

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

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15 **Keywords:** *Aspergillus carbonarius*; ATMT; Green Fluorescent Protein;  
16 Confocal microscopy; Ochratoxin production; Grapes; Wine.

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20

## 21 **Abstract**

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

## 46 **1. Introduction**

47 Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, carcinogenic,  
48 immunotoxic, genotoxic and teratogenic effects (Creppy, 1999; Kuiper-  
49 Goodman and Scott, 1989; Petzinger and Ziegler, 2000; Pfohl-Leszkowicz and  
50 Manderville, 2007). Grapes and wines have been reported among the food  
51 commodities contaminated by OTA with the greatest OTA intake, second only  
52 to cereals (Bau *et al.*, 2005; Belli *et al.*, 2004). Several studies in Europe, South  
53 America, Australia and the North of Africa have determined that OTA is  
54 produced during infection of grapes in vineyards by mycotoxigenic strains of  
55 *Aspergillus* species belonging to section *Nigri* (Battilani *et al.*, 2006; Chulze *et*  
56 *al.*, 2006; Leong *et al.*, 2006; Martínez-Culebras *et al.*, 2009; Oueslati *et al.*,  
57 2010). It is now widely accepted that OTA contamination of wine and other  
58 grape products is mainly due to *Aspergillus carbonarius*. Although *A.*  
59 *carbonarius* is less common than other Black *Aspergillus* species, such as  
60 *Aspergillus niger* and *Aspergillus tubingensis*, it is the main species responsible  
61 for OTA in grapes because almost all strains are high OTA producers (Martinez-  
62 Culebras and Ramon, 2007; Perrone *et al.*, 2007).

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

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

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

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

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

## 104 **2. Materials and methods**

### 105 **2.1. Strains and culture conditions**

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

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

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

121

## 122 **2.2. Construction of plasmid pRFHUE-eGFP**

123 The enhanced green fluorescent protein encoding gene (*egfp*) was  
124 amplified from plasmid pEGFP-C3 (Clontech, Mountain View, USA) with  
125 primers EGFP03 (5'-GGACTTAAUGGTGAGCAAGGGCGAGGAGCTGT-3')  
126 and EGFP04 (5'-GGACTTAAUGGTGAGCAAGGGCGAGGAGCTGT-3'), which  
127 incorporate an uracil near the 5' end, and the high fidelity Pfu Turbo HotStart  
128 DNA Polymerase (Agilent Technologies, Santa Clara, USA). Cycling conditions  
129 consisted of an initial denaturation step at 94 °C for 2 min, 30 cycles of 94 °C for  
130 2 min, 58 °C for 30 sec and 72 °C for 1 min and a final elongation step at 72 °C  
131 for 10 min. The amplified fragment was cloned into the plasmid vector pRF-HUE  
132 (Frandsen *et al.*, 2008), a binary vector designed to be used with the USER  
133 friendly cloning technique (New England Biolabs). Digestion of this plasmid with  
134 the restriction enzyme *PacI* followed by treatment with the nicking enzyme  
135 *Nt.BbvCI* generates 9-nucleotide long 3' single-stranded ends that are  
136 complementary to those present in the PCR-amplified fragment. DNA insert and  
137 treated vector were mixed together and treated with the USER (uracil-specific  
138 excision reagent) enzyme (New England Biolabs), which excises the uracil  
139 residues originating a single-stranded end PCR fragment that can anneal to the  
140 one generated in the vector, to obtain plasmid pRFHUE-eGFP. An aliquot of the  
141 mixture was used to transform chemical competent *E. coli* XL1-Blue cells.  
142 Kanamycin resistant transformants were screened by PCR. Proper fusion was

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

145

### 146 **2.3. *Agrobacterium tumefaciens*-mediated transformation of *A.*** 147 ***carbonarius***

148 *A. tumefaciens* AGL-1 carrying plasmid pRFHUE-eGFP was grown at  
149 28 °C for 72 h in Luria Bertani (LB) agar supplemented with kanamycin (50  
150 µg/mL), rifampicin (20 µg/mL) and carbenicillin (75 µg/mL). A single colony was  
151 used to inoculate a starter culture of 5 mL of LB medium containing the  
152 aforementioned antibiotics during 24 h. Bacterial cells were centrifuged, washed  
153 with induction medium (IM) (10 mM K<sub>2</sub>HPO<sub>4</sub>, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM NaCl, 2  
154 mM MgSO<sub>4</sub>, 0.6 mM CaCl<sub>2</sub>, 9 µM FeSO<sub>4</sub>, 4 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM glucose, 40  
155 mM 2-[N-morpholino] ethanesulfonic acid, pH 5.3, 0.5% glycerol) (De Groot *et*  
156 *al.*, 1998) and diluted in the same medium amended with 200 µM  
157 acetosyringone (IMAS) to an OD<sub>600</sub> = 0.15. Cells were grown at 28 °C and 200  
158 rpm until they reached an OD<sub>600</sub> of 0.5-0.75. An equal volume of this culture  
159 was mixed with a conidial suspension of *A. carbonarius*. Three conidial  
160 suspension concentrations were tested (10<sup>7</sup>, 10<sup>6</sup> and 10<sup>5</sup> conidia per mL). 100  
161 µl of these mixtures were spread onto cellulose nitrate membrane filters (0.45  
162 µm pore and 47 mm diameter, Albet, Dassel, Germany) that were placed on  
163 agar plates containing the co-cultivation medium (same as IMAS, but containing  
164 5 mM instead of 10 mM of glucose). After co-cultivation at 26 °C for 40 h, the  
165 membranes were transferred to CYA plates containing Hyg B as the selection  
166 agent for fungal transformants, and cefotaxime (200 µg/mL) to inhibit growth of  
167 *A. tumefaciens* cells. Although hygromycin sensitivity to *A. carbonarius* has

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

174

#### 175 **2.4. Genomic DNA extraction**

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

181

#### 182 **2.5. PCR analysis**

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

189

#### 190 **2.6. Determination of cultural characteristics**

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



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

196

## 197 **2.7. Pathogenicity tests**

198 Before infection, grape berries were separated from bunches by cutting  
199 the stem with the aid of scissors at approximately 0.5 cm from each grape.  
200 Table grapes (var. Dominga) not physically damaged were used in this study.  
201 They were surface-sterilised by dipping them into a NaClO solution (0.1% Cl)  
202 for 1 min, followed by one minute incubation in ethanol (70%). Excess of water  
203 was removed by placing berries in a laminar flow bench. For inoculation, conidia  
204 suspensions ( $10^3$  conidia/mL) from MEA cultures of the wild type and a selected  
205 eGFP transformant were prepared in distilled water containing Tween 80  
206 (0.005%). Thirty berries were dipped into 500 mL of each conidia suspension  
207 for 1 min. Berries were wounded before inoculation by puncturing them with a  
208 sterile needle and placed on the top of a rack previously disinfected with ethanol  
209 (96%), preventing any contact among them. Each test was conducted in  
210 triplicate with conidial suspensions obtained from independent cultures. Berries  
211 were incubated in a storage room with controlled temperature and relative  
212 humidity (30 °C - 90% HR) during seven days.

213

## 214 **2.8. Confocal laser scanning microscopy of grapes infected with *A.*** 215 ***carbonarius***

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

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

221

## 222 **2.9. Extraction and detection of OTA from culture**

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

239

## 240 **2.10. Statistical analyses**

241 Comparisons of growth rate, OTA production and percentage of infection  
242 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, flanked 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

267 transference to the selection medium. *A. tumefaciens*-mediated transformation  
268 of *A. carbonarius* was very efficient. The frequency of resistant colonies was  
269 894 per  $10^4$  conidia, 193 per  $10^5$  conidia and 200 per  $10^6$  conidia. Therefore, the  
270 most efficient transformation occurred when the co-cultivation was done with  
271  $10^4$  conidia due to higher frequency of resistance colonies and less background  
272 obtained. Transformation frequency obtained in our study was significantly  
273 higher than that achieved by Morioka *et al.*, (2006) in *A. carbonarius* (25 to 101  
274 transformants per  $10^5$  conidia) and much higher than that described for  
275 *Aspergillus awamori* (20 to 90 transformants per  $10^5$  conidia) and *Aspergillus*  
276 *niger* (5 transformants per  $10^7$  conidia), both reported by De Groot *et al.* (1998).

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

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

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

### 304 **3.2. Cultural characteristics, OTA production and pathogenicity in grapes** 305 **of the eGFP-tagged *A. carbonarius* strain**

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

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

358

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

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

371

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378 Figure 1. Physical map of plasmid pRFHUE-eGFP. eGFP = enhanced green  
379 fluorescent protein, P<sub>gpdA</sub> = glyceraldehyde-3-phosphate dehydrogenase  
380 promoter from *A. nidulans.*, p<sub>TrpC</sub> = Tryptophan promoter form *Aspergillus*  
381 *nidulans*, hph = hygromycin phosphotransferase, T<sub>trpC</sub> = Tryptophan terminator  
382 from *A. nidulans*, Kan<sup>R</sup> = kanamycin resistance.

383

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



387

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

392

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

398

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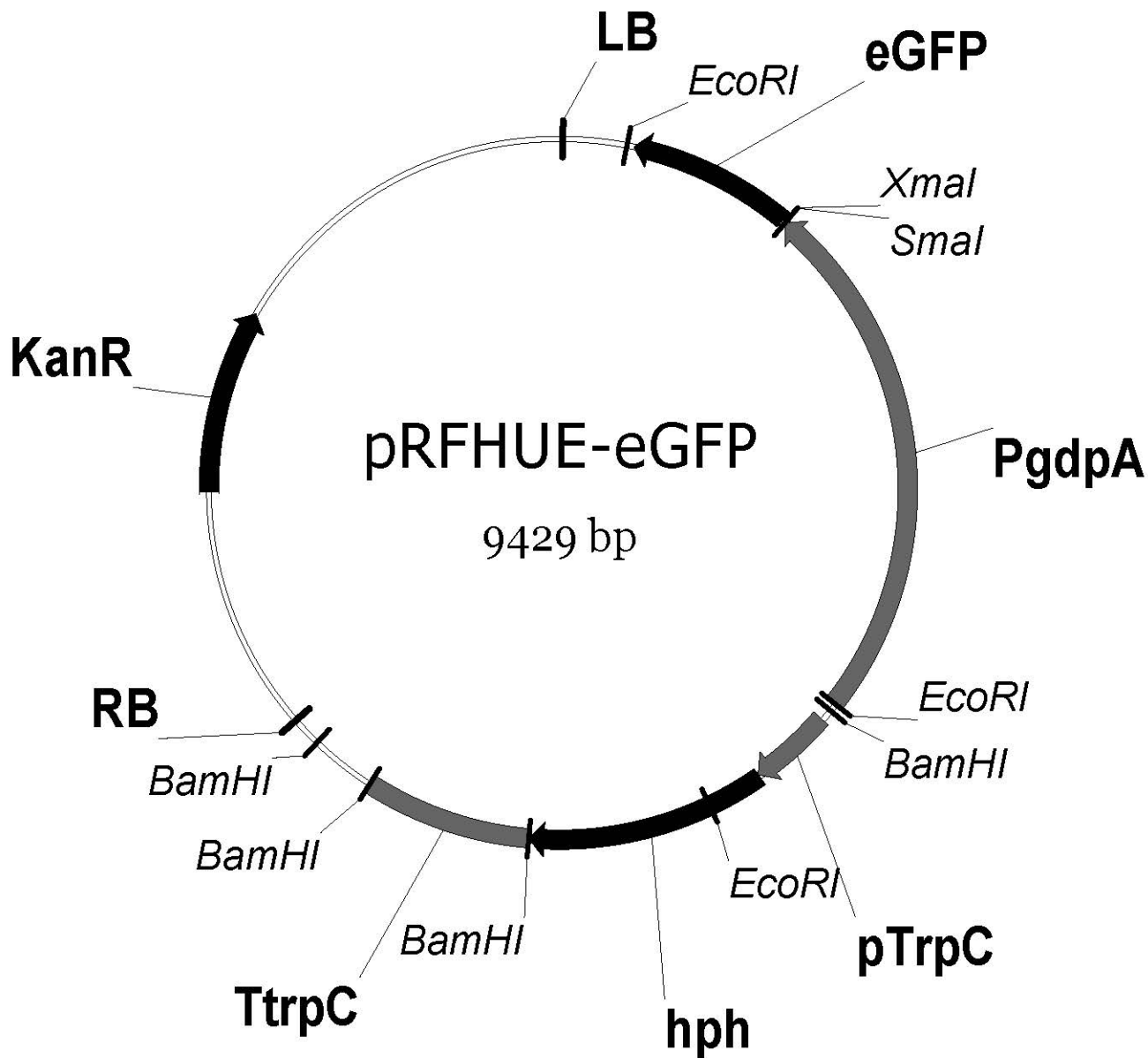
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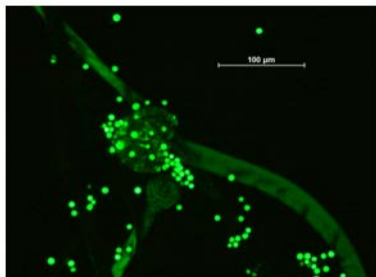
	Growth (mm)			OTA ( $\mu\text{g/g}$ cultura media)	% infected grapes	
	Day 2	Day 3	Day 4		Day 4	Day 7
<i>Wild type</i>	28.6 $\pm$ 1.0a	45.7 $\pm$ 0.5a	68.7 $\pm$ 1.2a	1.12 $\pm$ 0.18a	97.8 $\pm$ 1.9a	100a
<i>eGFP mutant</i>	27.0 $\pm$ 0.6b	43.7 $\pm$ 0.8b	67.0 $\pm$ 0.6b	3.31 $\pm$ 0.33b	78.9 $\pm$ 11.7b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



a)



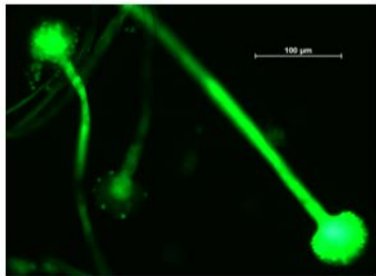
b)



c)

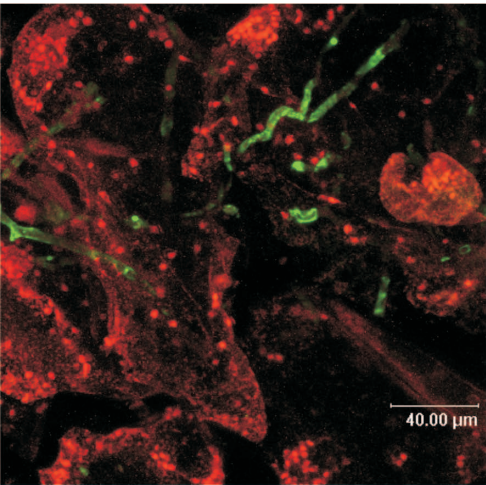


d)





a)



b)

