Eur J Plant Pathol (2011) 129:567–577
DOI 10.1007/s10658-010-9720-z

# Infection and colonization of strawberry by *Gnomonia fragariae* strain expressing green fluorescent protein

**Inga Moročko-Bičevska · Jamshid Fatehi**

Accepted: 15 November 2010 / Published online: 23 December 2010


**Abstract** *Gnomonia fragariae* is a poorly studied ascomycete, which was recently demonstrated to be a cause of severe root rot and petiole blight of strawberry. The pathogen was genetically transformed with the GFP as a vital marker and hygromycin resistance gene. Several stable transformants were obtained, which did not differ in their phenotype from the wild type isolate. Using one of the GFP-tagged isolates the infection process and colonization of roots and petioles of host plant by the pathogen were studied. Fluorescence microscopy examinations of the inoculated plants at different time points showed that plant infection occurs 24 h after inoculation and intensively continues during first 3 days. The specific penetration sites on epidermal cells and preferences in colonization for certain root and petiole tissues were observed. The pathogen intensively colonized and destroyed cortex of roots and petioles and spread rapidly longitudinally within intercellular spaces. The petioles were colonized by the hyphae, which grew mostly in the intracellular spaces of the cortical cells while in the roots the intracellular growth of hyphae occurred only in the later stages of infection. The fungus was also capable to infect the vascular tissues of petioles although these were not the primary tissues colonized by the pathogen. The mature ascomata were formed on the infected petiole bases several weeks after the inoculation. This study presents a genetic transformation method for *Gnomonia fragariae* and it demonstrates details on infection process and colonization of root, crown and petiole tissues of strawberry by the pathogen.

**Keywords** *Diaporthales* · *Fragaria x ananassa* · Fungi · Pathogenic interactions

I. Moročko-Bičevska · J. Fatehi
MASE Laboratories AB,
Box 148, 751 04 Uppsala, Sweden

I. Moročko-Bičevska
Department of Forest Mycology and Pathology,
Swedish University of Agricultural Sciences,
Box 7026, 750 07 Uppsala, Sweden

*Present Address:*
I. Moročko-Bičevska (✉)
Latvia State Institute of Fruit-Growing,
Graudu str. 1,
LV-3701 Dobele, Latvia
e-mail: Inga.Morocko@lvai.lv

## Introduction

*Gnomonia fragariae* Kleb. is a poorly studied ascomycete belonging to the order *Diaporthales,* with no anamorphic state known and with apparently limited distribution to Europe. The fungus has been found on dead tissues of various species of *Potentilla* and *Fragaria* plants, including cultivated strawberries (Bolay 1972). *Gnomonia fragariae* was originally found and described as a saprophyte living on petioles of dead leaves during the screening for causes of diseased strawberry in Germany (Klebahn 1918). At that time, Klebahn (1918) did not succeed to establish the

pathogenicity of *G. fragariae* on strawberry. Recently the fungus was repeatedly isolated during surveys for possible fungal causes of severe strawberry decline observed in several fields in Latvia and Sweden, and it was proved to be the cause of root rot and petiole blight of strawberry (Morocko et al. 2006). The root system of plants infected with this pathogen is usually poorly developed, with very few lateral roots and covered with irregular shaped, black lesions, which enlarge and may cover entire roots. The growth of diseased plants is severely affected and infection may also result in plant death. Outer leaves collapse due to black rot of petiole bases that progresses upwards, but younger leaves often are bluish green in colour and wilted.

*Diaporthales* includes a number of plant pathogenic fungi, which are mostly considered as facultative and few as systemic pathogens, causing cankers and dieback symptoms (Barr 1978). Parasitic species belonging to the genus *Gnomonia* usually attack aerial parts of the host plants (Barr 1978) and so far besides *G. fragariae* only *G. radicicola* has been described as a root pathogen causing severe root rot on roses (Noordeloos et al. 1989; Amsing 1995). Moreover, the recent studies have demonstrated that genus *Gnomonia* is polyphyletic and species ascribed to *Gnomonia* should be placed in *Sydowielaceae*, the family comprising fungi with diverse biology (Moročko and Fatehi 2007; Rossman et al. 2007; Sogonov et al. 2008).

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been developed and widely used as a reporter gene to study the plant-microbe interactions, to monitor the growth and colonization of the host by microbes and the behaviour of microorganisms within plant tissues (Lorang et al. 2001). The GFP technology has also been used in filamentous fungi and oomycetes to study the various aspects of interactions among host plant, pathogen and biocontrol agents, where it was used alone or in combination with other fluorescent markers (Lorang et al. 2001; Bolwerk et al. 2005; Olivain et al. 2006). The use of such marker has advantage of not requiring staining, cofactors or substrates and the microorganisms tagged with fluorescent markers can be directly monitored and visualized in their complex natural environments. The GFP has been successfully used as a vital marker for broad range of plant pathogens to study both leaf and root infections (Maor et al. 1998; Lorang et al. 2001; Horowitz et al. 2002; Nonomura et al. 2003; van West et al. 2003; Visser et al. 2004).

The aim of the present work was to study the infection process and colonization pattern of the fungal pathogen *G. fragariae* on the strawberry plant host. In this study *G. fragariae* was genetically transformed with the green fluorescent protein (GFP) gene and a GFP tagged mutant capable of infecting host plant similar to the wild type strain was subsequently used for inoculation of strawberry plants and the colonization of host tissues was monitored by using fluorescence microscopy.

## Materials and methods

### Fungal strain and plasmids for transformation

*Gnomonia fragariae* strain UN22 isolated from diseased strawberry roots during collection in Latvia (Morocko et al. 2006) was used in this study. The fungus was maintained on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) slants and as mycelial plugs in sterile distilled water at 4°C.

Two plasmids were separately used for transformation of *G. fragariae*. The plasmid pIGPAPA, containing the hygromycin resistance gene (hph) as a selective marker and the synthetic green fluorescence protein gene (sGFP – TYG) as a reporter marker flanked with the constitutive expression promoter of the isocitrate lyase gene (IL) of *Neurospora crassa*, was kindly provided by B. Gillian Turgeon (Cornell University, USA). The plasmid pCT74, holding hph and sGFP – TYG genes and promoter ToxA from *Pyrenophora tritici-repentis*, was provided by Lynda M. Ciuffetti (Oregon State University, USA). Plasmid propagation was performed in *Escherichia coli*, strain DH5α according to Sambrook and Russell (2001). Purification of plasmid DNA was carried out with QIAGEN Hispeed Plasmid Purification kit (Qiagen Inc., Crawley, UK) as described in the manufacturer's instructions.

### Protoplast preparation and transformation

The DNA-mediated genetic transformation of *G. fragariae* was performed using a polyethylene glycol and $CaCl_2$ procedure according to the protocol of Kistler and Benny (1988) modified by Mes et al. (1999) with some additional modifications.

For protoplast preparation Petri plates containing 25 ml of potato carrot broth (PCB) (Dhingra and Sinclair 1995) were inoculated with 5 mycelial plugs (4 mm diam.) from 9 day-old fungal cultures grown on potato carrot agar (PCA; Dhingra and Sinclair 1995) and plates were incubated stationary at room temperature for 5 days. Then the liquid was decanted and youngest mycelial mats were collected (ca. 2.5 ml) in 50 ml Falcon tubes and washed 3× in 15 ml of 1 M sorbitol solution (1 M sorbitol; 20 mM MES; pH 5.8). The mycelium was digested in 15 ml of 1 M sorbitol solution containing 10 mg $ml^{-1}$ of driselase (Sigma-Aldrich Co., Deissenhofen, Germany, cat. no. D-8037), 1 mg $ml^{-1}$ of chitinase (Sigma-Aldrich Co., cat. no. 2272) and 10 mg $ml^{-1}$ of lysing enzyme (Sigma-Aldrich Co., cat. no. 62815) at 30°C for 3 h 25 min. The protoplasts were separated by filtration through one layer of Miracloth (Calbiochem, San Diego, USA), washed in 1 M sorbitol solution and collected by centrifugation. After centrifugation they were washed 2 times in 4 volumes of 1 M sorbitol, 10 mM $CaCl_2$, 10 mM TrisHCl pH 8.0. Protoplasts were centrifuged and re-suspended in 200 µl of the same buffer at a density ca. $4\times10^4$ $ml^{-1}$. After digestion, protoplasts were kept on ice in all the procedures and all centrifugation steps were carried out with 2000 g at 4°C for 10 min.

Twenty µl of plasmid DNA solution (1.5 µg $\mu l^{-1}$) and 200 µl of protoplast suspension were mixed and incubated on ice for 30 min. Then 1.2 ml of PEG solution (60% *w/v* PEG 6000; 50 mM CaCl2; 10 mM Tris, pH 7.5) was gradually added and the mixture was incubated on ice for another 30 min. Finally, the protoplasts were washed with 2 ml of 0.8 M sorbitol in 0.5 X PCB, collected by centrifugation and re-suspended in 600 µl of the same solution. Aliquots of 100 µl protoplast suspension were spread on PCA medium containing 0.8 M sorbitol, 10 mM Tris-HCl (pH 8.0) and 50 µg $ml^{-1}$ hygromycin B (Gibco BRL, Grand Island, NY, USA). The plates were incubated in darkness at 20°C and checked daily for protoplast regeneration and germination.

## Selection of a stable transformant

The growing colonies on the selective medium were screened under Aristoplan epifluorescence microscope (Leitz, Germany) equipped with Endow GFP Long Pass filter set (470/40 nm excitation filter and 500 nm emission filter; Chroma Technology Corp.) and the colonies expressing GFP were transferred on oatmeal agar (OA; Difco, Detroid, USA) containing 50 µg $ml^{-1}$ hygromycin B. In total 16 transformants were selected and maintained on PDA slants at 4°C for several months. The stability of the transformants for expression of GFP was evaluated by the inoculation on PCA or OA (without antibiotic) for at least seven subcultures and colonies were examined under epifluorescence microscope.

In order to select a stable GFP-tagged strain similar to the wild type strain for plant inoculation, the mycelial plugs (4 mm in diam.) from the edge of actively growing cultures of transformants and wild type strain were transferred onto PDA, PCA and OA with autoclaved birch toothpicks and incubated at 19°C with 12 h daily illumination under black light-blue (Sylwania F15W/BLB-T8, Japan) and cool-white (Osram L 15W/840, Germany) lamps. The strains were evaluated after 1 month of incubation for the growth, colony morphology and ascospore formation and compared with the wild type strain. A detached leaf assay was also performed as described by Morocko et al. (2006) in order to test pathogenicity of the transformants and to obtain re-isolated strains of both GFP-tagged and wild type *G. fragariae* for plant inoculation experiment.

## Inoculum preparation and plant inoculation

Micropropagated strawberry plants of cultivar 'Korona' (MTT, Agrifood Research, Finland) were inoculated by root dipping for 2 h in ascospore suspension ($10^5$ per ml) prepared from 1 month-old cultures of wild type and GFP-tagged strain on OA (Morocko et al. 2006). The control plant roots were dipped in sterile distilled water. In total, 87 plants were used for each treatment. The treated plants were planted in pots containing 300 ml of the sterilized potting soil mixture, consisting 50/50 (*v/v*) of the commercial garden soil (Hasselfors Garden AB, Sweden) and washed building sand. The soil mixture was steam treated at 80°C for 24 h. The pots were randomly arranged in a growth chamber and grown for 8 weeks with a 14 h light regime and temperature range from 17 to 28°C.

### Plant sampling, disease evaluation and microscopic observations

The evaluation of infection development and the final assessment of diseased plants were performed as described in our previous study (Morocko et al. 2006). Plant sampling for microscopic examinations started 24 h after inoculation and continued daily for the first week, every second day in the second week, once per week in the third and fourth weeks, and once per 2 weeks until the 8 week when the final assessment of disease severity was done. Soil around the roots was carefully removed and roots were rinsed in sterile distilled water before they were placed on glass slides, covered with cover glasses and examined under light and epifluorescence microscope. Seven plants were sampled at each time point and 2–4 structural roots (including lateral roots) were examined per plant. Until appearance of obvious symptoms, which occurred 3 weeks after inoculation, the entire root was examined under the microscope. Crowns were also examined under the microscope both in surface and as lengthwise thin sections made by hand using a sharp razor blade. When the disease developed and symptoms appeared, the symptomatic parts of roots, crowns and petioles were selected for microscopic examinations.

## Results

### Transformation of *G. fragariae* with gfp gene and selection of a stable transformant

Sufficient numbers of regenerable protoplasts of *G. fragariae* UN22 were obtained and further used for the genetic transformations with two different vectors pIGPAPA and pCT74, which were used separately. The regenerated transformed protoplasts were germinated and the green fluorescence was detectable 3–4 days after transformation. Transformations with both plasmids were successful, resulting in stable transformants capable to grow on the selective PCA medium. In total, 16 stable transformants expressing GFP were obtained. After 1 month of incubation the colonies of transformants and wild type strain were submerged, grimy brown with distinct irregular margins on PDA, submerged and grey on PCA with toothpicks and brown with more aerial mycelium on OA with toothpicks. Nine transformant strains, which had covered the entire PDA plate, same as the wild type, after 1 month of incubation were selected for further tests. On PCA and OA with toothpicks, these transformants formed mature ascomata and revealed intense green fluorescence in mycelia, ascomata, asci and ascospores. Pathogenicity of selected GFP-tagged strains was confirmed using the detached leaf assay. Clear necrotic and black lesions developed on strawberry leaflets around the mycelial plugs 3 days after inoculation and reached ca. 3 cm diam. within 1 week in all nine GFP-tagged strains and wild type. The fungus was re-isolated from infected tissues. The GFP expression remained stable during subsequent sub-culturing on non-selective agar media and after re-isolation from plant tissues. One of the transformants, named pIG-2, obtained from the transformation with pIGPAPA was selected for further experiments on host plant.

### Early stages of the disease

All plants examined during the first 2 days after inoculation were healthy and no root discolouration was observed. In 24 h after inoculation, ascospores of GFP-tagged *G. fragariae* strain were visualized under fluorescence microscope and they were randomly attached to all the primary and lateral roots examined. The ascospores were predominantly attached to the middle and the younger region of the primary roots, approximately 1–2 cm from root apices. At this stage, the majority of ascospores had germinated and the germination tubes either penetrated the root epidermis, preferentially at the junctions between root epidermis cells, or they continued to elongate and grow on the surface mostly along the cell junctions (Fig. 1a, b, c). Ascospores were mostly germinated from both ends, but in some cases they formed only one germ tube from one cell. The hyphae often became swollen at penetration sites and formed hyphopodia and infection pegs, which enter the root epidermal cells (Fig. 1b, c). Several germinated ascospores were also found intermingled with root hairs on upper parts of roots, but root hairs were not penetrated by the hyphae at this stage.

Over the next 5 days, several plants inoculated with pathogen revealed slightly brown discolouration at root tips and brownish spots on primary roots. Such discolouration was also observed on roots of control plants. The discoloured tissues of about half of the examined roots from inoculated plants were intensively

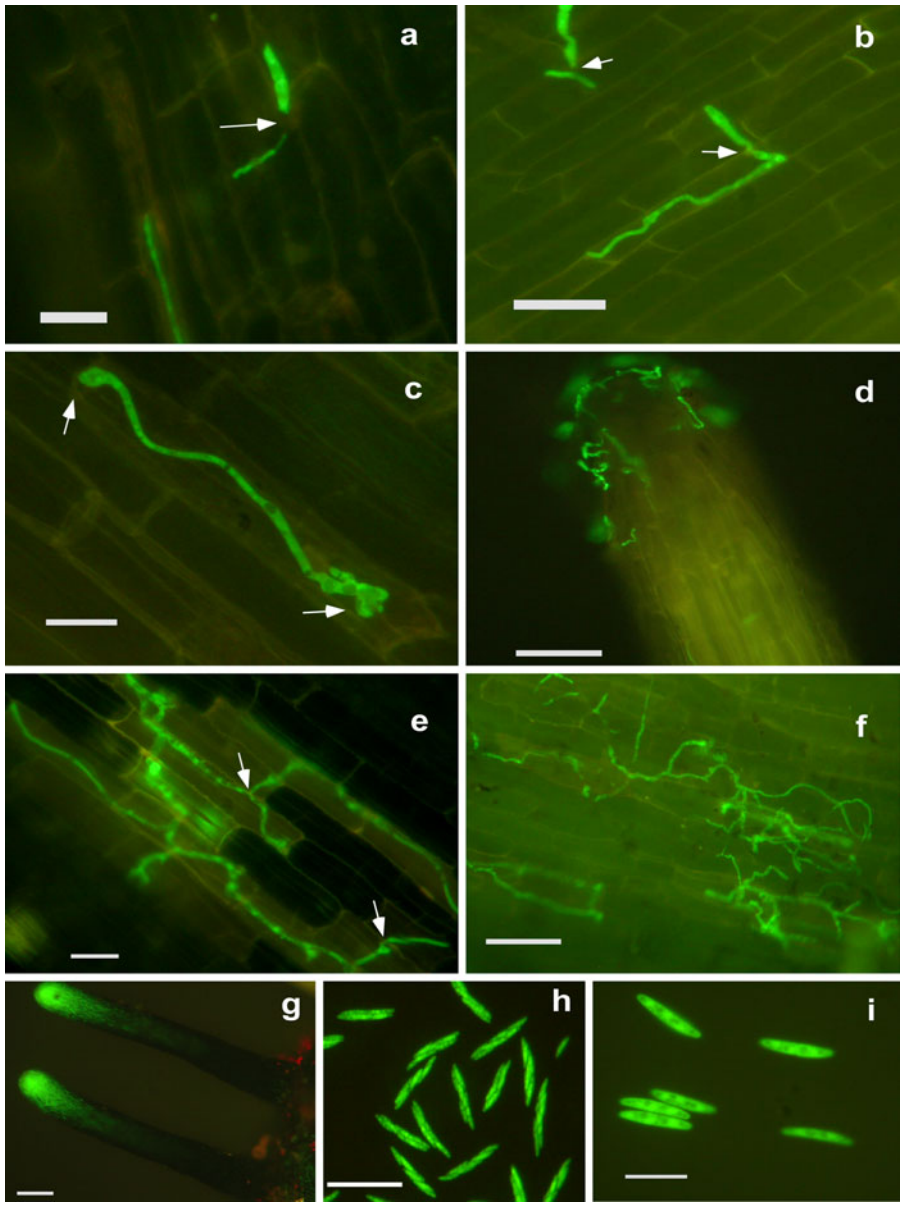


**Fig. 1** Early stages of strawberry root infection by GFP-tagged *Gnomonia fragariae* and perithecia, asci and ascospores of the fungus expressing GFP: **a** Attachment of fungal spore on the root surface and penetration (arrow) 24 h after inoculation. **b** Penetration of germinating spores (arrows) and hyphal growth along the junctions of epidermal cells 48 h after inoculation. **c** Hyphal swellings (arrows) at penetration points on surface of epidermal cell 48 h after inoculation. **d** Germinating ascospores attached and infecting apex of lateral root 72 h after inoculation. **e** Intracellular hyphae swollen at cell walls with constrictions (arrows) in places where they cross the cell walls 4 days after inoculation. **f** Invasive growth of the fungus forming an expanding network within epidermal tissues 5 days after inoculation. **g–i** Perithecia, asci and ascospores of the fungus formed on petiole bases 52 days after inoculation. Bar=20 μm for Figures a–c, e, i; 100 μm for Figures d, f–h

colonized by *G. fragariae* and hyphae penetrated and colonized the necrotic root tips (Fig. 1d).

Three days after inoculation, hyphae were inside the cells and continued to grow longitudinally and laterally in the epidermis of primary roots, and lateral roots close to primary root (Fig. 1e). Hyphae developed further and formed sparse mycelial networks inside the epidermis of roots (Fig. 1f). In general, no extensive and dense hyphal network was formed on the surface of the root system. The non-germinated ascospores were still found to germinate and penetrate the root tissues. The intracellular hyphae swelled on cell walls and became constricted at places where they crossed the cell wall entering to the neighbouring cells (Fig. 1e). After crossing the cell wall normal diameter of hyphae was retained. Ascospores were also found attached and they were germinated at the root hair zone while the penetration and colonization of the root hairs by the hyphae was seldom.

## Advanced stages of the disease

Ten to 12 days after inoculation, the disease symptoms on roots of plants inoculated with both wild type

and GFP transformed strains, became apparent as small, dark brown or black lesions on primary and lateral and as blackened root tips. The development of roots and shoots of inoculated plants was delayed in comparison to the controls. The microscopic examination of the discoloured root segments confirmed that the symptomatic roots were extensively colonized by the fungus (Fig. 2a). The hyphae were found deep inside in the root cortex close to the stele and spreading longitudinally in the cortical tissues. At the places, where hyphae spread from one cell to another, lysing of cell walls was also observed (Fig. 2b). Invasion of vascular tissues was not observed at this stage.

Nineteen days after inoculation more than 50% of strawberry plants, inoculated with both wild type and

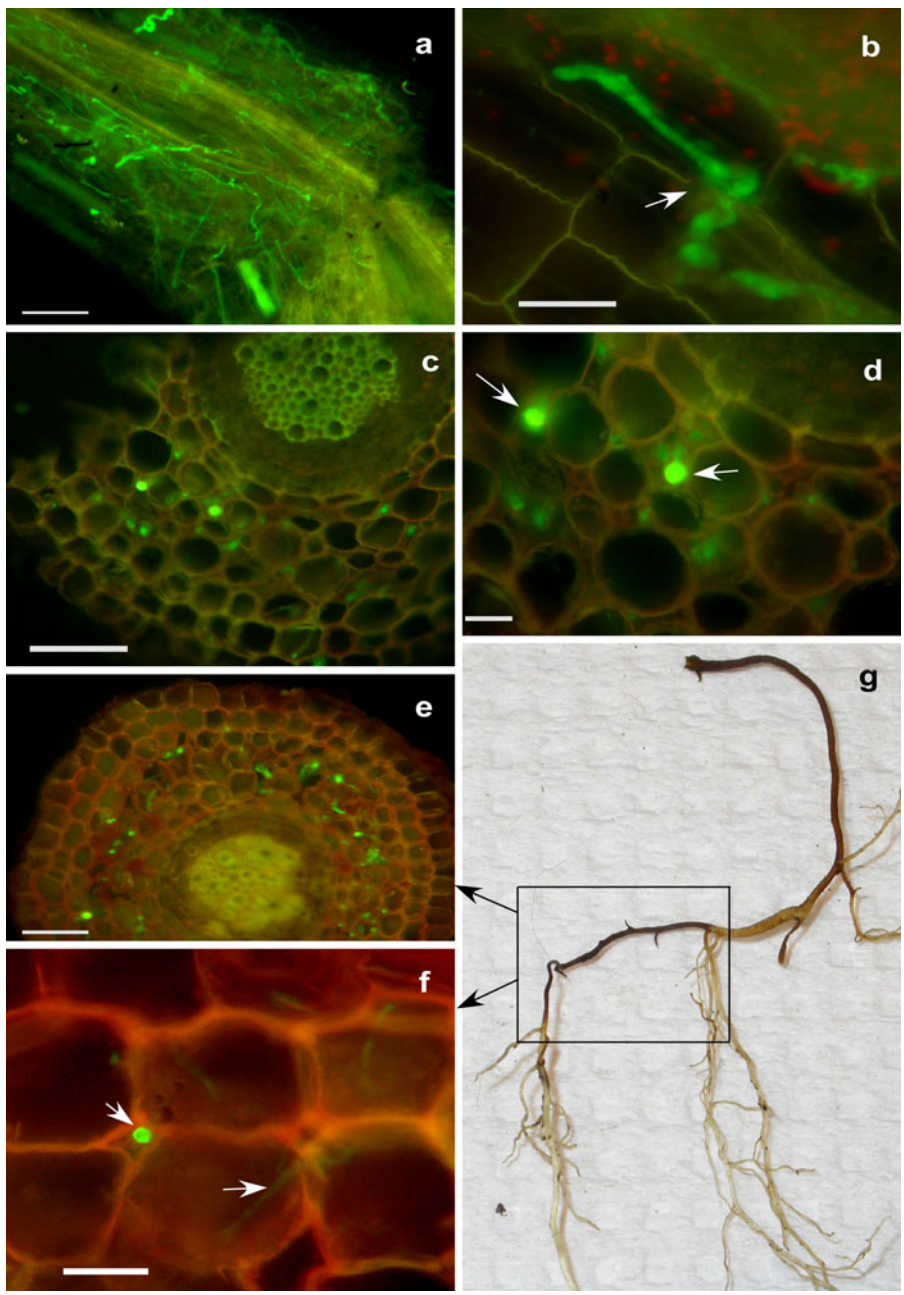


**Fig. 2** Advanced stages of strawberry root infection and colonization by *Gnomonia fragariae* marked with GFP: **a** Extensive colonization of discoloured tissues above the apex of lateral root 12 days after inoculation. **b** Spread of hyphae from one cell to another by disruption of the cell wall (arrow) 12 days after inoculation. **c–d** Intercellular growth of hyphae (arrows) in the cortex, cross-section of the primary root 19 days after inoculation. **e** Mycelium in the cortex, cross-section of necrotic primary root 37 days after inoculation. **f** Intra- and intercellular growth of the hyphae (arrows) in the cortex, cross section of necrotic primary root 37 days after inoculation. **g** Symptoms on strawberry root caused by *Gnomonia fragariae* 37 days after inoculation. Bar=100 μm for Figures a, c, e; 20 μm for Figures b, d, f

GFP tagged strain had obvious and typical disease symptoms on roots and stems. Primary roots, approximately 1 cm from crown downwards, were reddish brown and middle portions of roots were covered with 1–2 cm elongated black lesions, which sometimes girdled the roots and expanded further on lateral roots. Black rot developed upwards on tips of lateral and primary roots. In some cases, even if above ground part was well developed, roots were severely affected and covered with many black lesions. Microscopic observations of root segments taken within and around lesions confirmed that mycelium was present in and around the discoloured areas. Microscopic examinations of cross sections of infected roots revealed that fungal hyphae were present in the cortex between epidermal cell layers and stele. Mycelia grew from the primary roots towards the lateral roots and they were also present in the healthy tissues around the lesions. At this stage of root infection, hyphae were predominantly present in intercellular spaces of the inner cell layers of cortex and they expanded longitudinally in the roots (Fig. 2c, d) while they were seldom found inside the cortical cells. Petiole bases of infected plants, either with wild type or transformant, had black necrotic lesions, 0.5–2 cm from crown upwards. The examination of the longitudinal and cross sections made from these infected petioles with fluorescence microscope showed that cortex tissues between epidermis and vascular tissues were heavily colonized by the pathogen (Fig. 3a). Contrary to root infection pattern, intracellular growth was predominant in cortical tissues of the infected petioles (Fig. 3b) and hyphae were also present in vessels.

The epidermal cells of the invaded petioles were less affected and the fungus was mostly present in the intercellular spaces (Fig. 3b). On lesions, from inside of infected tissues hyphae frequently grew outside through stomata on the petiole surface (Fig. 3i). Longitudinal sections of crowns showed that they were also infected with pathogen. Fungal mycelia were found inside the crown at the places where petioles were attached to the crown and invasive growth continued deeper inside the crown and up into petioles (Fig. 3c, h).

Twenty five days after inoculation more than 80% of remaining plants inoculated with GFP-tagged strain and wild type were affected. Damage on roots and petiole bases had progressed resulting in severe stunting of plants and collapse of outer leaves. At this stage symptomatic tissues were heavily colonized by the pathogen as it was described above, and no apparent changes in pathogen growth were observed.

Thirty seven days after planting, more than 90% of inoculated plants showed disease symptoms including severe stunt, bluish green colour of younger leaves, yellowing and collapse of outer leaves, and black rot on petiole basis. Necrosis on primary roots near the crown and in middle parts had expanded approximately 5 cm in length (Fig. 2g). In cross sections mycelium predominantly was present in root cortex both intra- and intercellular (Fig. 2e, f). At this stage mycelium was not anymore visible on necrotic tips of primary and lateral roots, but the fungus extensively colonized tissues above the necrosis. Bases of infected petioles were heavily affected and necrosis covered at least 2/3 of length of petioles (Fig. 3h). The necrotic lesions of petioles reached the leaf and expanded in main veins of leaflets in several cases (Fig. 3h). Cortex of such severely affected petioles was disrupted and hyphae were present in vascular tissues (Fig. 3j, k). However mycelium was not present in tissues outside the lesions. In the cross sections made from less damaged petiole segments, the mycelia were mostly in the outer layers of the cortex though they were also found farther inside even in the endodermis. (Fig. 3e, f, g). The fluorescence microscopy examinations of longitudinal sections of crown tissues showed that infection of petioles predominantly had started at soil level, where petioles are attached to the crown as it was also observed in earlier stages (Fig. 3c, h). The hyphae developing inside the crown tissues grew mostly along the plant cell walls and followed the shape of the plant cells, and formed swellings where they crossed the cell walls (Fig. 3d). In some cases epidermal layers at the crown bases were also infected and fungus developed deeper inside the crown. At this stage, ascomata of fungus were formed on petiole bases of plants infected with wild type strain.

The experiment was ended 52 days after inoculation, when all remained plants were taken out from pots and evaluated. All inoculated plants exhibited typical disease symptoms and were severely affected while control plants remained healthy. The mean values of disease severity score were comparable for wild type and GFP-tagged strain (3.0 and 2.9, respectively). Mature perithecia with ascospores dis-

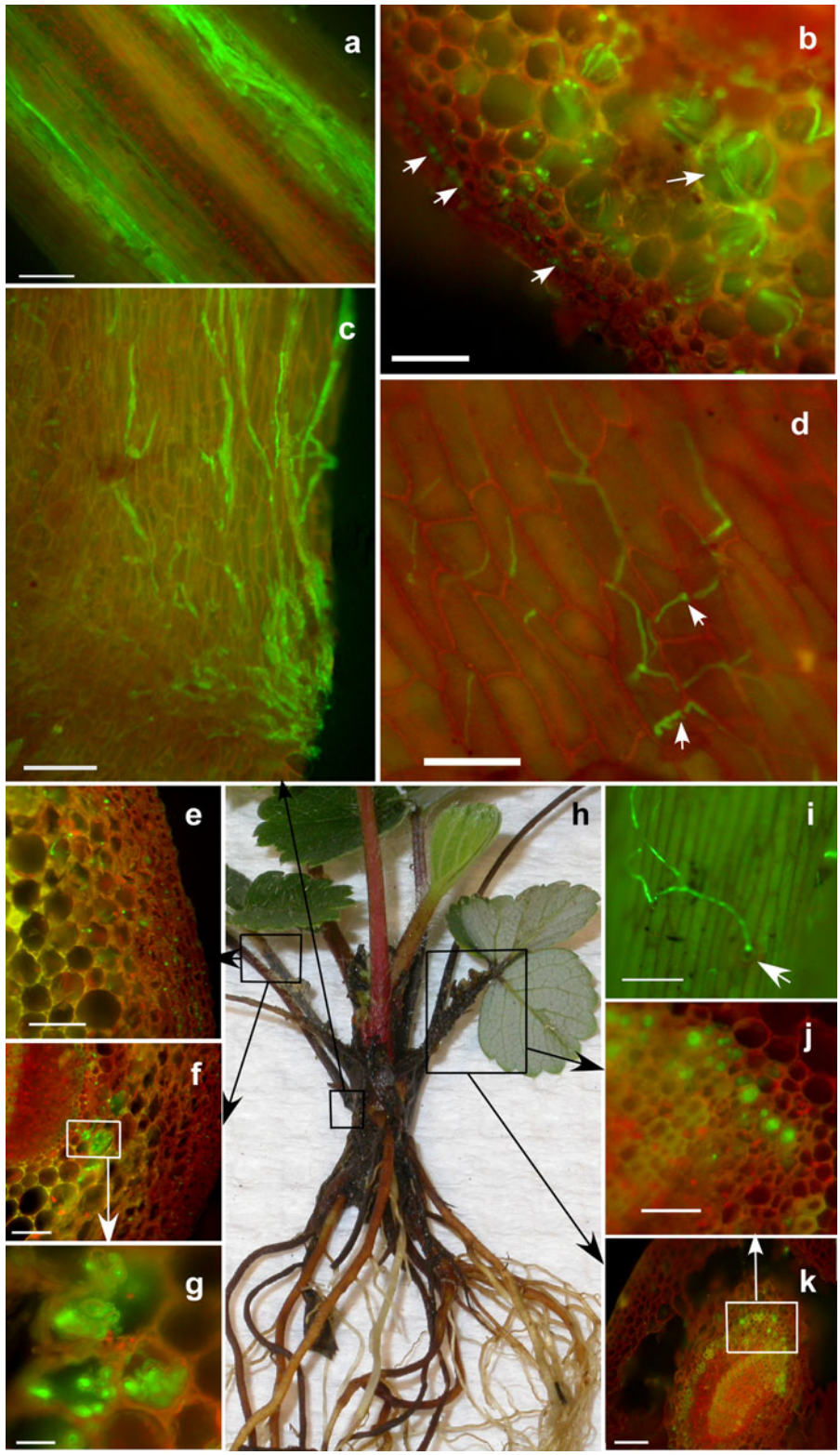


**Fig. 3** Advanced stages of strawberry petiole infection and colonization by *Gnomonia fragariae* marked with GFP: **a** Heavy colonization of cortex tissues between epidermis and vascular tissues, longitudinal section of symptomatic petiole base 19 days after inoculation. **b** Intracellular growth of pathogen (arrow) in the cortex tissues and intercellular (arrows) in the epidermal cell layers, cross-section of petiole 19 days after inoculation. **c** Infection of crown and spread of hyphae upwards into the petiole, longitudinal section of crown 19 days after inoculation. **d** Growth pattern of mycelium inside the crown tissues and swellings (arrows) were they cross the cell walls, longitudinal section of crown 37 days after inoculation. **e** Hyphae in outer layers of cortex, cross section of petiole segment between necrotic and healthy tissues 37 days after inoculation. **f–g** Hyphae in cells close to the vascular tissues, cross section of necrotic petiole segment 37 days after inoculation. **h** Black necrosis on petiole bases expanding upwards. **i** Hypha from infected petiole growing out through stomata (arrow) on the petiole surface, 19 days after inoculation. **j–k** Destroyed cortex cells and hyphae present in vascular tissues, cross section of necrotic petiole 37 days after inoculation. Bar=100 μm for Figures a–f, j–k; 20 μm for Figures g, j

charged on tip of necks were present on petiole bases of 36% and 25% of the inoculated plants with the wild type and transformant strain, respectively. Perithecia, asci and ascospores formed on plants inoculated with the GFP-tagged strain revealed bright green fluorescence when examined under the microscope (Fig. 1g–i). At this time point, the pathogen appeared to be not metabolically active at the necrotic lesions of roots and petioles since GFP expression was not detected by microscopic examination; however mycelia with bright green fluorescence were found in adjacent tissues.

## Discussion

Studies of pathogenic interactions, including plant infection and colonization by pathogenic fungi, have been greatly enhanced by the development of GFP as a vital marker for filamentous fungi (Lorang et al. 2001). In this study we describe the transformation of a recently reported strawberry pathogen, *Gnomonia fragariae*, with the GFP gene and the process by which the pathogen invades and colonizes the roots and petioles of its host.

*Gnomonia fragariae* was successfully transformed by a polyethylene glycol/$CaCl_2$ procedure with the plasmid pIGPAPA that has been previously used for constitutive expression of GFP in several plant pathogenic ascomycetes (Horwitz et al. 1999; Lorang et al. 2001; Jansen et al. 2005). Several transformants with homogenous and strong GFP signals were obtained, but GFP intensity varied among some of the transformants that might be a result of integration of plasmid into different chromosomal sites (Lorang et al. 2001; Lagopodi et al. 2002; Visser et al. 2004). The expression of fluorescent proteins itself does not affect the pathogenicity or phenotypes of the transformed fungi (Maor et al. 1998; van West et al. 1999; Nonomura et al. 2001; Lorang et al. 2001; Lagopodi et al. 2002; Horowitz et al. 2002; Nahalkova and Fatehi 2003; Visser et al. 2004; Sarrocco et al. 2006) and this was also the case of *G. fragariae* in this study.

The germination of ascospores, formation of germ tubes, hyphopodia, penetration pegs and infection of root epidermis rapidly occurred and could be detected by fluorescence 24 h after root inoculation. The hyphopodia (hyphal swellings), which are typical for root-infecting pathogens and are important for the penetration of root epidermis (Mendgen et al. 1996; Sesma and Osbourn 2004) were formed by *G. fragariae* on strawberry roots. In the inoculation method we applied the entire root system was dipped into an ascospore suspension; however, at an early stage after inoculation the majority of ascospores were attached at the middle part and younger regions of roots, which later correlated with appearance of symptoms. To conclude whether these tissues are more prone to infection by *G. fragariae* or whether this was a coincidence, more studies are needed in order to compare results with experiment where soil is infested with ascospores. Similar to *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato (Lagopodi et al. 2002), a preferential growth of hyphae along the intercellular junctions and penetration into epidermal cells at these sites was observed. However unlike several *Fusarium spp*. and other root infecting pathogens that colonize the root surface by dense and extensive hyphal networks (Lagopodi et al. 2002; Sesma and Osbourn 2004; Olivain et al. 2006), the root surface colonization by *G. fragariae* was very sparse and it was discontinued when hyphae penetrated into the root tissue. This pattern of the root colonization indicates a parasitic rather than saprophytic behaviour of *G. fragariae.*

The disrupted cell walls observed in the infected root cortical cells, where pathogen crossed the walls and entered into the adjacent cells, may be an indication for production of cell wall lyzing enzymes by *G. fragariae*, but this needs to be further verified by biochemical analysis. In roots hyphae grew mostly in intercellular spaces and only in the very late stages of the disease were they extensively found inside the cells. This pattern of infection and colonization of roots appears to be different from those described for *Fusarium spp*. and *Magnaporthe grisea* (Lagopodi et al. 2002; Sesma and Osbourn 2004; Olivain et al. 2006). Moreover these pathogens are able to colonize the root vessels in infected plants, while *G. fragariae* was not found in vascular tissues of roots. It has been observed in other fungal pathogens that fungi colonized the vascular system in roots and spread systemically to stem and other above ground tissues (Nonomura et al. 2001; Sukno et al. 2008). In the case of *G. fragariae*, root inoculation led to infection of above ground tissues including crowns and petioles, but so far we have not detected a continuity of hyphae from roots to above ground tissues. On the basis of

our microscopic observations, infection ability of ascospores, susceptibility of petiole tissues to the infection and specific sites of the formation of ascomata on the petioles at the ground level, it could be hypothesized that in the field the fungus spreads by mean of ascospore dispersal and infects petioles at the ground level where petioles are attached to the crown, and rapidly spreads upwards causing collapse of outer leaves. The strawberry root rot and petiole blight caused by *G. fragariae* is a newly discovered disease and so far there is no information available on epidemiology of pathogen in field. Further studies are needed to investigate the infection ability of the ascospores directly on petioles and above ground parts of the crowns and to assess the potential of the airborne inoculum for causing a petiole blight and possible systemic infection of root system in the field.

**Acknowledgements** The research was financially supported by the Swedish Institute and Strategic Foundation for Environmental Research (MISTRA). We are grateful to B. Gillian Turgeon, Cornell University, USA for kindly providing plasmid pIGPAPA and to Lynda M. Ciuffetti, Oregon State University, USA for providing plasmid pCT74. The preparation of the manuscript was supported by the grant 2009/0228/1DP/1.1.1.2.0/09/APIA/VIAA/035

## References

Amsing, J. J. (1995). *Gnomonia radicicola* and *Phytophthora* species as causal agents of root rot on roses in artificial substrates. *Acta Horticulturae, 382*, 203–211.

Barr, M. E. (1978). The *Diaporthales* in North America with emphasis on *Gnomonia* and its segregates. *Mycologia Memoir, 7*, 1–232.

Bolay, A. (1972). Contribution a la connaissance de *Gnomonia comari* Karsten (syn. G. fructicola [Arnaud] Fall). Etude taxonomique, phytopathologique et recherches sur sa croissance in vitro. Berichte der Schweizerischen Botanischen Gesellschaft. *Bulletin de la Société botanique Suisse, 81*, 398–482.

Bolwerk, A., Lagopodi, A. L., Lugtenberg, B. J. J., & Bloemberg, G. V. (2005). Visualization of interactions between a pathogenic and beneficial *Fusarium* strain during biocontrol of tomato foot and root rot. *Molecular Plant Microbe Interactions, 18*, 710–721.

Dhingra, O. D., & Sinclair, J. B. (1995). *Basic plant pathological methods*. USA: CRC.

Horowitz, S., Freeman, S., & Sharon, A. (2002). Use of green fluorescent protein-transgenic strains to study pathogenic and non-pathogenic lifestyles in *Colletotrichum acutatum*. *Phytopathology, 92*, 743–749.

Horwitz, B. A., Sharon, A., Lu, S.-W., Ritter, V., Sandrock, T. M., Yoder, O. C., et al. (1999). A G protein alpha subunit from *Cochliobolus heterostrophus* involved in mating and appressorium formation. *Fungal Genetics and Biology, 26*, 19–32.

Jansen, C., von Wettstein, D., Schäfer, W., Kogel, K.-H., Felk, A., & Maier, F. J. (2005). Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proceedings of the National Academy of Sciences USA, 102*, 16892–16897.

Kistler, H. C., & Benny, U. K. (1988). Genetic transformation of the fungal wilt pathogen, *Fusarium oxysporum*. *Current Genetics, 13*, 145–149.

Klebahn, H. (1918). *Haupt - und Nebenfruchtformen der Askomuyzeten*. Leipzig: Verlag von Gebrüder Borntraeger.

Lagopodi, A. L., Ram, A. F. J., Lamers, G. E. M., Punt, P. J., van den Hondel, C. A. M. J. J., Lugtenberg, B. J. J., et al. (2002). Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as marker. *Molecular Plant-Microbe Interactions, 15*, 172–179.

Lorang, J. M., Tuori, R. P., Martinez, J. P., Sawyer, T. L., Redman, R. S., Rollins, J. A., et al. (2001). Green fluorescent protein is lighting up fungal biology. *Applied and Environmental Microbiology, 67*, 1987–1994.

Maor, R., Puyesky, M., Horwitz, B. A., & Sharon, A. (1998). Use of green fluorescent protein (GFP) for studying development and fungal-plant interaction in *Cochliobolus heterostrophus*. *Mycological Research, 102*, 491–496.

Mendgen, K., Hahn, M., & Deising, H. (1996). Morphognesis and mechanisms of penetration by plant pathogenic fungi. *Annual Review of Plant Pathology, 34*, 367–386.

Mes, J. J., Wit, R., Testerink, C. S., de Groot, F., Haring, M. A., & Cornelissen, B. J. C. (1999). Los of avirulence and reduced pathogenicity of a gamma-irradiated mutant of *Fusarium oxysporum* f. sp. *lycopersisci*. *Phytopathology, 89*, 1131–1137.

Moročko, I., & Fatehi, J. (2007). Molecular characterization of strawberry pathogen *Gnomonia fragariae* and its genetic relatedness to other *Gnomonia* species and members of *Diaporthales*. *Mycological Research, 111*, 603–614.

Morocko, I., Fatehi, J., & Gerhardson, B. (2006). *Gnomonia fragariae*, a cause of strawberry root rot and petiole blight. *European Journal of Plant Pathology, 114*, 235–244.

Nahalkova, J., & Fatehi, J. (2003). Red fluorescent protein (DsRed2) as a novel reporter in *Fusarium oxysporum* f. sp. *lycopersici*. *FEMS Microbiology Letters, 225*, 305–309.

Nonomura, T., Matsuda, Y., Takasugi, M., Ootani, T., Hasegawa, T., Miyajima, K., et al. (2001). A monitoring system for green fluorescence protein gene-transformed *Fusarium oxysporum* in melon seedlings. *Journal of General Plant Pathology, 67*, 273–280.

Nonomura, T., Tajima, H., Kitagawa, Y., Sekiya, N., Shitomi, K., Tanaka, M., et al. (2003). Distinguishable staining with neutral red for GFP-marked and GFP-nonmarked *Fusarium oxysporum* strains simultaneously colonizing root surfaces. *Journal of General Plant Pathology, 69*, 45–48.

Noordeloos, M. E., van Kesteren, H. A., & Veenbaas-Rijks, J. W. (1989). Studies in plant pathogenic fungi I: *Gnomonia radicicola*, sp. nov., a new pathogen on roses. *Persoonia, 14*, 47–49.

Olivain, C., Humbert, C., Nahalkova, J., Fatehi, J., Haridon, F. L., & Alabouvette, C. (2006). Colonization of tomato root by pathogenic and nonpathogenic *Fusarium oxysporum* strains inoculated together and separately in the soil. *Applied and Environmental Microbiology, 72*, 1523–1531.

Rossman, A. Y., Farr, D. F., & Catlebury, L. A. (2007). A review of the phylogeny and biology of the *Diaporthales*. *Mycoscience, 48*, 135–144.

Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual*. New York: Cold Spring Harbor Laboratory Press.

Sarrocco, S., Mikkelsen, L., Vergara, M., Jensen, D. F., Lübeck, M., & Vannacci, G. (2006). Histopathological studies of sclerotia of phytopahtogenic fungi by GFP transformed *Trichoderma virens* antagonistic strain. *Mycological Research, 110*, 179–187.

Sesma, A., & Osbourn, A. E. (2004). The rice blast pathogen undergoes developmental processes typical pf root-infecting fungi. *Nature, 431*, 582–586.

Sogonov, M. V., Castlebury, A. Y., Mejia, L. C., & White, J. F. (2008). Leaf-inhabiting genera of the *Gnomoniaceae*, *Diaporthales*. *Studies in Mycology, 62*, 1–77.

Sukno, S. A., García, V. M., Shaw, B. D., & Thon, M. R. (2008). Root infection and systemic colonization of maize by *Colletotrichum graminicola*. *Applied and Environmental Microbiology, 74*, 823–832.

van West, P., Reid, B., Cambell, T. A., Sandrock, R. W., Fry, W., Kamoun, S., et al. (1999). Grenn fluorescent protein (GFP) as a reporter gene for the plant pathogenic oomycete *Phytophthora palmivora*. *FEMS Microbiology Letters, 178*, 71–80.

van West, P., Appiah, A. A., & Gow, N. A. R. (2003). Advances in research on oomycete root pathogens. *Physiological and Molecular Plant Pathology, 62*, 99–113.

Visser, M., Gordon, T. R., Wingfield, B. D., Wingfield, M. J., & Viljoen, A. (2004). Transformation of *Fusarium oxysporum* f. sp. *cubense*, causal agent of *Fusarium* wilt of banana, with the green fluorescent protein (GFP) gene. *Australasian Plant Pathology, 33*, 69–75.