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Keywords: ATMT; population monitoring; ecophysiology; pathogenicity; apple; orange.

Abstract: Penicillium digitatum and P. expansum are responsible of green and blue moulds in citrus and pome fruits, respectively, and cause important economical losses over the world. In order to study their infection process in fruits, we successfully introduced a Green Fluorescent Protein (GFP) encoding gene into wild type P. digitatum and P. expansum isolates, by means of the Agrobacterium tumefaciens-mediated transformation (ATMT) technique, using hygromycin B resistance as the selectable marker. To our knowledge, this is the first report describing the transformation of P. digitatum and P. expansum with GFP and the use of transformed strains to study compatible and non-host pathogen interactions of these two important postharvest pathogens. The transformation did not affect the pathogenicity and ecophysiology in P. digitatum and P. expansum transformants as compared to their respective wild type strains. Therefore, these GFP-tagged strains were used for in situ analysis in compatible and non-host pathogen interactions on oranges and apples. Knowledge of the infection process of apples and oranges by their pathogens and non-host pathogens is essential for the design of novel strategies to control these postharvest diseases and determine the response of P. digitatum and P. expansum on/in plant surface and tissues to different postharvest treatments.

RESEARCH HIGHLIGHTS

1. GFP-tagged strains of *P. digitatum* and *P. expansum* were successfully obtained
2. Transformation did not affect the pathogenicity ~~and~~ ecophysiology of ~~both strains~~
3. *P. expansum* was able to infect oranges under specific conditions
4. *P. digitatum* was able to cause a limited infection around the apple wounded tissue

Use of GFP-tagged strains of *Penicillium digitatum* and *P. expansum* to study ~~their infection process in~~ host-pathogen interaction[1]

G. Buron-Moles [a, 1], M. López-Pérez [b, 1], L. González-Candelas [b], I. Viñas [a], N. Teixidó [c], J. Usall [c], R. Torres [c,*]

[a] *University of Lleida, XaRTA-Postharvest, Rovira Roure 191, 25198 Lleida, Spain*

[b] *Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Av. Agustín Escardino 7, Paterna 46980-Valencia, Spain*

[c] *IRTA, XaRTA-Postharvest, Rovira Roure 191, 25198 Lleida, Spain*

* Corresponding author. Tel.: + 34 973003429; fax: + 34 973238301.
E-mail address: rosario.torres@irta.cat (R. Torres).
[1] Both authors have contributed equally to the work.

ABSTRACT

Penicillium digitatum and *P. expansum* are responsible ~~of~~ green and blue moulds in citrus and pome fruits, respectively, ~~and cause important economical losses over the world~~. In order to study their infection process in fruits, we successfully introduced a Green Fluorescent Protein (GFP) encoding gene into wild type *P. digitatum* and *P. expansum* isolates, ~~by means of the~~ *Agrobacterium tumefaciens*-mediated transformation (ATMT) ~~technique, using~~ hygromycin B resistance as the selectable marker. To our knowledge, this is the first report describing the transformation of ~~*P. digitatum* and *P. expansum*~~ with GFP and the use of transformed strains to study compatible and non-host pathogen interactions ~~of these two important postharvest pathogens~~. ~~The~~ transformation did not affect the pathogenicity ~~and~~ ecophysiology ~~in *P. digitatum* and *P. expansum* transformants as~~ compared to their respective wild type strains. ~~Therefore, these~~ GFP-tagged strains were used for *in situ* analysis ~~in~~ compatible and non-host pathogen interactions on oranges and apples. Knowledge of the infection process of apples and oranges ~~by their pathogens and non-host pathogens is essential for~~ the design of novel strategies to control these postharvest diseases and determine the response of *P. digitatum* and *P. expansum* on/in plant surface and tissues to different postharvest treatments.

Keywords: ATMT; population monitoring; ecophysiology; pathogenicity; apple; orange.

1. Introduction

Oranges and apples are both essential food crops cultivated in Spain and largely exported to Europe and other countries. *Penicillium digitatum* and *P. expansum* are responsible ~~of~~ green and blue moulds in citrus and pome fruits, respectively, ~~and cause important economical losses during their postharvest handling over the world.~~

~~Because of~~ the development of resistance to fungicides ~~in fungal pathogens~~ Viñas et al. ~~(~~1993), as well as concerns about the environment and consumer's health, the use of synthetic fungicides is becoming increasingly restricted. In spite of ~~the application of fungicides~~ and the increased implementation of new alternative strategies, both~~,~~ green mould in citrus and blue mould in pome fruit, continue ~~exhibiting high infection pressure on~~ stored fruits worldwide. The development of a fungal disease ~~during postharvest~~ depends ~~among other factors~~ on storage conditions~~,~~ the physiological ~~age and the defense mechanisms of the host~~. These factors are intimately related, as fruit tends to become more susceptible to infection with physiological age (Su et al., 2011; Torres et al., 2003; Vilanova et al., 2012b). While the etiology of *Penicillium* rots are well understood, the physiological and biochemical bases of their host specificity is much less clear. Both *P. digitatum* and *P. expansum* are wound pathogens. The primary infection court~~s~~ ~~are wounds on the surface of the fruit,~~ where nutrients and volatiles stimulate conidia germination, which is followed by ~~penetration and~~ colonization of the fruit tissue (Droby et al., 2008; Eckert and Brown, 1986). This ~~fact~~ suggests that adaptation to a particular host plays an important role in pathogenicity. ~~It~~ has been shown that the presence of some oils facilitate infection in citrus (Rodov et al., 1995; Stange et al., 2002).

~~To our knowledge, the interactions~~ *P. digitatum*-orange and *P. expansum*-apple are considered compatible~~. On the contrary,~~ *P. digitatum* has not been shown to cause postharvest disease on pome fruits~~;~~ ~~consequently, it is considered non-pathogen on apples. The same holds true for the *P. expansum*-citrus fruit interaction, where *P. expansum* can be regarded as a non-pathogen of citrus fruit.~~ However, Vilanova et al. (2012b) ~~demonstrated that from the commercial harvest, a~~ non-host pathogen interaction can become compatible if ~~favourable conditions are present~~.

P. digitatum is a very specific pathogen that under natural conditions only infects citrus fruits (Adams and Moss, 2000), whereas *P. expansum* has been isolated from a wide range of other fruits, including tomatoes, strawberries, avocados, grapes and a variety of others, indicating that it is a broad spectrum pathogen (Snowdon, 1990). The basis of this host specificity remains unknown, but a deeper knowledge on compatible and non-host pathogen interactions could help in the development of new and safer control strategies to control green and blue moulds on fruit produce.

The use of fungal transformants expressing the green fluorescent protein (GFP) has enhanced our knowledge on the fungus-host interaction, constituting a very useful molecular tool to study compatible, incompatible and non-host pathogen interactions and detect and visualize the infection process *in situ* (Horowitz et al., 2002). GFP-tagged fungal transformants have been obtained from a variety of postharvest pathogens (de Silva et al., 2009; Isshiki et al., 2003; Li et al., 2007). However, there are few reports on the genetic transformation of these two important postharvest pathogens, and to the best of our knowledge GFP-tagged strains of *P. digitatum* and *P.expansum* have not been reported so far.

The aim of this study was to introduce the *gfp* gene into wild type *P. digitatum* and *P. expansum* isolates by means of the *Agrobacterium tumefaciens*-mediated transformation (ATMT) technique using hygromycin B resistance as the selectable marker. ATMT has been recently described for *P. digitatum* (Wang and Li, 2008), but has not been reported before for *P. expansum*. In order to check whether transformants maintained their pathogenicity, germination and growth capacity compared to wild type strains ecophysiological studies were conducted before use them to visualize the infection process in compatible and non-host pathogen interactions on oranges and apples.

2. Materials and methods

2.1. Plasmids

Two different plasmids containing two variants of the GFP were used for *Penicillium* transformation. The binary plasmid pRFHUE-eGFP (Crespo-Sempere et al., 2011) contains the eGFP, which differs from the native GFP from *Aequorea victoria* in a double amino acid substitution of Phe-64 to Leu, Ser-65 to Thr. The *egfp* gene included in this plasmid was obtained from plasmid pEGFPC3 and was cloned in plasmid pRF-HUE (Frandsen et al., 2008) under the control of the *Aspergillus nidulans gpdA* promotor. The second plasmid used was pCAMBgfp (Sesma and Osbourn, 2004). This plasmid contains the sGFP variant, in which there is a single amino acid substitution of Ser-65 to Thr with respect to the original GFP. In plasmid pCAMBgfp the expression of the *sgfp* gene is under control of *ToxA* gene promoter from *Pyrenophora tritici-repentis*. These two vectors were introduced into electrocompetent *Agrobacterium tumefaciens* AGL-1 cells.

2.2. Fungal strains

Isolate Pd1 of *P. digitatum* (Pers.:Fr.) Sacc was obtained from a rotten "Navelina" orange at IVIA orchards that were not treated with fungicides and *P. expansum* Link CMP-1 was isolated from a decayed "Golden" apple after several months in storage. Wild type strains, *P. digitatum* and *P. expansum*, were grown on Petri dishes containing Potato Dextrose Agar medium (PDA: 200 mL/L boiled potato extract; 20 g/L dextrose, 20 g/L agar, pH 5.5) in the dark at 25 ºC for 7-10 days to achieve conidia production. Transformed strains, *P. digitatum* and *P. expansum* were maintained on PDA containing 100 and 200 µg/mL of hygromycin B (Hyg B; Invivogen, San Diego, USA), respectively. Transformed strains were also incubated at 25 ºC in the dark during 7-10 days to obtain heavily ~~sporulated~~ cultures. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-day-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. The final conidia concentration was adjusted using a haemacytometer and diluted to different concentration depending on each assay.

2.3. *Agrobacterium tumefaciens*-mediated transformation (ATMT)

A. tumefaciens AGL-1 carrying the plasmid of interest was inoculated at 28 ºC for 24 h in LC liquid medium (Hooykaas et al., 1977) with kanamycin (50 µg/mL), rifampicin (20 µg/mL) and carbenicillin (75 µg/mL). 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 $(NH4)_2SO_4$, 10 mM glucose, 40 mM 2-[N-morpholino] ethanesulfonic acid, pH 5.3, 0.5% glycerol) and diluted to an OD_{600} of 0.15 in the same medium amended with 200 µM acetosyringone (AS). The culture was inoculated at 28 ºC and 200 rpm until it reached an OD_{600} of 0.7-1.0.

Conidial suspensions of *P. digitatum* and *P. expansum* were washed twice with induction medium (IM) and adjusted to a concentration of 10^5 conidia/mL. Then, equal volumes of conidia and *A. tumefaciens* cells were mixed and spread onto nitrocellulose membrane filters (0.45 µm pore and 47 mm diameter, Albet, Dassel, Germany) that were placed on agar plates containing IM (containing 5 mM instead of 10 mM of glucose). After co-cultivation at 24 ºC for three days, the filters were transferred to PDA plates containing hygromycin B (100 µg/mL or 200 µg/mL for *P. digitatum* and *P. expansum*, respectively) as the selection agent for fungal transformants, and 200 µg/mL of cefotaxime (Serva, Heidelberg, Germany) to inhibit growth of *A. tumefaciens* cells. Hygromycin resistant colonies obtained after 4 to 5 days of incubation were transferred to PDA plates containing hygromycin B and incubated at 24 ºC for sporulation.

2.4. Genomic DNA extraction and PCR analysis

Conidia from transformants were transferred with a toothpick to a 1.5 mL Eppendorf tube containing 0.5 mL of Glucose Peptone Yeast (GPY: glucose 10 g/L, peptone 5 g/L, yeast extract 2 g/L, pH 7.5) medium supplemented with hygromicyn B and incubated with shaking at 24 ºC for 48 h. The culture was centrifuged for 5 min at 12000 rpm, the supernatant was removed and the pellet was resuspended with 300 µl of TNES (50 mM Tris HCl pH 8.0, 20 mM EDTA, 100 mM NaCl, 1% SDS). The sample was shaken during 2 min in a cell disruptor (BeadBeater, Biospec, Bartlesville, USA) with five stainless steel balls of 2.7 mm and centrifuged for 10 min at 12,000 rpm. Then, DNA was purified following the protocol described by Cenis (1992) and dissolved in 100 µL of TE.

To confirm the integration of the T-DNA in the genome, several transformants were randomly selected to analyze the presence of the hygromicin resistance gene. PCR was conducted using oligonucleotides HMBR1 (5'-CTGATAGAGTTGGTCAAGACC-3') and HMBF1 (5'-CTGTCGAGAAGTTTCTGATCG-3'). DNA amplification was done under the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 62 °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were separated in an agarose gel and were visualized under UV light.

2.5. Detection of GFP gene expression in transformants

To visualize the fluorescence of *P. digitatum* and *P. expansum* transformants obtained with each vector, they were grown on PDA plates with hygromicyn B at 24 ºC for several days. A piece of mycelium from these plates was washed and resuspended in 20% glycerol. The fluorescence was visualized using a fluorescence microscope 90i (Nikon) equipped with a FITC B-2E/C filter (excitation from 450 to 490 mm and emission from 515 to 565 nm). Images were acquired with the Nikon's NIS-elements software.

2.6. Pathogenicity studies on fruit

Oranges (*Citrus sinensis* L. cv Lanelate) were obtained from a commercial orchard in Tortosa (Catalonia, Spain) and apples (*Malus domestica* L. cv Golden Delicious) from Lleida (Catalonia, Spain). Fruits were selected by hand ~~and used in the experiments before any~~ commercial postharvest treatment ~~was applied~~. Fruit were stored at an optimal storage temperature for each fruit, 4 ºC for oranges and 0 ºC for apples, until used. Prior to inoculation, the fruits were randomized, washed with tap water and allowed to air-dry at room temperature. Each fruit was artificially wounded once with a nail (3 mm wide and 3 mm deep) on the equator and inoculated with 15 µL of aqueous conidia suspension~~s~~ of pathogen at $1 \cdot 10^6$ conidia/mL ~~concentration~~. The trial was performed with the wild type strains of *P. digitatum* and *P. expansum*, and the transformed strains eGFP-*P. digitatum* and sGFP-*P. expansum*, with their host, oranges and apples, respectively. This methodology was carried out individually for each pathogen and host. The treated fruit were incubated seven days at 20 ºC and 85-90% relative humidity (RH). After the incubation period, the percentage of infected wounds (incidence) and

the lesion diameters (severity) caused by wild type and transformed strains were measured. Five fruits constituted a single ~~replicate and each treatment was repeated four times~~.

2.7. Sporulation assessment

~~To express the intensity of sporulation, a sporulation index was used which represents the percentage of the fruit surface covered with conidia.~~ The degree of *Penicillium* sporulation on the surface of decayed fruits was evaluated on a 0-5 scale described by Palou et al. (2003). A quantitative sporulation index was used in which the numbers indicated: 0, soft lesion but no conidia or mycelium present; 0.5, mycelium but no conidia present; 1, < 5%; 2, 5-30%; 3, 31-60%; 4, 61-90% and 5 > 91% of the fruit surface covered with conidia. The index value for each fruit ~~is treated as a replication~~.

2.8. Ecophysiological characterization of strains

Conidia obtained from actively growing 7-10 day-old colonies of each strain grown on PDA media (wild types) and PDA media containing hygromycin B (transformants) were used for all ecophysiological studies.

Synthetic and semi-synthetic media were used for all ecophysiological studies. Synthetic media were PDA and Orange Serum Agar (OSA) with a pH of 5.5. Semi-synthetic Apple-based medium (AM) contained 10% of apple juice sterilized through a nitrocellulose membrane filter (0.22 µm pore and 25 mm diameter, Millipore, Billerica, U.S.A) glucose (4 g/L) and 2% agar with a final pH of 4.3.

2.8.1. Germination studies *in vitro*

Ten µL droplets of the conidia suspensions adjusted to $5 \cdot 10^5$ conidia/mL were inoculated on PDA and OSA for *P. digitatum* strains and PDA and AM for *P. expansum* strains. Petri dishes were incubated at 25 ºC (*P. digitatum* and *P. expansum* strains), 4 ºC (*P. digitatum* strains) and 0 ºC (*P. expansum* strains). Periodically, depending on the temperature, three agar discs (5 mm diameter) coinciding with each of the placed drops were aseptically removed from each replicate using a cork borer. At each sampling time, discs from the same temperature and medium were placed into a sterile empty Petri dish, and conidia germination was immediately stopped by adding 3 mL of ammonia (NH_3 25%) onto a filter paper placed on the cover of each

plate. Then, Petri dishes were stored at 4 ºC until microscopic examination. Fifty single conidia per disc (150/replicate; 450/treatment) were microscopically examined (Leica DM5000B). Conidia were considered germinated when the germ tube was equal to or longer than the diameter of the conidia (Casals et al., 2010; Plaza et al., 2003). The variable measured was the percentage of germination at different temperatures and culture media against time. Experiments were carried out with three replicates per treatment.

2.8.2. Growth studies *in vitro*

Ten µL droplets of the conidia suspensions adjusted to $5 \cdot 10^5$ conidia/mL were single-point inoculated in the middle of Petri plates with different media. Wild type and transformed strains of *P. digitatum* were inoculated on PDA and OSA and *P. expansum* strains on PDA and AM. *P. digitatum* strains were incubated at 25 ºC and 4 ºC and *P. expansum* strains at 25 ºC and 0 ºC. Strains incubated at 25 ºC were examined daily meanwhile strains incubated at cold conditions were examined every seven days. Measurements were carried out for a maximum of 12 days at 25 ºC and 77 days at 4 ºC or 0 ºC depending on the pathogen. Colony diameters were measured in two directions at right angles to each other (Marín et al., 2006) until the plate was fully covered. The variable measured was the colony diameter at different temperatures and culture media against time. Experiments were carried out with three replicates per treatment.

2.9. Visualization of fruit infected with *Penicillium* strains

Six fruit discs (16 mm diameter and 5 mm thickness) were removed from oranges and apples using a cork borer and placed into sterile Petri plates. One set of discs remained intact and another set was wounded once with a nail (3 mm wide and 3 mm deep) at the center. Both, intact and wounded fruit discs were inoculated with 15 µL of a conidia suspension of each strain. For compatible interactions, orange-*P. digitatum* and apple-*P. expansum*, 10^5 conidia/mL of *P. digitatum* or eGFP-*P. digitatum* and 10^4 conidia/mL of *P. expansum* or sGFP-*P. expansum* were inoculated. For non-host pathogen interactions, orange-*P. expansum* and apple-*P. digitatum*, the concentration in both cases was 10^7 conidia/mL. Discs inoculated with wild type and transformed strains of *P. digitatum* were stored at 20 ºC and 4 ºC. In the case of discs inoculated with *P. expansum* strains they were stored at 20 ºC and 0 ºC. The experiment was

conducted for a maximum of 96 hours at 20 ºC and 32 days at 0 ºC and 4 ºC. After the incubation period, samples were examined using a stereoscope (Leica MZ16F) equipped with external light source and appropriate filter sets (excitation from 460 to 500 nm and 510 nm emission). Images were captured using the Leica's DFCTwain software.

2.10. Statistical analyses

Differences on the percentage of infected wounds (incidence), lesion diameters (severity), germination percentage, growth rate and sporulation index between the wild type and the transformed strains were evaluated by the t-test using the statistical package SAS (Microsoft). Differences between mean values were considered significant when $P \leq 0.05$.

Scores in the sporulation index were considered as a quantitative variable. In order to homogenize variances, each value in the sporulation data set was transformed to the square root of the value plus 0.5.

For the growth studies, growth rates (mm/day) were obtained from the growth data using linear regression of the linear parts of the temporal growth curves.

3. Results and discussion

3.1. Transformation

The major aim of this work was to obtain GFP-tagged *P. digitatum* and *P. expansum* transformants in order to study compatible (orange-*P. digitatum* and apple-*P. expansum*) and non-host pathogen (orange-*P. expansum* and apple-*P. digitatum*) interactions~~ of these two important postharvest pathogens~~. In many fungal species transformation with the native *gfp* gene have resulted in non-fluorescent transformants (Fernandez-Abalos et al., 1998), probably due to inadequate codon usage. As *P. digitatum* transformants expressing this native version of the *gfp* gene did not show any fluorescence (data not shown), we used the modified versions encoded by *egfp* and *sgfp* genes for GFP tagging of *P digitatum* and *P. expansum*. These two GFP variants are present in plasmids pRFHUE-eGFP and pCAMBgfp, respectively. ATMT was used to transform *P. digitatum* and *P. expansum* with these two plasmids. Randomly selected hygromycin resistant colonies were analyzed by PCR to detect the presence of the hygromycin resistance gene and all of them were positive (data not shown). Microscopic analysis of GFP-

252 tagged strains revealed homogeneity of the fluorescent signal, which was clearly visible in the
253 conidia and hyphae and stable for several hours during observations. No green autofluorescent
254 background was observed in the wild type strains (Fig. 1).
255 Despite the economic importance of these two postharvest pathogens little attention has been
256 paid to their physiological and genetic characterization. Genetic transformation of *P. expansum*
257 has only been described twice (Dias et al., 1999; Sanzani et al., 2012), but to our knowledge this
258 is the first time that ATMT has been described in *P. expansum*. On the other hand, genetic
259 transformation of *P. digitatum* has been used to study the mechanisms of fungicide resistance
260 (Hamamoto et al., 2001; Nakaune et al., 1998; Nakaune et al., 2002) and ATMT has been
261 described recently for this fungus (Wang and Li, 2008).
262 GFP-tagged fungal pathogens have been used to study ~~the~~ different stages of the progress of the
263 fungus within ~~the~~ host (Horowitz et al., 2002; Isshiki et al., 2003; Lagopodi et al., 2002; Pliego
264 et al., 2009; Talhinhas et al., 2008). Thus, the availability of GFP-tagged *P. digitatum* and *P.*
265 *expansum* transformants constitutes a powerful tool for studying the interactions between *P.*
266 *digitatum* and *P. expansum* and their fruit hosts. ~~They~~ are also very useful in determining the
267 response of *P. digitatum* and *P. expansum* on/in plant surface and tissues to different
268 postharvest treatments.
269 3.2. Pathogenicity studies
270 To determine whether the transformants are altered in their ability to cause disease~~s~~ in ~~both~~
271 hosts as a result of integration of the transforming DNA within the genome, assessment of
272 pathogenicity of wild type and transformed strain was conducted. For this purpose one
273 transformant strain from each species was chosen and compared to the non-transformed wild
274 type strain.
275 Seven days after inoculation in oranges, there was no significant difference~~s~~ in percentage of
276 infected wounds (%) ~~and~~ lesion diameter~~s~~ (cm) at 20 ºC ($P > 0.05$) (data not shown). For the
277 wild type and the eGFP-*P. digitatum* strains, the *Penicillium* rot was 95% and 100%, and the
278 lesion diameter was 12.5 and 11.5 cm, respectively. As in oranges, no difference~~s~~ were found in
279 the ~~apple~~ percentage of infected wounds (%) ~~and~~ lesion diameter~~s~~ (cm), after 7-days incubation

at 20 ºC ($P > 0.05$) (data not shown). For the wild type and the sGFP-*P. expansum* strains, blue mould was observed in 100% inoculated apples, and lesion diameter was 3.4 cm.

The GFP has been successfully used as a vital marker for broad range of plant pathogens to study both leaf and root infections (Horowitz et al., 2002; Lorang et al., 2001; Maor et al., 1998; Morocko-Bicevska and Fatehi, 2011; van West et al., 1999; Visser et al., 2004). As other authors described previously for other fungal species such as *Botrytis cinerea* in strawberry (Li et al., 2007), *Aspergillus carbonarius* in grapes (Crespo-Sempere et al., 2011), *Fusarium oxysporum* in tomato (Lagopodi et al., 2002) and *Rosellinia necatrix* in avocado (Pliego et al., 2009), the expression of fluorescent proteins itself does not affect the pathogenicity of the transformed fungi. In agreement with these results, pathogenicity tests on oranges and apples revealed that the GFP-tagged strains of *P. digitatum* and *P. expansum* express the GFP in hyphae and conidia of the fungus both *in vitro* and *in vivo*, and maintain the characteristics of the wild type strains. Therefore, these GFP-tagged strains for *P. digitatum* and *P. expansum* allow their utilization as a powerful tool for *in situ* analysis of infection in fruit-pathogen interactions between the pathogens and their fruit hosts.

3.3. Sporulation index

The results obtained for the sporulation index in oranges and apples were similar between wild type and transformed strains. Inoculated oranges reached a sporulation index of 2 indicating that 5-30% of the fruit surface was covered with conidia (data not shown). On inoculated apples, less than 5% of the fruit surface was covered with conidia which correspond to a sporulation index of 1 (data not shown). There were not significant differences on the sporulation index between wild type and GFP-tagged strains on oranges and apples ($P > 0.05$) (data not shown). Our results confirm that GFP transformation did not affect significantly the sporulation of both pathogens on their hosts.

3.4. Ecophysiology studies

3.4.1. Germination studies *in vitro*

The results obtained in synthetic (PDA and OSA) media inoculated with wild type and transformed strains of *P. digitatum* are shown in Fig. 2. Overall patterns of germination were

similar for *P. digitatum* and eGFP-*P. digitatum* strains. For instance, after 12 hours of incubation on PDA at 25 ºC, the percentage of germination for both strains was 85% and 88%, respectively. In addition, after 96 hours of incubation at 4 ºC, the percentage of germination also showed similar values (77% and 79%, respectively). In both cases, ~~these~~ differences were not significant ($P > 0.05$) (Fig. 2A-B). After 12 hours of incubation on OSA at 25 ºC, the percentage of germination ~~showed significant differences ($P < 0.05$);~~ for *P. digitatum* ~~was 76%, whereas for~~ eGFP-*P. digitatum* was 90% (Fig. 2C). The wild type strain of *P. digitatum* germinated slightly ~~latter~~ than eGFP-*P. digitatum* at 4 ºC on OSA. However, after 96 hours no significant differences were observed (70% and 75% for *P. digitatum* and eGFP-*P. digitatum*, respectively) ($P > 0.05$) (Fig. 2D). It is interesting to note that the percentage of germinated spores at both temperatures reached similar values, although there was a delay in germination ~~rate~~ at 4 ºC.

~~The patterns of g~~ermination percentage ~~were~~ also similar for the wild type and transformed *P. expansum* strains in synthetic (PDA) and semi-synthetic (AM) media (Fig. 3). For example, after 12 hours of incubation on PDA at 25 ºC, germination percentage reached 100%, and no significant differences were observed between both strains ($P > 0.05$) (Fig. 3A). Only in a few cases the differences were significant ($P < 0.05$): first, after 144 hours of incubation on PDA at 0 ºC, the germination percentage was 90% for *P. expansum*, and 78% for sGFP-*P. expansum* (Fig. 3B). Second, after 12 hours of incubation on AM at 25 ºC, it was 84% and 70%, for the wild type and sGFP-*P. expansum* strains, respectively (Fig 3C). Lastly, sGFP-*P. expansum* also germinated to a lower extent than *P. expansum* at 0 ºC (Fig 3D). However, ~~it is noteworthy~~ the high germination capability exhibited by *P. expansum* and sGFP-*P. expansum* at 0 ºC.

These results suggest that transformation with the *gfp* gene did not modify the overall germination pattern in *P. digitatum* and *P. expansum* transformants as compared to their respective wild type on different incubation media and temperatures.

In this study OSA and AM media were used because they are reasonably similar in composition to orange and apple fruit. Wyatt and Parish (1995) demonstrated that *P. digitatum* conidia ~~also~~ did not germinate at 0 or 3 ºC on Orange juice serum agar. Low temperatures used in this study

are commonly used in storage rooms, used to store oranges (4 ºC) and apples (0 ºC) for several months. Our results showed 70% of *P. digitatum* germination after 96 hours of incubation at 4 ºC on OSA (Fig. 2D). These results differ from those obtained by Plaza et al. (2003), in which the germination percentage for *P. digitatum* was around 20% after 96 hours of incubation at 4 ºC on OSA. These differences could be due to the different isolates used in both studies.

The comparison of germination percentages at two different temperatures suggests that *P. digitatum* and *P. expansum* may be more difficult to control in susceptible foods stored at 4 and 0 ºC, respectively, because both fungi show a high germination rate at low temperatures, thus cold storage does not prevent spoilage but only retards it.

3.4.2. Growth studies *in vitro*

No differences in colony morphology on synthetic and semi-synthetic media were observed at 25 ºC, 4 ºC and 0 ºC. The effects of temperature and culture medium on the growth rate for wild type and GFP-tagged strains are presented in Table 1 and 2. There were no significant differences between the wild type and the eGFP-*P. digitatum* strains, in any studied condition ($P > 0.05$) (Table 1). For instance, both wild type and eGFP-*P. digitatum* strains had similar growth rates when cultured on PDA (8.3 and 8.5 mm/day, respectively) and OSA (8.8 and 8.4 mm/day, respectively) at 25 ºC. These values are higher than reported by Plaza et al. (2003) for another isolate of *P. digitatum* incubated at the same temperature (3.5 mm/day). As in the germination study, these differences could be due to the different isolates used in both studies. Growth rates of both strains were reduced when temperature varied from 25 to 4 ºC. In such case, the growth rates were 0.4 and 0.3 mm/day on PDA, and 0.6 and 0.4 mm/day on OSA (Table 1).

Statistical analysis revealed that the growth rate was not different between the wild type and the transformed *P. expansum* strains, in any studied condition ($P > 0.05$) (Table 2). At 25 ºC, growth rates of *P. expansum* and sGFP-*P. expansum* strains were 8 mm/day on PDA, and 7.9 and 7.8 mm/day on AM. Growth rates of both strains were reduced when temperature varied from 25 to 0 ºC. At 0 ºC, the growth rates were 0.5 mm/day on PDA, and 0.6 mm/day on AM (Table 2). These results are in agreement with those obtained by Baert et al. (2007), who

demonstrated that shortened growth rates were found when the temperature increased from 2 ºC to 25 ºC.

These results suggest that transformation with the *gfp* gene did not modify the growth rate in *P. digitatum* and *P. expansum* transformants as compared to their respective wild type strains on different incubation media and temperatures. It is difficult to extrapolate the results obtained *in vitro* to natural environment because other factors, such as pH, antifungal compounds of the peel, essential oils, may influence the development of the fungus within the host. Moreover, further studies are needed in order to provide detail knowledge on the ecological requirements of these species for colonizing and infecting the surface of oranges and apples.

3.5. Fluorescence visualization *in vivo*: compatible and non-host pathogen interaction

Once determined *in vitro* that from the ecophysiological point of view transformant and wild type are equivalent, other confirmation on fruit-pathogen interaction were done *in vivo*.

Fruits were inoculated with wild type and transformant strains to test whether GFP can help to visualize the pathogen on the fruit surface and the behavior of both transformants on host and non-host fruits. Colonization of the hosts by the pathogens in relation to the development of green and blue mould in compatible and non-host pathogen interactions was determined using GFP-tagged strains (Figure 4 and 5). Overall, wounded orange and apple discs with the transformed strains showed green fluorescence under the fluorescence stereomicroscope, whereas no fluorescence was observed in intact fruit discs confirming that both are wound pathogens. Additionally, re-isolation of GFP-tagged from inoculated wounded discs fruits on PDA with hygromycin B confirmed microscopic observations.

To visualize pathogens ~~in plant~~ it is important ~~that~~ whole fungi ~~have been labeled with~~ GFP. ~~This fact is possible thanks to~~ constitutive expression of GFP, as described above, which typically results in a cytoplasmically located protein occurring in all fungal morphotypes (hyphae, conidia, etc) with no obvious effects on fungal growth or pathogenicity (Lorang et al., 2001; Maor et al., 1998; Spellig et al., 1996; van West et al., 1999). Compatible interactions are shown in figure 4 A-C and 5 A-C, whereas non-host pathogen interactions are shown in figures 4 B-D and 5 B-D. In this work, GFP transformants of phytopathogenic fungi were easily

detected with fluorescence microscopy (Figure 4 C-D and 5 C-D). In addition, images of *P. expansum* in orange revealed that primary infection takes place in a similar way as in the *P. digitatum*-orange interaction. Vilanova et al. (2012b) has previously shown that depending on the combination of factors (maturity stage and inoculum concentration), the *P. expansum*-orange interaction can change from non-host pathogen to compatible in Valencia and Navelina varieties. We have observed the same phenomenon in Lanelate oranges, supporting this previous finding. The maturity stage of the oranges we have used was mature to over-mature (data not shown). Therefore, maturity stage could affect the infection capacity in a non-host interaction in oranges. However, rot infection did not develop in the apple-*P. digitatum* interaction, although, as shown in Fig 5B and D, *P. digitatum* is able to germinate inside the wounded apple tissue and produce a limited infection only in surrounding cells. It seems that apple is able to avoid the progress of *P. digitatum* by expressing an efficient defence response. The passive or preexisting defence mechanisms involve structural barriers such as waxy cuticle, or strategically positioned reservoirs of antimicrobial compounds that function to prevent colonization of the tissue (Jackson and Taylor, 1996; Osbourn, 1996). In addition, lignin formation may play an important role in the defence mechanisms. Lignification was apparently more important in immature fruits than in commercial mature fruits, and lignin-like material was not observed in over matured oranges (Vilanova et al., 2012b).

The phenomenon of selective stimulation of fruit pathogens by volatile compounds is known in other fruit pathogen systems. Droby et al. (2008) demonstrated that *P. digitatum* and *P. italicum* are uniquely adapted to and stimulated by the volatile environment associated with citrus wounds, whereas this same environment is inhibitory to other non-citrus pathogens such as *P. expansum* and *B. cinerea*. However, the relative importance of each volatile component separately is not clear.

We have confirmed the capacity of *P. expansum* to infect oranges (non-host pathogen) under specific conditions. Similarly, *P. digitatum* is able to germinate inside apple wounded tissue (non-host pathogen), but only causing a limited infection around the wound (Vilanova et al., 2012a).

As well as the specificity of *P. digitatum* to orange, even though is able to germinate inside apple wounded tissue and cause a limited infection around the wound. Other studies in strawberries (Li et al., 2007) and grapes (Crespo-Sempere et al., 2011) demonstrated that the GFP-tagged strains could be a powerful tool for future studies on the interactions between the pathogens and fruits. The availability of *P. digitatum* and *P. expansum* GFP-tagged strains will allow us to conduct a more detailed *in situ* analysis of both compatible and non-host pathogen interactions involving orange and apple fruits.

The characterization of each transformant compared with the wild type strain demonstrated the suitability of the use of GFP as a marker for *P. digitatum* and *P. expansum* in monitoring both pathogens in fruit-pathogen interaction studies.

To our knowledge, this is the first study that reports the transformation of *P. digitatum* and *P. expansum* with GFP and the use of transformed strains to study compatible and non-host pathogen interactions of these two important postharvest pathogens. Knowledge of these interactions is essential to offer novel insights into *P. digitatum* and *P. expansum* pathogenicity from the early stages of tissue colonization to advanced stages of the disease. In addition, knowledge of the infection process of apples and oranges by their pathogen and non-host pathogen is essential for the design of novel strategies to control these postharvest diseases and determine the response of *P. digitatum* and *P. expansum* on/in plant surface and tissues to different postharvest treatments.

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Table

Table 1. Comparison of growth rate (mm/day) between the wild type and the eGFP-*P. digitatum* at different media (PDA and OSA) and temperature (25 ºC and 4 ºC).

		Growth rate (mm/day)*		
		P. digitatum	eGFP-*P. digitatum*	*P-value*
25 ºC	PDA	8,30	8,48	0,7033
	OSA	8,77	8,41	0,3791
4 ºC	PDA	0,38	0,32	0,3410
	OSA	0,64	0,45	0,1051

*Rate was determined as the slope of the linear portion of a curve produced by plotting distance of growth diameter (mm) vs time (days). Each result is the mean of three replicates. Significant differences ($P < 0.05$) were evaluated using t-test.

Table 2. Comparison of growth rate (mm/day) between the wild type and the *sGFP-P. expansum* at different media (PDA and AM) and temperature (25 ºC and 0 ºC).

		Growth rate (mm/day)*		
		P. expansum	sGFP-*P. expansum*	*P-value*
25 ºC	PDA	8,019	8,002	0,9567
	AM	7,878	7,844	0,5404
0 ºC	PDA	0,524	0,516	0,6282
	AM	0,627	0,629	0,5870

*Rate was determined as the slope of the linear portion of a curve produced by plotting distance of growth diameter (mm) vs time (days). Each result is the mean of three replicates. Significant differences ($P < 0.05$) were evaluated using t-test.

FIGURES

Figure 1. Microscopy analysis of transformant strains eGFP-*Penicillium digitatum* (A) sGFP-*P. digitatum* (B), eGFP-*P. expansum* (C) and sGFP-*P. expansum* (D). Differential interference contrast (left) and fluorescence (right) images of conidiophores and conidia (A-D). A, C, and D, microscope magnification, ×400; B, microscope magnification, ×200.

Figure 2. Effect of temperature on the germination percentage of *Penicillium digitatum* and eGFP-*P. digitatum* strains on PDA and Orange Serum Agar (OSA) media. Strains are (■) *P. digitatum* and (▲) eGFP-*P. digitatum*. Values are the means of three replicates and 150 conidia per replicate. Vertical bars are the standard deviation.

Figure 3. Effect of temperature on the germination percentage of *Penicillium expansum* and sGFP-*P. expansum* strains on PDA and Apple-based medium (AM) media. Strains are (■) *P. expansum* and (▲) sGFP-*P. expansum*. Values are the means of three replicates and 150 conidia per replicate. Vertical bars are the standard deviation.

Figure 4. *In situ* visualization of wild type and GFP-tagged strains on wounded oranges. (A) *Penicillium digitatum*, (B) *P. expansum*, (C) eGFP-*P. digitatum*, (D) sGFP-*P. expansum*. Light (A-B) and fluorescence (C-D). Scale bar = 2 mm.

Figure 5. *In situ* visualization of wild type and GFP-tagged strains on wounded apples. (A) *Penicillium expansum*, (B) *P. digitatum* (C) sGFP-*P. expansum*, (D) eGFP- *P. digitatum*. Light (A-B) and fluorescence (C-D). Scale bar = 1 mm.

Figure 1
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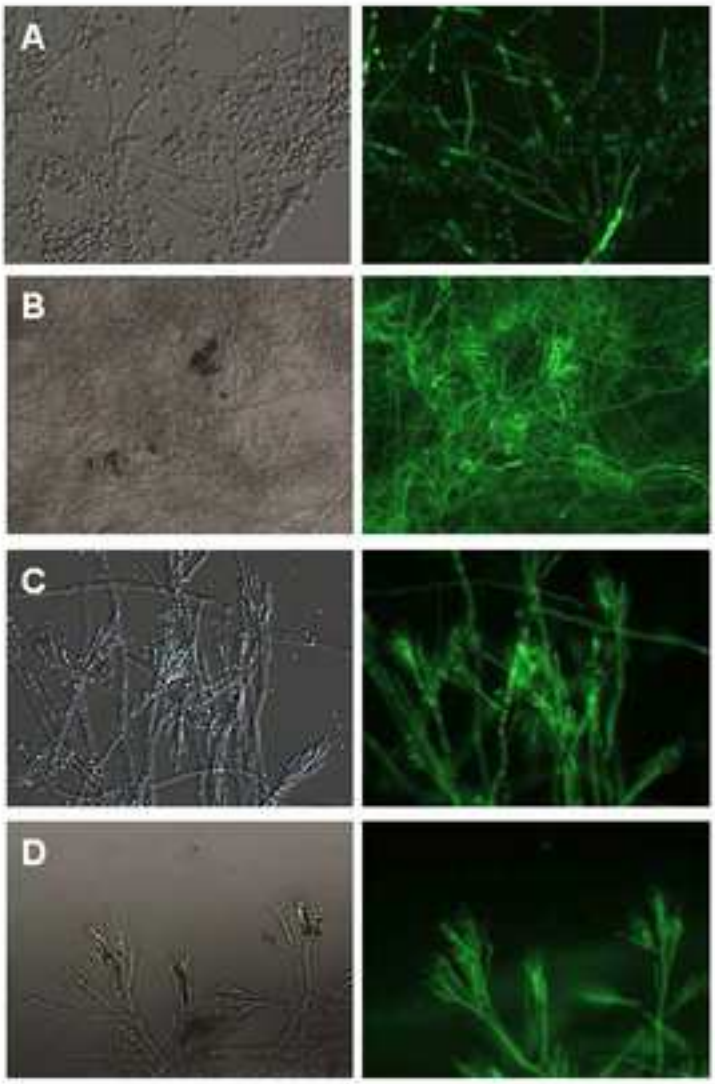

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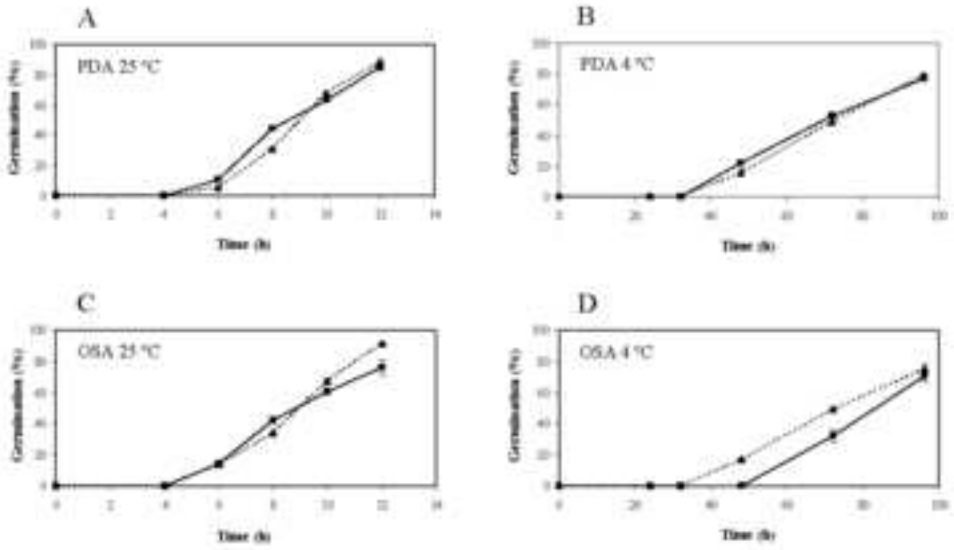

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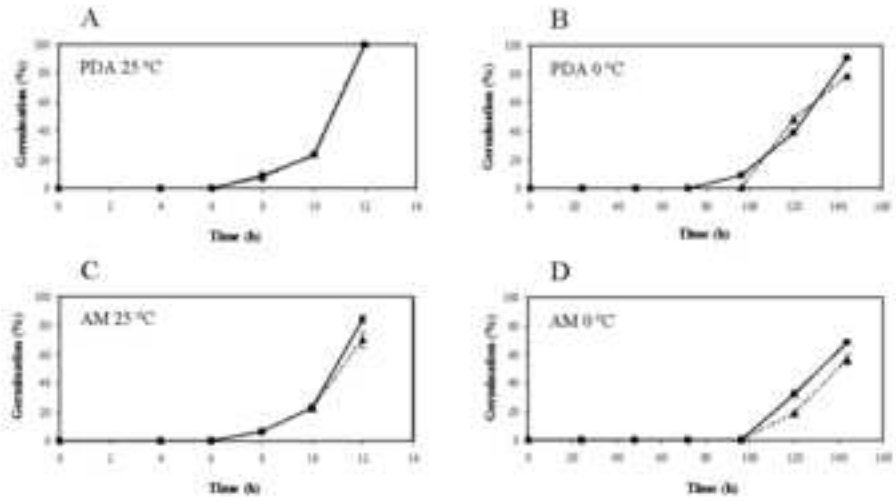

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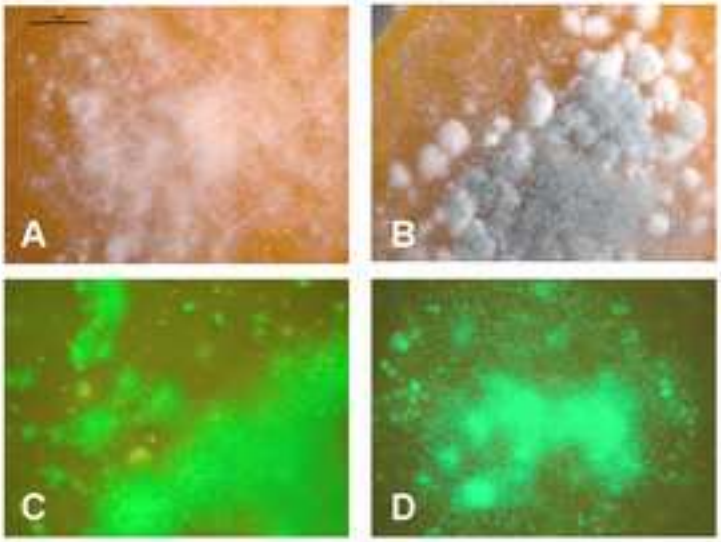

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