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## INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin that can affect human health and its presence in food and food products is undesirable. *Aspergillus carbonarius* is an important OTA-producing fungus that is responsible for toxin contamination of grapes and wine, coffee and cocoa. 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. *A. carbonarius* is an invasive fungus, able to colonise and penetrate berries even without skin damage in artificially inoculated grapes (Belli *et al.*, 2007). Little is known about infection processes of berries through the stigma, pedicels, natural openings or by direct penetration of the cuticle.

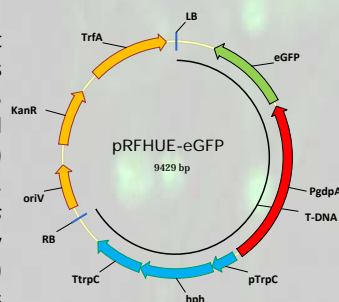


## OBJECTIVE

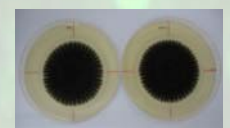
Use of a green fluorescent tagged strain of *A. carbonarius* for infection studies of grapes.

## METODOLOGY

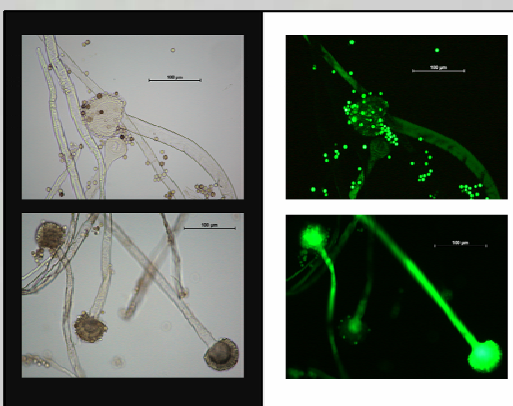
The enhanced green fluorescent protein encoding gene (eGFP) was amplified from plasmid pEGFP-C3 (Clontech) and cloned into the plasmid vector pRFHUE (Frandsen *et al.*, 2008) to obtain plasmid pRFHUE-eGFP. Transformation of *A. carbonarius* (W04-40) was mediated by *Agrobacterium tumefaciens* (AGL-1) and kanamycin resistant transformants were screened by PCR.



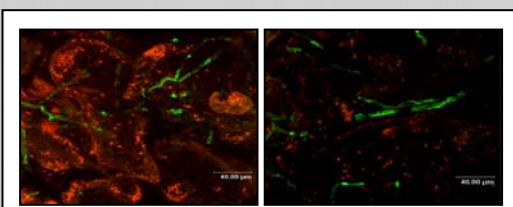
For growth assessment, two perpendicular diameters of the growing colonies were measured daily. OTA was determined by HPLC from cultures in CYA plates. Ten berries were inoculated with conidia suspensions ( $10^3$  conidia mL<sup>-1</sup>) from the wild type and eGFP transformant. Each treatment was performed in technical and microbiological triplicates. Berries were incubated in a storage room at 30 °C - 90% HR.



## RESULTS



Microscopy analysis of an eGFP-tagged *A. carbonarius* strain. Differential interference contrast and fluorescence images of mycelia, conidiophores and conidia.



In situ visualization of eGFP-tagged *A. carbonarius* mycelia colonizing a grape berry after four days of inoculation. The green fluorescence emitted by the grape tissues were visualized using CLSM. Proliferation of *A. carbonarius* mycelia throughout a grape berry.

	Growth (mm)			OTA (µg/g cultura media)	% grapes infected	
	Day 2	Day 3	Day 4		Day 4	Day 7
<i>Wild type</i>	28.66 ± 1.03	45.66 ± 0.51	68.66 ± 1.21	1.12 ± 0.18	97.77 ± 1.92	100
<i>GFP mutant</i>	27 ± 0.63	43.66 ± 0.81	67 ± 0.63	3.31 ± 0.33	78.88 ± 11.7	100

## CONCLUSIONS

- The most efficient transformation occurred when the co-cultivation was done with  $10^4$  conidia due to higher frequency of resistant colonies (894 per  $10^4$  conidia) and lower background obtained.
- 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.
- 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.

## REFERENCES

- Belli, N., Marín, S., Coronas, I., Sanchis, V., Ramos, A.J., 2007. Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes. Food Control 18, 1343-1349.
- Frandsen, R., Andersson, J., Kristensen, M., Giese, H., 2008. Efficient four fragment cloning for the construction of vectors for targeted gene replacement in filamentous fungi. BMC Molecular Biology 9, 70.