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ORIGINAL RESEARCH ARTICLE

Agrobacterium-Mediated Transformation of *Diaporthe schini* Endophytes Associated with *Vitis labrusca* L. and Its Antagonistic Activity Against Grapevine Phytopathogens

Aretusa Cristina Felber[1] · Julio Cesar Polonio[1] · Ravely Casarotti Orlandelli[1] · Alessandra Tenório Costa[1] · Eliane Papa Ambrosio-Albuquerque[1] · Renata Assis Castro[2] · Maria Carolina Quecine-Verdi[2] · João Lúcio de Azevedo[2] · João Alencar Pamphile[1] · Káthia Socorro Mathias Mourão[3]

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Abstract Fungus-caused diseases are among the greatest losses in grapevine culture. Biological control of pathogens by endophytes may be used to decrease fungicide application rates and environmental impacts. Previously, *Diaporthe* sp. B46-64 and C27-07 were highlighted as antagonists of grapevine phytopathogens. Herein, molecular multigene (ITS-TUB-TEF1) identification and phylogenetic analysis allowed the identification of these endophytes as belonging to *Diaporthe schini* species. *Agrobacterium tumefaciens*-mediated transformation was employed for obtaining 14 stable and traceable *gfp*- or *DsRed*-expressing transformants, with high transformation efficiency: 96% for the pFAT-GFP plasmid and 98% for pCAM-DsRed plasmid. Transformants were resistant to hygromycin B with gene *hph* confirmed by polymerase chain reaction and proved to be mitotically stable, expressing the fluorescent phenotype, with morphological differences in the colonies when compared with wild strains. In vitro antagonism tests revealed an increased antagonistic activity of some transformant strains. The current genetic transformation of *D. schini* mediated by *A. tumefaciens* proved to be an efficient technique within the randomized insertion of reporter genes for the monitoring of the strain in the environment.

Keywords Green fluorescent protein · Red fluorescent protein · Molecular multigene identification · Phylogeny · Biological control

Introduction

Grapevine culture is economically important worldwide: in 2016, cultivated area reached ~ 7 million hectares and grape production was over 77 million tons [1]. However, most grapevine cultivars are greatly sensitive to several fungal pathogens [2]. Biological control through microorganisms that inhibit or antagonize plant pathogens can reduce or eliminate the use of chemical fungicides. Fungal endophytes (that inhabit asymptomatically the interior of plants) are effective antagonists that compete with pathogens within the same ecological niches, providing the host several advantages as decrease in herbivory, protection against pests and systemic resistance against pathogens [3].

Several studies on the biocontrol of grapevine pathogens by endophytes have been undertaken [4–6] but in vivo investigations about endophytes-grapevine interactions are rare. One of the main strategies for monitoring in situ marked strains consists of using fluorescent protein that are highly stable, do not require an exogenous substrate nor do they affect the examined tissue and only ultraviolet light and oxygen are needed for the fluorescence emission [7]. *Agrobacterium tumefaciens*-mediated transformation (ATMT), widely employed for the insertion of genes that encode fluorescent proteins, is facilitated by the bacterial

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✉ João Alencar Pamphile
japamphile@uem.br

[1] Department of Biotechnology, Genetics and Cell Biology, State University of Maringá, Maringá, São Paulo 87020-900, Brazil

[2] Department of Genetics, College of Agriculture Luiz de Queiroz, University of São Paulo, Piracicaba, São Paulo 13418-900, Brazil

[3] Department of Biology, State University of Maringá, Avenida Colombo, 5.790, Jd. Universitário, Maringá, Paraná 87020-900, Brazil

capacity to transfer part of their DNA to intact fungal cells as spores, mycelia and fructification bodies [8]. Easy execution, high transformation efficiency and sole insertion of genetic material in the host genome are some of its advantages [8, 9].

Diaporthe (= *Phomopsis*) is a dominant genus in the endophyte community of tropical plants [6, 10, 11]. Previously, *Diaporthe* sp. B64-46 and *Diaporthe* sp. C27-07, isolated from grape cultivars Bordô and Concord (*Vitis labrusca* L.) have been highlighted as promising antagonists of grapevine pathogens [6]. Herein, we identify these endophytes at species level; establish an efficient ATMT methodology for *Diporthe* genus, describe the development of stable and traceable transformed strains in the environment through green (*gfp*) and red fluorescent (*DsRed*) protein-encoding genes; and compare the antagonistic activity of transformed and wild fungi against phytopathogens.

Materials and Methods

Strains and Plasmids

Diaporthe sp. B64-46 (GenBank code KM362383.1) and *Diaporthe* sp. C27-07 (KM362392.1), respectively isolated as endophytes from leaves of grape cultivars Bordô and Concord (*V. labrusca*), were retrieved from the Collection of Endophytic and Environmental Microorganisms (CMEA/LBIOMIC-UEM), Laboratory of Microbial Biotechnology, Universidade Estadual de Maringá, Brazil. Previous molecular identification was based on sequence of ITS1-5.8S-ITS2 regions of rDNA [6].

Disarmed strains of *A. tumefaciens* EHA105 containing plasmids pFAT-GFP and pCAM-DsRed and the binary transformation vector pFAT-GFP [9] with 18,422 bp. were kindly given by Dr. Carlos Labate (Department of Genetics, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Brazil) and by Kim M. Plummer (CSIRO Plant Industry, Australia), respectively. The T-DNA region featured hygromycin B-resistance gene from *Escherichia coli* (*hph*), led by promoter of glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) from *Glomerella cingulata*, and the gene of green fluorescent protein (*gfp*) from the jellyfish *Aequorea victoria*. The binary transformation vector pCAM-DsRed with 12,008 pb [12], was retrieved from the Fungal Genetics Stock Center, and contains the T-DNA region, which comprised *hph* gene from *E. coli* (*hph*), led by promoter of glyceraldehyde-3-phosphate dehydrogenase gene (*PgpdA*) from *Aspergillus nidulans*, and the gene of red fluorescent protein (*DsRed*) from the coral *Discosoma* sp.

Molecular Multigene Identification of *Diaporthe* sp. Endophytes

Molecular multigene identification was employed to identify *Diaporthe* endophytes at species level. First, genomic DNA was extracted as previously reported by Felber et al. [6]. Primers and sequencing conditions were performed as described in Polonio et al. [13]: EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) were used to amplify the elongation factor gene 1-α (TEF1); T1 (5′-AACATGCGTGAGATTGTAAGT-3′) and Bt-2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′), for amplification of the β-tubulin (TUB) gene; ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCCCGCTTATTGATATGC-3′) were used to amplify the ITS1-5.8S-ITS2 regions of rDNA.

Polymerase chain reaction (PCR) was performed in a final volume of 25 µL, comprising: buffer 1×, 25 µM of dNTPs, 0.6 µM of each primer, 0.04 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, USA), and 10 ng of DNA. $MgCl_2$ concentrations for reactions comprised: 3.75 mM for gene ITS; 2 mM for TEF1; and 1.5 mM for TUB. PCR conditions for TEF1 and TUB: initial denaturation at 94 °C during 4 min, followed by 40 cycles with denaturation at 94 °C for 30 s; annealing at 55 °C for 1 min; extension at 72 °C for 1 min; final extension at 72 °C for 3 min. PCR conditions for ITS gene: 92 °C for 4 min (initial denaturation); 35 cycles at 92 °C for 40 s (denaturation); 52 °C for 1 min and 30 s (annealing); 72 °C for 2 min (extension); 72 °C for 5 min (final extension). Amplicons were analyzed in 1.5% agarose gel with standard for DNA molecular weight latter 1 kb (Kasvi, São José dos Pinhais, Brazil) at 70 Volts for 90 min. DNA sequencing and multigenic (ITS + TUB + TEF1) phylogenetic analysis based on the Maximum Likelihood and Bayesian inference were also performed according to Polonio et al. [13].

Sensitivity Test to Hygromycin B

The detection of minimum inhibition concentration (MIC) of hygromycin B was required to select transformants after ATMT, considering *hph* gene present in plasmids. To establish the MIC, *Diaporthe* sp. B64-46 and C27-07 were incubated in PDA dishes plus hygromycin B (Invitrogen; 0, 5, 25, 50, 75, 100, 150 and 200 µg/mL) in the dark for 7 days at 28 °C, with three replicates.

A. tumefaciens-Mediated Transformation (ATMT) of *Diaporthe* Endophytes

ATMT was performed according to Bernardi-Wenzel et al. [14], with modifications. *A. tumefaciens* strains with vector pFAT-GFP or pCAM-DsRed were prepared as previously described [14]; except that 10 mL (instead of 25 mL) of YEP medium was used. For strain with pFAT-GFP, YEP medium was supplemented with 300 μg/mL of streptomycin (Sigma-Aldrich, St. Louis, USA) and 100 μg/mL rifampicin (Sigma); for strain with pCAM-DsRed, 100 μg/mL of kanamycin (Sigma) and 100 μg/mL of rifampicin were used. Fragments of mycelial growth (1 cm^2) from seven-day-old cultures of *Diaporthe* endophytes (previously grown in PDA) were transferred to potato dextrose broth (PDB; pH 6.6), incubated at 28 °C for 6 h, and then centrifuged (4000 rpm, 7 min) and diluted in liquid inducing medium (IM) [14] until the OD_{600} reached 0.6.

Suspensions of *A. tumefaciens* with vector pFAT-GFP and *Diaporthe* sp. B64-46 were mixed; the same was performed for *A. tumefaciens* with pCAM-DsRed and *Diaporthe* sp. C27-07. For this, 20 μL microbial suspension was placed on sterilized 1.5-cm^2 filter papers one over the other (J. Prolab, São José dos Pinhais, Brazil). Fifty fragments for each transformation event placed on Petri dishes with IM and incubated at 28 °C for 48 h were used. Filter paper fragments were incubated for 72 h in PDA dishes with 25 μg/mL hygromycin (to select transformants) and 300 μg/mL cefoxitin sodium (Kefox-ABL) to eliminate bacterial cells; then fungi were peaked in PDA dishes under the same culture conditions. Finally, fungi were transferred to PDA dishes with 25 μg/mL hygromycin B. Colonies that grew under such conditions were considered transformants.

Expression of GFP and DsRed Proteins

Heterologous genes were determined 7 days after the growth of transformed fungi in PDA dishes with 25 μg/mL hygromycin B. Seven transformants for each gene were randomly chosen and mycelia were directly inserted in laminas with sterilized distilled water. Fluorescence emission was observed under a 40× fluorescence microscope (Olympus Bx51) with FITC filter (480 nm) or TRITC filter (545 nm), for transformants carrying pFAT-GFP or pCAM-DsRed plasmids, respectively. Images were captured by camera locked to the microscope, by Spectral Imagining (Carl Zeiss). Wild strains were negative controls.

Molecular Analyses of Transformants

DNA of wild and *gfp*- or *DsRed*-expressing strains (14 transformants) was extracted according to Felber et al. [6]; except that transformants were initially grown in PDB dishes with 25 μg/mL hygromycin B at 28 °C for 7 days. DNA concentration and purity were verified in 1% agarose gel premixed with 0.1 μg/mL ethidium bromide with DNA ladder 1 kb (Kasvi) as molecular weight standard. Firstly, *hph* gene of each plasmid was amplified with primers hph1 (5′-TTCGATGTAGGAGGGCGTGGAT-3′) and hph2 (5′-CGCGTCTGCTGCTCCATACAAG-3′). PCR reaction was performed as previously described [20]. Amplicons were visualized on a 1% agarose gel premixed with 0.1 μg/mL ethidium bromide.

Primers glGFP5 (5′GCCGGAATTCATGAGCAAG GGCGAGGAACTGTTC-3′) and glGFP3 (5′-GCCGAGC TCAGATCTCACTTGTACAGCTCGTCCATGCC-3′) [9] were employed to amplify the *gfp* gene. For *DsRed* gene, primers DsRedF (5′-AGGACGTCATCAAGGAGTTC-3′) and DsRedR (5′-CAGCCCATAGTCTTCTTCTG-3′) [15] were employed. Both reactions were similarly prepared in a final volume of 25 μL: buffer for PCR 1×, 0.2 mM of dNTPs, $MgCl_2$ (1.4 mM for *gfp*, 3 mM for *DsRed*), 0.2 mM of each primer, 0.05 U of Platinum Taq DNA Polymerase (Invitrogen), 10 ng of genomic DNA and ultrapure water. PCR conditions were: initial denaturation at 94 °C for 5 min (*gfp*) or 4 min (*DsRed*); 32 (*gfp*) or 25 cycles (*DsRed*) with denaturation at 92 °C for 30 s; annealing for 30 s at 58 °C (*gfp*) or 58.4 °C (*DsRed*); extension at 72 °C for 30 s and final extension at 72 °C for 7 min. Amplicons were visualized on a 1% agarose gel premixed with 0.1 μg/mL ethidium bromide to confirm the presence of the plasmid-specific genes in the transformed strains.

Mitotic Stability of Transformants

Fourteen transformants were cultured on PDA without hygromycin B for five successive cultures incubated for seven days at 28 °C in the dark, and then transferred to PDA dishes with 25 μg/mL of hygromycin B. Transformants with continuous growth were considered stable [9]. Strains were analyzed under a microscope to confirm the permanence of heterologous genes.

In Vitro Antagonism and Competitive Interactions Between Endophytes and Pathogens

Wild endophytes and 14 transformants were tested against the grapevine pathogens *Alternaria* sp. CNPUV 674 (leaf blight) and *Glomerella* sp. CNPUV 378 (rottenness of mature grapes) provided by EMBRAPA Uva e Vinho,

Table 1 *Diaporthe* endophytes isolated from grape cultivars Bordô and Concord and sequences with greatest identity when aligned to GenBank database (NCBI)

Endophyte	Gene	Fungus with highest similarity	GenBank code	Identity (%)
Diaporthe sp. B64-46 (KM362383.1)	ITS	*Diaporthe terebinthifolii* CBS 133180	KC343216.1	99
	TUB	*Diaporthe schini* CBS 133181	KC344159.1	99
	TEF1	*Diaporthe schini* CBS 133181	KC343917.1	98
Diaporthe sp. C27-07 (KM362392.1)	ITS	*Diaporthe schini* CBS 133181	KC343191.1	99
	TUB	*Diaporthe schini* CBS 133181	KC344159.1	97
	TEF1	*Diaporthe schini* CBS 133181	KC343917.1	99

Bento Gonçalves, Brazil. The dual culture method was performed as Orlandelli et al. [16]. As control, dishes were inoculated only with phytopathogen plugs. Competitive interactions (CI), antagonism indexes (AI) and the statistical analysis were performed in according with methodologies described in Felber et al. [6].

Results and Discussion

Multigene Molecular Identification of *Diaporthe* Endophytes

The DNA sequences comparison with other sequences in Genbank, including those available in TreeBASE database (S13943), revealed that endophytes had high similarity to *Diaporthe schini* (Table 1); except for the ITS analysis of *Diaporthe* sp. B64-46, which resulted in higher identity to *D. terebinthifolii.* Phylogenetic data revealed that a relative evolutionary closeness existed between both species; however, Bayesian analysis of ITS, TEF1 and TUB genes revealed specific alignment at 100% of Bayesian probability between endophytes and *D. schini* strains, by forming a specific clade for the species (Fig. S1). Previously employed for *Diaporthe* identification [6], the ITS gene is a robust tool for identification at genus level, even though the inclusion of additional specific genes for species identification required for some taxonomic groups [17]. Consequently, recent researches have underscored multigene phylogenetic analysis for the precise identification at species level [13, 18].

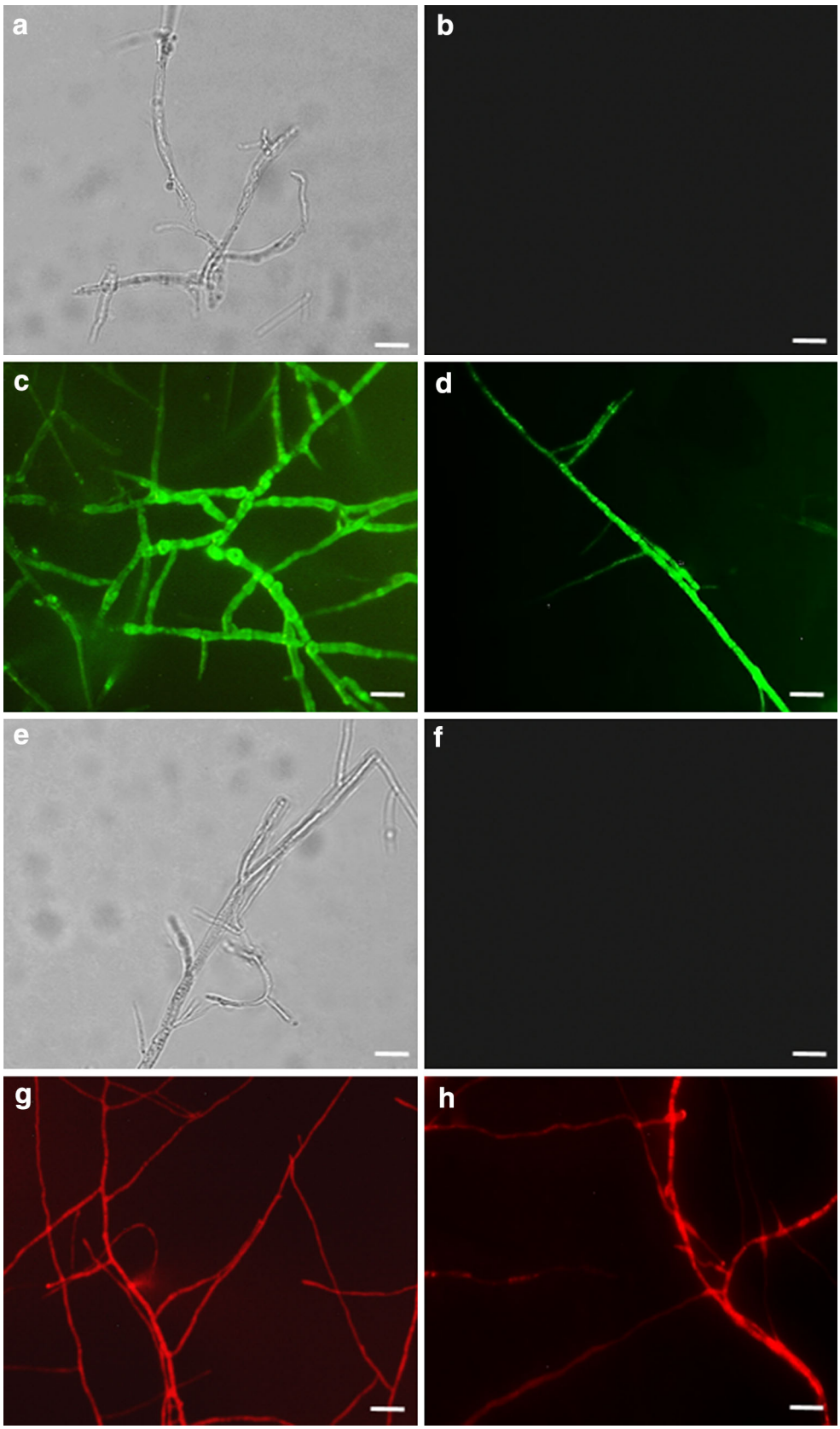
Sensitivity of *D. schini* Endophytes to Hygromycin B and Analysis of Genetic Transformation

The growth of *D. schini* B64-46 and C27-07 was partially inhibited at 5 mg/mL and completely inhibited at concentrations $\geq$ 25 mg/mL. Thus, 25 mg/mL hygromycin B was the MIC selected for transformants. Resistance to hygromycin B is variable in different *Diaporthe* species. As example, *Phomopsis* (= *Diaporthe*) *viticola* was totally inhibited by 5 mg/L; but to provide stringent selection of transformants, 10 mg/l of hygromycin B was selected [19]. Interestingly, 0,1 mg/ml was the MIC selected for endophytes *D. phaseolorum* [20], *D. endophytica* and *D. terebinthifolii* [21].

ATMT represents an efficient tool for insertion mutagenesis in filamentous fungi including *Diaporthe* species [20, 21]. Herein, the transformation efficiency was 96% for the pFAT-GFP plasmid (producing 48 transformants of *D. schini* B64-46) and 98% for pCAM-DsRed plasmid (49 transformants of *D. schini* C27-07). This slight difference in transformation rates may be associated to differences between plasmids or intraspecies diversity of fungal strains. Despite our successful transformation results, literature reports that mycelial tissue is lower efficacious than spores in transformation processes since a greater number of transformants is generally associated to an increase in the number of fungal cells [19].

PCR analysis confirmed the presence of *hph* gene in transformants through the amplification of a gene fragment of approximately 600 bp. This gene was integrated to the fungal genome within all transformants with pFAT-GFP or pCAM-DsRed plasmids. Analysis revealed the absence of gene fragment in wild strains. Further analyses revealed that the same technique showed *gfp* gene in the seven transformants with plasmid pFAT-GFP through the amplification of a gene fragment of $\sim$ 700 bp; also, *DsRed* gene was shown in the seven transformants with plasmid pCAM-DsRed through the amplification of a $\sim$ 414 bp fragment. Fragments of these genes were not observed in wild strains.

Fig. 1 *Expression of GFP and DsRed proteins by D. schini.* **a** *D. schini* B64-46 mycelium on bright background; **b** in fluorescence; **c**, **d** *D. schini* B64-46 transformants with green fluorescence; **e** *D. schini* C27-07 mycelium on bright background; **f** in fluorescence. **g**, **h** *D. schini* C27-07 transformants with red fluorescence. Bar = 10 μm ▶


a
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The introduction of visual marker genes may trigger changes in fungal characteristics [8]. In fact, transformant colonies showed macroscopic differences related to growth, color and morphology (Fig. S2). Variations may be due to changes in culture conditions and to insertion site of T-DNA within the fungal genome. Fluorescence genes integrated the genome of *D. schini* endophytes by recombination, then some genes may have been disrupted since the process is randomized [8]. Such as reported herein, the mentioned contrasts were observed in previous studies involving the insertion of the same genes in other *Diaporthe* strains [19] and other fungal genera [8, 22].

Mitotic Stability of Transformants and Expression of GFP and DsRed Proteins

All transformants showed 100% mitotic stability, thereby confirming stable maintenance of *hph* gene in the genome across five successive subcultures and confirmed by PCR technique. Microscopic analysis of transformants hyphae confirmed the heterologous gene expression in all transformants.

Hyphae of wild strains failed to show any fluorescence, whereas all transformants emitted stable fluorescence throughout their hyphae, albeit not uniformly (hyphae segments with reduced or no expression of heterologous could be observed in Fig. 1). The fluorescence maintenance indicates that the synthesis and translation of mRNA remain constant even after the removal of selective pressure. In the case of *Fusarium verticillioides* strains with GFP and DsRed, lack of fluorescence emission was restricted to vacuoles [23], suggesting that heterogeneity in fluorescence during the analyses of the hyphae of *D. schini* transformants may be related to sites in which these organelles occurred. Also, hyphae of *D. schini* transformants featuring decreased or total absence of gene expression in microscopic analysis may have been retrieved from an older region of the mycelium, as reported by Eckert et al. [12].

In Vitro Antagonism of Fungal Strains Against Grapevine Pathogens

The CI between *D. schini* B64-46 and C27-07 or their transformants and phytopathogens belong to the type A = deadlock with mycelial contact, corroborating our previous study where most endophytes isolated from the Bordô and Concord cultivars showed inhibition of *Alternaria* sp. CNPUV 674 and *Glomerella* sp. CNPUV 378 per mycelial contact [6].

Regarding AI, most of the transformants presented higher in vitro antagonism when compared with wild strains. For *Alternaria* sp. CNPUV 674, wild fungi and transformants had in vitro antagonism ranging from ~ 36 to 41% (Table 2); however, results did not differ statistically, revealing that randomized insertion of exogenous

Table 2 Antagonistic activity of *Diaporthe schini* endophytes (and their transformants) against grapevine phytopathogens in dual culture

Strains	Antagonism Index (AI)*	
	Alternaria sp. CNPUV 674	*Glomerella* sp. CNPUV 378
D. schini B64-46	35.96[a]	39.45[b]
TGFP 1	36.40[a]	43.84[b]
TGFP 2	41.02[a]	48.99[a]
TGFP 5	37.13[a]	43.11[b]
TGFP 6	39.95[a]	51.48[a]
TGFP 7	36.31[a]	51.66[a]
TGFP 8	39.33[a]	47.50[a]
TGFP 10	37.00[a]	45.07[b]
D. schini C27-07	37.64[a]	38.28[b]
TDsRed 1	37.20[a]	42.70[b]
TDsRed 2	37.64[a]	44.56[b]
TDsRed 3	40.65[a]	41.17[b]
TDsRed 4	40.00[a]	45.01[b]
TDsRed 5	40.00[a]	42.32[b]
TDsRed 7	40.87[a]	44.25[b]
TDsRed 10	38.26[a]	39.66[b]
Control	0.00[b]	0.00[c]

TGFP gfp-expressing transformants, *TDsRed DsRed*-expressing transformants

*For each column, means of triplicates followed by same letters indicate that the AI intervals (calculated according to Ribeiro et al. [10] do not differ statistically by Scott-Knott test ($p < 0.05$)

genes failed to change the antagonistic activity of transformants when contrasted to wild endophytes. In relation to *Glomerella* sp. CNPUV 378, indexes varied between ~ 38.2 and 51.6%. Statistical analysis demonstrated that some transformants were more efficient antagonists than wild strains.

Since *D. schini* wild strains (B64-46 and C27-07) have previously shown in vitro potential for biological control [6], this current assay aimed to detect whether transformants maintained their ability in inhibiting the grapevine phytopathogens, and verify whether transformation had silenced the biological control genes or, alternatively, it became a traceable marker of strains that may be used in the control of phytopathogens. ATMT may recover mutants without certain function or produce genetically improved mutants for a specific interest [8]. In current assay, the genetic transformation process by randomly inserting a gene may provide an improved phenotype and thus a promising alternative for the mutation- and recombination-based genetic improvement of fungi. Other studies assessed whether the marking of fungi with GFP and DsRed interfered in antagonism action. Santos et al. [21] employed the dual culture to evaluate the antagonism of *D. endophytica* and *D. terebinthifolii* transformants against the citrus pathogen *Phyllosticta citricarpa*, verifying that tested transformants failed to show any change in the antagonistic activity when compared with wild strains. Bitsadze et al. [24] reported similar results in the control of *Sclerotinia sclerotiorum*, where the mortality of sclerotia was evaluated by mycoparasite activity. GFP transformants of *Microsphaeropsis ochracea* and DsRed transformants of *Coniothyrium minitans* did not differ from wild strains regarding their potential for biological control. The two studies mentioned above also report that transformants did not show any morphological and physiological differences when compared to wild strains.

The results of this study contribute to knowledge and development of *A.* tumefaciens-mediated transformation by the insertion of fluorescent-marker genes. Transformation was highly successful and reproducible, whilst transformants may be applied in further studies, including the genic prospection of biotechnological interest. Further, the stable expression of fluorescent proteins GFP and DsRed will monitor the mechanisms of host colonization by endophyte. Their capacity in biological control may be assessed by in vivo tracing since transformants maintained their antagonistic activity against the grapevine phytopathogens.

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