Artigos

Molecular characterization and vegetative compatibility groups of *Fusarium* spp. pathogenic to *Ilex paraguariensis* A. St.-Hil.

Caracterização molecular e grupos de compatibilidade vegetativa de *Fusarium* spp. patogênicos à *Ilex paraguariensis* A. St.-Hil.

Ricardo Mezzomo[I]
Jéssica Mengue Rolim[II]
Tales Poletto[III]
Clair Walker[IV]
Paola Mendes Milanesi[V]
Marlove Fátima Brião Muniz[III]

[I]Freelance Researcher, Santa Maria, RS, Brazil
[II]Universidade Federal de Pelotas, Pelotas, RS, Brazil
[III]Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[IV]Universidade Estadual de Londrina, Londrina, PR, Brazil
[V]Universidade Federal da Fronteira Sul, Erechim, RS, Brazil

ABSTRACT

In the central-western and southern regions of Brazil, the cultivation of yerba mate (*Ilex paraguariensis*) is considered an important source of forest extraction, besides providing an alternative income, especially for families. The yerba mate, like other forest species, is infected by several pathogens, such as fungi of the genus *Fusarium,* causing root rot. Pathogens have wide genetic variability, even within a single genus and species, resulting from adaptations to their environments. This study aimed at characterizing molecularly isolated *Fusarium* spp. through the sequencing of the ITS and β-tubulin genome regions, identifying the species level as well as the formation of vegetative compatibility groups (VCG) among these isolates of the root rot pathogen in yerba mate. The sequencing of the ITS and β-tubulin regions, defined the separation between the seven pathogenic isolates of *F. solani* and *F. oxysporum*. For the same isolates, the technique to obtain *nit* mutants was suitable for the phenotypic classification, obtaining five VCGs for species of *Fusarium,* root rot in yerba mate

Keywords: ITS; β-tubulin; *nit*

RESUMO

Nas regiões Centro-Oeste e Sul do Brasil, a cultura da erva-mate (*Ilex paraguariensis*) constitui-se uma importante fonte de extrativismo florestal, além de diversificar a renda, principalmente na agricultura familiar. A erva-mate, como outras espécies florestais, é infectada por diversos patógenos, como o gênero *Fusarium,* causador da podridão-de-raízes. Os patógenos possuem ampla variabilidade genética, mesmo dentro de um único gênero e dentro de uma única espécie, resultado de adaptações ao meio em que vivem. Este estudo teve como objetivo avaliar o sequenciamento das regiões do genoma ITS e β-tubulina na identificação em nível de espécie dos isolados de *Fusarium* spp. patogênicos à erva-mate, e identificar a formação de grupos de compatibilidade vegetativa (VCG) entre os isolados de *Fusarium* spp. patogênicos à erva-mate. O sequenciamento das regiões ITS e β-tubulina consolidou a separação entre os sete isolados patogênicos em *F. solani* e *F. oxysporum*. Para esses isolados, a técnica para obtenção de mutantes *nit* foi adequada para a classificação fenotípica, fundamentais nos testes de VCG para espécies de *Fusarium*, sendo obtidos cinco VCG.

Palavras-chave: ITS; β-tubulina; *nit*

1 INTRODUCTION

The cultivation of yerba mate (*Ilex paraguariensis*) is an alternative for diversifying the production of small properties mainly in the southern region of Brazil, due to the low initial costs and the ease of using agro-silvopastoral systems, enabling the strengthening of family farming. In Rio Grande do Sul state (RS), southern Brazil, the production chain involves 35 thousand hectares with about 14 thousand properties, in addition to 300 nurseries and 250 herbaceous industries, generating a gross income of approximately R$ 1 billion/year (FUNDO DE DESENVOLVIMENTO E INOVAÇÃO DA CADEIA PRODUTIVA DE ERVA-MATE, 2014). However, yerba mate is subject to attack by pathogens, such as fungi of the genus *Fusarium*, known to cause diseases, such as root rot, that leads to reduced productivity.

Root rot interferes directly with the plant nutrient intake. The symptoms are observed in the shoot, including leaf spots, yellowing, leaf fall, reduction in growth, wilting and dryness and even to the death of the seedling (GRIGOLETTI JUNIOR; AUER; MASCHIO, 1996).

For control and preventive strategies to be implemented, therefore, studies are required to understand the correct identification and genetic variability of the fungus that causes the disease.

A precise way to identify pathogen variability is the use of molecular techniques, allowing the development of rapid, sensitive, and specific methods in the diagnosis of phytopathogens. In addition, its wide usage is mainly due to its simplicity, speed, safety, and amplitude of the obtained results (TEIXEIRA; VIEIRA; MACHADO, 2004).

However, in some cases, the use of molecular techniques is insufficient to detect intraspecific variability among species of *Fusarium.* Thus, the identification of vegetative compatibility groups (VCG) is commonly employed in the characterization of diversity among fungal isolates, allowing the distinction between populations (ROSALEE; COELHO; DHINGRA, 1999). In this context, vegetative compatibility is employed as a resource capable of identifying the genetic variability within fungal populations. This technique is based on the anastomosis of hyphae expressing homologous alleles, leading to the formation of stable heterokaryon (GLASS; JACOBSON; SHIU, 2000).

In view of the above, the objective of this study was to characterize molecular isolates of *Fusarium* spp. through the sequencing of the ITS and β-tubulin genome regions, identifying them at the species level, as well as in the formation of vegetative compatibility groups (VCG) among the isolates obtained from yerba mate.

2 MATERIAL AND METHODS

2.1 Isolates from *Fusarium* spp.

The experiment was conducted at the "Elocy Minussi" Phytopathology Laboratory of the Center of Rural Sciences of the Federal University of Santa Maria, Santa Maria, Rio Grande do Sul state (RS), Brazil. For the molecular characterization and the VCG test, a *Fusarium oxysporum* (I6AR2) and six other isolates from *Fusarium solani* (I1AR1, I8AR1, M3AR2, M4, M5AR2 and M7C1) pathogenic to mate, as described by Mezzomo *et*

al. (2018), collected in RS state, and deposited in the mycology collections of the same laboratory, where they remain stored in refrigeration in Eppendorff *tubes,* containing saline solution 0.85% NaCl (Table 1).

Table 1 – Areas for the collection of *Fusarium* spp. isolates pathogenic to yerba mate

Isolate	Collection date	Location	Coordinates (GMS)
I1AR1	January 2015	Ilópolis/RS	-28°55'42.5"S, -52°08'32.6"W
I6AR2	January 2015	Ilópolis/RS	-28°53'58.1 "S, -52°04'48.0"W
I8AR1	January 2015	Ilópolis/RS	-28°55'48.6"S, -52°10'40.1"W
M3AR2	March 2015	Machadinho/RS	-27°32'46.6"S, -51°40'47.7"W
M4	March 2015	Machadinho/RS	-27°32'45.9"S, -51°40'48.1"W
M5AR2	March 2015	Machadinho/RS	-27°32'16.1"S, -51° 42'32.4"W
M7C1	March 2015	Machadinho/RS	- 27°33'25.0"S, -51°40'04.2"W

Source: Authors (2017)

2.2 Molecular characterization of pathogenic isolates of *Fusarium* spp.

The isolates from *Fusarium* spp. were submitted to DNA extraction according to the CTAB method described by Doyle and Doyle (1991). The extracted genomic DNA samples were submitted to Chain Polymerase Reaction (PCR) for amplification of the ITS region (Internal Transcribed Spacer) of the rDNA with the primers ITS1 (5 "TTCCGTAGGTGAACCTGCGG 3") and ITS4 (5 "TCCTCCGCTTATTGATATGC 3") (WHITE *et al*., 1990), as well as the beta-tubulin region (β-tubulin) amplified with the pair of Btub-F primers (AAGGGHCAYTAYACYGARGG) and Btub-R (CATGTTGGACTCDGCCTC), developed at the Biological Institute - São Paulo, SP state. The reaction was performed with 30 ng of DNA, 10X buffer, 2.5 µM of each dNTP, 20 nM $MgCl_2$, 25 poles of each of the oligonucleotide primers, five units of the enzyme Taq polymerase and ultrapure water to complete the reaction volume. The reactions were carried out in a thermal cycler MJ Research, INC. PTC - 100MT, under the following thermal conditions: 94ºC for 2 min, 30 cycles of 94ºC for 45 s, 55°C for 30 s, 72°C for 35 s and 72ºC for 10 min. At

the end of the reaction, the PCR products were maintained at 4°C. A negative control without DNA was included in PCR amplifications. Amplified fragments and control were separated by 1.2% agarose gel electrophoresis in 1X TBE buffer (10.8 g of tris base, 5.5 g of boric acid, 4 mL of 0.5 M EDTA and 4 mL of distilled water), containing ethidium bromide, viewed under ultraviolet light. The PCR products were purified with 13% PEG 8000 and, in the sequencing reactions, the oligonucleotides ITS1 and ITS4 were used. The sequences generated contained 400 to 900 bp, the size related to the segment corresponding to regions ITS1, 5.8S and ITS2 (HALL, 1999).

The sequencing of the genomic regions was performed on a Mega BACE 500 sequencer (Amersham) and the sequenced fragments were analyzed through the BioEdit program (HALL, 1999). The obtained nucleotide sequences were compared with those already existing in GenBank, so that the pathogens isolated and the GenBank sequences that had the highest coverage scores and similarity could be selected and aligned together with the sequences obtained in the ClustalW algorithm sequencing. The phylogenetic analysis was performed using the Neighbor-joining statistical method with 1000 replicates, by the MEGA version 4 program (TAMURA *et al*., 2007). The model used was chosen according to the available online program, FindModel, which establishes the best model after supplying data showing the chosen sequences aligned through BioEdit, Hall (1999), or another program. The similarity of the nucleotide sequences among the isolates was calculated using the Basic Local Alignment Search Tool - BLAST procedure.

2.3 Obtaining the *nit* mutants

The isolates from *Fusarium* spp were transferred to Petri dishes containing minimal (MM) medium and incubated in the dark for seven days at 24°C. The trace element solution was composed of citric acid, 5 g $ZnSO_4.6H_2O$, 1 g $Fe\ (NH_4)_2SO_4.6H_2O$, 50 mg $MnSO_4$, 50 mg H_3BO_3, 50 mg $NaMoO_4.2H_2O$, 95 mL of distilled water. Afterwards,

disks of mycelia agar (5 mm) arranged in a triangular arrangement were transferred to 10 Petri dishes per isolate containing minimal medium plus potassium chlorate (MMC), in the concentration of 1,5, 2 and 2.5% plus 1,6 g/L of L-asparagine for the induction of mutants, which are manifested in sections in the colony. The plates were kept in the dark at 25 ± 2 °C for 14 to 21 days (PUHALLA, 1985; CORRELL; PUHALLA; SCHNEIDER, 1986). After the formation of the sectors, a single sector of each mycelial disc was removed and transferred to plates containing minimal (MM) medium. The isolates that presented thin growth in MM were classified as *nit* mutants. However, the sectors that presented robust growth were discarded (LESLIE, 1993).

2.4 Phenotypic characterization of *nit* mutants

The *nit* mutants which showed low growth in MM were transferred to Petri dishes containing culture medium with different nitrogen sources: sodium nitrate (2 g/L); sodium nitrite (0.5 g/L) and hypoxanthine (0.2 g/L) (PUHALLA, 1985; CORRELL; PUHALLA; SCHNEIDER,1986). After spiking in the different media, the plates were kept in the dark at 25 ± 2 ° C for a period of 3-4 days (LESLIE; SUMMERELL, 2006). Three classes designate mutant phenotypes *nit* in the genus *Fusarium*, which are based on mutation in the nitrogen assimilation route (Table 2) (PUHALLA, 1985; CORRELL; PUHALLA; SCHNEIDER, 1986).

Table 2 – Use of different nitrogen sources in the phenotyping tests

Mutant	Nitrate	Nitrite	Hypoxanthine
Wild	+	+	+
*nit*1	-	+	+
nit 3	-	-	+
nit M	-	+	-

Source: Authors (2017)

2.5 Vegetative compatibility test

The pairings were performed in MM and allocated in combinations containing the *nit* mutants 1 in the center of the Petri dish and the M/*nit* 3 mutants at the ends. In *nit*s of the same species studied the positive control was observed with aerial mycelium growth in the line of intersection between *nit* 1 and *nit* M/n*it 3*. Among the different isolates, the positive reactions follow as described above; that is, they were observed through aerial mycelium growth in the line of intersection between *nit* 1 and *nit* M/ni*t* 3, confirming the classification of these isolates as belonging to the same VCG. Negative reactions were observed through thin growth in the line of intersection between *nit* 1 and *nit* M/*nit* 3 of different species (PUHALLA, 1985; CORRELL; PUHALLA; SCHNEIDER, 1986; LESLIE; SUMMERELL, 2006).

3 RESULTS AND DISCUSSION

In the sequencing of the ITS region (ITS1, 5.8S and ITS2) (Figure 1), the M3AR2 and M4 isolates were allocated in a large clade (branch) together with the sequences of *Fusarium solani* used in the comparison with *bootstrap* of 99%. Isolates I1AR1, I8AR1, as well as M5AR2 and M7C1 were allocated in two distinct clades with *bootstrap* of 61 and 63%, respectively. However, when compared to the other *F. solani*, the support of *bootstrap* increases to 99% similarity.

Even with *bootstrap* values (61, 63 and 65%), it was observed that the sequencing of the ITS region allowed the separation of the *F. solani* (M1AR2, M4, M5AR2 and M7C1) assigned to different branches to those collected in Ilópolis - RS state (I1AR1 and I8AR1). During alignment, the I6AR2 isolate was assigned to a single *clado* added to the other 10 sequences used, with the support of *bootstrap* of 99% similarity, identified as *F. oxysporum*.

Figure 1 – Phylogenetic dendrogram based on the method *Neighbor-joining* from DNA sequences of the ITS region. The numbers on the branches indicate the percentage of repetitions of the *bootstrap* analysis in which the repetitions were observed (1,000 repetitions). A sequence of *Lasiodiplodia subglobosa* was used as *outgroup*
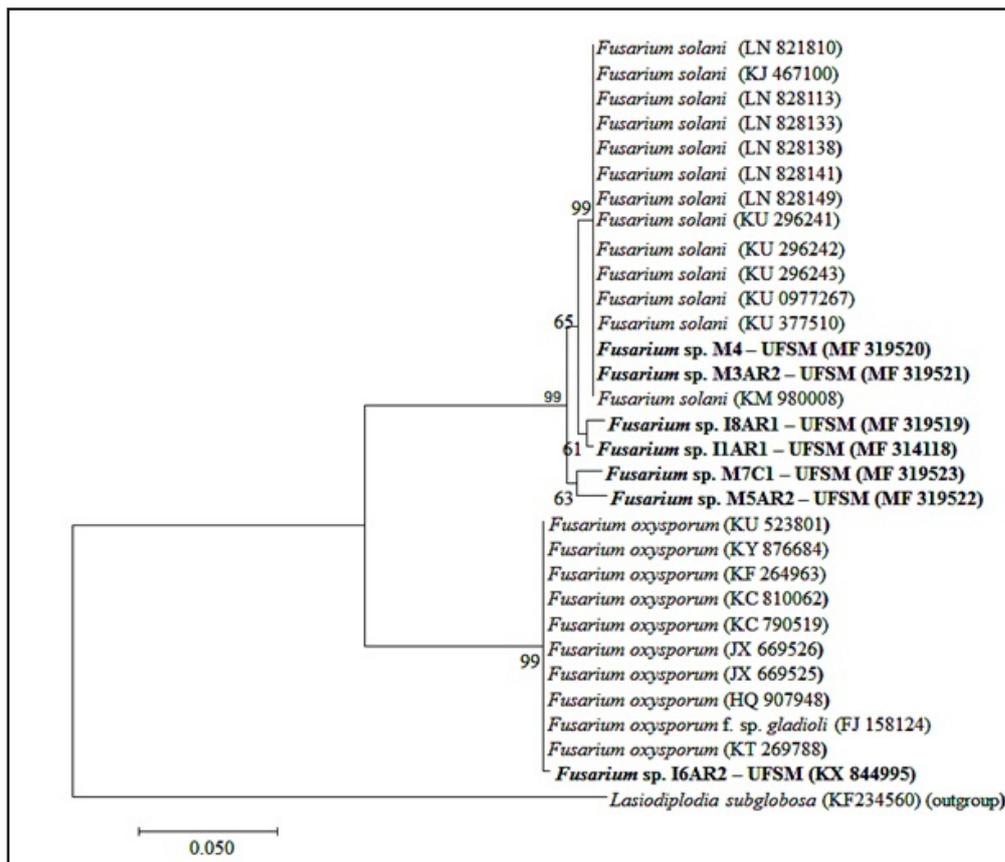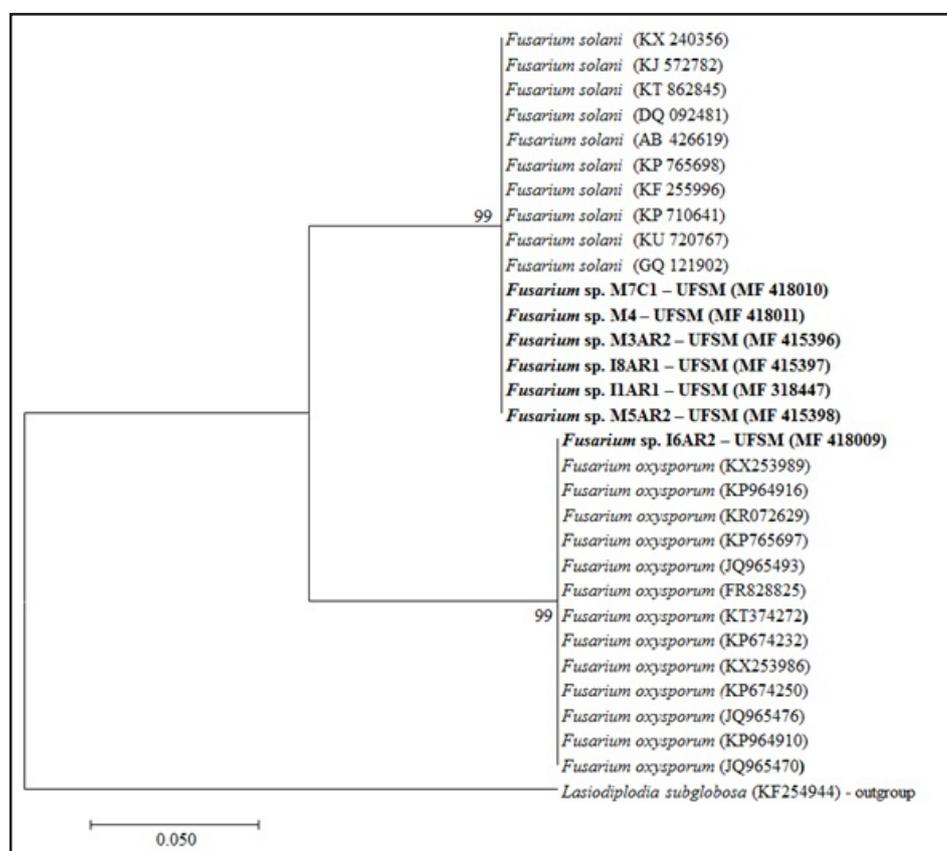

Source: Authors (2017)

Together with the sequencing of the ITS region and the dendrogram resulting from the analysis, it was possible to determine the species in question. Corroborating the results of the present study, the dendrogram obtained by Shahnazi *et al*. (2012) clearly identifies the formation of two clades, discriminating the species of *F. solani* and *F. proliferatum*. In addition, this result was supported by a high *bootstrap* value (100%). According to Visentin *et al*. (2009), the sequencing of the ITS region was effective in distinguishing *Fusarium verticillioides* and *Fusarium proliferatum*, collected in maize producing regions in northwestern Italy. According to Datta *et al*. (2011) the use of

specific primers for the ITS1 and ITS2 regions was effective in distinguishing 12 isolates from *Fusarium* spp. obtained from different regions of India. However, the authors highlighted a slight presence of polymorphism in the amplified region of rDNA, making it difficult to separate some isolates into three distinct groups. The sequencing of the β-tubulin region (Figure 2) confirmed and consolidated the identification of *F. solani* (I1AR1, I8AR1, M3AR2, M4, M5AR2, M7C1) and *F. oxysporum* (IAR2). In both cases, the formation of a single clade per species was verified. Despite the high *bootstrap* (99%), this analysis did not provide the intraspecific differentiation of *F. solani* observed in the previous sequencing.

Figure 2 – Phylogenetic dendrogram based on the method *Neighbor-joining* from β-tubulin region DNA sequences. The numbers on the branches indicate the percentage of repetitions of the *bootstrap* analysis in which the repetitions were observed (1000 repetitions). A sequence of *Lasiodiplodia subglobosa* was used as *outgroup*
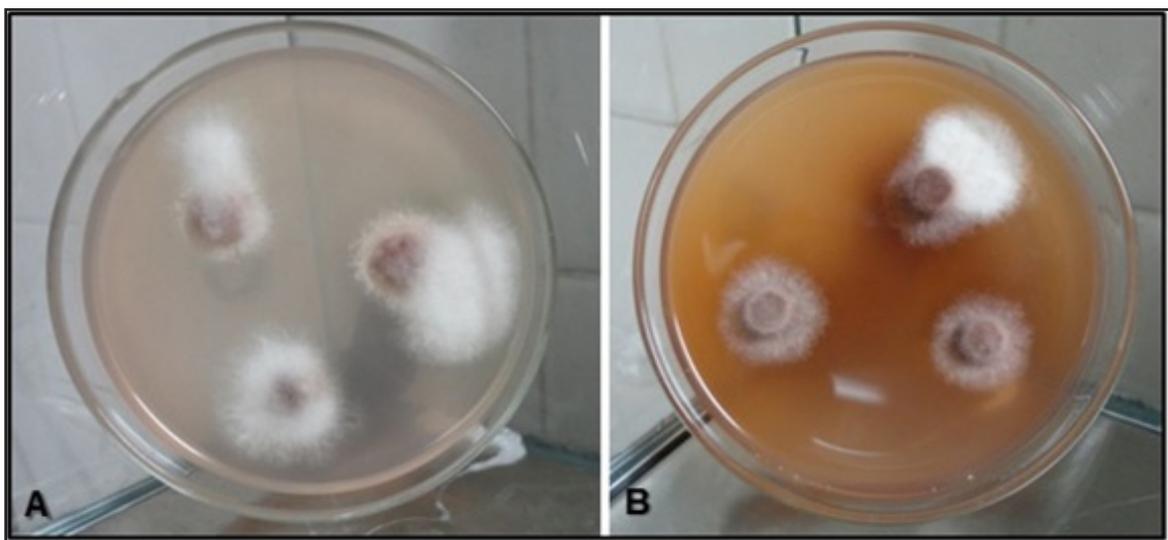

Source: Authors (2017)

Even with the formation of single clades supported by a high *bootstrap,* the sequencing of the β-tubulin region has consolidated the species-level identification of pathogenic *Fusarium* spp. Jost *et al*. (2004), emphasize that introns studies are fundamental in species-level identification, since these elements are essential and specific for the expression and regulation of β-tubulin genes. For Bogale *et al*. (2009), the β-tubulin region allowed the separation of *Fusarium solani* in two distinct clades. According to Jacobs *et al*. (2010), isolates from *F. ananatum* and *F. guttiforme* of pineapple fusariosis were clearly separated into different clades using the β-tubulin gene region.

Considering from the seven pathogenic isolates *Fusarium* spp., only I1AR1 and M7C1 did not form resistant sectors when cultured in minimal medium supplemented with mutagenic agent potassium chlorate (MMC). The other isolates had resistant sections and were classified as *nit* mutants (Figure 3). The isolate from *F. oxysporum* (I6AR2) was the only one to form more than one sector per block, but not necessarily the most expressive number of sectors, since for the analysis, only one sector was used per block.

Figure 3 – Production of resistant sectors of $KClO_3$ isolates of *Fusarium solani*. (A): M3AR2 and (B): M5AR2

Source: Authors (2017)

The formation of sectors constitutes the first step for the identification of VCGs. For Saabale and Dubey (2014) the first signs of sectors were confirmed between the second and third week of incubation. In their studies, the authors analyzed 499 sectors resistant to $KClO_3$ from 39 isolates of *F. oxysporum* f. sp. *ciceri*, causal agent of chickpea wilt (*Cicer arietinum*), collected in 13 regions of India. Kiprop *et al*. (2002) report similar results for the emergence of sectors between the first and second week of culture in MMC of *F. udum* from pigeon pea (*Cajanus cajan*) in plantations in Kenya, India and Malawi.

From the total of 38 sectors identified as resistant to potassium chlorate, the highest number was obtained at the concentration of 1.5%. However, the concentration of 2.5% was responsible for the formation of 20% of them. The 2% concentration did not contribute to the mutation of the isolates. Twelve sectors indicated robust growth in MM and were discarded. There was a difference between the isolates of *F. solani* and *F. oxysporum* in the production of sectors resistant to $KClO_3$, despite the various concentrations used. According to Altinok, Can and Çolak (2013) the concentration of 2% provided the highest number of mutants generated by *F. oxysporum* f. sp. *melongenae* eggplant isolates (*Solanum melongena*). For Alcázar *et al*. (2006) the addition of 30 g/L of potassium chlorate in the MM provided the best composition of the isolate *F. oxysporum* f. sp. *radicis-cucumerinum* from cucumber plantations (*Cucumis sativus* L.) in the province of Almería in Spain. Dias, Abreu and Resende (2014) was successful in restoring sectors using the 50 g/L concentration and the mean frequency was 0.50 to 1.40 sectors of *F. oxysporum* f. sp*. cubense* when grown in MMC.

The growth in MM containing nitrate (NO_3^-), nitrite (NO_2^-) and hypoxanthine (HP) as sources of nitrogen, resulted in 10 *nit* mutants 1 (38.46%), 10 *nit* M (38.46%) and 6 *nit* mutants3 (23.08%) (Table 3).

Three groups designate mutant phenotypes that are based on mutations in the nitrate assimilation pathway. Mutations may occur in the structural locus of nitrate reductase (*nit* 1), in the specific structural locus of the nitrate assimilation

metabolic pathway (*nit* 3) or at the locus that affects the pathways of the enzyme co-factor containing molybdenum required for the activity of nitrate reductase and purine dehydrogenase (*nit* M) (LESLIE, 1993). According to Ilić *et al*. (2013), the *nit* 3 phenotype (43%) prevailed among the 230 mutants recovered from 16 isolates from *F. oxysporum* collected in four regions of Croatia. Alvarado, Resendes and Fernandez (2012), consolidated the hegemony of *nit* 1 (36.91%) of the 149 *nit* restored from 15 isolates of *F. mexicanum,* cause of hose malformation *(Mangifera indica)*, in Jalisco, Mexico. According to the authors, the percentages of phenotypes *nit* Mine *nit* 3 were 28.18 and 19.46%, respectively, differing from the results of the present study, in which the *nit* 3 had the lowest percentage among the identified phenotypes.

Table 3 – Phenotypic classification of *nit* mutants obtained from *Fusarium* spp. pathogenic to yerba mate

| Isolate	Phenotype of mutants *nit*			N°. mutants *nit*
	nit 1	*nit* 3	*nit* M	
I6AR2	1	-	4	5
I8AR1	2	1	2	5
M3AR2	3	-	1	4
M4	1	3	-	4
M5AR2	3	2	3	8
N°. phenotypes	10	6	10	26

Source: Authors (2017)

In the self-compatibility test the *nit* mutants were found to be compatible in all isolates; that is, they formed stable heterocyclic (Figure 4).

According to the results obtained in the complementation test, the different isolates were allocated to five different VCGs. Each VCG was composed of one isolate. The distribution of the isolates in different VCGs suggests the existence of genetic variability, mainly among the isolates of *F. solani*, as pointed out in the sequencing of the ITS region.

According to Leslie (1993), mutants that do not form a stable heterocyan are classified as self-incompatible and cannot be used for the determination of VCGs. Reinforcing the results of the present study, Jusoh, Zin and Nagao (2013) obtained 12 VCGs composed of only one isolate, from the pairing of 12 isolates of *F. solani* (5 pathogenic and 7 non-pathogenic) obtained from tobacco (*Nicotiana tabacum* L.) in five producing regions in the eastern coast of Malaysia. The authors attributed the sexual reproduction to the degree of genetic diversity of the isolates. Similar results were reported by Crespo (2010) where isolates from the same region were classified in different VCGs, supporting local variability. Still, according to the author, the presence of isolates from different regions in the same VCG would indicate the spread of the pathogen from one region to another. According to Elena and Pappas (2002) of the 23 isolates of *F. oxysporium* f. sp. *phaseoli* obtained in different regions of Greece, six were classified as single VCGs. According to these authors, the presence of 15 isolates in the same VCG may be related to the genetic homogeneity present in the population of *F. oxysporum*f.sp. *phaseoli* studied. Puhalla (1985) states that isolates belonging to the same VCG are genetically similar, which reinforces the hypothesis of the existence of genotypic variability among the isolates used in the present study.

Figure 4 – Test of compatibility in minimal medium (MM). (A) and (B): self-compatibility of isolates M4 and I8AR1, respectively; (C): pairing between I6AR2 and I8AR1 isolates
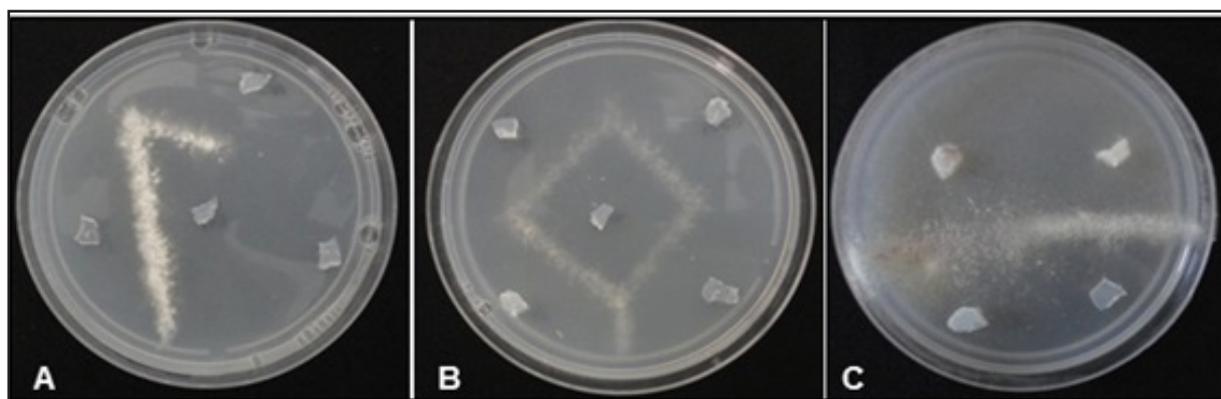
Source: Authors (2017)

4 CONCLUSION

The regions ITS and β-tubulin when used together are efficient and give greater reliability in the identification at the level of species within the genus *Fusarium.* The results suggest the existence of genetic variability among populations, but they point out the need for additional studies on the number and distribution of VCGs present in the patho system *Fusarium* x yerba mate.

ACKNOWLEDGEMENTS

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Authorship Contribution

1 – Ricardo Mezzomo

Forestry Engineer, Work Safety Engineer, Dr., Freelance Researcher
https://orcid.org/0000-0002-7392-9588 • mezzomoricardo@hotmail.com
Contribution: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing

2 – Jéssica Mengue Rolim

Forestry Engineer, MSc.

https://orcid.org/0000-0003-2737-7599 • eng.jessicarolim@gmail.com

Contribution: Data curation, Formal Analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – review & editing

3 – Tales Poletto

Forestry Engineer, MSc.

https://orcid.org/0000-0002-6162-4445 • tecnicotales@hotmail.com

Contribution: Data curation, Formal Analysis, Visualization, Writing – original draft, Writing – review & editing

4 – Clair Walker

Forestry Engineer, Dr., Professor

https://orcid.org/0000-0002-6707-3963 • clairwalker@gmail.com

Contribution: Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing

5 – Paola Mendes Milanesi

Agronomist, Dr., Professor

https://orcid.org/0000-0001-7319-916X • paola.milanesi@uffs.edu.br

Contribution: Formal Analysis, Methodology Software, Validation, Visualization, Writing – review & editing

6 – Marlove Fátima Brião Muniz

Agronomist, Dr., Professor

https://orcid.org/0000-0001-7436-9589 • marlovemuniz@yahoo.com.br

Contribution: Conceptualization, Supervision, Validation, Visualization, Writing – review & editing

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