GENETIC TRANSFORMATION WITH THE *gfp* GENE OF *Colletotrichum gloeosporioides* ISOLATES FROM COFFEE WITH BLISTER SPOT

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ABSTRACT

Blister spot (*Colletotrichum gloeosporioides*) is now widespread in most coffee producing states of Brazil, becoming a limiting factor for production. The lack of data relating to the reproduction of typical symptoms (light green, oily patches) leaves a gap within the pathosystem, forcing the search for new methodologies for monitoring the disease. Monitoring of genetically modified organisms has proven to be an effective tool in understanding the host x pathogen interactions. Thus, the present study was carried out to evaluate the effectiveness of two systems of genetic transformation in obtaining mutants using the *gfp* reporter gene. Using the two transformation systems (PEG and electroporation) revealed the efficiency of both, confirmed by fluorescence microscopy and resistance to the antibiotic hygromycin-B, when incorporated into the culture medium. The fungus maintained its cultural and morphological characteristics when compared to wild strains. When inoculated on coffee seedlings, it was found that the pathogenicity of the processed isolates had not changed.

Key words: *Colletotrichum gloeosporioides*. Fluorescent proteins. Pathogenicity.

INTRODUCTION

Blister spot, which is related to the plant pathogen *Colletotrichum gloeosporioides* in *Coffea arabica* was reported for the first time in Brazil in 1958, by Bitancourt (4). Currently, it is widespread in most coffee producing states (Minas Gerais, Espirito Santo, Sao Paulo, Parana and Rondonia), in the species *Coffea arabica* L. and *Coffea canephora* Pierre (9), where field observations have revealed their escalation, leading to a decline in plant vegetation and consequently production.

The disease presents symptoms of buttery-like, light-green oily patches lighter than the surface of the leaf. In later stages these spots become pale greenish-yellow with irregular borders, which coalesce and cause premature leaf drop. In branches and fruits, the lesions appear as smaller, depressed, necrotic light brown in color and irregular borders. Intense attacks are usually observed on leaves and new shoots of adult plants, causing necrosis and the drying out of branches at the

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apex, potentially leading to plant death in descending order (8).

Despite the efforts of recent research, it is still uncertain as to the reproducibility of the typical symptom of the disease in leaves (light green, oily patch). Only positive results have been obtained demonstrating hypocotyls death, necrosis of fruits and leaves when they are inoculated with isolates from plants showing symptoms of blister spot (18). Lack of data relating the pathogen with the main symptom of the disease, combined with the few studies focusing on the histopathological aspects when interacted with the pathogen-host, leaves a doubt as to the real importance of controlling the pathogen within the culture.

One alternative that has resulted in better understanding the interaction of pathogenic fungi with their host is the transformation of these pathogens with genes that allow the traceability of tissues and organs within the plant (25). Several marker genes have been employed; including the ones that encode fluorescent proteins which have been considered important tools in revolutionizing cell biology, especially in the study of living cells (10). The reason these have been regarded as excellent molecular markers is that the display of their products does not require substrates or cofactors and can be used in any organism that is genetically modified (7). Current literature has described several plasmids containing genes that encode proteins that fluoresce in different wavelengths.

The green fluorescent protein (GFP), encoded by the *gfp* gene, a protein with 238 amino acids, which is intrinsically fluorescent, emits a green light when exposed to ultraviolet irradiation (5). Its main feature is the fluorescence provided by their tertiary structure, called beta-can, which has in its interior, the chromophore region responsible for fluorescence. This property allows this protein, in noninvasive detection, to be used as an efficient marker gene in vivo. GFP has become important because it allows a noninvasive detection due to its small size, besides being highly stable and easily detectable (12).

The *gfp* gene has been widely used to study the interaction between host plants as well as pathogenic and non-pathogenic microorganisms, to study the penetration, colonization and dissemination, such as; *Erwinia amylovora* in apple (3); pathogenic and non-pathogenic isolates of *Colletotrichum acutatum* (11), *Fusarium virguliforme* in soybean (15), *Phomopsis viticola* in grapevine (1), *Rosellinia necatrix* in avocado (20), ectomycorrhizal fungi (*Pisolithus tinctorius*) and endomycorrhizal (*Oidiodendron Maius*) in angiosperms (22, 16) as well as quantification of *Trichoderma harzianum* in soil (19).

The attainment of *Colletotrichum gloeosporioides* transformed with *gfp* can be an important step in the elucidation of relevant aspects of the blister spot pathosystem, clarifying whether the real potential of the isolates are related to symptoms observed in the plant.

Currently there are a number of strategies available that can be used to obtain transgenic fungi which may vary according to the organism used, the objective of the study and available resources. Thus, it is necessary to use techniques that are suitability for each species studied with the purpose of obtaining an efficient transformation. Therefore, the study was conducted to evaluate two techniques for the transformation of this fungus with the GFP gene, and verify whether it interferes with the pathogenicity of the isolate.

MATERIALS AND METHODS

The isolate of *Colletotrichum gloeosporioides* (IS2) used for genetic transformation was selected from the mycological collection of Laboratory Diagnosis and Control of Plant Diseases, Department of Plant Pathology, Federal University of Lavras, which is derived from spore cultures, which had previously confirmed their pathogenicity.

Transformation Vector

The transformation plasmid vector used was pCT74 (pSC001) (Figure 1), containing the sgfp, with the promoter

gene PtOx from the fungus *Aspergillus nidulans*, containing a resistance gene (HPR) to the antibiotic hygromycin-B. The plasmid was provided by researcher Dr. Theo van der Lee (Plant Research International, The Netherlands).

Obtaining protoplasts

Following the standard procedure for production of protoplasts, for every 100 mg of wet mycelium, 3 mL of osmotic buffer of 0.7 M KCl pH 5.8 were used with the addition of the combined lytic enzymes (Lysing enzymes, Sigma # L 1393) at a concentration of $10\mu g.mL^{-1}$ of osmotic stabilizer. After preparation, this was incubated at room temperature with agitation at 75 rpm, for four hours (2). After the release of protoplasts, the suspension was centrifuged, subjected to microscopic analysis and quantified in a Neubauer chamber, with the aim of obtaining a final concentration adjusted at 10^7 protoplastos.mL^{-1}.
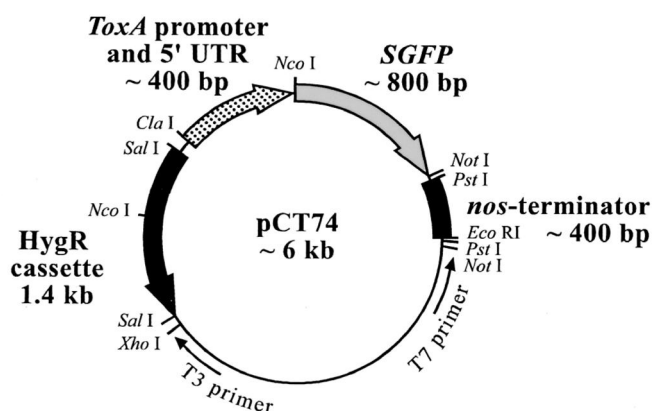

Figure 1. A GFP expression vector for filamentous fungi, pCT74 (14).

Chemical transformation mediated by polyethyleneglycol (PEG)

After the acquisition, the protoplasts were resuspended in storage buffer, containing four parts STC (0.8 M sorbitol, 50 mM Tris HCl pH 8.0 and 50 mM CaCl$_2$) and one part SPTC (0.8 M sorbitol, 40% PEG 4000, 50 mM Tris HCl pH 8.0 and 50 mM CaCl$_2$). After adding $10\mu L$ of plasmid DNA at a concentration of 1.66 $\mu g.\mu L^{-1}$, the suspension was incubated on ice for 30 minutes. As a control, sterile distilled (autoclaved) water was used in place of DNA. Then it was added to a suspension of SPTC 1 mL and incubated for 20 minutes at room temperature. Then it was poured into 100 mL of regeneration medium, previously autoclaved, and added to 50 $\mu g.mL^{-1}$ of hygromycin-B. The experiment was performed in duplicate.

Transformation by electroporation

For transformation by electroporation, $10\mu L$ of plasmid DNA at a concentration of 1.66 $\mu g.\mu L^{-1}$, was added to 1mL of protoplast suspension (10^7protoplastos.ml^{-1}). As a control, we used sterile distilled (autoclaved) water in place of plasmid DNA. The suspensions were placed in disposable cuvettes on ice for 10 minutes. After this period, samples were subjected to electroporation in the electroporated Porator Cell®, using internal resistance of 400 ohms, 50 mF capacitance and field strength of 1.5 kV / cm. After they were placed on ice for 15 minutes. Later on they were mixed with 1mL of the osmotic stabilizer KCl by gently shaking the suspension and then poured into 100 mL of regeneration medium, with 50 $\mu g.ml^{-1}$ hygromycin-B, used for selection of transformants. The experiment was performed in duplicate.

Transformant Rating

After seven days of growth, observations were made under epifluorescence microscopy (EMF) (Zeiss Axio Observer Z.1) with a 470 to 490 nm filter and peak transmission at 510 to 560 nm.

After transformation, the isolates were evaluated using MEA (malt extract 2% - Agar) supplemented with hygromycin-B, as well as coloring, and mycelial growth index (MGI). Additionally, testing was performed on the mitotic stability of transformants in which only colonies grown in MEA were transferred successively seven times, with the last subculture containing the antibiotic hygromycin-B($50\mu g.mL^{-1}$).

To evaluate the pathogenicity of the transformed isolate, inoculation was carried out by spraying 15 hypocotyls, obtained from embryo culture during "match-stick" stage, with suspensions of spores at a concentration of $2x10^6$ conidia mL^{-1}. The hypocotyl remained in a growth chamber for a photoperiod of 12 hours at 25 ° C ± 1 for 15 days. Symptoms were assessed according to the Van DenVossen et al. (23) scale and interpretation of results made by the disease intensity index (DII = Σ(nº of hypocotyls in each class x numeric value of each class) / (nº of hypocotyls in all classes x 4)).

RESULTS AND DISCUSSION

The genetic transformation using the plasmid pCT74, expressing *gfp*, was successful in both cases. Confirmation of transformation of *C. gloeosporioides* was performed by resistance to the hygromycin-B antibiotic and green fluorescence in the transformed strains since the wild isolates and control, when under epifluorescence microscopy, do not emit fluorescence.

Fluorescence intensity was variable among the transformed isolate as when observed in conidia and hyphae cytoplasm. In general, the highest fluorescence intensities were observed in conidia of *C. gloeosporioides*. It was also observed that the quality of fluorescence between the two techniques were different. In conidia of *C. gloeosporioides* transformed by electroporation, it was noted that the fluorescence was uniform. On the other hand, failures were seen in the emission of fluorescence in conidia transformed by PEG. The opposite was observed in the mycelium, because those transformed by the PEG method, showed a higher number of hyphae with high fluorescence. According to Mansouri *et al.* (15), differences in the level of expression of GFP may occur due to the positioning of the GFP gene in different regions of the genome of the fungus, or due to multiple integrations of the vector transformation

When transformed by electroporation (Figure 2) 18 transformed colonies were obtained. This is the first report of transformation of *Colletotrichum gloeosporioides*, causal agent of blister spot on coffee plants in Brazil by electroporation. For the chemical transformation mediated by PEG (Figure 2) 21 transformed colonies were obtained. The transformation efficiency varies depending on the system used and the microorganism to be transformed. When working with systems of genetic modification, you should stick to the fact that some microorganisms naturally incorporate exogenous DNA under certain conditions, and almost all cells are transformed. However, in some populations, only a few cells become competent, that is, capable of receiving the DNA, making it possible that only a small number of cells have a plasma membrane permeable to transforming DNA, limiting the frequency of transformation (13).
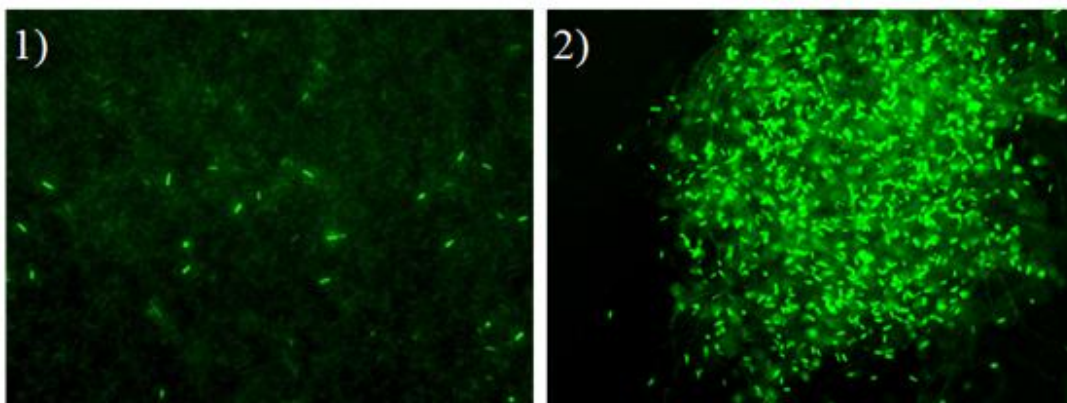
Figure 2. Conidia of *C. gloeosporioides* transformed by electroporation (1) and transformed by PEG (2), when observed under an epifluorescent microscope with 10x magnification. The difference in fluorescence between conidia transformed by electroporation (3) and PEG (4). Hypocotyls of coffee with symptoms of stem necrosis caused by *C. gloeosporioides* and healthy subjects (controls): A) infection by the transformed strain (indicated by the arrows); B) infection by isolated pathogenic wild type (indicated by the arrows), C) isolated nonpathogenic wild type; D) control inoculated with water; E) conidia isolated from hypocotyls of coffee inoculated with transformed isolates.

Robinson & Sharon (21) managed to obtain more than 80 stable transformants of *Colletotrichum gloesporioides* f. sp. *aeschynomene*. Whereas, Chen, Hsiang and Goodwin (6), had nine transformants of *C. destructivum* and 8 *C. orbiculare*. Oren *et al.* (17), working with *Fusarium verticillioides*, had two transformants per μg of plasmid DNA and West *et al.* (24) generated 64 transformants of *Phytophthora palmivora*.

No difference was observed between the transformed isolate and the wild type as far as the shape and color of the colonies, ranging from gray-white to salmon. Regarding the mycelial growth rate index (MGI), there was a slight reduction in growth between the transformed isolate and the wild type (Figure 3 and 4).
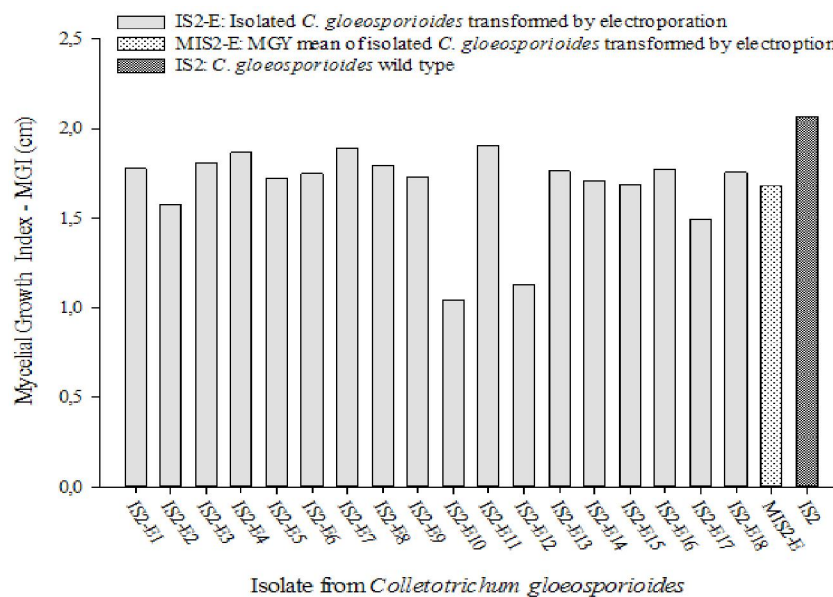

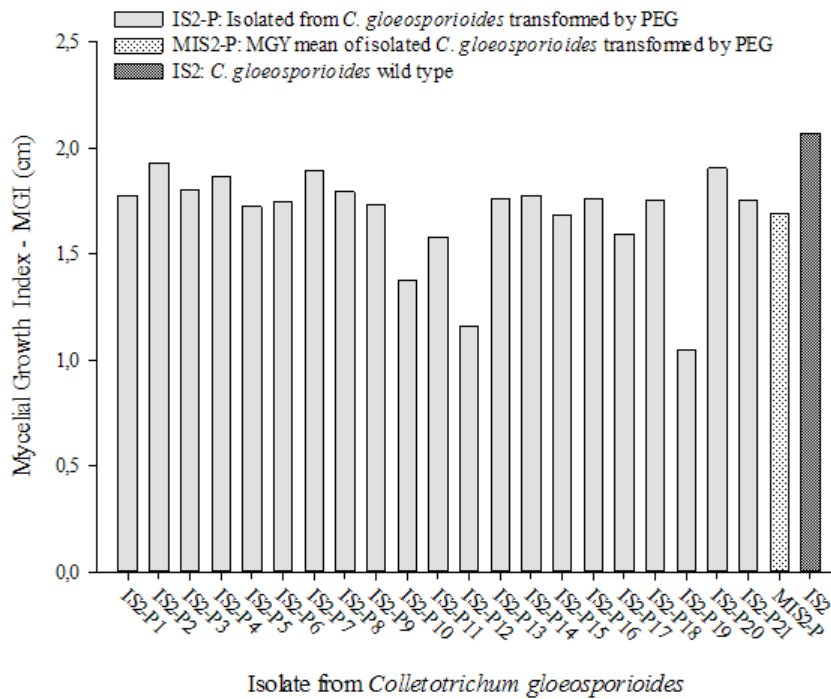**Figure 3.** Grow rate of *C. gloeosporioides* transformed by electroporation

Figure 4. Grow rate of *C. gloeosporioides* transformed by PEG.

Chen, Hsiang and Goodwin (6) succeeded in transforming *Colletotrichum destructivum* and *Colletotrichum orbiculare*, noting that even after several transfers in selective and nonselective media, there was maintenance of stable expression of fluorescence. Transformants showed the same growth rate and the same cultural characteristics of the wild type, as one can observe the expression of *gfp* in all fungal structures during leaf infection of *Nicotiana benthamiana*.

In relation to mitotic stability, when the transformed fungi were transferred successively six times in MEA, they showed normal growth. However, for those obtained by electroporation, when transferred for the seventh time to MEA containing hygromycin-B (50 μg.mL^{-1}), the transformed showed no growth after seven days and did not maintain their fluorescence. As for the transformed obtained with PEG showed a slight growth when transferred to selective medium, they too did not maintain fluorescence. However, when both were transferred to medium containing hygromycin-B, on average, fifty percent of the isolate transformed with *gfp* maintained their fluorescence.

According to Lorang *et al.* (14), this can be explained by the fact that some fungi form multinucleate protoplasts, which can lead to transformants with fluorescence in only a few fungal cells, which then requires many transfers in selective medium to maintain fluorescence, in these cases, the nucleus does not maintain the information required for the production of fluorescence during the successive transfers.

By means of the pathogenicity test of isolate transformed to *C. gloeosporioides* on coffee seedlings, it was evident that these isolates retained their ability to infect and cause damage (Table 1). The virulence of the isolate transformed with the *gfp* marker in this study was comparable to the wild type. This suggests that the presence or expression of the gene did not affect significantly the fitness of the transformed fungus, nor did it interrupt genes potentially involved in the processes of recognition and establishment of the relationship.

Table 1. Results of pathogenicity test presented by transformed isolate   according to Vossen *et al.* (23).

Isolated	Disease Intensity Index (DII)
IS2	0,90
IS2-P	0,45
IS2-E	0,66
IH	0,00
ADE	0,00

SI2 - isolated from *C. gloeosporioides* pathogenic
IS2-P - isolated from *C. gloeosporioides* transformed by PEG
IS2-E - isolated *C. gloeosporioides* transformed by electroporation
IH - Isolated *C. gloeosporioides* nonpathogenic
ADE - inoculated with sterile distilled water

During the pathogenicity test the first symptoms were observed seven days after inoculation, however the wild type isolate showed greater changes in the lesions, resulting in overturning and consequent death of the hypocotyls on the ninth day of evaluation. For the transformants (PEG and electroporation), such results could only be seen on the thirteenth day after inoculation. This fact may be related to the reduction of MGI after transformation of the isolate, delaying the process of infection and consequently the manifestation of symptoms.

After the test, was carried out the isolation of the pathogen in synthetic medium containing hygromycin-B, confirming that it was the cause of the injuries, this was also observed in fluorescence microscopy, confirming the presence of those transformed.

CONCLUSIONS

The isolate transformed by both methods exhibited normal phenotypes for the species in both synthetic medium and during infection, indicating that the *gfp* gene did not alter the morphology or pathogenicity of the isolate, proving that this can be an effective marker in monitoring the pathogen in processes of infection and colonization of the host plant. The number of mutant colonies obtained in this study, as well as the expression of GFP was not a factor in the choice of methodology. Thus the two techniques showed that they are able to obtain the mutants.

This work demonstrates the success achieved in two methods of genetic transformation in *Colletotrichum gloeosporioides* using the plasmid pCT74, which is effective in the expression of the marker gene *gfp*.

In view of the progress achieved and of how relevant it is, for the *Colletotrichum* pathosystem in coffee, additional work is still needed, enabling the direction of future work for monitoring and quantification of the pathogen, as well as studies of transmissibility of the pathogen.

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