Development of a green fluorescent tagged strain of *Aspergillus carbonarius* to monitor fungal colonization in grapes

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Abstract

An enhanced green fluorescent protein has been used to tag an OTA-producing strain of *Aspergillus carbonarius* (W04-40) isolated from naturally infected grape berries. Transformation of the fungus was mediated by *Agrobacterium tumefaciens*. The most efficient transformation occurred when the co-cultivation was done with 10^4 conidia due to higher frequency of resistance colonies (894 per 10^4 conidia) and lower background obtained. To confirm the presence of the *hph* gene in hygromycin resistant colonies, 20 putative transformants were screened by PCR analysis. The *hph* gene was identified in all the transformants. Variation on the expression levels of the eGFP was detected among the transformants and 50% of them appeared bright green fluorescent under the microscope. Microscopic analysis of all the bright fluorescent transformants revealed homogeneity of the fluorescent signal, which was clearly visible in the hyphae as well as in the conidia. eGFP expression in *A. carbonarius* was shown to be stable in all transformants. Confocal Laser scanning microscopy images of grape berries infected with the eGFP transformant demonstrated fungal penetration into the berry tissues. OTA production was importantly increased in the eGFP transformant in comparison with the wild type strain and pathogenicity on grape berries was slightly decreased after four days of inoculation. However, no differences in virulence were found after seven days of inoculation, thus allowing utilization of this eGFP mutant for *in situ* analysis of *A. carbonarius* infection of grape berries. To our knowledge, this is the first report describing the construction of a GFP-tagged strain belonging to *Aspergillus* section *Nigri* for monitoring *Aspergillus* rot on grape berries.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, carcinogenic, immunotoxic, genotoxic and teratogenic effects (Creppy, 1999; Kuiper-Goodman and Scott, 1989; Petzinger and Ziegler, 2000; Pfohl-Leszkowicz and Manderville, 2007). Grapes and wines have been reported among the food commodities contaminated by OTA with the greatest OTA intake, second only to cereals (Bau *et al.*, 2005; Belli *et al.*, 2004). Several studies in Europe, South America, Australia and the North of Africa have determined that OTA is produced during infection of grapes in vineyards by mycotoxigenic strains of *Aspergillus* species belonging to section *Nigri* (Battilani *et al.*, 2006; Chulze *et al.*, 2006; Leong *et al.*, 2006; Martínez-Culebras *et al.*, 2009; Oueslati *et al.*, 2010). It is now widely accepted that OTA contamination of wine and other grape products is mainly due to *Aspergillus carbonarius*. Although *A. carbonarius* is less common than other Black *Aspergillus* species, such as *Aspergillus niger* and *Aspergillus tubingensis,* it is the main species responsible for OTA in grapes because almost all strains are high OTA producers (Martinez-Culebras and Ramon, 2007; Perrone *et al.*, 2007).

Soil and vine trash on soil are the primary sources of *A. carbonarius* in vineyards. Wind-borne spores from the soil are deposited onto the surface of vines, including berry surfaces. Black *Aspergillus* species appear to be secondary invaders that infect grapes only after they have been damaged by pre-harvest rain, mechanical impacts, insects and other fungal pathogens such as *Botrytis cinerea*. However, several studies have reported the ability of *A. carbonarius* strains to colonise and penetrate berries even without skin damage in artificially inoculated grapes (Battilani *et al.*, 2004; Bellí *et al.*, 2007). The way

A. carbonarius penetrates the fruit in undamaged grapes is still unknown. Additionally, the presence of OTA in healthy berries has been reported by Serra *et al.*, (2006), which suggest that *A. carbonarius* may infect berries at earlier stages of berry development. Actually, the study of Kazi *et al*. (2008) showed that berry infections can be established as early as flowering and persist in berries until harvest. These studies highlighted the need to study more deeply the entrance of *A. carbonarius* into berries, whether it is through the stigma, pedicels, natural openings or by direct penetration of the cuticle.

The use of fungal transformants expressing cytoplasmic fluorescent proteins has greatly enhanced the ability to visualize and analyze the colonization and infection processes. The majority of studies utilising this technology in fungi have used modified forms of the green fluorescent protein (GFP) (Chalfie *et al.*, 1994), such as enhanced green fluorescent protein (eGFP) (Cormack *et al.*, 1996), which confer higher levels of fluorescence without major effects on fungal growth or pathogenicity. Although a large number of GFP expression vectors have been developed for most important fungal pathogens (Czymmek *et al.*, 2004; Lorang *et al.*, 2001), GFP tagging of an ochratoxigenic fungus has only been described for *Penicillium nordicum* (Schmidt-Heydt *et al.*, 2009). GFP tagging of a specific fungal strain depends on both the development of an efficient transformation protocol and the stable expression of the *gfp* gene in the fungus under natural environmental conditions.

The genetics of the *A. carbonarius* is poorly studied and currently only one study has reported the transfer of exogenous genes to *A. carbonarius.* Morioka *et al.* (Morioka *et al.*, 2006) transformed a coffee isolate of *A.*

carbonarius with the hygromycin phosphotransferase gene (*hph*) using *Agrobacterium tumefaciens*-mediated transformation (ATMT). ATMT has been considered advantageous over “direct DNA transfer” because it generates high percentage of transformants. On the other hand, GFP tagging of *A. carbonarius* has not been reported yet. In this article, we describe an ATMT protocol for the efficient expression of the eGFP in *A. carbonarius* in order to facilitate microscopic visualisation of the infection process of grapes of this ochratoxigenic species.

2. Materials and methods

2.1. Strains and culture conditions

The OTA-producing *A. carbonarius* strain W04-40 was isolated from a Spanish vineyard by Martinez Culebras and Ramón (2007) and deposited in the Institute of Agrochemistry and Food Technology of the Spanish National Research Council (IATA-CSIC). *Agrobacterium tumefaciens* AGL-1 strain was kindly provided by L. Peña (Instituo Valenciano de Investigaciones Agrarias, Valencia, Spain)

A. carbonarius was grown on Petri dishes containing Malt Extract Agar (MEA) medium in the dark at 30 °C for 6 days to achieve conidia production. Conidia were collected with a sterile solution of 0.005% (v/v) Tween 80 (J.T. Baker, Deventer, Holland) and were adjusted to 10^6 conidia/mL using a haemocytometer. 100 µL of the conidial suspension was homogeneously spread on Petri dishes containing Czapeck Yeast Extract Agar (CYA) medium and sub-cultured in the dark at 30 °C. Routinely growth of transformed strains

was performed on media containing 100 µg/mL of hygromycin B (Hyg B; Invivogen, San Diego, USA).

2.2. Construction of plasmid pRFHUE-eGFP

The enhanced green fluorescent protein encoding gene (*egfp*) was amplified from plasmid pEGFP-C3 (Clontech, Mountain View, USA) with primers EGFP03 (5'-GGACTTAAUGGTGAGCAAGGGCGAGGAGCTGT-3') and EGFP04 (5'-GGACTTAAUGGTGAGCAAGGGCGAGGAGCTGT-3'), which incorporate an uracil near the 5' end, and the high fidelity Pfu Turbo HotStart DNA Polymerase (Agilent Technologies, Santa Clara, USA). Cycling conditions consisted of an initial denaturation step at 94 ºC for 2 min, 30 cycles of 94 ºC for 2 min, 58 ºC for 30 sec and 72 ºC for 1 min and a final elongation step at 72 ºC for 10 min. The amplified fragment was cloned into the plasmid vector pRF-HUE (Frandsen *et al.*, 2008), a binary vector designed to be used with the USER friendly cloning technique (New England Biolabs). Digestion of this plasmid with the restriction ezyme *Pac*I followed by treatment with the nicking enzyme *Nt.Bbv*CI generates 9-nucleotide long 3' single-stranded ends that are complementary to those present in the PCR-amplified fragment. DNA insert and treated vector were mixed together and treated with the USER (uracil-specific excision reagent) enzyme (New England Biolabs), which excises the uracil residues originating a single-stranded end PCR fragment that can anneal to the one generated in the vector, to obtain plasmid pRFHUE-eGFP. An aliquot of the mixture was used to transform chemical competent *E. coli* XL1-Blue cells. Kanamycin resistant transformants were screened by PCR. Proper fusion was

confirmed by DNA sequencing. Then, plasmid pRFHUE-eGFP was introduced into electrocompetent *Agrobacterium tumefaciens* AGL-1 cells.

2.3. *Agrobacterium tumefaciens*-mediated transformation of *A. carbonarius*

A. tumefaciens AGL-1 carrying plasmid pRFHUE-eGFP was grown at 28 ºC for 72 h in Luria Bertani (LB) agar supplemented with kanamycin (50 µg/mL), rifampicin (20 µg/mL) and carbenicillin (75 µg/mL). A single colony was used to inoculate a starter culture of 5 mL of LB medium containing the aforementioned antibiotics during 24 h. Bacterial cells were centrifuged, washed with induction medium (IM) (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.6 mM $CaCl_2$, 9 µM $FeSO_4$, 4 mM $(NH4)_2SO4$, 10 mM glucose, 40 mM 2-[N-morpholino] ethanesulfonic acid, pH 5.3, 0.5% glycerol) (De Groot *et al.*, 1998) and diluted in the same medium amended with 200 µM acetosyringone (IMAS) to an OD600 = 0.15. Cells were grown at 28 ºC and 200 rpm until they reached an OD600 of 0.5-0.75. An equal volume of this culture was mixed with a conidial suspension of *A. carbonarius*. Three conidial suspension concentrations were tested (10^7, 10^6 and 10^5 conidia per mL). 100 µl of these mixtures were spread onto cellulose nitrate membrane filters (0.45 µm pore and 47 mm diameter, Albet, Dassel, Germany) that were placed on agar plates containing the co-cultivation medium (same as IMAS, but containing 5 mM instead of 10 mM of glucose). After co-cultivation at 26 ºC for 40 h, the membranes were transferred to CYA plates containing Hyg B as the selection agent for fungal transformants, and cefotaxime (200 µg/mL) to inhibit growth of *A. tumefaciens* cells. Although hygromycin sensitivity to *A. carbonarius* has

been previously reported by Morioka *et al.* (2006), a prerequisite for the use of hygromycin resistance gene as a selection marker is to determine the sensitivity of the host strain to this drug. *A. carbonarius* did not grow at concentrations equal or higher than 50 µg/mL. For selecting Hyg B transformants, 100 µg/mL Hyg B was enough to prevent growth of untransformed conidia. Hygromcycin resistant colonies appeared after 3 to 4 days of incubation at 30 ºC.

2.4. Genomic DNA extraction

All strains were grown on MEA medium for 4 days. Mycelium was collected from the plates, frozen in liquid nitrogen and ground to a fine powder. DNA extractions were performed using 100 mg of powder and the commercial EZNA Fungal DNA kit (Omega Bio-Tek, Doraville, USA) according to the manufacturer's instructions.

2.5. PCR analysis

Primers Hmbr1 (5'-CTGATAGAGTTGGTCAAGACC-3') and Hmbf1 (5'-CTGTCGAGAAGTTTCTGATCG-3') were used in polymerase chain reaction (PCR) analysis to amplify a fragment of the *hph* gene in putative transformants. The cycling conditions were as follows: an initial denaturation step (95 ºC, 2 min), 35 cycles of denaturation (95 ºC, 30 s), annealing (62 ºC, 45 s), and elongation (72 ºC, 1 min), and a final elongation step (72 ºC, 10 min).

2.6. Determination of cultural characteristics

For growth assessment, CYA plates were inoculated centrally with 5 µl of conidia suspensions (10^6 conidia/mL) from the wild-type strain of *A. carbonarius*

and the eGFP transformant. Two perpendicular diameters of the growing colonies were measured daily during four days until the colony reached the edge of the Petri dish. Assay was performed in triplicate.

2.7. Pathogenicity tests

Before infection, grape berries were separated from bunches by cutting the stem with the aid of scissors at approximately 0.5 cm from each grape. Table grapes (var. Dominga) not physically damaged were used in this study. They were surface-sterilised by dipping them into a NaClO solution (0.1% Cl) for 1 min, followed by one minute incubation in ethanol (70%). Excess of water was removed by placing berries in a laminar flow bench. For inoculation, conidia suspensions (10^3 conidia/mL) from MEA cultures of the wild type and a selected eGFP transformant were prepared in distilled water containing Tween 80 (0.005%). Thirty berries were dipped into 500 mL of each conidia suspension for 1 min. Berries were wounded before inoculation by puncturing them with a sterile needle and placed on the top of a rack previously disinfected with ethanol (96%), preventing any contact among them. Each test was conducted in triplicate with conidial suspensions obtained from independent cultures. Berries were incubated in a storage room with controlled temperature and relative humidity (30 °C - 90% HR) during seven days.

2.8. Confocal laser scanning microscopy of grapes infected with *A. carbonarius*

Berries were subsequently sliced using a microtome with freezing capabilities. Segments were placed directly onto glass slides immersed in a

drop of water and examined using a confocal laser scanning microscope (CLSM) (LEICA, TCS-SP) equipped with filter blocks with spectral properties matching those of eGFP (488 nm excitation and emission from 500 to 560 nm).

2.9. Extraction and detection of OTA from culture

Ochratoxin A was extracted using a variation of a simple method described previously (Bragulat *et al*., 2001). The isolates were grown on CYA and incubated at 30 ºC for 3 days (Pitt and Hocking, 1997). Separation, detection and quantification were performed by injecting 20 μl of extract from each vial into an HPLC system consisting of a Dionex model P680A pump (Sunnyvale, USA) connected to a Dionex model RF-2000 programmable fluorescence detector and to a Dionex PDA-100 photodiode array detector. For the determination of OTA, a C18 reversed-phase column (150 x 4.6 mm i.d., 5 μm particle size Kromasil C18 (Análisis Vínicos S.L., Tomelloso, Spain), connected to a precolumn Kromasil C18 (10 x 4.6 mm i.d., 5 μm particle sizes, Análisis Vínicos S.L.) were used. For chromatographic separation of OTA, the mobile phase was acetonitrile: water: acetic acid, (57:41:2 v/v/v) under isocratic elution during 10 min, at a flow rate of 1 mL/min. OTA was determined by fluorescence detection at an excitation wavelength of 330 nm and an emission wavelength of 460 nm. The ochratoxin standard was obtained from *A. ochraceus* (Sigma-Aldrich, St. Louis, USA).

2.10. Statistical analyses

Comparisons of growth rate, OTA production and percentage of infection between the wild type and the eGFP transformant strains were performed by

243 the t-test using Statgraphics Plus 5.1 (Manugistics, Rockville, USA). Differences
244 between mean values were considered significant when $P < 0.05$. Percentage
245 of infection data were converted into Bliss angular values (arcsin $\sqrt{\%}$) before
246 analysis.

247 3. Results and discussion

248 3.1. Transformation of *A. carbonarius* with the *egfp* gene

249 The aim of this study was to develop an ATMT protocol for the efficient
250 transformation of *A. carbonarius* to obtain an eGFP-tagged strain as a tool for
251 studying the infection process of berries by *A. carbonarius*. Transformation was
252 performed on one OTA-producing *A. carbonarius* strain (W04-40) previously
253 identified and tested for its ability to produce OTA (Martinez-Culebras and
254 Ramon, 2007). Recently, the same *A. carbonarius* strain was used to identify
255 differentially expressed genes putatively involved in OTA production (Crespo-
256 Sempere *et al.*, 2010). Transformation of the fungus was mediated by *A.*
257 *tumefaciens* (AGL-1). Plasmid pRFHUE-eGFP (Figure 1) was constructed by
258 inserting the *egfp* gene, encoding the enhanced green fluorescent protein,
259 under the control of the *Aspergillus nidulans* glyceraldehyde-3-phosphate
260 dehydrogenase (*gpdA*) gene promoter, into the binary plasmid pRF-HUE. This
261 plasmid contains the *hph* gene, which encodes a hygromycin
262 phosphotransferase, flanqued by the *TrpC* promoter and terminator, allowing
263 selection of Hyg B resistant fungal transformants

264 Co-cultivation of *A. carbonarius* with *A. tumefaciens*, harbouring the
265 binary vector, onto induction medium in the presence of acetosyringone (IMAS)
266 during 40 hours resulted in hygromycin-resistant colonies after 3-4 days of the

transference to the selection medium. *A. tumefaciens*-mediated transformation of *A. carbonarius* was very efficient. The frequency of resistant colonies was 894 per 10^4 conidia, 193 per 10^5 conidia and 200 per 10^6 conidia. Therefore, the most efficient transformation occurred when the co-cultivation was done with 10^4 conidia due to higher frequency of resistance colonies and less background obtained. Transformation frequency obtained in our study was significantly higher than that achieved by Morioka *et al*., (2006) in *A. carbonarius* (25 to 101 transformants per 10^5 conidia) and much higher than that described for *Aspergillus awamori* (20 to 90 transformants per 10^5 conidia) and *Aspergillus niger* (5 transformants per 10^7 conidia), both reported by De Groot *et al.* (1998).

To confirm the presence of the *hph* gene in the hygromycin resistant colonies, 20 putative transformants were screened by PCR analysis. Using Hmbr1 and Hmbf1 oligonucleotide primers, the expected 799-bp PCR fragment was detected in all transformants, which was not amplified from the untransformed strain (data not shown). All of the isolated transformants exhibited green fluorescence, although the emission intensity varied among these transformants and 50% of them appeared bright green fluorescent under the microscope. Microscopic analysis of all the bright fluorescent transformants revealed homogeneity of the fluorescent signal, which was clearly visible in the hyphae as well as in the conidia and stable for several hours during observations (Figure 2). No green autofluorescence background was observed for the parental strain W04-40 neither on CYA medium nor during infection of grapes (data not shown). To test the stability of *egfp* expression in transformants, these transformants were subcultured successively 10 times on

CYA without selection pressure. Transformants exhibited stable expression of the *egfp* gene.

One eGFP-tagged *A. carbonarius* strain was artificially inoculated on grape berries. Four days after inoculation, infected grape berries were examined using CLSM. Regarding grapes surface colonisation and index of infection, fungal growth was mainly observed near the puncture produced in the skin and also around the stalk of each berry. Sections of grape berries samples revealed that mycelial penetrated into the grape tissues among the epidermal cells. Mycelial penetration into the berry tissues was mainly observed through the artificially produced wound and was followed by proliferation of hyphal strands in several directions (Figure 3). Further and more detailed microscopic studies are needed to known the way *A. carbonarius* penetrates the fruit in undamaged grapes.

3.2. Cultural characteristics, OTA production and pathogenicity in grapes of the eGFP-tagged *A. carbonarius* strain

No differences in colony morphology were observed in eGFP-tagged transformants when compared with the wild type strain on non-selective media (data not sown). However, the growth rate of the selected eGFP transformant was slightly, although significantly, slower than the observed for the wild type (Table 1). This result has been previously reported for other fungal species (Pliego *et al.*, 2009; Zhang *et al.*, 2008). The slower growth may be caused by factors such as some type of stress due to exogenous DNA transfer. In contrast to the growth rate, the eGFP mutant produced a significant ($p = 3.9 \times 10^{-9}$) higher amount of OTA in comparison to the wild-type (Table 1). The wild type

315 produced 1.12 µg/g of OTA, whereas the eGFP mutant produced 3.31 µg/g. In
316 comparison with other GFP mutants developed in mycotoxin producing fungi,
317 aflatoxin production were not increased in a GFP mutant of *Aspergillus flavus*
318 (Rajasekaran *et al.*, 2008). It is difficult to explain this important increase in the
319 OTA production observed for the eGFP mutant. It has been previously reported
320 that oxidative stress stimulates mycotoxin biosynthesis (Reverberi *et al.*, 2010).
321 It is likely that this increase in the OTA production as well as the slower growth
322 observed might be caused by a pleotropic effect on fungal fitness due to the
323 transformation itself. Thus, the reduced fitness of the transformant, reflected by
324 its slower growth rate, would be indicative of a stressful situation, which would
325 trigger a higher production of OTA. It is worth noting that different *A.*
326 *carbonarius* transformants affected in other genes not related to the *egfp* gene
327 also show a slower growth rate and higher OTA production (data not shown).
328 These results support the hypothesis that changes in fungal fitness due to the
329 transformation process might be responsible of the slower growth and the
330 increase in OTA production levels.

331 Pathogenicity tests of the eGFP transformants on grape berries in
332 comparison to the wild type were performed on artificially inoculated grapes
333 (Table 1). Severity of rots was assessed by scoring the proportion of infected
334 berries. A total of 180 berries corresponding to three microbiological replicates
335 of thirty berries and two treatments (wild type and eGFP mutant) (see Material
336 and Methods) were analyzed. Berries were colonized by the fungus within 4-7
337 days. Four days after inoculation, *Aspergillus* rot was observed in 88 of 90
338 (97.7%) and 71 of 90 (78.8%) inoculated berries with the wild type and the
339 eGFP mutant, respectively. These results indicate that virulence of the wild type

was slightly higher that of the eGFP mutant (p = 0.0346, Table 1), which would also point to a slightly reduced fitness of the transformant strain. However, when pathogenicity tests were analysed seven days after inoculation *Aspergillus* rot was observed in all the berries analyzed in the wild type as well as in the eGFP mutant. Therefore, after seven days of inoculation no differences in virulence between these two strains were found, thus allowing utilization of this eGFP mutant for *in situ* analysis of *A. carbonarius* infection of grape berries. It has been previously suggested that mycotoxin production could be related to pathogenicity by facilitating fungal penetration and development. Actually, it has been previously reported that OTA is able to induce necrotic lesions in detached leaves from *Arabidopsis thaliana* (Peng *et al.*, 2010). Although this fact seems logical, the results from this study where the eGFP-tagged transformant showed a three fold increase in OTA production and slightly reduced virulence suggest that mycotoxin production is not directly related to the infection capability. Additional and more detailed pathogenicity studies together with the evaluation of OTA contamination in berries are necessary in order to confirm that OTA production is not involved in the infection process of *A. carbonarius* in grapes.

In conclusion, *A. carbonarius* transformation with eGFP is a potentially useful tool for monitoring the infection process of grapes of this ochratoxigenic species. To our knowledge, this is the first report describing the construction of a GFP-tagged strain belonging to *Aspergillus* section *Nigri* for monitoring *Aspergillus* rot on grape berries. It would be interesting to evaluate the

possibility that transformants can survive longer in order to elucidate whether this fungus is able to infect the plant at the time of flowering, remaining quiescent for a long period of time. This aspect might be essential for the design of novel strategies to control *Aspergillus* infections. Furthermore, eGFP tagging of both OTA-producing and non OTA-producing strains represent a good starting point for future visualisation and characterisation of different lifestyles and interactions of *A. carbonarius* strains in grape vines.

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Figure 1. Physical map of plasmid pRFHUE-eGFP. eGFP = enhanced green fluorescent protein, PgpdA = glyceraldehyde-3-phosphate dehydrogenase promoter from *A. nidulans*., pTrpC = Tryptophan promoter form *Aspergillus nidulans*, hph = hygromycin phosphotransferase, TtrpC = Tryptophan terminator from *A. nidulans*, KanR = kanamycin resistance.

Figure 2. Microscopy analysis of an eGFP-tagged *A. carbonarius* strain. Differential interference contrast (panels a and c) and fluorescence (panels b and d) images of mycelia, conidiophores and conidia.

Figure 3. In situ visualization of eGFP-tagged *A. carbonarius* mycelia colonizing a grape berry after four days of inoculation. The green fluorescence emitted by the fungal hyphae was visualized using CLSM. Proliferation of *A. carbonarius* mycelia throughout a grape berry (panels a and b).

Table 1. Comparison of growth rate, OTA production and pathogenicity tests between the wild type and the eGFP-tagged *A. carbonarius* transformant. Mean values within the same column followed by different letters are significantly different according to *t*- test ($p < 0.05$). *p* values are shown for each comparison.

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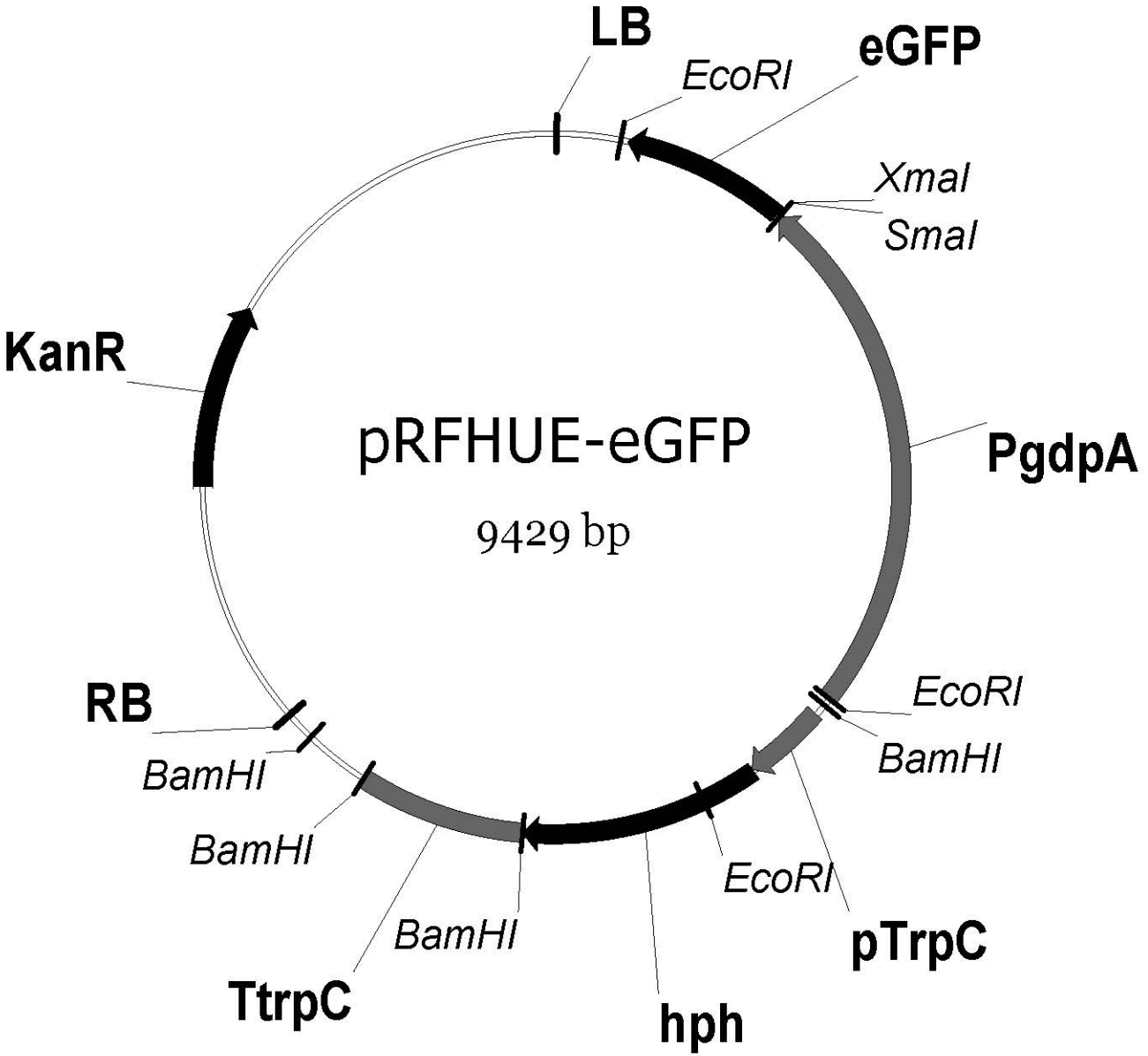
	Growth (mm)			OTA (µg/g cultura media)	% infected grapes	
	Day 2	Day 3	Day 4		Day 4	Day 7
Wild type	28.6 ± 1.0a	45.7 ± 0.5a	68.7 ± 1.2a	1.12±0.18a	97.8±1.9a	100a
eGFP mutant	27.0 ± 0.6b	43.7 ± 0.8b	67.0 ± 0.6b	3.31±0.33b	78.9±11.7b	100a
p value	0.0071	0.0005	0.01361	3.99E-8	0.0346	


LB
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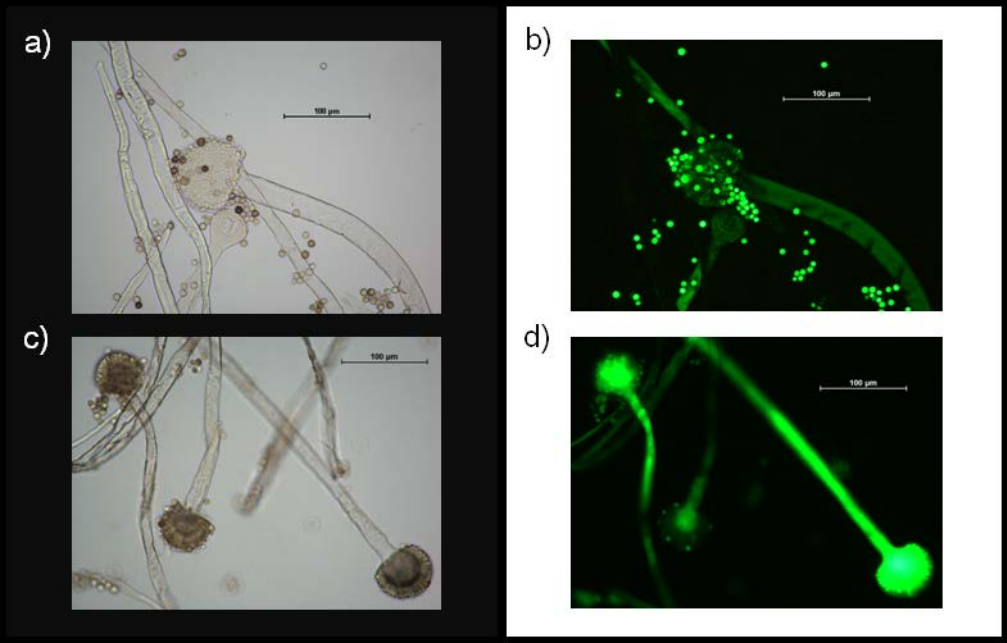
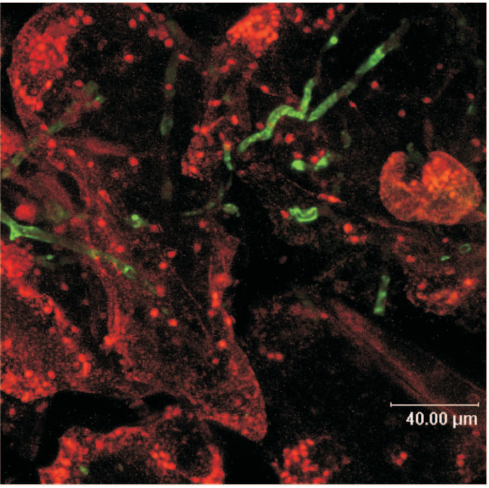

a)
100 µm
b)
100 µm
c)
100 µm
d)
100 µm

a)



b)