [e]Mating type: 1 – *MAT1-1*, 2 – *MAT1-2*.

Supplementary Table 2 Analysis of mycotoxins of 54 rice samples.

| Samples | States | Harvest year | Mycotoxins (ng/g)[a] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FB1 | | FB2 | | MON | | BEA | | ENNA | | ENNA1 | | ENNB | | ENNB1 | |
| | | | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour |
| 1 | GO | 2015 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | 40,5 | 52,9 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | < LOQ |
| 2 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 38,1 | 43,3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 3 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 24,6 | 32 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 4 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 24,5 | 20,1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 5 | GO | 2015 | < LOQ | n.d. | n.d. | n.d. | n.d. | n.d. | 23,2 | 54,2 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | n.d. | n.d. |
| 6 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 15,3 | 14 | n.d. | n.d. | n.d. | n.d. | 2,6 | < LOQ | 1,2 | < LOQ |
| 7 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 9,7 | 11,7 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 8 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 9,5 | 10,7 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 9 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 8,7 | 10 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 10 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 6,7 | 29,9 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 11 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 6,4 | 22,2 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | n.d. | n.d. |
| 12 | GO | 2015 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | 6 | 12,6 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 13 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 3,2 | 27,4 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | 0,5 | n.d. |
| 14 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2,9 | 7,4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 15 | GO | 2015 | n.d. | n.d. | n.d. | < LOQ | n.d. | n.d. | 2,8 | 4,2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 16 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2,8 | 3,3 | < LOQ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 17 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2,5 | 15 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2,1 | 4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 19 | GO | 2015 | n.d. | < LOQ | n.d. | n.d. | n.d. | n.d. | 2 | 1,8 | n.d. | n.d. | n.d. | n.d. | n.d. | < LOQ | n.d. | n.d. |
| 20 | GO | 2015 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | 1,8 | 3,8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

Continue 1/3

Supplementary Table 2 Analysis of mycotoxins of 54 rice samples.

| Samples | States | Harvest year | Mycotoxins (ng/g)[a] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FB1 | | FB2 | | MON | | BEA | | ENNA | | ENNA1 | | ENNB | | ENNB1 | |
| | | | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour |
| 21 | GO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1,6 | 5,8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 22 | MT | 2015 | n.d. | n.d. | n.d. | n.d. | 13,3 | 28,1 | 110,4 | 810,1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 23 | MT | 2015 | n.d. | n.d. | < LOQ | n.d. | n.d. | < LOQ | 57,6 | 91 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 24 | MT | 2015 | n.d. | n.d. | < LOQ | n.d. | n.d. | < LOQ | 39,7 | 100 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 25 | MT | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 9,9 | 6,8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 26 | MT | 2016 | n.d. | < LOQ | n.d. | n.d. | n.d. | < LOQ | 8,1 | 30,4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 27 | MT | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 5,5 | 7,5 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 28 | MT | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1,2 | 6,2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 29 | RR | 2016 | n.d. | 4,1 | n.d. | n.d. | n.d. | 2,6 | 8,3 | 68,3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 30 | RS | 2016 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | 11,1 | 30,2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 31 | RS | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 3,6 | 13 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 32 | RS | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 3,5 | 7,6 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 33 | RS | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 3,4 | 25,1 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | n.d. | 0,5 |
| 34 | RS | 2015 | n.d. | n.d. | 2,9 | n.d. | n.d. | n.d. | 2,9 | 2 | < LOQ | n.d. | < LOQ | n.d. | n.d. | n.d. | n.d. | n.d. |
| 35 | RS | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2,8 | 7,8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 38 | RS | 2015 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | 2,7 | 5,7 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | n.d. | n.d. |
| 39 | RS | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2,6 | 6,1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 40 | RS | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2,5 | 3,1 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | n.d. | n.d. |
| 41 | RS | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2,5 | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 42 | RS | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1,8 | 7,8 | n.d. | n.d. | n.d. | n.d. | n.d. | < LOQ | n.d. | n.d. |

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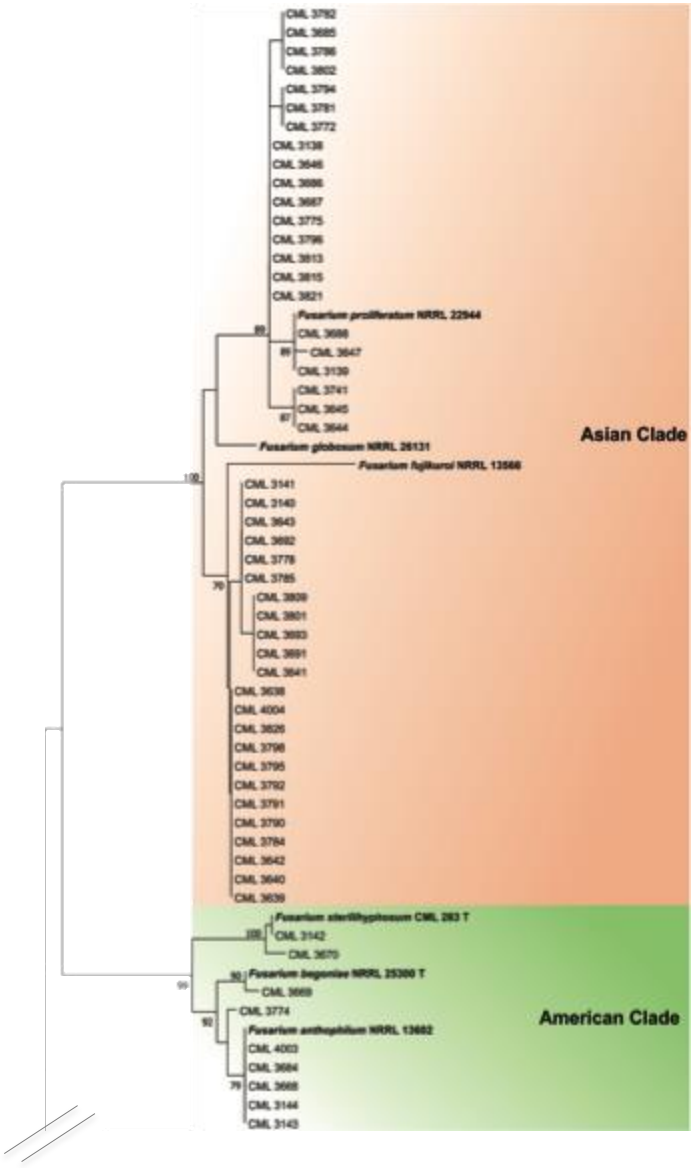
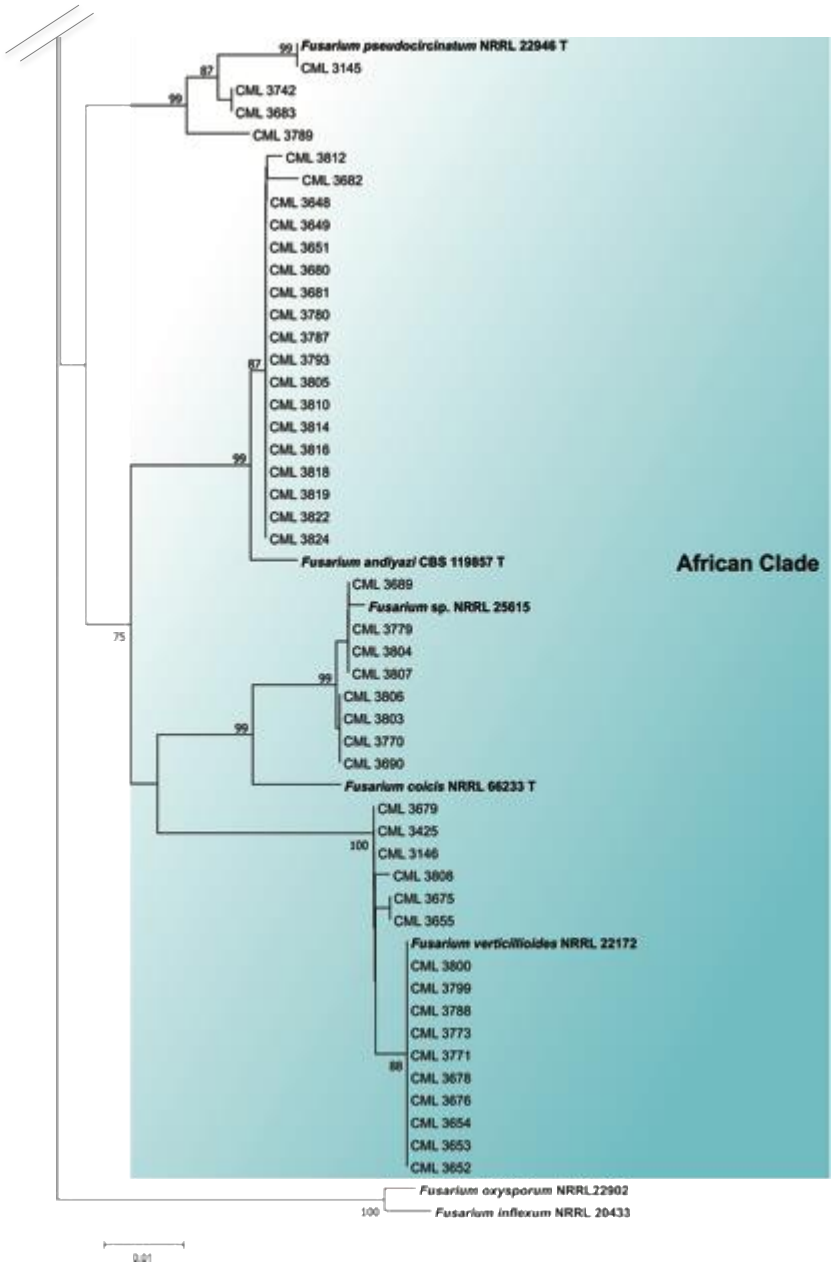
Supplementary Table 2 Analysis of mycotoxins of 54 rice samples.

| Samples | States | Harvest year | Mycotoxins (ng/g)[a] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FB1 | | FB2 | | MON | | BEA | | ENNA | | ENNA1 | | ENNB | | ENNB1 | |
| | | | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour | Hull | Flour |
| 43 | SC | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 8,2 | 1,5 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 44 | SC | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2,5 | 5,3 | n.d. | n.d. | n.d. | n.d. | n.d. | < LOQ | n.d. | n.d. |
| 45 | SC | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1,1 | 1,7 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 46 | SP | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 6,8 | 19,3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 47 | SP | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1,6 | 14,2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 48 | SP | 2016 | n.d. | < LOQ | n.d. | n.d. | n.d. | n.d. | 1,5 | 7,4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 49 | SP | 2016 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | 1,3 | 8,2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 50 | TO | 2015 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 21,7 | 39,7 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 51 | TO | 2016 | n.d. | n.d. | < LOQ | n.d. | n.d. | n.d. | 3,4 | 10,1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 52 | TO | 2015 | n.d. | n.d. | n.d. | < LOQ | n.d. | n.d. | 2,4 | 5,5 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 53 | TO | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1,3 | 8,5 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 54 | TO | 2016 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0,2 | 4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

[a]Abbreviations: Mycotoxins: FB1 = fumonisin B1; FB2 = fumonisin B2; MON = moniliformin; BEA = beauvericin; n.d. = not detectable; LOQ = low limits of detection:FB1 = 3,9 μg/g, FB2 = 2,0 μg/g; MON = 1,9 μg/g; BEA = 1,0 μg/g; ENNA = 0,5 μg/g; ENNA1 = 1,0 μg/g; ENNB = 1,0 μg/g; ENNB1 = 0,5 μg/g.




Suppl. Figure 1 Phylogenetic tree inferred from partial of *TEF* sequences (649 bp) from all members of the *Fusarium fujikuroi* species complex isolates analyzed in this study. The evolutionary distances were computed using the Maximum Composite Likelihood method and the numbers on branches are bootstrap values based on 1000 replicates (Continue).




Suppl. Figure 1 Phylogenetic tree inferred from partial of *TEF* sequences (649 bp) from all members of the *Fusarium fujikuroi* species complex isolates analyzed in this study. The evolutionary distances were computed using the Maximum Composite Likelihood method and the numbers on branches are bootstrap values based on 1000 replicates.

# ARTICLE 2 - ANALYSIS OF *FUM* GENES AND FUMONISIN PRODUCTION ABILITY IN *FUSARIUM FUJIKUROI* SPECIES COMPLEX FROM BRAZILIAN RICE

*Manuscript prepared for submission to Tropical Plant Pathology*

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**ABSTRACT**

Fumonisins (FBs) are the main mycotoxins produced by species of *Fusarium* belonging to *Fusarium fujikuroi* species complex (FFSC). The fumonisin biosynthetic pathway has been well recognized and described for some species. Since the lack of information about the mycotoxin ability of species isolated from Brazilian rice since 2008, the main aim of this study was to evaluate the mycotoxigenic potential of species of FFSC isolated from Brazilian rice grains. Sixty-one FFSC strains from rice were analyzed for the occurrence of genes involved in fumonisin metabolic pathways as *FUM1*, *FUM8* and *FUM14*. The data obtained confirmed that at least one strain of all the tropical species of FFSC isolated from rice grains in Brazil, had *FUM* genes. The strains were also investigated for their ability to produce fumonisins *in vitro* rice cultures. Among the tropical species, *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* produced FB1, FB2 and FB3. *F. pseudocircinatum*, *Fusarium* sp. 1 and *Fusarium* sp. 2 produced only FB1 while *F. anthophilum* and *F. sterilihyphosum* did not produce FBs. In this study we investigated for the first time the fumonisin ability of FFSC members associated to rice grains in Brazil. As rice is a staple crop for the population in Brazil, the importance of fumonisin occurrence in rice needs to be further clarified. In particular, since strains of the new lineages identified on rice grains produced fumonisins *in vitro* cultures, more detailed analysis on the occurrence of *FUM* genes in their genomes is needed. Our results contribute to generate new knowledge on the ability of tropical members of FFSC associated with rice grains, to produce mycotoxins which is of value for improving models for assessing the risk of mycotoxin production on rice in Brazil.

**Key words:** *Oryza sativa*, *FUM* genes, phylogeny analysis, fumonisins.

## 1 INTRODUCTION

Rice is a crop to tropical and subtropical areas of Brazil, where it holds an economic importance, mainly in the south Brazil, where is the greater production of rice (FAO, 2018). Bakanae disease has been recorded almost in all countries where paddy rice is grown commercially (Zainudin et al., 2008; Hossain et al., 2016). In Brazil, the rice culture is susceptible to a number of fungal diseases, of which those caused by *Fusarium* species are not the most severe and important (Prabhu et al., 2007). On the other hand, the importance of *Fusarium* diseases is related to the ability of *Fusarium* species to be micotoxigenic, being able to produce a wide array of secondary metabolites in several agro-food products (Marasas et al., 2004). Based on the in vitro and in planta mycotoxin synthesis comparison of the *Fusarium fujikuroi* species complex (FFSC), the causal agent of bakanae disease, one should consider fumonisins (FBs) as likely major secondary metabolites (Kvas et al. 2009). To date, there is little information on secondary metabolites produced by *Fusarium* species in infected rice grains in Brazil (Majeed et al., 2018) and there is less information about secondary metabolites produced by FFSC on rice (Becker-Algeri et al., 2013). However, at the worldwide level, the fumonisins are frequently associated to rice (Majeed et al., 2018).

The fumonisin biosynthetic gene pathway has been well recognized and described (Proctor et al. 2013) and *FUM* genes (particularly *FUM1*, *FUM8* and *FUM14*) have often been used for studies on fumonisin producing fungi (Waalwijk et al. 2004, Proctor et al. 2008). The progress in the studies focused on the fumonisin biosynthetic gene pathway has been recently reported (Proctor et al., 2013). The species like *F. verticillioides, F. proliferatum* and *F. fujikuroi* are the main species able to synthesize fumonisins (Proctor et al., 2013). However, others species members of FFSC can producer fumonisin type C, as *F. anthophilum* (Proctor et al., 2013). In lack information about the ability of species isolated of Brazilian rice since 2008, the main aim of this study was to evaluate the mycotoxigenic potential of strains of tropical *Fusarium fujikuroi* species isolated from Brazilian rice grains. To develop the latter information, the steps of this study were (i) identify the key genes of fumonisin metabolic pathway in the different species isolated from rice grains in Brazil; (ii)

evaluate the evolutionary relationships among *FUM* genes occurring in different species; and (iii) to detect the ability of species of the FFSC to produce FB1, FB2 and FB3 in vitro rice cultures.

## 2 MATERIAL AND METHODS

### 2.1 Collection of strains

Sixty-one strains of *Fusarium fujikuroi* species complex were isolated from rice grains samples from states of Brazil and characterized morphologically and identified by phylogenetic relationships with housekeeping genes *TEF*, *CAL* and *TUB* (Nicolli et al., 2019). Among that strains there are six species and two new lineages: *F. anthophilum*, *F. fujikuroi*, *F. proliferatum*, *F. pseudocircinatum*, *F. sterilihyphosum*, *F. verticillioides*, *Fusarium* sp. 1 and *Fusarium* sp. 2. All the strains are deposited in the Mycological Collection of Lavras (CML), Department of Plant Pathology of the Federal University of Lavras, Brazil and ITEM Microbial Culture Collection of Institute of Sciences and of Food Production (ISPA), Italy.

### 2.2 DNA extraction, primers and PCR assays

Mycelia of the strains studied were grown on solid PDA. DNA was extracted and purified from fresh using the "Wizard® Magnetic DNA Purification System for Food" kit (Promega, USA) according to the manufacturer's protocol. Phylogenetic relationships of strains were investigated by amplifying and sequencing the fumonisin biosynthetic genes: *FUM1*, *FUM8* and *FUM14* (Proctor et al., 2004). Primers used to amplify and sequence *FUM1*, *FUM8* and *FUM14* were described sequences for these genes (Proctor et al., 2004), using the following PCR program: denaturation at 98 °C for 5 min; 40 cycles of the denaturation at 98 °C for 5 s, annealing at 55°C for 20s, extension at 72 °C for 1 min; and final extension at 72 °C for 1 min.

### 2.3 Sequence and phylogenetic analysis

Before sequencing, PCR products were purified with the enzymatic mixture EXO/FastAP (Exonuclease I, FastAP thermosensitive alkaline phosphatase, Thermo Scientific, Lithuania, Europe). Sequence reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit for both strands, after which they were purified by gel filtration through Sephadex G-50 (5%) (Sigma Aldrich), before they were analyzed on the 3730xl DNA Analyzer (Applied Biosystems – HITACHI). In order to evaluate the phylogenetic relationships of biosynthesis of fumonisins among the strains studied, DNA sequences and aligned were determined by BioNumerics Seven software platform (Applied Maths) and phylogenetic analysis by the Clustal W algorithm (Thompson et al., 1994) with MEGA 7.0 software (Tamura et al., 2013). All gene sequences were analyzed using the maximum likelihood approach and the bootstrap with 1,000 replicates. The sequences of strains obtained were compared with sequences from strains of reference species in the FFSC already available in GenBank from National Center for Agricultural Utilization Research, Peoria, Illinois, USA (NRRL) (*F. ramigenum* NRRL 25208, *F. anthophilum* NRRL 25214, *F. fujikuroi* HKM 41, *F. proliferatum* ITEM 2287). The sequences of *Fusarium oxysporum* O-1890 and *Fusarium bulbicola* NRRL 13618 were outgroup.

**2.4 Mycotoxins analysis**

*Preparation of rice cultures.* Erlenmeyer flasks (250 mL) were filled with 30 g rice kernels, 13 mL distilled water and autoclaved for 30 min at 121 °C. After cooling each flask was inoculated with 5 plugs of 0.6 cm of diameter of each culture. Flask cultures were incubated in the dark 21 days at 25 °C. During the first 3 days flaks were shaken once daily to distribute the inoculum more evenly. After 21 days the culture were transferred into aluminum bags and were dried for 48 hours at 65 °C and then crushed. Controls were treated the same way, except that they were not inoculated. The mycotoxins extraction and the analysis were performed according to each mycotoxin bellow.

*Determination of FB1, FB2 and FB3.* One gram of rice culture was extracted with 5 mL of methanol/water (75:25, v/v). Samples were placed for 60' in an orbital shaker, then were filtered using whatman no. 4 filters (Maidstone, UK). Five hundred microlitters was

diluted with 500 µL ultrapure water (Millipore, Bedford, MA). 50 µL of the extract was derivatized with 50 µL of o-phtaldialdehyde (OPA) mixed for 50 sec. using the HPLC autosampler Agilent 1100 (Agilent, Waldbronn, Germany) equipped with a binary pump, column thermostat set at 30°C, 100 µl was injected by full loop at 3 min after adding the OPA reagent for fumonisins analysis. The analytical column was a SymmetryShield RP18 15 cm x 4,6 mm, 5 µm (Waters) with a guard column inlet filter (0.5 µm x 3 mm diameter, Rheodyne Inc. CA, USA) and the mobile phase consisted of a binary gradient was applied as follows: the initial composition of the mobile phase 57% of (A) water/ acetic acid (99:1, v/v) / 43% of (B) acetonitrile/ acetic acid (99:1, v/v) was kept constant for 5 min, then B solvent was linearly increased to 54% in 21 min, then up to 58% at 25 min and kept constant for 5 min. The flow rate of the mobile phase was 0.8 mL/min. The fluorometric detector was set at wavelengths, ex = 335 nm, em = 440 nm. Retention time $FB_1$ was about 16.6 min., $FB_2$ 24.6 min. and $FB_3$ 26.0 min. $FB_s$ were quantified by measuring peak areas, and comparing them with a calibration curves obtained with standard solutions. Detection limit was 0.02 µg/g based on a signal-to-noise ratio of 3:1 for $FB_1$ and $FB_2$.

## 3 RESULTS

### 3.1 Identification of fumonisin biosynthetic genes

The presence of *FUM1* (encoding the polyketide synthase), *FUM8* (PLP-dependent aminotransferase from the fumonisin biosynthetic gene cluster) and *FUM14* genes (catalyzed esterification of CoA-activated tricarballylic acid to the C-14 and C-15 hydroxyls of the fumonisin backbone) was confirmed in at least one strain of all the tropical *Fusarium fujikuroi* species that we found in association with Brazilian rice grains (Table 1). *FUM14* was the gene that showed the minor exceptions. Namely, the *F. anthophilum* CML 3684, *F. fujikuroi* CML 3801, *F. pseudocircinatum* CML 3145, *F. sterilihyphosum* CML 3142, *F. verticillioides* CML 3800 and *Fusarium* sp. 1 CML 3804 and CML 3806 strains gave no amplification for *FUM14*. On the other hand, the genes *FUM1* and *FUM8* for more than one strain did not amplify for the species *F. anthophilum*, *F. fujikuroi*, *F. proliferatum*, *F. pseudocircinatum* and *Fusarium* sp. 1.

Table 1 Sixty-one strains of eigth members of *Fusarium fujikuroi* species complex used in this study: their codes collection, geographical origin, year of isolation, the presence of *FUM* genes from the fumonisin biosynthetic pathway and amounts of mycotoxins synthesized in rice culture.

| **Code CML**[a] | **Code ITEM**[b] | **Phylogenetic species**[c] | **Origin**[d] | **Year** | **Fumonisin genes**[e] | | | **Mycotoxins (µg/g)**[f] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | ***FUM1*** | ***FUM8*** | ***FUM14*** | **FB1** | **FB2** | **FB3** |
| 3144 | 17551 | *F. anthophilum* | Capivarí do Sul RS | 2012 | + | + | + | n.d. | n.d. | n.d. |
| 3684 | 17585 | *F. anthophilum* | Formoso do Araguaia TO | 2016 | - | - | - | n.d. | n.d. | n.d. |
| 3668 | 17572 | *F. anthophilum* | Itajaí SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 4003 | 17651 | *F. anthophilum* | Itajaí SC | 2016 | + | - | + | n.d. | n.d. | n.d. |
| 3669 | 17573 | *F. anthophilum* | Nova Veneza SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3774 | 17599 | *F. anthophilum* | Nova Veneza SC | 2016 | - | + | + | n.d. | n.d. | n.d. |
| 3143 | 17550 | *F. anthophilum* | Palmares do Sul RS | 2009 | + | + | + | n.d. | n.d. | n.d. |
| 3784 | 17609 | *F. fujikuroi* | Garuva SC | 2016 | - | + | + | 1.018,1 | 966,0 | 38,5 |
| 3791 | 17616 | *F. fujikuroi* | Jacinto Machado SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3691 | 17590 | *F. fujikuroi* | Nova Veneza SC | 2016 | + | + | + | 1.662,3 | 4.139,1 | 37,6 |
| 3692 | 17591 | *F. fujikuroi* | Nova Veneza SC | 2016 | - | + | + | 486,8 | 82,9 | 20,9 |
| 3798 | 17623 | *F. fujikuroi* | Nova Veneza SC | 2016 | + | + | + | 6.335,4 | 5.611,1 | 50,1 |
| 3809 | 17634 | *F. fujikuroi* | Nova Veneza SC | 2016 | - | - | + | 1.107,1 | 2.643,1 | 44,3 |
| 3785 | 17610 | *F. fujikuroi* | Santo Antônio do Goiás GO | 2015 | + | + | + | 115,6 | 29,3 | n.d. |
| 3801 | 17626 | *F. fujikuroi* | Sinop MT | 2015 | - | + | - | 14.745,0 | 2.073,6 | n.d. |
| 3796 | 17621 | *F. proliferatum* | Brazabrantes GO | 2015 | - | + | + | 4.637,9 | 1.233,6 | 3.491,5 |
| 3775 | 17600 | *F. proliferatum* | Nova Veneza SC | 2016 | + | + | + | 1.683,3 | 809,7 | 179,1 |

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Table 1 Sixty-one strains of eigth members of *Fusarium fujikuroi* species complex used in this study: their codes collection, geographical origin, year of isolation, the presence of *FUM* genes from the fumonisin biosynthetic pathway and amounts of mycotoxins synthesized in rice culture.

| **Code CML**[a] | **Code ITEM**[b] | **Phylogenetic species**[c] | **Origin**[d] | **Year** | **Fumonisin genes**[e] | | | **Mycotoxins (µg/g)**[f] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | ***FUM1*** | ***FUM8*** | ***FUM14*** | **FB1** | **FB2** | **FB3** |
| 3815 | 17640 | *F. proliferatum* | Nova Veneza SC | 2016 | - | + | + | 1.348,7 | 3.650,1 | 63,2 |
| 3685 | 17586 | *F. proliferatum* | Sinop MT | 2015 | - | + | + | 2.897,3 | 2.054,2 | 39,8 |
| 3772 | 17597 | *F. proliferatum* | Sinop MT | 2015 | - | + | + | 3.254,3 | 2.269,7 | 66,9 |
| 3686 | 17587 | *F. proliferatum* | União do Sul MT | 2015 | + | - | + | 1.640,0 | 401,2 | 542,3 |
| 3741 | 17593 | *F. proliferatum* | União do Sul MT | 2015 | - | + | + | 1.830,2 | 727,5 | 86,5 |
| 3794 | 17619 | *F. proliferatum* | União do Sul MT | 2015 | - | + | + | 2.383,6 | 1.846,1 | 48,1 |
| 3742 | 17594 | *F. pseudocircinatum* | Boa Vista RR | 2016 | - | + | + | n.d. | n.d. | n.d. |
| 3683 | 17584 | *F. pseudocircinatum* | Nova Veneza SC | 2015 | - | + | + | 70,5 | n.d. | n.d. |
| 3145 | 17552 | *F. pseudocircinatum* | Palmares do Sul RS | 2009 | + | + | - | n.d. | n.d. | n.d. |
| 3789 | 17614 | *F. pseudocircinatum* | Paraibano MA | 2016 | - | + | + | n.d. | n.d. | n.d. |
| 3142 | 17549 | *F. sterilihyphosum* | Pelotas RS | 2012 | + | + | - | n.d. | n.d. | n.d. |
| 3670 | 17574 | *F. sterilihyphosum* | São Sepé RS | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3771 | 17596 | *F. verticillioides* | Boa Vista RR | 2016 | + | + | + | 2.826,4 | 1.399,8 | 220,3 |
| 3773 | 17598 | *F. verticillioides* | Boa Vista RR | 2016 | + | + | + | 1.385,8 | 572,8 | 106,3 |
| 3676 | 17576 | *F. verticillioides* | Brazabrantes GO | 2015 | + | + | + | 4.317,4 | 2.169,7 | 154,9 |
| 3800 | 17625 | *F. verticillioides* | Brazabrantes GO | 2015 | + | + | - | 3.119,3 | 1.961,0 | 594,0 |
| 3808 | 17633 | *F. verticillioides* | Paraibano MA | 2016 | + | + | + | 4.012,3 | 2.157,7 | 435,4 |
| 3799 | 17624 | *F. verticillioides* | Quaraí RS | 2015 | + | + | + | 5.426,4 | 3.835,6 | 18,8 |
| 3652 | 17568 | *F. verticillioides* | Uruguaiana RS | 2015 | + | + | + | 3.655,2 | 2.390,5 | n.d. |

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Table 1 Sixty-one strains of eigth members of *Fusarium fujikuroi* species complex used in this study: their codes collection, geographical origin, year of isolation, the presence of *FUM* genes from the fumonisin biosynthetic pathway and amounts of mycotoxins synthesized in rice culture.

| **Code CML**[a] | **Code ITEM**[b] | **Phylogenetic species**[c] | **Origin**[d] | **Year** | **Fumonisin genes**[e] | | | **Mycotoxins (µg/g)**[f] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | ***FUM1*** | ***FUM8*** | ***FUM14*** | **FB1** | **FB2** | **FB3** |
| 3689 | 17581 | *Fusarium* sp. 1 | Sinop MT | 2015 | + | + | + | 70,2 | n.d. | n.d. |
| 3690 | 17582 | *Fusarium* sp. 1 | Sinop MT | 2015 | + | + | + | 69,4 | n.d. | n.d. |
| 3770 | 17595 | *Fusarium* sp. 1 | Sinop MT | 2015 | - | + | + | n.d. | n.d. | n.d. |
| 3803 | 17628 | *Fusarium* sp. 1 | Sinop MT | 2015 | - | + | + | n.d. | n.d. | n.d. |
| 3804 | 17629 | *Fusarium* sp. 1 | Sinop MT | 2015 | - | - | - | n.d. | n.d. | n.d. |
| 3806 | 17631 | *Fusarium* sp. 1 | Sinop MT | 2015 | + | + | - | n.d. | n.d. | n.d. |
| 3807 | 17632 | *Fusarium* sp. 1 | Sinop MT | 2015 | - | + | + | n.d. | n.d. | n.d. |
| 3651 | 17567 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | + | + | + | n.d. | n.d. | n.d. |
| 3682 | 17583 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | + | + | + | 175,3 | n.d. | n.d. |
| 3780 | 17605 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | + | + | + | n.d. | n.d. | n.d. |
| 3787 | 17612 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | + | + | + | 191,6 | n.d. | n.d. |
| 3819 | 17643 | *Fusarium* sp. 2 | Brazabrantes GO | 2015 | + | + | + | n.d. | n.d. | n.d. |
| 3816 | 17641 | *Fusarium* sp. 2 | Garuva SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3822 | 17646 | *Fusarium* sp. 2 | Garuva SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3824 | 17648 | *Fusarium* sp. 2 | Garuva SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3648 | 17565 | *Fusarium* sp. 2 | Itajaí SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3649 | 17566 | *Fusarium* sp. 2 | Itajaí SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3680 | 17579 | *Fusarium* sp. 2 | Itajaí SC | 2016 | + | + | + | 195,1 | n.d. | n.d. |
| 3681 | 17580 | *Fusarium* sp. 2 | Itajaí SC | 2016 | + | + | + | 98,6 | n.d. | n.d. |

Continuation 3/4

Table 1 Sixty-one strains of eigth members of *Fusarium fujikuroi* species complex used in this study: their codes collection, geographical origin, year of isolation, the presence of *FUM* genes from the fumonisin biosynthetic pathway and amounts of mycotoxins synthesized in rice culture.

| **Code CML**[a] | **Code ITEM**[b] | **Phylogenetic species**[c] | **Origin**[d] | **Year** | **Fumonisin genes**[e] | | | **Mycotoxins (µg/g)**[f] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | ***FUM1*** | ***FUM8*** | ***FUM14*** | **FB1** | **FB2** | **FB3** |
| 3793 | 17618 | *Fusarium* sp. 2 | Itajaí SC | 2016 | + | + | + | 135,4 | 6,3 | n.d. |
| 3805 | 17630 | *Fusarium* sp. 2 | Itajaí SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3810 | 17635 | *Fusarium* sp. 2 | Itajaí SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3814 | 17639 | *Fusarium* sp. 2 | Itajaí SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3818 | 17642 | *Fusarium* sp. 2 | Itajaí SC | 2016 | + | + | + | n.d. | n.d. | n.d. |
| 3812 | 17637 | *Fusarium* sp. 2 | Tangará da Serra MT | 2016 | + | + | + | n.d. | n.d. | n.d. |

[a,b]Culture collection abbreviations: CML – Coleção Micológica de Lavras, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil; ITEM – Microbial Culture Collection of Institute of Sciences and of Food Production (ISPA), Puglia, Bari, Italy; Results for phylogenetic species identification already published in Nicolli et al. (2019): [c]Phylogenetic identification by three housekeeping genes: TEF – elongation factor 1-α, CAL – calmudolin and TUB – β – tubulin; [d]Brazilian cities and states: RS – Rio Grande do Sul, SC – Santa Catarina, GO – Goiás, MT – Mato Grosso, RR – Roraima, TO – Tocantins, MA – Maranhão; [e]Fumonisin genes by Proctor et al., 2004; [f]Abbreviations: n.d. = not detectable; Low limits of detection (LOD): FB1, FB2, FB3 = 0,02 µg/g.

### 3.2 Phylogenetic analysis of *FUM* genes

The partial sequences of the *FUM1*, *FUM8* and *FUM14* genes from 61 isolates studied were amplified. PCR products were used directly for sequencing the fragments. In some cases, we were not successful in obtaining the sequence of good quality, despite having the fragment amplified. The obtained sequences were used for phylogeny reconstruction. The *FUM1* dataset were analyzed 569 characters, of which 144 sites were polymorphic and 86 sites were parsimony informative (Figure 1). The individual *FUM1* tree into a maximum likelihood analysis with kimura 2-parameter model + invariant sites (K2+I) showed that the two lineages have the different evolution *FUM1* gene that *Fusarium ramigenum* NRRL 25208 with 100% of bootstrap (Figura 1). The *FUM8* dataset were analyzed 802 characters, of which 225 sites were polymorphic and 204 sites were parsimony informative. Maximum likelihood tree generated from individual nucleotide sequences of *FUM8* analysis with kimura 2-parameter model + gamma distributed (K2+G) substitution model showed the two new lineages and *F. verticillioides* strains are different evolution *FUM8* gene that *Fusarium ramigenum* NRRL 25208 with 100% of bootstrap (Figura 2). The evolution of *FUM8* gene between *F. fujikuroi* and *F. proliferatum* strains are the same with 100% of bootstrap and those species are different evolution of *F. anthophilum* NRRL 25214 with are 100% of bootstrap (Figure 2). Finally, for the *FUM14* were analyzed 836 characters, of which 232 sites were polymorphic and 185 sites were parsimony informative and the maximum likelihood analysis with K2+G substitution model resulted in the same evolution of *FUM8* gene for all species that were analyzed (Figure 3).

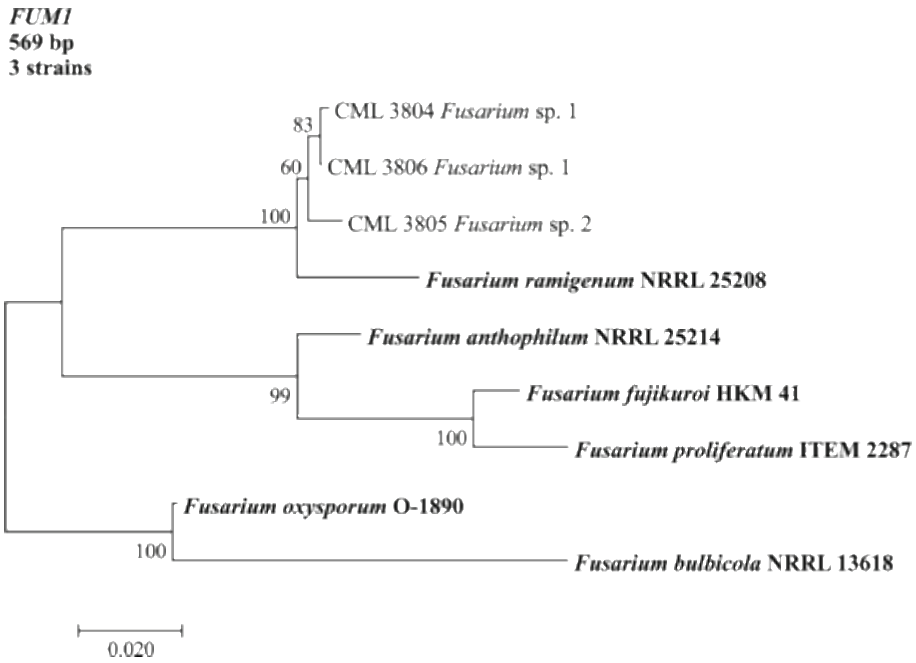


Figure 1 Maximum likelihood tree generated from individual nucleotide sequences of *FUM1* from strains of two new lineages of FFSC. Numbers near branches are bootstrap values based on 1,000 replicates. The analysis employed the K2+I substitution model.

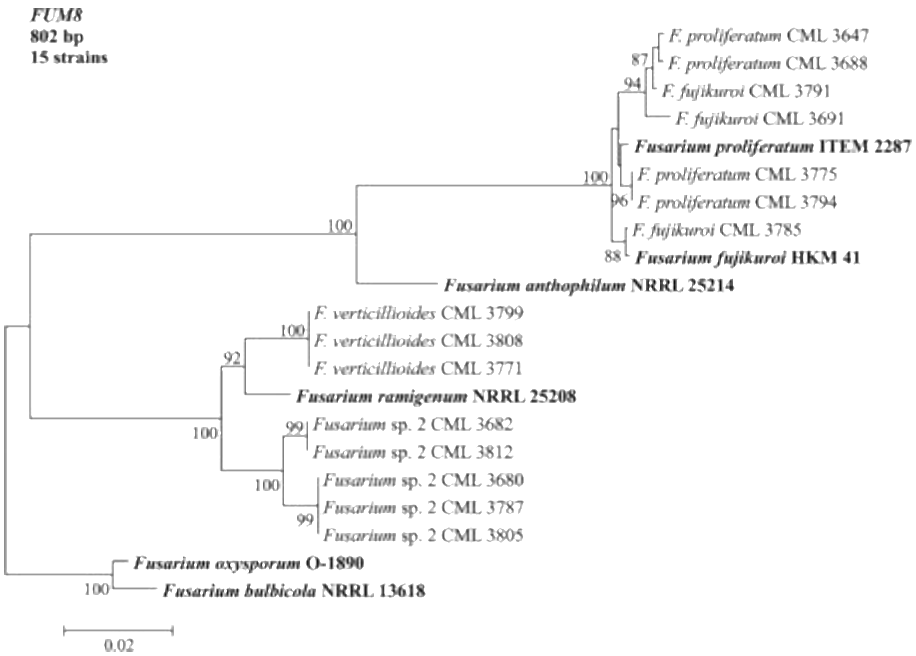


Figure 2 Maximum likelihood tree generated from individual nucleotide sequences of *FUM8* from strains of three FFSC species and one new lineage of FFSC. Numbers near branches are bootstrap values based on 1,000 replicates. The analysis employed the K2+G substitution model.

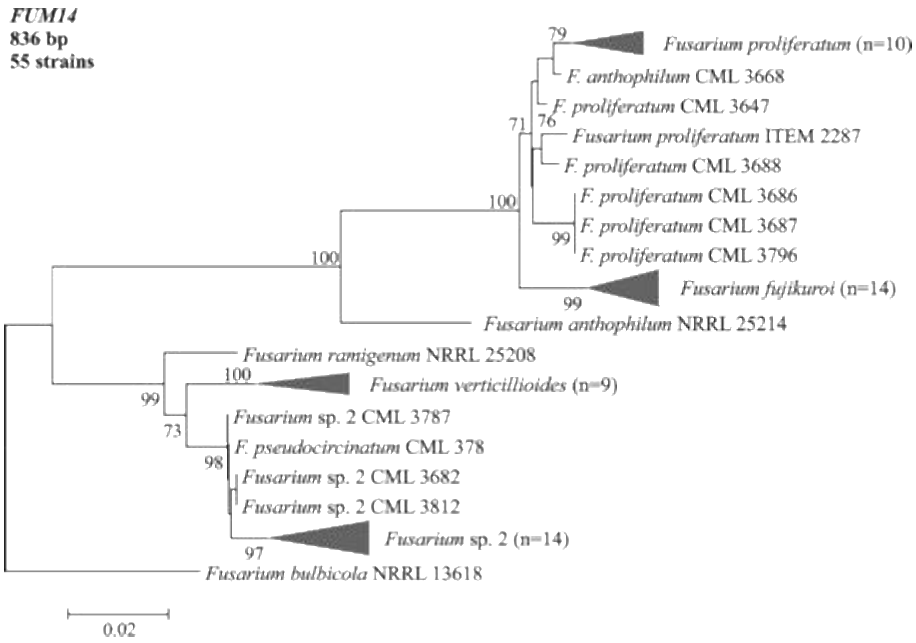


Figure 3 Maximum likelihood tree generated from individual nucleotide sequences of *FUM14* from strains of three FFSC species and one new lineage of FFSC. Numbers near branches are bootstrap values based on 1,000 replicates. The analysis employed the K2+G substitution model.

### 3.3 Mycotoxin analysis

Based on the results of the analysis of 61 strains of FFSC from Brazilian rice, the species of FFSC can be distinguished in three groups (Table 1). The group that produced FB1, FB2 and FB3 composed by *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* species. The group that produced only FB1 composed by *F. pseudocircinatum*, *Fusarium* sp. 1 and *Fusarium* sp. 2 and the group that does not produce FBs composed by *F. anthophilum* and *F. sterilihyphosum*.

## 4 DISCUSSION

In this study we investigated for the first time the fumonisins ability of tropical diversity of *Fusarium fujikuroi* species associated with rice grains in Brazil, using a set of strains representative of eight members of FFSC (six species and two lineages). We also examined the strains for fumonisin production and presence of *FUM* genes in their genomes. Fumonisin production was detected in six species, *F. fujikuroi*, *F. verticillioides*, *F. proliferatum*, *F. pseudocircinatum*, *Fusarium* sp. 1 and *Fusarium* sp. 2. Chemical analyses were confirmed by the detection of the *FUM* genes in the same six species. Moreover, the FUM genes were also detected in *F. anthophilum* and *F. sterilihyphosum*, although these two species could not produce fumonisins *in vitro* cultures. The fumonisin production was not detected in the species in which the genes were not detected.

Proctor et al. (2013) that showed the occurrence of five different genomic contexts among species of FFSC. We confirmed the presence of the three key genes for fumonisin production. A more detailed analysis of gene sequences may facilitate the development of more accurate tools to detect the mycotoxins production, contributing thus to the improvement of strategies to manage the contamination on rice grains. The results of this study represent the first investigation to correlate fumonisin production with the presence of a fumonisin biosynthetic gene in species of FFSC that occur associated with rice in Brazil. Among the species that are not able to produce fumonisins, we reported *F. anthophilum* that, on the contrary, was reported in previous studies as fumonisin C producer (Proctor et al., 2004; Proctor et al., 2013) and *F. sterilihyhphosum*, that was reported as only moniliformin producer (Waffa et al., 2011).

On the other hand, *F. pseudocircinatum* is a fumonisin producer according to our data, although this species was reported as only moniliformin and fusaproliferin producer (Marasas et al., 2004; Fraeyman et al., 2017). Furthermore, we reported that two new lineages isolated from rice grains were both fumonisin producers. Thus, this is the first report on fumonisin production by *F. pseudocircinatum*, *Fusarium* sp. 1 and *Fusarium* sp. 2 isolated from rice grains in Brazil. All of the strains reported here were studied for their phylogenetic relationships by analyzing sequences of the highly conserved genes *TEF*, *TUB* and *CAL*

(Nicolli et al., 2019). We compared for each species identified the primary metabolic phylogeny with the phylogeny of the *FUM1*, *FUM8* and *FUM14* genes.

The eight members of FFSC reported above include representatives of each of the three clades of the FFSC described by O'Donnell et al. (1998). Based on the housekeeping genes analysis the *F. verticillioides*, *F. pseudocircinatum*, *Fusarium* sp. 1 and *Fusarium* sp. 2 are in the African clade; *F, fujikuroi* and *F. proliferatum* are in the Asian Clade; and *F. anthophilum* and *F. sterilihyphosum* are in the American clade. The *FUM* genes were detected in all species but not in all strains, thus the analyzed of *FUM* genes were based on the strains that obtained sequences of good quality. By *FUM* genes analysis the topology of the species were almost the same of that of housekeeping genes, with the exception of the *F. fujikuroi* and *F. proliferatum* that were genetically sister species of *F. anthophilum*. This result also agrees with analysis of *FUM* genes of members of FFSC by Proctor et al. (2013), where all fumonisin-producing African and Asian-clade species produce predominatly fumonisins type B, whereas all fumonisin-producing American-clade species that have been examined produce predominantly fumonsins type C (Proctor et al., 2004; Sewram et al., 2005).

Our dataset of mycotoxin production *in vitro* rice cultures showed exactly that the group that produced FB1, FB2 and FB3 composed by *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* species are in the Asian and African clades. On the other hand,, *F. pseudocircinatum*, *Fusarium* sp. 1 and *Fusarium* sp. 2 are placed in the African clade. The American-clade represented by *F. anthophilum* does not produce fumonisin type B as showed our data.

In conclusion, our data confirm the ability to produce fumonisins of several species isolated from rice in Brazil and the potential risk due to fumonisin contamination of rice grains by members of FFSC in Brazil. Fungal contamination of rice grains samples with these members of FFSC remains a clear problem for crops in many developing countries. The notorious production of fumonisin in vitro by our strains of *F. verticillioides, F. proliferatum*, *F. fujikuroi* is consistent with observations of Desjardins et al. (1997) and Proctor et al. (2013). In addition to the above mentioned species, other species members of FFSC were detected in a recent study (Nicolli et al., 2019) and we prove that they are also fumonisin producers. As rice is a staple crop for the population in Brazil, the importance of fumonisins

in rice needs to be further investigated. Finally, since also strains belonging to new *Fusarium* lineages were able to produce fumonisins, more further investigations on their mycotoxigenic ability and biosynthetic *FUM* gene patheway are needed.

## 5 CONCLUSION

Our results provide new knowledge on the ability of tropical members of FFSC associated with rice grains, which is of value for improving models for assessing the risk of mycotoxin contamination on rice in Brazil.

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***Camila Primieri Nicolli***

My academic career started at Universidade Federal do Rio Grande do Sul (UFRGS) where I became Agronomist. During my undergraduate studies I was invited by Professor Emerson Del Ponte to work in the Laboratory of Plant Epidemiology. The main research subject was the *Fusarium fungal genus*, in association with cereals grains. As I was fascinated by that research, I started a Master degree in 2013. The main research subject was to compare biology of different species belonging to the *F. graminearum* complex causing Fusarium Head Bligth in wheat. These work allowed to present the article entitle "Fitness traits of deoxynivalenol and nivalenol-producing *Fusarium graminearum* species complex strains from wheat" available (https://doi.org/10.1094/PDIS-12-17-1943-RE). These work motivated me to continue my professional career on Research and the next logical step was to start a PhD course. Thus, I moved to Universidade Federal de Lavras, Minas Gerais and started my PhD studies under the supervision of Prof. Ludwig H. Pfenning. As part of my academic formation during my PhD studies, I had visiting student at the Institute of Sciences of Food Production (ISPA) in Italy. Under supervision of Dr. Antonio Moretti, I performed morphological, biological and phylogenetic identification of species of the *Fusarium fujikuroi* species complex occurring on rice. I also worked on the determination of toxigenic potential of these pathogens through molecular techniques and high performance liquid chromatography (HPLC). Currently, I am a Professor at Universidade Federal de Goiás (UFG) where I teaching on Plant pathology with special focus on Plant Protection.