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Artigo Completo

DESENVOLVIMENTO DE LINHAGEM SELVAGEM E TRANSFORMANTE DE *Fusarium verticillioides* ENDOFÍTICO APÓS COLONIZAÇÃO EM *Zea mays* L.

Lenita Zayatz[1], Ravely Casarotti Orlandelli[2], João Alencar Pamphile[3]

RESUMO

O gênero de fungos com maior ocorrência nas culturas de milho brasileiras é o *Fusarium* e a espécie predominante é a *F. verticillioides*, que pode viver em associação sintomática ou assintomática. Nesta última, ele age como um endófito, um microrganismo que vive no interior da planta hospedeira sem causar nenhum dano, sendo benéfico ao hospedeiro. Este estudo teve por objetivo analisar o comportamento morfológico de uma linhagem de *F. verticillioides* selvagem e transformante e comparar o crescimento micelial e a produção de conídios, antes e após a sua inoculação na planta hospedeira. O diâmetro e peso úmido da colônia da linhagem transformante não apresentaram diferenças significativas antes e após a inoculação, sendo ligeiramente maiores para a linhagem selvagem reinoculada. O resultado indicou que fungos endofíticos geneticamente manipulados podem colonizar eficientemente os tecidos hospedeiros, servindo como um vetor alternativo interessante para introdução de genes de interesse. Além disso, a planta hospedeira pode influenciar as características morfológicas dos endófitos espécie-específicos.

Palavras-chave: *endófitos; colonização; desenvolvimento; milho.*

DEVELOPMENT OF WILD AND TRANSFORMANT STRAIN OF ENDOPHYTIC *Fusarium verticillioides* AFTER COLONIZATION IN *Zea mays* L.

ABSTRACT

The fungal genus with the highest occurrence in Brazilian maize crops is *Fusarium* and the predominant specie is *F. verticillioides*, which can live in symptomatic or asymptomatic association. In this last one, it acts as an endophyte, a microorganism that live inside of host plant without causing any damage and being beneficial. This study analyzed the morphological behavior of a wild and a transfomant endophytic strain of *F. verticillioides* and compared the mycelial growth and conidial production before and after their inoculation in host plant. As result, it was observed a systemic colonization in leaves and roots. The conidial production of wild strain was larger before inoculation, while it was larger after inoculation of transformant strain. The colony diameter/wet weight of transformant strain before and after inoculation do not showed large differences, being slightly larger in reinoculated wild strain. This study indicated that a genetically manipulated endophyte can colonize efficiently the host tissues, serving as an alternative vector to introduce interesting genes. Also, the endogenous host environment could influence the morphological characteristics of specie-specific endophytes.

Keywords: *endophytes; colonization; development; maize.*

INTRODUÇÃO

The *Fusarium* genus is characterized by its fast colonies growth, with aerial and diffuse mycelium, pale or colored (1).

Most of *Fusarium* species are compound by soil fungi with a cosmopolitan distribution, acting in the decomposition of cellulosic substrates of plants. Some strains are parasites of plants. Human pathogenicity caused by this genus is rare, but many species produce toxins (2-4).

Fusarium verticillioides (= *F. moniliforme*) is often found in maize (*Zea mays*

[1] Graduada em Tecnologia em Alimentos pelo CEFET (Centro Federal de Educação Tecnológica) de Campo Mourão, Paraná. Especialista em Biotecnologia com Ênfase em Meio Ambiente e Saúde pela Universidade Estadual de Maringá, UEM.

[2] Bióloga. Especialista em Biotecnologia Aplicada à Agroindústria pela UEM. Mestre em Biologia Comparada pela UEM. Doutoranda em Biologia Comparada pela UEM.

[3] Docente da Universidade Estadual de Maringá, UEM. Laboratório de Biotecnologia Microbiana Departamento de Biologia Celular e Genética Áreas de atuação: Genética Molecular e de Microrganismos Microbiologia Biotecnologia.

L.) in asymptomatic or symptomatic association and constitutes an important source of inoculum in soil (5). When this fungus acts as a phytopathogen, it is reported to produce mycotoxins such as beauvericin, enniatins, fumonisins, fusaproliferin, fusaric acid, fusarins, moniliformin, trichothecenes and, zearalenones (6). But more commonly is its life style as an endophyte (7).

Endophyte is a microorganism that lives inside tissues of living plants (7). Endophytic fungi are those capable of beneficial infections in plants, after colonization of host tissues, regardless of its systemic nature (8). It is relatively unstudied their potential to produce novel natural products for exploitation in agriculture, industry and medicine (9).

As an endophyte, *F. verticillioides* facilitates the growth of other fungi by degrading maize antimicrobial compounds (10). However, the greatest interest in this microorganism has been its use in disease control and its capacity to reduce mycotoxin contamination (11).

Some authors have reported that the genus with the highest occurrence in Brazilian maize crops is *Fusarium*, with high specificity at genotypic level (12). *F. verticillioides* has been showed as predominant among *Fusarium* species on maize (13).

A commonly occurrence of endophytic *F. verticillioides* in maize enhances the effects of phytopathogen *Ustilago maydis*, by interfering in the early infection process and limiting disease development, resulting in increase of maize growth (14).

In their study with a tropical endophytic strain of *F. verticillioides*, Pamphile et al. (5) created a genetic transformation system using the *Fusarium oxysporum* nitrate reductase *nia* gene and plasmid pNOM 102 carrying the *Escherichia coli* β-glucuronidase *gus*A gene. They obtained a transformation frequency between 30 and 60 transformants per µg of vector DNA. According to these authors, this system proved to be efficient, because the co-transformant expressed the gene in maize, demonstrating its effectiveness as vector for the introduction of characteristics with biotechnological or agricultural interest into tropical host plants.

This study aimed to analyze the morphological behavior of two strains of *Fusarium verticillioides*, 25 strain (wild) and A3 strain (transformant), and to compare the mycelial growth and conidial production before and after their inoculation in *Zea mays*, in order to verify whether the colonization of the host plant can alter the characteristics of an endophyte that shows specificity to this plant.

MATERIAIS E MÉTODOS

Fungal strains and culture media

Wild endophytic *Fusarium verticillioides* (25 strain) was isolated from maize by Pamphile and Azevedo (12) and the transformant *F. verticillioides* GUS^+ (A3 strain) was obtained by Pamphile *et al*. (5)

Murashige and Skoog medium (MS) (15) and solid Complete Medium (CM) (16) were employed.

Maize (*Zea mays* L.)

Seeds of self-fertilized maize cultivar AsT214 (03/2004) were donated by Dr. Alberto José Prioli (Departamento de Biologia Celular e Genetica, Universidade Estadual de Maringá).

Sterilization, inoculation and cultivation of maize seeds

This process was performed in accordance with Pamphile et al. (5). Maize seeds were washed in running tap water and brushed. Then the seeds were immersed into 0.01% Tween 80 and agitated for 30 min. The solution was replaced after 15 minutes.

Afterwards, the surface disinfection was made by immersion in 70% ethanol (1 min), 3% sodium hypochlorite (25 min) and 70% ethanol (30 sec). The disinfected seeds were washed in distilled sterile water, transferred to sterilized Petri dishes containing moist filter paper and germinated for 32 h at 26° C under aseptic conditions.

Seeds at the beginning of germination were aseptically imersed in a suspension of *F. verticilliodes* wild and transformant conidia at a concentration of 1.6 x 10^7 conidia/ mL, transferred to flasks containing MS medium and incubated at 26° C using a 13 h-light and

11 h-dark photoperiod. After 10 days the plantules were collected.

Reisolation of *Fusarium* strains from maize plantules

Infected vegetal samples (leaves and roots) were cut into 1 cm^2 fragments and deposited on Petri dishes (22 fragments per dish) containing CM medium supplemented with 100 $\mu g.mL^{-1}$ of antibiotic Terramycin (to inhibit bacterial growth) and incubated at 28º C. The dishes were supervised daily.

The colonization frequency (CF) was calculated as following: CF = number of leaf fragments with fungal growth/ total of sampled fragments x 100. The endophytic isolates were transferred to dishes containing CM medium and incubated at 28º C for 10 days.

Estimate of radial growth, conidial production and wet weight

After incubation, conidia of each strain were diluted in 9 mL of 0.01% Tween 80 solution, concentrated at 10^3 conidia/mL and spread (100 µL) onto Petri dishes containing CM medium. All dishes were incubated at 28º C for 48 h.

Afterwards, monosporic colonies were inoculated on dishes containing CM medium and autoclaved cellophane membrane. The dishes were incubated at 28º C for 4 days, then the colony diameters were measured, the sporulation and wet weights were quantificated.

The measurements of radial growth were taken on the back of dishes. To quantify conidial production, 1 cm^2 of colony fragments were diluted in saline solution (9 mL) and conidia were counted with a hemacytometer. The wet weight was quantificated by mycelial mass weight. Each counting was made in triplicate and the results were recorded considering the average values of the triplicates.

RESULTADOS E DISCUSSÃO

Reisolation of *Fusarium* strains from maize plantules

We employed the surface disinfection of seeds before isolation of *F. verticillioides* strains. This reisolation was performed with young plantules (12 days of development), using 66 fragments of plant tissues corresponding to seeds infected with the transformant strain and also 66 fragments of plant tissues corresponding to seeds infected with the wild strain.

It was possible to observe the systemic colonization of endophytic strains (A3 and 25) in maize leaves and roots (Table 1).

Table 1. Colonization frequency (%) of endophytes in maize.

Fusarium strains	Vegetal samples	
	Leaves	**Roots**
A3	31.18	45.45
25	59.10	50.00

Similarly, Pamphile and Azevedo (12) isolated endophytic *Fusarium* species from maize seeds after sufaced disinfection. Practically the same colonization frequency was obtained in the two field sites (around 60%). Among the genera found inside of this host plant, the most frequent was *Fusarium*.

These authors collected 21 isolates of *F. verticillioides* and they evaluated the plant-endophyte interactions and the genetic variability. Variability analysis of endophytic isolates via RAPD showed genome polymorphism taxa of species around 60%. The presence of different genotypes of *F. verticillioides* in maize seeds indicates an intimate association among them, which can be specific to the host plant genotype.

Based on our results, it was possible to observe that *Fusarium* strains have a small difference in their action way in roots. The leaf colonization frequency was higher for wild strain than for transformant one. With individual analyses, we observed that the two strains act in host tissues with significant differences.

The colonization frequency of endophytes and their distribution inside vegetal tissues may be influenced by plant-endophyte interactions. The *Fusarium* strains used in this study showed capacity to colonize different maize tissues.

Souza et al. (17) made an interesting research about the interactions between maize

and endophytic bacteria using Scanning Electron Microscopy (SEM), observing a preferential colonization of the adaxial epidermal cells of maize.

In their study, Silva and Bettiol (18) demonstrated the efficiency of non-pathogenic *Fusarium oxysporum* isolates inoculated in tomato roots seedlings in controlling vascular wilt caused by *F. oxysporum* f. sp. *lycopersici* in tomato.

Mattos et al. (19) analyzed the ability of endophytic bacterium *Burkholderia kururiensis* to colonize rice after roots reinfection. SEM and 16S rDNA analysis revealed a predominant colonization on root hair zones, demonstrating that the endophytic colonization occur primarily through the endodermis, followed by spreading into xylem vessels, reaching the aerial parts.

Conidial production, mycelial diameter and wet weight

The conidial production by each strain before and after plant inoculation is shown in Table 2. Comparing the 25 strain before and after reinfection of maize, a lower conidial production was observed after this reinfection. On the contrary, a larger production was showed by A3 strain after reinfection of maize. A3 strain increased considerably its conidial production and it kept its capacity to infect maize tissues.

This difference may be explained by the fact of the non-transgenic endophytic strain presents an endogenous condition in the interaction with *Zea mays*. Differently, although the capacity of infection has been preserved in the transgenic endophytic strain, some conditions of vegetative growth were altered and reduced. The recolonization process inside host plant could positively alter these parameters.

Table 2. Conidial production by wild (25) and transformant strain (A3) of *Fusarium verticillioides* before and after reinfection of maize tissues (mean of triplicates).

Conidial concentration (conidia/mL)	*F. verticillioides* strains	
	25	**A3**
Before maize reinfection	8.35×10^8	1.10×10^8
After maize leaves reinfection	4.87×10^8	5.68×10^8
After maize roots reinfection	1.20×10^8	2.60×10^8

One hypothesis for the increase of conidial production by the transformant strain is that the variation of this production may be influenced by: any intrinsic condition to the fungus, the genotype of host or the plant-fungal interactions. Plant-fungal interaction are much dynamic and complex, involving biotic and abiotic factors that can exercise pressure on inoculated fungi, causing changes in their genome.

Our results showed that the transformant *F. verticillioides* increased its expression inside maize, changing significantly its conidial production after maize leaves reinfection. This fact indicates its potential to the biological control, genetic improvement and its use as vector for genetic transformation of plants.

Glenn (20) examined the spore germination phenotypes of *F. verticillioides* and other *Fusarium* species, observing that *F. verticillioides* form germ tubes that rapidly penetrate into agar. Such invasive germination was the predominant growth phenotype among 22 field isolates of *F. verticillioides*. Their study with corn seedling blight showed that the surface germinating strains of *F. verticillioides* were less virulent than invasively germinating strains.

According to this author, the invasive germination is proposed as the dominant form of spore germination among *Fusarium* species. Conidia were not necessary for development of corn seedling disease, but invasive conidia germination may have enhanced the virulence of conidiating strains.

In this study, the wild strain reisolated from leaves showed a minor vegetative development than the wild non-inoculated strain, although the wild strain reisolated from roots presented a major vegetative development than the wild non-inoculated strain. The wet weight and estimate mycelial

diameter of strains are shown in Table 3 and 4, respectively.

The A3 strain, after reisolation from leaves and roots, do not showed large difference in diameter/wet weight relation when compared with A3 strain (without reinoculation in maize). On the contrary, the 25 strain presented slightly differences between reisolated strains.

When the weight of strains was compared, those reisolated from leaves showed a higher weight than those strains that do not have been reinoculated in maize.

Table 3. Wet weight and diameter/wet weight relation of *F. verticillioides* strains before and after reinoculation of maize (mean of triplicates).

Strains	Wet weight (g)	Diameter/ Wet weight (g/cm)
25[(1)]	1.58	5.43
25 L[(2)]	1.85	4.25
25 R[(3)]	1.48	5.74
A3[(1)]	1.50	5.71
A3 L[(2)]	1.63	5.34
A3 R[(3)]	1.58	5.42

[(1)]Strains non-inoculated in maize; [(2)]Strains reisolated from leaves; [(3)]Strains reisolated from roots.

Table 4. Radial growth velocity of *F. verticillioides* strains before and after reinoculation of maize (average of triplicates).

Strains	Colony diameter (cm) in relation to the growth time (h)			
	24 h	48 h	72 h	110 h
25[(1)]	1.65	3.20	4.60	8.58
25 L[(2)]	1.60	2.77	4.73	7.87
25 R[(3)]	1.60	3.00	4.67	8.50
A3[(1)]	1.50	3.50	6.85	8.57
A3 L[(2)]	1.40	5.00	6.80	8.70
A3 R[(3)]	1.43	3.27	6.57	8.57

[(1)]Strains non-inoculated in maize; [(2)]Strains reisolated from leaves; [(3)]Strains reisolated from roots.

Our results showed that wild and transformant strains are capable of colonizing different maize tissues. The growth velocity showed to be an efficient parameter to value the response of microbial strains after reinoculation of host plants.

Strains showed not much difference in the growth velocity at the first 72 hours, however, comparing the growth of 25 strain before and after its reinoculation in maize, there was no difference in its growth (Figure 1). The A3 strain showed slow growth velocity at the first 48 hours when it was compared with the reisolated strain (Figure 2).

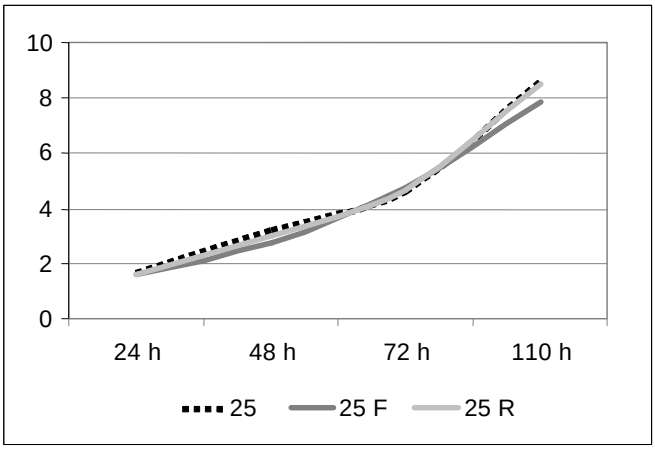

Figure 1. Growth velocity of wild *F. verticillioides* before (25) and after reinfection of maize leaves (25 F) and roots (25 R).

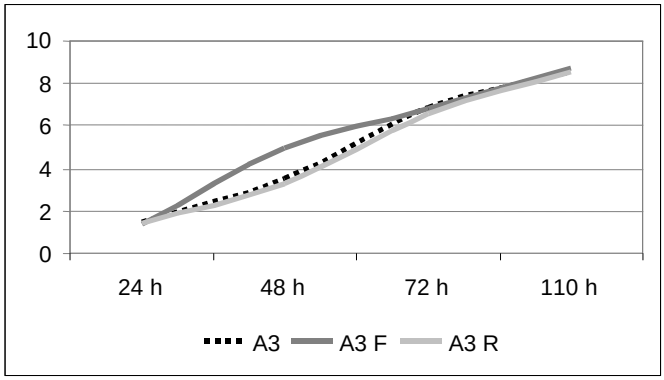

Figure 2. Growth velocity of transformant *F. verticillioides* before (A3) and after reinfection of maize leaves (A3 F) and roots (A3 R).

In their study about pathogenic strains of *F. verticillioides*, Suárez-Jiménez et al. (21) showed that the methanolic extracts of *Braccharis glutinosa* and *Larrea tridentata* can reduce the spore germination percentage, radial growth extesion and biomass production of this pathogen in more than 90%, indicating their potential for the control of this pathogen during its different development phases.

Studies involving morphological analysis are important to collaborate with preliminary studies about specificity among fungi and host plant, as well as they contribute with the understanding of microbial evolution, specially endophytic *F. verticillioides*. Because of the incorrect and confused application of *Fusarium* species names, there are limitations of morphological recognition of them and their applications.

The fungal behavior after passage in hosts has been also observed by several research groups. Feijó et al. (22) analyzed behavior and cytology of *Beauveria bassiana* after passage in eggs, larvae and adults *Chrysomya albiceps.* The germination percentage was elevated in the reisolates from the larval phase. Also, the number of colonies increased for all reisolates, as well as the conidia number of the reisolates from the adult phase, suggesting the capacity of these fungi for controlling *C. albiceps*, an important causer of secondary myiais.

The same authors (23) also employed the same methodology to evaluate the behavior and cytology of *Metarhizium anisopliae* var. *anisopliae* and *M. flavoviride* var. *flavoviride* after passage in eggs, larvae and adults *C. albiceps*. According to them, no significant differences were observed in the cytological aspects of the life cycle of the fungi after passage in host. But the germination percentage, quantity of conidia, quantity and diameter of colonies was greater post-passage. In agreement with previous study, the results showed the potential of *Metarhizium* species to control this pest.

A similar study was made by Almeida et al. (24), in order to verify the viability of *B. bassiana* reisolated from eggs, larvae and adults of *Anthonomus grandis*. The conidial suspension caused high death index of *A. grandis* and a high viability of germination, colonial growth and conidiogenesis was also observed. Thus, *B. bassiana* is able to be used as a control agent against this insect.

Wang et al. (25) evaluated the evolution of virulence in *Fusarium oxysporum* f. sp. *vasinfectum* after serial passage through cotton, observing an increased virulence in the offspring isolates generated on cotton. It suggests that the evolution of virulence in this fungus occurs in a continuous process and is associated with the presence of cotton.

Therefore, the results obtained in our research are important information that may contribute with assays using transformant microorganisms, comparing them with wild strains and comparing the interactions among both of them with host plants.

This study indicated that a genetically manipulated fungal endophytes can colonize different tissues of the host plant efficiently, serving as an alternative vector to introduce interesting genes in plants. Also, the endogenous host environment could influence morphological characteristics of endophytes that are specie-specific with them.

Lenita Zayatz , Ravely Casarotti Orlandelli , João Alencar Pamphile

Endereço para correspondência: Laboratório de Biotecnologia Microbiana
Departamento de Biologia Celular e Genética
Universidade Estadual de Maringá
CEP 87020-900, Maringá, Paraná
Departamento de Biologia Celular e Genética, Universidade Estadual de Maringá, CEP 87020-900, Maringá, Paraná.
Tel: (44) 3011-4342
Fax: (44) 3011-4893.
E-mail: prof.pamphile@gmail.com

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