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Colonization of *Vitis* spp. Wood by sGFP-Transformed *Phaeomoniella chlamydospora*, a Tracheomycotic Fungus Involved in Esca Disease

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Accepted for publication 10 November 2011.

ABSTRACT

Landi, L., Murolo, S., and Romanazzi, G. 2012. Colonization of *Vitis* spp. wood by sGFP-transformed *Phaeomoniella chlamydospora*, a tracheomycotic fungus involved in Esca disease. Phytopathology 102:290-297.

To evaluate wood colonization and interactions with *Vitis* spp. of *Phaeomoniella chlamydospora*, a fungal agent involved in Esca disease, isolate CBS 229.95 was transformed using a pCT74 construct which contained the genetic markers for synthetic green fluorescent protein (sGFP) and hygromycin B phosphotransferase. Nine stable *P. chlamydospora* fungal transformants (*Pch*-sGFP lines) were obtained using polyethyleneglycol-mediated transformation of protoplasts. These were characterized for *sgfp* and hygromycin B phosphotransferase (*hph*) genome insertions and for sGFP fluorescence emission, using quantitative polymerase chain reaction and fluorimetric systems, respectively. No correlation was observed between *sgfp* copy number genome insertion and sGFP fluorescence expression. Cuttings of *Vitis vinifera* 'Montepulciano', 'Verdicchio', 'Sangiovese', 'Biancame', and 'Cabernet Sauvignon'; and the grapevine rootstocks 'Kober 5BB', 'SO4', '420A', '1103P', and *V. rupestris* were inoculated by immersion in a conidial suspension of the selected fungal *Pch*-sGFP71 line and incubated at 4 ± 1 and 25 ± 1°C. Wood colonization was estimated through epifluorescence microscopy and was affected by incubation temperature. After 6 months at 4 ± 1°C, the fungal growth was completely inhibited. At 25 ± 1°C, the highest extent of wood colonization was recorded in Montepulciano and Verdicchio, with the lowest in the rootstocks SO4 and *V. rupestris*. The expression of the *Pch*-sGFP71 transformed line was localized in the xylem area, primarily around the vessels. The use of sGFP-transformed *P. chlamydospora* helped to clarify different aspects associated with the location of this pathogen in grapevine tissue, before disease symptom expression.

Additional keywords: quantitative real-time polymerase chain reaction.

Esca is one of the most destructive grapevine trunk diseases (9). The complexity of Esca disease is due to its etiology. Fungi associated with Esca symptoms include the hyphomycetes *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., which are associated with black streaking and brown-red wood (12,19). It is the combination of these fungi with the wood-rotting basidiomycete *Fomitiporia mediterranea* that causes "Esca proper", affecting mostly older vines (40), whereas young vines showing Esca foliar symptoms, wood black streaking, and necrosis are mainly infected by *P. chlamydospora* or *Phaeoacremonium* spp. Recently, symptomatic vines have been frequently recorded in 2- to 3-year-old vineyards and, from such plants, *P. chlamydospora* and *P. aleophilum* are consistently isolated (30,35). A histochemical study of necrotic wood tissue of vines infected with *P. chlamydospora* revealed that the fungus was spread through the xylematic area (43,44). On the other hand, *P. chlamydospora*, when artificially inoculated, did not completely succeeded in reproducing the syndrome of young Esca (40). *P. chlamydospora* may even be isolated from apparently healthy rootstock mother plants (15,35,46) and from cuttings (5,17). This, indeed, raises questions about the possible role of nurseries in propagating pathogens associated with Esca in the field.

Studies of fungal colonization in plants using fungal transformation with a vital marker can allow the fungal structures to be traced in the plant host, independent of the expression of disease symptoms. Monitoring fungal colonization – in particular, *P. chlamydospora* – in grapevine cuttings can provide information to better understand disease epidemiology. The synthetic green fluorescent protein (*sgfp*) gene from the jellyfish *Aequorea victoria*, which encodes GFP, has been widely used as a reporter gene in a large number of organisms. Such fluorescent genes are useful for tracking proteins in living cells; as reporters of promoter activity; as labels to visualize specific tissues, whole cells, or subcellular organelles; and for monitoring of gene expression and protein localization. This protein is a successful reporter because only UV or blue light and oxygen are needed for its visualization, and not cofactors or substrates, as is often the case for other reporters. Indeed, this gene and its variants are the most widely used reporters in biological research (22). Expression of GFP has been described in many different filamentous fungal genera, including *Fusarium*, *Botrytis*, *Pyrenophora*, *Alternaria*, *Cochliobolus*, *Sclerotinia*, *Colletotrichum*, and *Verticillium* (24). *P. chlamydospora* has been transformed with GFP previously and characterized according to its morphological and pathogenetic features (7,28). However, host colonization of *P. chlamydospora* (*Pch*)-sGFP on grapevine cuttings has not yet been investigated.

The aim of this study was to develop a procedure for in situ analysis of the spread of *P. chlamydospora* in grapevine and rootstock cuttings before the disease symptoms become apparent, using this sGFP reporter. To reach this main objective, we developed a fungal transformation procedure and a protocol to characterize the fungal sGFP transformants, both by a molecular method (quantitative real-time polymerase chain reaction [PCR]), and in vivo by a fluorimetric method (fluorescence emission).

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains one supplemental figure.

http://dx.doi.org/10.1094/PHYTO-06-11-0165
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MATERIALS AND METHODS

Preparation of protoplasts, transformation, and selection of *P. chlamydospora* transformants. Transformation was performed on *P. chlamydospora* CBS 229.95 type strain. To obtain protoplasts, *P. chlamydospora* was grown on potato dextrose agar (PDA) (Micropoli, Legnano, Milan, Italy) at 23 ± 1°C for 20 days; then, 300 mg of fungal mycelium was scraped from the plates and inoculated into 250-ml flasks containing 100 ml of potato dextrose broth. These were incubated for 7 days in the dark at 25 ± 1°C in an orbital shaker at 150 rpm. The mycelia were pelleted by centrifugation at 2,500 × *g* for 10 min, rinsed with sterile distilled water, resuspended in a petri dish with lysis buffer (15 ml of 1.4 M $MgSO_4$ and 10 mM Na_2HPO_4, pH 5.8) containing lysozyme (L7651; Lysozyme from chicken egg white) (Sigma-Aldrich, St. Louis) at 10 mg/ml, and incubated at 30 ± 0.5°C for 4 h. The enzyme-protoplast solution was then filtered through a nylon membrane (50 µm). Aliquots of 5 ml were placed in centrifuge tubes and overlaid with 2 ml of 1 M sorbitol and 50 mM Tris-HCl, pH 8.0. After centrifugation at 800 × *g* for 5 min, the protoplasts were collected from the interface and resuspended in 1.2 M sorbitol, 50 mM $CaCl_2$, and 50 mM Tris-HCl, pH 8.0. The viability and quantity of the protoplasts were assessed using the fluorescein diacetate staining method (47) and observed in a hemocytometer chamber using epifluorescent Nikon Diaphot TMD inverted microscope (Nikon, Tokyo), equipped with a blue filter block B-2A (excitation filter, EX450-490; barrier filter, BA 520) (Nikon).

The pCT74 plasmid vector was kindly provided by Prof. L. Ciuffetti (Oregon State University) and contained the *sgfp* driven by the *ToxA* gene promoter of *Pyrenophora tritici-repentis* and the hygromycin B phosphotransferase (*hph*) gene, which can induce hygromycin B resistance (24). This vector was used to transform the fungus using a polyethylene glycol (PEG)-mediated system.

In all, ≈10 µg (1 µg/µl) of plasmid DNA was added to 200 µl of 1.2 M sorbitol, 50 mM $CaCl_2$, and 50 mM Tris-HCl, pH 8.0, containing ≈10^8 protoplasts that showed 80 to 90% viability, with 10 µl of 20 mM aurintricarboxylic acid (Sigma-Aldrich), 5 µl of 50 mM spermidine (Sigma-Aldrich), and 100 µg of heparin (Sigma-Aldrich) added to improve the PEG-mediated transformation (16). After an incubation of 30 min on ice and the addition of 60% PEG 6000 in 1.2 M sorbitol, 50 mM $CaCl_2$, and 50 mM Tris-HCl, pH 8.0, in three successive steps of 250, 250, and 850 µl, the mixture was kept for 30 min at 25 ± 1°C and plated on PDA supplemented with 0.8 M sucrose. The cultures were incubated for 7 days at 28°C. Experiments were repeated three times.

Fungal tip colonies grown on PDA supplemented with 0.8 M sucrose were transferred onto PDA amended with hygromycin B at 100 mg/liter (PDAhyg) for 20 days. The stability of the transformants was tested by subculturing three times on a nonselective medium (PDA without the antibiotic) and then checking again for their ability to grow on PDAhyg. To evaluate the stability of the *sgfp* gene insertion into the fungal genome, preliminary analyses were performed for the emission of fluorescence from mycelium of sGFP-transformed colonies, using an epifluorescent Nikon Diaphot TMD inverted microscope (Nikon) equipped with a blue filter block B-2A (Nikon). To confirm the presence of both the *sgfp* and *hph* genes, PCR was performed on DNA from putative transformants and the CBS 229.95 wild-type strain using gene-specific primers (Table 1). Genomic DNA was isolated from the mycelia of the *Pch*-sGFP lines using a previously published cetyltrimethylammonium-bromide (CTAB)-based DNA extraction procedure (31), with minor modifications. Briefly, 100 mg of mycelium, ground in liquid nitrogen, was suspended in 1 ml of extraction buffer (100 mM Tris-HCl [pH 8.0], 1.4 M NaCl, 20 mM EDTA, 2% CTAB, and 0.4% β-mercaptoethanol) and incubated at 65°C for 20 min. The samples were extracted with an equal volume of chloroform/octanol (24:1) and centrifuged at 7,500 × *g* for 10 min. The supernatants were transferred to fresh tubes and nucleic acids were precipitated by adding 0.6 volume of isopropanol. After centrifugation (13,000 × *g* for 15 min), the supernatants were discarded and the DNA pellets were rinsed with 70% ethanol, and resuspended in Tris-EDTA buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0). PCR conditions for detection of *sgfp* and *hph* comprised one cycle at 95°C for 8 min; followed by 35 cycles at 95°C for 50 s, 55°C for 30 s, and 72°C for 50 s; and a supplementary extension of 5 min at 72°C. The *P. chlamydospora* species-specific primer $OPA13_{844}$ (1,35) was used to confirm the identity of the transformants. All the PCR reactions contained 10 µl of 2× Taq PCR Master Mix kit (Qiagen, Hilden, Germany), 1 µM each primer, and 50 ng of total DNA. The transformants were subcultured five more times on PDAhyg. After this selection step, nine transformant colonies that showed similar morphological growth and features compared with wild-type *P. chlamydospora* were selected and characterized by quantitative (q)PCR and for sGFP expression using a Nikon P101 microscopy photometer (Nikon).

***Pch*-sGFP lines characterized by qPCR molecular analysis of gene integration.** Insertion of the *sgfp* and *hph* genes into the fungal genome was checked by qPCR using SYBR Green technology. The relative copy numbers of the *sgfp* and *hph* target genes were calculated using the comparative quantification ΔCq method (23). As a reference, the *β-tubulin* and *elongation factor-1α* (*EF1-α*) genes were used (Table 1), because they have been previously described in other filamentous fungi as single-copy regions within the genome (25,33,38). All of the primers were selected using the Primer3 software (36).

The *sgfp* and *hph* gene copy numbers were determined according to the equation $10^{-(\text{Cq reference} - \text{Cq target})/\text{slope})}$, where *Cq* is the quantification cycle and reflects the cycle number at which the fluorescence generated within the reaction of each sample crosses the threshold. The transgene targets were the *sgfp* and *hph* genes, the references were the *β-tubulin* and *EF1-α* genes, and the slope was calculated according to the standard curves, which were

TABLE 1. Primer sequences, reference accession numbers (National Center for Biotechnology Information [NCBI]), and polymerase chain reaction product sizes for confirming the presence and copy number of each gene in *Phaeomoniella chlamydospora*

Gene	NCBI accession number	Primer sequence (5′→3′)	TM (°C)[w]	Amplicon (bp)
sgfp[x]	GU045599.1	TATATCATGGCCGACAAGCA	60.5	219
	…	GAACTCCAGCAGGACCATGT	60.0	…
hph[y]	AY142483.1	ATCTTAGCCAGACGAGCG	59.0	360
	…	GTCTGCTGCTCCATACAAGC	60.5	…
β-tubulin	AF253968.1	AACCGGCCAATGTGTAAGTC	60.2	156
	…	TGAGCATCGTTCTTGTCTGG	59.8	…
EF1-α[z]	EU158825.1	CTCAAAACGACCCTTGCTTC	60.5	165
	…	GCCTTGAGCTTGTCCAAAAC	59.4	…

[w] Predicted melting temperature.
[x] Synthetic green fluorescent protein.
[y] Hygromycin B phosphotransferase.
[z] Elongation factor-1α.

specific for each gene (Supplementary Figure S1). Standard curves for the target and reference genes were obtained from the *Pch*-sGFP51 transformant, because this transformant showed the highest *Cq* value according to our preliminary qPCR analysis, using 10-fold serial dilutions of 10 to 10^{-2} ng. The standard curve efficiencies were 100.2, 101.5, 99.3, and 101.7% for the *sgfp*, *hph*, *β-tubulin*, and *EF1-α* genes, respectively, with slopes of –3.318, –3.286, –3.340, and –3.283, respectively. The high efficiency for each gene allowed the assumption that the genes are amplified with the same efficiency, and an average slope of –3.306 was used in the equation. DNA from the *Pch*-sGFP lines and from *P. chlamydospora* wild-type (negative control) was quantified with the VersaFluor Fluorometer system (Bio-Rad, Hercules, CA). The study was carried out in an iCycler iQ Multicolor Real-Time PCR Detection system (Bio-Rad). The reaction mixture was prepared in a final volume of 22 µl, including 11 µl of 2× SYBR Green I (Bio-Rad), 1 µl of forward and reverse primers (0.1 µM each), and 9 µl of diluted DNA (1.8 ng for each sample). The following thermal cycling conditions were used: one cycle at 95°C for 8 min followed by 40 cycles at 95°C for 40 s, 55°C 30 s, and 72°C for 40 s. Fluorescence was read during the annealing step or during the extension at 72°C. The specificity of the amplicons for all of the PCR reactions was verified by melting curves. These cycles were performed at temperature increases of 0.05°C/s, from 55 to 95°C. All of the genes were assayed in the same plate, with each in duplicate, and the experiments were repeated three times.

In vivo fluorescence emission of the *Pch*-sGFP lines. The fluorescence intensity of the *Pch*-sGFP mycelia was measured. Three replicates of selected *Pch*-sGFP lines were cultured for 10 days in PDAhyg media. The *P. chlamydospora* strain CBS 229.95 was included as a control. Mycelia (200 mg) was collected and resuspended in 1.0 ml of sterile distilled water. The suspensions obtained (three from each *Pch*-sGFP line) were placed in 30-mm-diameter petri dishes and their fluorescence emission was examined using a Nikon System P101S photometer linked to an epifluorescent Nikon Diaphot TMD inverted microscope (Nikon), equipped with a blue filter block B-2A (Nikon) and a Nikon ×20/5.0 NA (objective magnification/numerical aperture field diaphragm). Thirty single measurements were taken for each subculture replication. The exposure time for each measurement was <2 s. The fluorescence background was calculated according to the *P. chlamydospora* CBS 229.95 wild-type used as the control. Experiments were repeated twice.

Colonization of *Pch*-sGFP71 in *Vitis* spp. In winter 2007, cuttings from *Vitis vinifera* ('Montepulciano', 'Verdicchio', 'Sangiovese', 'Biancame', and 'Cabernet Sauvignon') and the rootstocks 'Kober 5BB' (*V. berlandieri* × *V. riparia*), 'SO4' (*V. berlandieri* × *V. riparia*), '420A' (*V. berlandieri* × *V. riparia*), '1103P' (*V. berlandieri* × *V. rupestris*), and *V. rupestris* were collected from a mother plant vineyard located in Ascoli Piceno, in the Marche region (central-eastern Italy). The sanitary status of mother plants was monitored by visual inspections twice in the year and assessed by immuno-enzymatic and molecular tools for the detection of the main grapevine viruses and fungi involved in Esca complex, respectively.

The *Pch*-sGFP71 line was grown on PDAhyg dishes for 2 weeks. Conidia and mycelia were collected by rinsing each petri dish and gently scraping the colony surface with a glass rod in the presence of 10 ml of 0.1% Triton X-100. The suspensions were filtered through sterile double-layer cheesecloth. The concentrations of the suspension were adjusted to 1×10^7 CFU/ml using a spectrophotometer reading at a 425-nm wavelength (= 1 = 1×10^8 CFU/ml) adapted from Romanazzi et al. (34). With the aim to reproduce nursery storage conditions of the cuttings, two different incubation temperatures at 4 ± 1 and 25 ± 1°C were tested for both inoculated cultivars and rootstocks. For each incubation temperature, four groups of 10 70-cm-long cuttings were prepared, and their bottom ends were aligned. Three of these groups were inoculated with the *Pch*-sGFP71 conidial suspension and one group with water (control) for 12 h (≤5 cm from the bottom of the cuttings). After inoculation, the bottom section of each cutting was covered with cotton soaked in water, enclosed in plastic bags, and stored for 6 months.

After incubation for 6 months at 4 and 25 ± 1°C, 20 30-µm-thick transverse sections were cut from each sample, using a cryo-microtome (HM505E microtome; Microm Laborgeräte, Walldorf, Germany). These were selected from along the stem (two sections for each 5 cm) and analyzed using an epifluorescent Diaphot TMD inverted microscope, with a blue filter block B-2A (Nikon). Thirty cuttings were analyzed per group, and the colonization levels were expressed as centimeters from the bottom of each cutting, according to the stem fragments that showed green fluorescence. In order to correlate the presence of the *Pch*-sGFP71 with the epifluorescence signal, *P. chlamydospora* and s*gfp* molecular detection was carried out by PCR, using specific primer pairs as previously described, on 30 randomly selected sections, 20 from portions of inoculated cuttings showing brilliant green fluorescence and 10 from portions of inoculated cuttings showing orange-yellow fluorescence, the typical color observed on control cutting tissue by an epifluorescent microscope. Moreover, to confirm the stability of *Pch*-sGFP71 line, the fungus was reisolated on PDA from 30 inoculated cutting sections showing green fluorescence by incubation in a humid chamber at 25 ± 1°C for 48 h, and observed under epifluorescent microscopy (Nikon).

Statistical analysis. Data collected from molecular analysis of *sgfp* and *hph P. chlamydospora* gene integration, in vivo fluorescence of the *Pch*-sGFP lines, and extent of colonization of Pch-sGFP71 in *Vitis* spp. were statistically evaluated for means comparison, and significant differences were separated using Duncan's multiple range tests ($P \le 0.05$) after analysis of variance. sGFP expression and the *sgfp* DNA copy numbers, which were calculated in *Pch*-sGFP lines as previously described, were correlated using Pearson linear correlation ($P \le 0.05$). Pearson correlation coefficients (r) and probabilities (P) of the significance test for correlation were calculated for the *β-tubulin* and *EF1-α* references genes.

RESULTS

Protoplast isolation and transformation efficiency. The *P. chlamydospora* isolate CBS 229.95 generated a high protoplast yield and was efficiently transformed using this PEG-mediated method and the pCT74 plasmid containing both the *sgfp* and *hph* genes. A mean of 6 to 8×10^6 protoplasts/ml was generated from 300 mg of mycelium. Based on vital staining with fluorescein diacetate, the viability of the *P. chlamydospora* protoplasts was 85 to 95%. The transformation experiments yielded ≈60 individual hygromycin-resistant transformants after the first subculture in PDAhyg. After three successive subcultures on PDA and one on PDAhyg, there were 24 (40%) stably transformed fungal colonies. The stability of these transformants was evaluated after five subsequent subcultures on PDAhyg. All of the *Pch*-sGFP transformants continued to express sGFP after 12 months of consecutive subculturing on PDA. From this group, nine *Pch*-sGFP lines were selected and characterized.

Molecular characterization of *Pch*-sGFP lines. PCR analysis confirmed the presence of both the *sgfp* and *hph* genes in all of the 24 transformants. No *sgfp*- and *hph*-specific amplicons were obtained in the wild-type *P. chlamydospora* used as the control. PCR with species-specific *P. chlamydospora* primers confirmed the species identity (data not shown).

The qPCR analysis was performed to determine the *sgfp* and *hph* copy numbers in each of the nine selected *P. chlamydospora* transformants and showed the same results using *β-tubulin* or *EF1-α* as the reference normalizing genes (Table 2). Although

equal amounts of genomic DNA were analyzed, different *sgfp* and *hph* relative copy numbers were observed across these different *P. chlamydospora* lines, which indicates that the integration of the construct occurred at multiple sites. The multiple-copy integration seen for *sgfp* and *hph* was from twofold to sixfold the reference genes. By comparing the results for each line, seven of nine *Pch*-sGFP lines showed the same number of integrated copies of *sgfp* and *hph*, while differences were seen for the *Pch*-sGFP62 line (six copies of *sgfp* and four copies of *hph*, compared with the reference genes) and for the *Pch*-sGFP11 line (four copies of *sgfp* and three copies of *hph*, compared with the reference genes).

***Pch*-sGFP line fluorescence intensities.** Epifluorescence microscopy analysis of all of the selected *Pch*-sGFP expressing lines showed uniform bright green fluorescence in the growing hyphal tips. Fluorescence measurements of mycelial plugs of selected *Pch*-sGFP lines showed higher fluorescence values with respect to the fluorescence background detected with *P. chlamydospora* wild-type ($P \leq 0.05$), which ranged from 23.5-fold (*Pch*-sGFP32) to 49.6-fold (*Pch*-sGFP71). However, significant differences in the fluorescence intensities were observed across the *Pch*-sGFP lines. The lowest sGFP fluorescence intensity values were recorded for *Pch*-sGFP81, *Pch*-sGFP82, and *Pch*-sGFP32 (<200), while the highest value was seen for *Pch*-sGFP71 (>350) (Fig. 1). Moreover, *Pch*-sGFP71 showed the same copy numbers of *sgfp* and *hph* genes (Table 2).

A low relationship between sGFP fluorescence expression and *sgfp* gene copy was observed in the *Pch*-sGFP lines analyzed (Fig. 2). The Pearson's correlation coefficients were $r = 0.41$, $P = 0.27$ using the *β-tubulin* reference gene and $r = 0.45$, $P = 0.22$ based on the *EF1-α* gene ($P \leq 0.05$).

Colonization of *Pch*-sGFP71 in *Vitis* spp. The *Pch*-sGFP71 line, showing both the highest sGFP fluorescence emission and the same copy number of *sgfp* and *hph* genes, was selected in our study on *Vitis* spp. colonization. Cultivars and rootstocks inoculated with *Pch*-sGFP71 showed wide black discoloration along the bases of the cuttings. However, the wood disease symptoms were not indicative of *P. chlamydospora* colonization, because sGFP-specific green fluorescence was observed in symptomless wood sections from inoculated cuttings of the cultivars and rootstocks. These symptomless sections from inoculated cuttings that showed green fluorescence were analyzed using the *P. chlamydospora* and *sgfp*-specific primers, and *Pch*-sGFP71 was detected by PCR (data not shown).

Direct epifluorescence microscopy analysis of sections obtained from inoculated cuttings showed different extents of *P. chlamydospora* colonization and it was affected by incubation temperature. A higher extent of growth was observed in cultivar and rootstock cuttings incubated at 25 ± 1°C after 6 months of incubation, compared with the cuttings incubated at 4 ± 1°C for the same period. Montepulciano and Verdicchio showed the highest fungal colonization at 31.8 and 30.2 cm from the bottom of the cuttings, respectively. The lowest levels of colonization were in Cabernet Sauvignon (12.0 cm) and the *V. rupestris* (10.4 cm) and SO4 (8.0 cm) rootstocks. The other cultivars (Sangiovese and Biancame) and rootstocks (Kober 5BB, 420A, and 1103P) analyzed showed intermediate *Pch*-sGFP colonization (Fig. 3). On the other hand, at 4 ± 1°C, all of the analyzed cultivars and rootstocks showed reduced *Pch*-sGFP colonization that was localized only in the basal section (data not shown). However, fungal viability was preserved, as demonstrated by the development of the mycelium on the cutting section from canes stored at 4 ± 1°C for 6 months and then incubated at 25 ± 1°C for 72 and 96 h (Fig. 4A and B).

After 6 months, in the inoculated cuttings stored at 25 ± 1°C, the epifluorescent microscopy revealed brilliant green fluorescence in the xylem tissue (Fig. 4C to F), while a uniform yellow-brown coloration in control samples was observed (Fig. 4G, 4H). Analysis of the transverse sections showed the *P. chlamydospora*-specific green fluorescence in the secondary xylem. In particular,

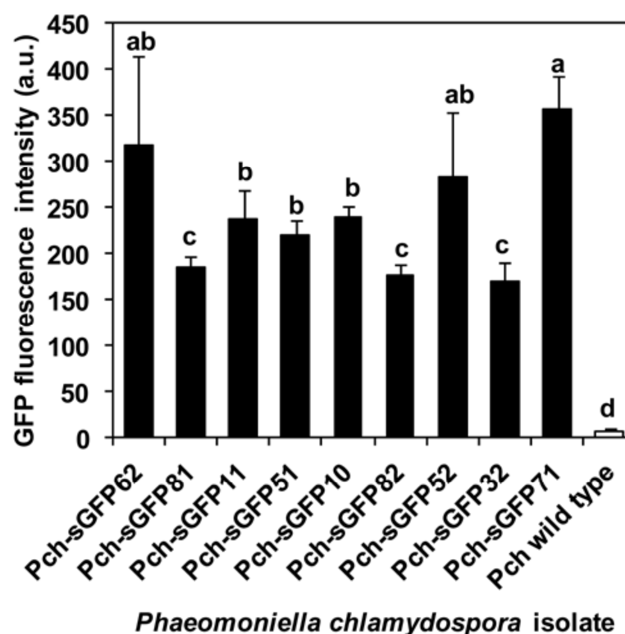

Fig. 1. Intensity of synthetic green fluorescent protein (sGFP) fluorescence (expressed as arbitrary units [a.u.]) of the mycelia of the nine *Phaeomoniella chlamydospora* (*Pch*)-sGFP lines and *P. chlamydospora* wild-type (as indicated). Data are means (± standard deviation) of 30 single measurements for each of three subcultured *Pch*-sGFP lines, with each repeated twice per *Pch*-sGFP line. Columns with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$).

TABLE 2. Average quantification cycles (Cq) and relative copy numbers of synthetic green fluorescent protein (*sgfp*) and hygromycin B phosphotransferase (*hph*) genes detected by quantitative polymerase chain reaction analyses and calculated with the ΔCq comparison method[x]

Isolates	*Cq* value				*β-tubulin*[y]		*EF1-α*[z]	
	β-tubulin	*EF1-α*	*sgfp*	*hph*	*sgfp*	*hph*	*sgfp*	*hph*
Pch-sGFP62	18.2 ± 0.05	18.7 ± 0.05	15.6 ± 0.10	16.2 ± 0.05	5.9 a	4.0 b	5.6 a	3.8 b
Pch-sGFP81	16.9 ± 0.05	16.9 ± 0.15	14.9 ± 0.15	15.1 ± 0.15	4.1 a	3.9 a	4.0 a	3.8 a
Pch-sGFP11	19.6 ± 0.10	19.6 ± 0.05	17.6 ± 0.15	17.9 ± 0.15	4.1 a	3.3 b	4.0 a	3.2 b
Pch-sGFP51	16.4 ± 0.20	16.4 ± 0.10	15.3 ± 0.05	15.4 ± 0.25	2.2 a	2.2 a	2.2 a	2.1 a
Pch-sGFP10	17.6 ± 0.05	17.5 ± 0.05	15.5 ± 0.10	15.5 ± 0.05	4.3 a	4.1 a	4.0 a	3.8 a
Pch-sGFP82	17.6 ± 0.20	17.5 ± 0.10	15.5 ± 0.15	15.6 ± 0.05	4.4 a	4.1 a	4.2 a	4.0 a
Pch-sGFP52	17.8 ± 0.05	17.9 ± 0.10	16.3 ± 0.05	16.3 ± 0.10	3.0 a	2.9 a	3.2 a	3.0 a
Pch-sGFP32	16.2 ± 0.05	16.2 ± 0.15	15.1 ± 0.10	15.2 ± 0.10	2.3 a	2.1 a	2.2 a	2.0 a
Pch-sGFP71	17.5 ± 0.05	17.4 ± 0.05	15.5 ± 0.05	15.4 ± 0.15	4.1 a	4.1 a	4.0 a	4.0 a

[x] Data reported relative to β-*tubulin* and elongation factor 1-α (*EF1-α*) genes were used as references. Means in each column followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).
[y] Gene copies with *β-tubulin* as calibrator.
[z] Gene copies with *EF1-α* as calibrator.

Pch-sGFP71 colonized tissues around vessels, including the xylem fibers and the paratracheal parenchyma cells surrounding the vessels. Moreover, it was noted that there were complete or partial occlusions of the vessel lumens by compounds characterized by an orange coloration, which was also observed in the axial parenchyma. In contrast, the vessel lumens of nonfluorescent areas were usually empty (Fig. 4C and D). Analysis of the longitudinal sections confirmed the Pch-sGFP71 colonization (Fig. 4E and F), and the presence of compounds which were responsible for the occlusion of the vessel lumens was detected near the vessel according to the intense green fluorescence (Fig. 4F). No specific green fluorescence was observed into the vessels of the longitudinal sections (Fig. 4F).

The molecular analysis performed on selected sections from inoculated cuttings revealed the presence of the *sgfp* and *P. chlamydospora* genes in all of the sections showing a brilliant green fluorescence, while none were detected in sections without specific green fluorescence (data not shown).

DISCUSSION

Fluorescent reporter proteins are useful tools for plant–microbe interaction studies (13,45). In the present study, efficient PEG-mediated transformation of protoplasts from hyphae with an sGFP vital marker gene was developed for *P. chlamydospora*, one of the fungal species involved in the grape Esca disease complex.

The selected transformant *Pch*-sGFP71 line was used to analyze in situ *P. chlamydospora* spread in grapevine cultivars and rootstock tissues. To select a stable *Pch*-sGFP line, the transformants were characterized for *sgfp* and *hph* genome insertion and sGFP fluorescence emission using qPCR and fluorimetric systems, respectively. It is worth noting that there was low correlation between the relative *sgfp* copy numbers and the sGFP fluorescence expressed across the nine *Pch*-sGFP lines selected. Thus, the levels of expression of sGFP in each transformant appeared to be determined not only by gene copy number but also by different factors, including genomic location and arrangements of the integrate genes (29,37). Transgene copy numbers and sGFP expression levels can be positively correlated or not correlated, as has been reported previously (18,33).

Using sGFP-transformed *P. chlamydospora*, we proposed a new strategy for the detection of this fungal species in inoculated grapevine cuttings. Unlike nucleic acid-based approaches, using sGFP-expressing *P. chlamydospora* provides topological information on the spread of the fungus within the grapevine plant. Moreover, we were able to detect the presence of *P. chlamydospora* before the expression of any internal Esca disease symptoms (dark streaking) on the cuttings. Therefore, our approach represents an efficient way for analyzing grapevine wood colonization in vivo. Moreover, the GFP technology applied to *P. chlamydospora* revealed that fungal presence was limited to the wood tissues in all of the cultivars and rootstocks analyzed. This is evidence that the sGFP-transformed *P. chlamydospora* maintained its pathogenicity and was able to express the sGFP protein in the hyphae of the fungus both in vitro and in vivo.

In our study, the specific green fluorescence was particularly concentrated around the vessels while no green fluorescence was detected in the vessel lumens. The vessel lumens inside the areas infected by *Pch*-sGFP71 line showed typical occlusion, which was visible under the microscope as an orange coloration. The production of these compounds, the source of which appears to be axial parenchyma cells, and as also demonstrated by Sun et al. (39), was observed only in areas showing brilliant green fluorescence. It is known that this pathogen induces the production of tyloses or gels that can result in the occlusion of the vessels (43). In particular, these substances might be related to the phytotoxic pullulans (exopolysaccharides) (3,8) or phenolic compounds (2,20,21,27,42,43) that can be produced in response to infection. In vascular diseases, tyloses and other occluding materials (gels or gums) have generally been considered to be active defense mechanisms that can limit the growth of pathogenic microorganisms (11). On the other hand, previous studies have suggested that the principal role of *P. chlamydospora* in the early stages of the disease could be to reduce the plant resistance through its toxic activity (2,8,20,32), thus opening the way to other fungi that are more strictly related to enzymatic wood degradation (44).

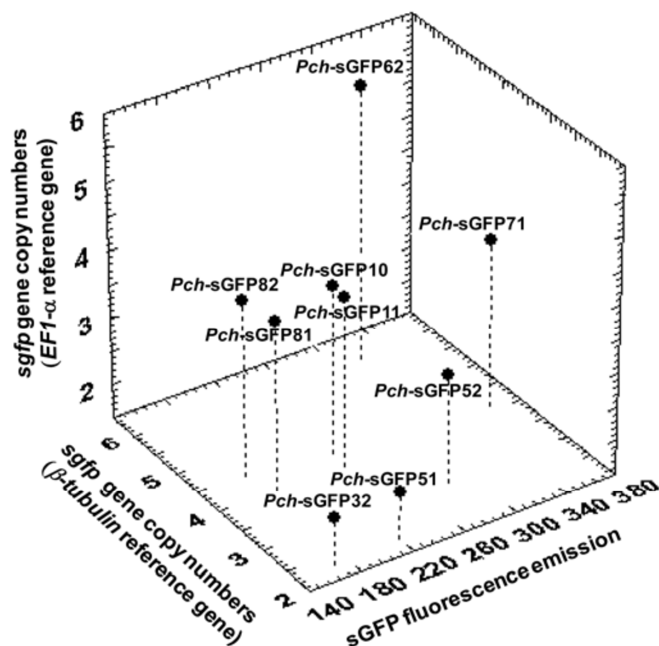

Fig. 2. Relationships between the number of synthetic green fluorescent protein (*sgfp*) gene copies in the transgenic *Phaeomoniella chlamydospora* (*Pch*)-sGFP lines, calculated using quantitative polymerase chain reaction and relative to the *β-tubulin* reference gene, the *elongation factor 1-α* (*EF1-α*) reference gene, and sGFP expression as measured by fluorescence microscopy. Data were correlated using the Pearson linear correlation method ($P \leq 0.05$).

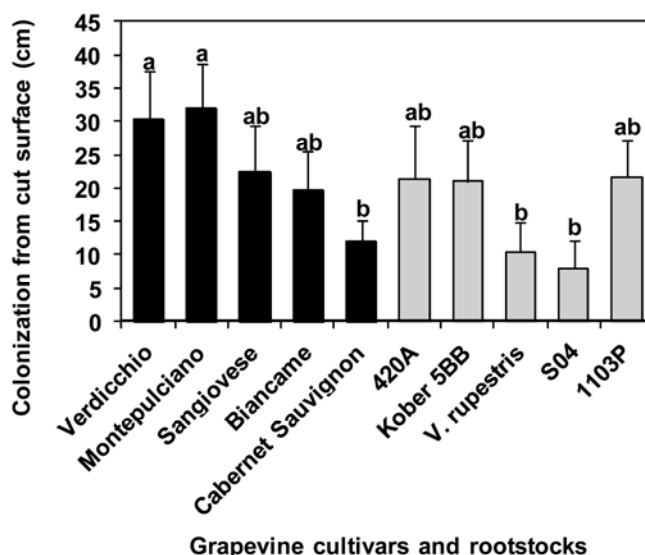

Fig. 3. *Phaeomoniella chlamydospora* synthetic green fluorescent protein (*Pch*-sGFP)71 colonization observed with inoculated cuttings of grapevine cultivars (*Vitis* spp.) and rootstocks (as indicated) incubated at 25 ± 1°C. Data are means (± standard deviation) of 30 different cuttings for each cultivar and rootstock. Values with different letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

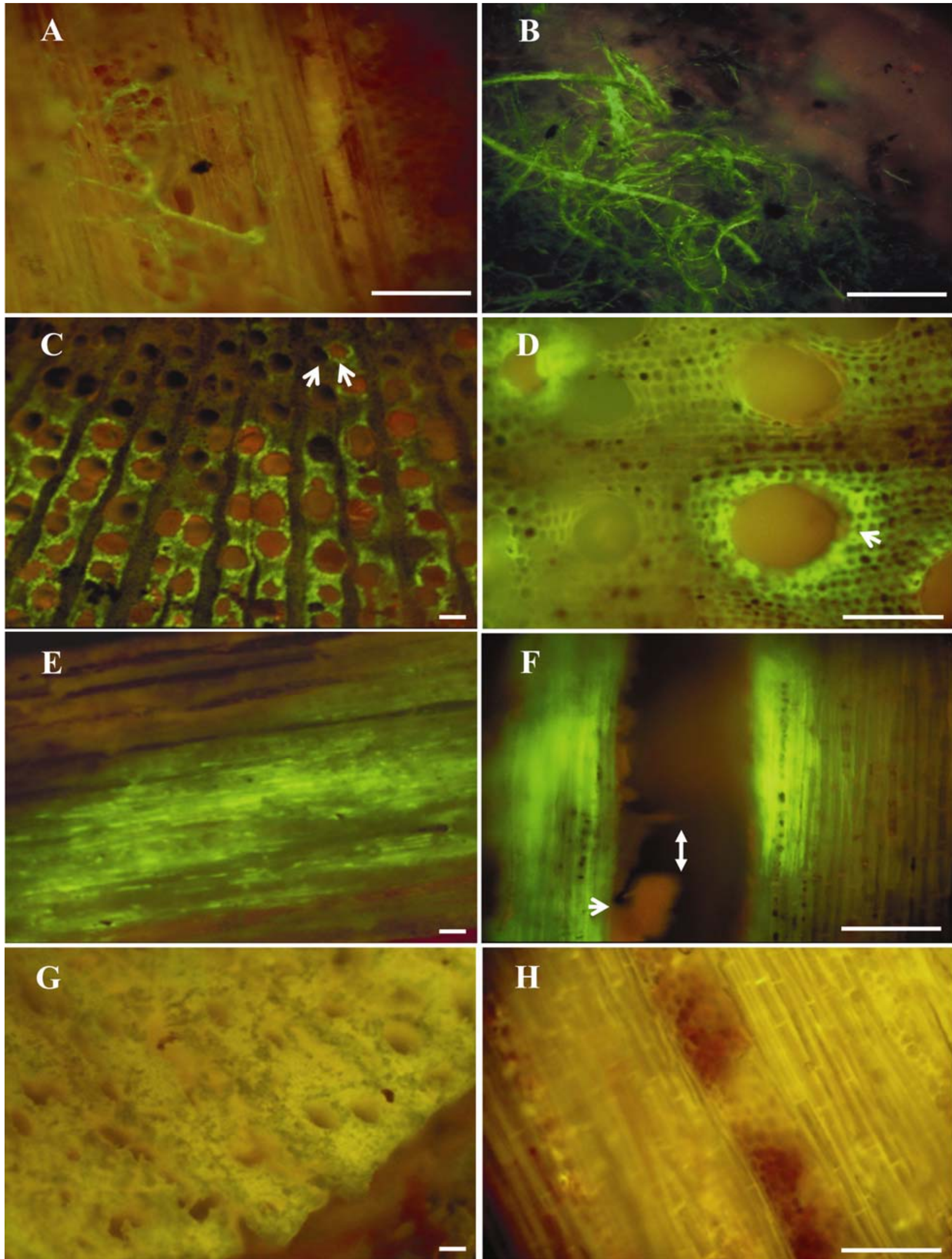

Fig. 4. Green fluorescence detection using an epifluorescent microscope (Diaphot TMD inverted microscope by blue filter block B-2A ex 450 to 490 em BA520) (Nikon, Tokyo) of the transgenic *Phaeomoniella chlamydospora* synthetic green fluorescent protein (*Pch*-sGFP)71 line in inoculated grapevine cuttings. Proliferation of *Pch*-sGFP71 mycelia obtained from the basal section of inoculated 'Sangiovese' grape cuttings kept for 6 months at 4 ± 1°C and then incubated at 25 ± 1°C for **A,** 72 h and **B,** 96 h, respectively. **C to F,** Sections from Sangiovese grape cuttings inoculated with the *Pch*-sGFP71 line and observed after incubation at 25 ± 1°C for 6 months show an intense sGFP green fluorescence in xylem area. **C,** Transverse section of inoculated cuttings showing obstructed vessels (orange) surrounded by green fluorescence, while other vessels without green fluorescence are empty (see arrows). **D,** sGFP green fluorescence localized around vessels in the parenchymal cells surrounding xylem fibers (arrow). **E,** Longitudinal section showing *Pch*-sGFP71 colonization of the wood. **F,** Longitudinal section showing the presence of orange material in xylem vessel surrounded by *Pch*-sGFP71 colonization. No sGFP expression was observed into the vessels of the longitudinal sections (arrows). Control = **G,** transverse section and **H,** longitudinal section of Sangiovese grape noninoculated cuttings. Scale bars = 100 µm.

In the present study, the *Pch*-sGFP71 line colonized the wood tissues and, after 6 months at 25 ± 1°C, different rates of colonization were seen across these cultivars and rootstocks of *Vitis* spp. that have considerable commercial interest. In particular, Verdicchio and Montepulciano showed the highest values for *Pch*-sGFP71 fungal invasion, while the lowest value was recorded for Cabernet Sauvignon. In the literature, however, Cabernet Sauvignon is reported as one of the most sensitive varieties, based on Esca foliar symptoms (6). As expected, it is not always possible to correlate Esca sensitivity based on wood colonization and foliar symptoms, whose severity depends on different factors (microorganisms, climate conditions, cultivar susceptibilities, vineyard characteristics, and rootstock) (26,35,40). The differences observed in the extent of colonization might depend on several factors. Among these are the differential abilities of the pathogen to colonize both cultivar or rootstock, and the variations in the structural and chemical characteristics of the host tissue. Some studies have reported a lower hydraulic conductance of rootstocks compared with the cultivars (10,41). This can justify the tendency toward an overall lower mean colonization extent observed in the rootstocks compared with the cultivars. Different degrees of *P. chlamydospora* colonization were also observed among grapevine genotypes (14).

A further observation was related to the different *Pch*-sGFP71 development responses observed relative to the incubation temperatures. As expected, despite a complete inhibition of the spread of *Pch*-sGFP71 as seen for the cuttings incubated at 4 ± 1°C, the in vitro reisolation and the development at 25 ± 1°C revealed that low temperatures do not affect the viability of the fungus. For this reason, these data highlight the need to check each step in the production of grapevine-propagating materials because, once they are contaminated, they represent an important carrier of the fungi involved in Esca disease into newly established vineyards, even when the cuttings are cold stored (4,46).

To our knowledge, these are the first data in which sGFP has been used to study *P. chlamydospora* colonization in grapevine cuttings. Thus, this information will help to provide a better understanding of the *P. chlamydospora* colonization levels of grapevine cultivars and rootstocks, with the aim of predicting the possible susceptibilities of plants to Esca disease. However, further trials using a wider range of grapevine cultivars are necessary to confirm these observations. The system also allowed us to detect *P. chlamydospora* plant colonization before the black streaking symptoms are expressed. Furthermore, the use of this sGFP-transformed *P. chlamydospora* has been helpful for the clarification of different aspects associated with the localization of this pathogen and for providing information to design a sanitation program in order to improve the quality of grapevine plant material.

ACKNOWLEDGMENTS

The research study was commissioned from ARSIA-Toscana (Regional Agency for Development and Innovation in Agriculture and Forest) by 14 administrative regions and one autonomous province, and financed with funds provided by the Ministero per le Politiche Agricole e Forestali (Ministry for Agriculture and Forestry Policy, Italy) to implement the inter-Regional Project Grapevine Esca: research and experiment in the nursery and in the field for prevention and cure. We thank G. Surico (University of Florence, Italy) for kindly providing CBS 229.95 isolate of *Phaeomoniella chlamydospora* and F. Faretra (University of Bari, Italy) for kindly providing the primers for the detection of *P. chlamydospora.*

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