

Characterization of the Strawberry Pathogen *Gnomonia fragariae*, and Biocontrol Possibilities

Inga Moročko

*Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Pathology
Uppsala*

**Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006**

Acta Universitatis Agriculturae Sueciae

2006: 71

ISSN 1652-6880

ISBN 91-576-7120-6

© 2006 Inga Moročko, Uppsala

Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Moročko, I. 2006. Characterization of the strawberry pathogen *Gnomonia fragariae*, and biocontrol possibilities. Doctoral dissertation.
ISSN 1652-6880, ISBN 91-576-7120-6

The strawberry root rot complex or black root rot is common and increasing problem in perennial strawberry plantings worldwide. In many cases the causes of root rot are not detected or it is referred to several pathogens. During the survey on strawberry decline in Latvia and Sweden the root rot complex was found to be the major problem in the surveyed fields. Isolations from diseased plants showed that several pathogens such as *Cylindrocarpon* spp., *Fusarium* spp., *Phoma* spp., *Rhizoctonia* spp. and *Pythium* spp. were involved. Among these well known pathogenic fungi a poorly studied ascomycetous fungus, *Gnomonia fragariae*, was repeatedly found in association with severely diseased plants. An overall aim of the work described in this thesis was then to characterize *G. fragariae* as a possible pathogen involved in the root rot complex of strawberry, and to investigate biological control possibilities of the disease caused.

In several pathogenicity tests on strawberry plants *G. fragariae* was proved to be an aggressive pathogen on strawberry plants. The pathogenicity of *G. fragariae* has been evidently demonstrated for the first time, and the disease it causes was named as strawberry root rot and petiole blight.

In order to investigate the process how *G. fragariae* invades and colonizes the roots and petioles of strawberry, the fungus was genetically transformed with the green fluorescent protein (GFP) gene. As revealed by studies with GFP-tagged strain, *G. fragariae* is an aggressive invader of both strawberry root and petiole tissues, and the cortex are the most affected tissues. Observations of the pathogen development in root and petiole tissues showed that the petioles are more sensitive to the pathogen infection resulting in more severe damages in shorter period of time. *Gnomonia fragariae* was also able to infect vascular tissues of petioles in later stages of the disease development. Ascospores were found to be highly infective suggesting that they could be one of the major means of disease spread in the field.

Gnomonia fragariae isolates were further characterized and phylogenetic relationships inferred with other *Gnomonia* species and members of *Diaporthales* based on the nucleotide sequence analyses of two loci (nuLSU rDNA and ITS/5.8S region) of the ribosomal rDNA array. In addition to molecular methods, morphological examination was also performed. Results showed that *G. fragariae* is genetically distinct from the genus type species, *G. gnomon*, and other members of *Gnomoniaceae*, although morphological evidences to support this discrepancy were not found. It was suggested, that *G. fragariae* together with *G. rubi*, *G. rosae* and *Hapalocystis* represents a genetically distinct group, possibly a new family, within *Diaporthales*.

In a series of biocontrol and root inoculation experiments carried out with two non-pathogenic *Fusarium* sp. strains originated from strawberry roots it was shown, that these strains have beneficial effect on strawberry plants and can reduce root rot on strawberry caused by *G. fragariae*. However, the evaluation of disease suppression efficiency and effect on strawberry productivity in actual culturing systems and elucidation of the modes of action of these *Fusarium* sp. strains need to be further studied.

Keywords: root rot complex, *Gnomonia*, *Diaporthales*, *Fragaria*, *Rubus*, green fluorescent protein, root pathogens, *Phytophthora*, *Fusarium*, biological control

Author's address: Inga Moročko, Department of Forest Mycology and Pathology, SLU, P.O. Box 7026, S-750 07, Uppsala, Sweden.
E-mail: Inga.Morocko@mykopat.slu.se

Contents

Introduction, 7

Gnomonia fragariae, 7

History, hosts and distribution, 7

Taxonomy and related pathogens, 7

Root rot complex of strawberry, 8

Interactions between plants and pathogens, 9

Plant infection by pathogenic fungi, 10

GFP as a vital marker to study plant infection by pathogenic fungi, 11

Control strategies for the strawberry root rot complex, 12

Aims of the study, 14

Materials and Methods, 15

Collection sites and plant sampling, 15

Isolation of fungi, 15

Morphological examination and identification, 15

Pathogenicity tests, 16

Molecular characterization of *Gnomonia fragariae*, 17

DNA extraction, PCR amplification and sequencing, 17

Sequence analyses, 18

Transformation of *Gnomonia fragariae* and study of plant infection, 19

Transformation with GFP gene, 19

Plant inoculation and microscopic examinations, 19

Screening of *Fusarium* spp. for biocontrol, 19

Results and Discussion, 21

Disease symptoms in the surveyed fields, 21

Fungi associated with strawberry root rot complex, 22

Gnomonia fragariae, a cause of strawberry root rot and petiole blight (I), 24

Molecular characterization of *Gnomonia fragariae* (II), 26

Transformation of *Gnomonia fragariae* with GFP gene and the study of plant infection (III), 29

Transformation and selection of a stable transformant, 29

Early stages of the disease and microscopic observations, 30

Advanced stages of the disease and microscopic observation, 31

Effect of non-pathogenic *Fusarium* spp. on strawberry root rot (IV), 32

Conclusions, 35

References, 36

Acknowledgements, 42

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

- I.** Moročko, 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.
- II.** Moročko, I. & Fatehi, J. Molecular characterization of strawberry pathogen *Gnomonia fragariae* and its genetic relatedness to other *Gnomonia* species and members of *Diaporthales*. Submitted manuscript.
- III.** Moročko, I. & Fatehi, J. Transformation of *Gnomonia fragariae*, the cause of strawberry root rot and petiole blight, with the green fluorescent protein (GFP) gene and the study of host infection and colonization. Manuscript.
- IV.** Moročko, I., Fatehi, J. & Gerhardson, B. Effect of non-pathogenic *Fusarium* sp. on strawberry root rot caused by *Gnomonia fragariae*. Manuscript.

Paper I is reproduced by kind permission of Springer Science and Business Media

Introduction

The strawberry cultivation in many regions of the world is constrained by serious diseases that can affect root system, crown, and the basal part of the petioles causing damages to the host and considerable reduction of the yield. Among these, root rot and crown rot caused by *Phytophthora* spp., *Verticillium* wilt, and disorders caused by *Colletotrichum* spp. are known as specific and lethal diseases in strawberry cultivation world wide (Paulus, 1990; Anonymous, 1996; Freeman *et al.*, 1997; Maas, 1998; Freeman, Katan & Shabi, 1998; Duncan, 2002). However, the field infected strawberries often present a complex disease profile and there are complexes of non-lethal fungal pathogens that contribute to the reduction of plant growth and subsequent yield.

Gnomonia fragariae

History, hosts and distribution

Gnomonia fragariae Kleb. is a poorly studied ascomycete which was first described in the beginning of last century by Klebahn (1918). *G. fragariae* was found on petioles of dead leaves of strawberry during the screening for causes of strawberry decline in Germany. Based on negative results obtained in pathogenicity tests, Klebahn (1918) considered this fungus as a saprophyte living on dead tissues of strawberry plants. Later, *G. fragariae* was recorded on dead tissues of pink barren strawberry (*Potentilla micrantha*), silverweed or goose grass (*P. anserina*), rock cinquefoil (*P. rupestris*), woodland strawberry (*Fragaria vesca*) and cultivated strawberry (*Fragaria x ananassa*) collected in several counties of Switzerland (Bolay, 1972). Since the first record, *G. fragariae* has been mentioned in association with strawberries in several other European countries, North America, Asia, Australasia and Oceania (Alexopoulos & Cation, 1952; Parikka, 1981; CMI Distribution maps of plant diseases, No. 438, 1982). However as it has been pointed out by Bolay (1972), Maas (1998) and Farr *et al.* (1989) it is misapplication of the name probably referring to *G. comari*. Considering this, so far the distribution of *G. fragariae* can be certainly assigned only to few countries including Germany (Klebahn, 1918), Switzerland (Bolay, 1972), United Kingdom (CMI, Map No.438, 1989; living culture IMI 100647, CBS 146.64).

Taxonomy and related pathogens

According to Monod (1983) the ascomycetous fungus *G. fragariae* belongs to the order *Diaporthales* Nannf., family *Gnomoniaceae* G. Winter, genus *Gnomonia* Ces. & De Not. *Diaporthales* includes a number of plant pathogenic fungi mostly considered as facultative pathogens, and a few systemic causing cankers and dieback on several hosts. The most destructive diseases are caused by *Cryphonectria parasitica* (Murrill) Barr (chestnut blight), *Diaporthe phaseolorum* (Cooke & Ell.) Sacc. (soybean stem canker) and *D. citri* F.A. Wolf (stem-end rot of citrus) (Farr *et al.*, 2001; Castlebury *et al.*, 2002). The members of *Diaporthales* are characterized by having brown to black perithecial ascomata immersed in

substrate or stroma, lack of true paraphyses at maturity, and unitunicate asci with refractive ring at the apex (Barr, 1978; Castlebury *et al.*, 2002). As summarized by Castlebury *et al.* (2002) eight families with several considerable rearrangements based on morphological grounds have been recognized within *Diaporthales* by various authors over the past 25 years. Recent phylogenetic studies on the diaporthalean taxa using the large subunit nuclear ribosomal DNA nucleotide sequences by Castlebury *et al.* (2002) strongly suggested the presence of *Gnomoniaceae* family and five other major lineages within this order and disagreed with Eriksson *et al.* (2001) concept to circumscribe *Diaporthales* only in three families including *Melanconidiaceae* G. Winter, *Valsaceae* Tul. & C. Tul., and *Vialaeaceae* P.F. Canon.

The family *Gnomoniaceae* is characterized by solitary, upright ascomata with usually central beaks which are immersed in substrate or aggregated in reduced stromata, having non-septate, one-septate or occasionally several septate ascospores (Barr, 1978; Monod, 1983). According to Monod (1983), the genus *Gnomonia* is characterized by ascomata immersed in, or liberated from host tissues at maturity, containing asci with two, four, and most often eight or 20-30 hyaline, straight or slightly curved ascospores. These spores are one septate at the middle or between middle and 2/5 of the length with little or no constriction at the septum, often with thread-like or spatuliform appendages at both ends. Most species of *Gnomonia* are saprophytic, but the genus also includes several plant pathogens which attack aerial constituents of herbaceous and woody hosts (Barr, 1978). In relatively recent studies by Noordeloos, Kesteren & Veenbaas-Rijks (1989) and Amsing (1995), *G. radicola* Noordel., Kest. & Veenb. was described in the Netherlands and shown to be a root infecting pathogen of roses in artificial substrates in greenhouses. There are also several other species of this genus that are associated with rosaceous plants. Among them *Gnomonia rubi* (Rehm) Winter and *G. rostellata* (Fr.) Wehm. are the causal agents of severe cankers and dieback of different cultivated and wild *Rubus* species and roses both in Europe and America (Schneider, Paetzholdt & Willer 1969; Ellis, Kuter & Wilson, 1984; Maas, Galleta & Ellis, 1989; Arsenijević & Veselić, 1995; Veselić & Arsenijević, 1998; Nordskog, Stensvand & Heiberg, 2003). Based on morphological grounds some authors have considered *G. rostellata* and *G. rubi* as synonyms (Monod 1983; Barr 1991).

The strawberry root rot complex

The strawberry root rot complex, also known as black root rot, is a common and increasing problem in perennial strawberry plantings worldwide. In general, symptoms assigned to the root rot complex or black root rot of strawberry are: patchy appearance of stunted plants in a field and decline of plant vigour; poor production and small berries; blackening of entire root system and discoloration of leaves that turn yellow or red (Wing, Pritts & Wilcox, 1994). In many cases the causes of root rot and plant decline are not detected or it is referred to several pathogens of which *Pythium* spp., *Rhizoctonia solani* Kühn and *R. fragariae* Husain & McKeen, *Cylindrocarpon* spp., *Phoma* spp., *Coniothyrium* sp., *Idriella*

lunata Nelson & Wilhelm, *Pezizella lythri* (Desm) Shear & Dodge and *Fusarium* spp. are the most common (Strong & Strong, 1931; Wilhelm *et al.*, 1972; Yuen *et al.*, 1991; Wing, Pritts and Wilcox, 1994; Wing, Pritts & Wilcox, 1995; Anonymous, 1996; Parikka & Kukkonen, 2002). Moreover, nematodes can greatly intensify the severity of black root rot and the disease is favoured by plant abiotic stresses (Chen & Rich, 1962; la Mondia & Martin, 1989; Wing, Pritts & Wilcox, 1994; Anonymous, 1996).

Gnomonia comari Karst. and *G. fragariae* are also listed among the fungi that may play role in the root rot complex of strawberry. *Gnomonia comari* is a worldwide strawberry pathogen which is well known as a cause of leaf blotch, fruit rot and stem end rot (Alexopoulos & Cation, 1948; Shipton, 1967; Bolay, 1972; Gubler & Feliciano, 1999). In addition, *G. comari* can cause petiole blight (van Adriechem and Bosher, 1958) and root rot of strawberry, particularly in a synergic interaction with nematodes (Kurppa and Vrain, 1989). However, *G. comari* is considered as a weak pathogen that rarely causes considerable losses (Bolay, 1972). *Gnomonia fragariae* was once listed among the fungi associated with the root rot of strawberry in Finland (Parikka, 1981), but pathogenicity of it and other isolated fungi was not evaluated in that study. Moreover, confusion remains on the identity of that fungus since the references listed by the author are related to *G. comari*, particularly to *G. fructicola* (Arnaud) Fall, a synonym of *G. comari*.

Interactions between plants and pathogens

Plants are hosts to many diseases caused by a wide range of pathogens including fungi, bacteria, viruses and nematodes. Only a relatively small portion of the fungi, approximately 10 %, can successfully invade plant hosts and cause disease (Baker *et al.*, 1997; Kahmann & Basse, 2001). Plant and pathogen species have co-evolved over time (Wan, Dunning & Bent, 2002), resulting in close and complex communications between these two organisms. During this communication pathogen activities are focused on infection, colonization of the host, and utilization of its resources, while plants are adapted to detect the presence of pathogens and to react with antimicrobial defences and other stress responses (van Etten *et al.*, 1995; Wan, Dunning & Bent, 2002).

Plant infectious diseases and their causes have been generally classified by the tissue types they affect such as root diseases and pathogens or foliar diseases and pathogens. However as it has been recently stressed for some pathogenic fungi, this could be misleading when considering disease development and epidemiology. Several important plant pathogens known of affecting aerial constituents such as *Magnaporthe grisea* (T.T. Hebert) M.E. Barr (rice blast disease), *Leptosphaeria maculans* (Desm.) Ces. & De Not. (stem canker of brassicas) and *Cercospora beticola* Sacc. (leaf spot of sugar beat) have been shown to attack their host roots and therefore it was hypothesised that soil borne inoculum and root infection may play an important role for the disease development (Dufresne & Osbourn, 2001; Sosnowski *et al.*, 2001; Osbourn & Sesma, 2004; Vereijssen, Schneider & Termorshuizen, 2004).

In every infectious disease there is a series of more or less distinct events, including adhesion, penetration, colonization, growth, reproduction, dissemination, and survival of the pathogen, that occur one after another and lead to development and spread of the disease. This chain of events is called a disease cycle (Agrios, 1997). Understanding the each action in detail and behaviour of plant pathogenic fungi involved in the disease process is of primary importance to provide basic knowledge for development of control strategies. For example, a very important requirement for fungicides is the selectivity of their mode of action. Non-target effects can be reduced by developing fungicides, which do not inhibit vegetative hyphal growth but specially interfere with developmental events involved in pathogenesis, such as spore germination, formation of penetration structures or sporulation (Thines, Anke & Weber, 2004).

Plant infection by pathogenic fungi

As mentioned above, most of interactions between plants and fungi do not result in invasion and colonization of plant tissues. Plants are capable to protect themselves from invasion by most of the fungi. Nevertheless, among the fungi that manage to overcome plant defence responses and invade plants, great diversity exists in lifestyle and in the diseases they cause. To infect and obtain nutrients from plants fungi have evolved amazing variations in the infection strategies.

Infection structures of pathogenic fungi usually are modified hyphae specialized for the invasion of plants tissues. The most advanced in this sense are biotrophic and hemibiotrophic fungi. Initial events of infection are adhesion to the plant surface and for some pathogens, e.g. *Magnaporthe grisea*, directed growth of the germ tube on the plant surface. At the site of penetration, which can be directly or through natural openings, appressoria are often formed that may have melanized walls which lead to development of high turgor pressure to break the cuticle and penetrate the cell (Mendgen, Hahn & Deising, 1996; Dean, 1997; Tucker & Talbot, 2001). Penetrating hyphae may also secrete a variety of cell-wall degrading enzymes in a highly regulated fashion in order to penetrate the cuticle, plant cell walls and to spread within host plant tissues (Walton, 1994; Mendgen, Hahn & Deising, 1996; Tucker & Talbot, 2001). After host invasion, fungi use different strategies to gain access to host nutrients. Obligate biotrophic rust fungi, for example, can form the most specialized hypha, namely haustoria, and obtain nutrients from plants without killing the cell (Bushnell, 1972; Mendgen & Hahn, 2002). In contrast, necrotrophs quickly kill plant cells to obtain nutrients subsequently as saprotrophs (Walton, 1994; Oliver & Osbourn, 1995; Kahmann & Basse, 2001).

Non-differentiated hyphae serve as means of penetration for most root-infecting fungi (e.g. *Fusarium* spp.) (Mendgen, Hahn & Deising, 1996; Olivain & Alabouvette, 1999). This is also true for certain leaf-infecting species such as *Ustilago* spp. or monokaryotic stages of rust fungi (Mendgen, Hahn & Deising, 1996). However hyphal swellings - simple penetration structures (hyphopodia) are also formed by several root infecting pathogens (Sesma & Osbourn, 2004). Root infecting pathogens, such as *Fusarium* spp. or *Rhizoctonia* spp., accumulate

hyphae that form infection cushions or completely colonize root surface before individual hyphae penetrate with minor modifications of their morphology (Wilhelm *et al.*, 1972; Wilhelm, 1984; Mendgen, Hahn & Deising, 1996; Recorbet *et al.*, 2003). Following the penetration, the mode of invasive growth inside the plant root tissues also differs among pathogenic fungi.

As summarized by Waugh, Ferrin & Stanghellini (2005), root infecting pathogens, in general, can be grouped in three categories such as non-specialized, ectotrophic and vascular pathogens of which each colonize plant roots in different ways. Non-specialized pathogens (e.g. *Rhizoctonia* and *Pythium*) produce extensive intra- and inter-cellular hyphae in the root cortex subsequent to penetration, and there is little extension of hyphae into the vascular tissues (Waugh, Ferrin & Stanghellini, 2005; Wilhelm *et al.*, 1972). Characteristics that define the group of ectotrophic pathogens are epiphytic growth of hyphae over the root surface as a network, the hyphae then rapidly penetrate the cortex and also the vascular system (e.g. the cause of wheat take-all, *Gaeumannomyces graminis* var. *tritici*) (Liu, Shang & Tan, 2000; Guilleroux & Osbourn, 2004; Waugh, Ferrin & Stanghellini, 2005). Well known representatives of the third group, the vascular pathogens, are *Fusarium oxysporum* (Sacc.) Snyder & Hans. and *Verticillium* spp., which cause wilts to broad range of plants as different specialized forms in case of *F. oxysporum* or due to the extensive host range in case of *Verticillium* spp. (Armstrong & Armstrong, 1981; Tjamos, 1981; Paulus, 1990; Domsch, Gams & Anderson, 1993; Recorbet *et al.*, 2003; Waugh, Ferrin & Stanghellini, 2005). Following penetration, growth coincided with rapid cell collapse occurs directly through the cortex and into the vascular tissue where these pathogens spread systemically (Domsch, Gams & Anderson, 1993; Olivain & Alabouvette, 1999; Salerno, Gianinazzi & Gianinazzi-Pearson, 2000; Bao & Lazarovits, 2001).

GFP as a vital marker to study plant infection by pathogenic fungi

Since the first cloning of cDNA for the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* in 1992, this gene or its derivatives were successfully expressed first in bacteria and *Caenorhabditis elegans* cells in culture, then followed by yeasts, mammals, *Drosophila*, *Dictyostelium*, plants and filamentous fungi (Howard, 2001; Lorang *et al.*, 2001). The tremendous success of GFP as a reporter can be attributed to unique qualities of this protein to absorb light at maximum of 395 and 475 nm and to emit light at a maximum of 508 nm (Lorang *et al.*, 2001).

The GFP has been developed and widely used to study diverse range of biological questions. For example, it is used as a reporter for gene expression and protein localization in living cells, as well as to assess dispersal and biomass of different organisms in complex environments (Howard, 2001; Lorang *et al.*, 2001; Jansen, 2005). In filamentous fungi GFP provides excellent means for identifying, visualizing and tracing the dispersal of a tagged strain within its natural environment (e.g. host plant tissue). In this sense, GFP has been used to study several aspects of plant-fungal interactions, including plant-pathogen (Lorang *et al.*, 2001; Lagopodi *et al.*, 2002; Nonomura *et al.*, 2003; Sesma & Osbourn, 2004;

Visser *et al.*, 2004), plant-biocontrol agent (Nonomura *et al.*, 2001) and plant-pathogen-biocontrol agent interactions involving other markers for simultaneous application of pathogen and biocontrol agent (Olivain *et al.*, 2006; Bolwerk *et al.*, 2005). The use of GFP tagged plant pathogenic fungi and oomycetes has greatly enhanced the studies on host plant infection processes. Visualization of spore attachment, hyphal elongation on root surface, penetration process and colonization of the host plant tissues by pathogen has been made possible without disturbance by staining and fixation of the sample. So far the GFP has been successfully used as a vital marker for a broad range of plant pathogens to study both leaf and root infections (Visser *et al.*, 2004; Nonomura *et al.*, 2003; van West, Appiah & Gow, 2003; Horowitz, Freeman & Sharon, 2002; Maor *et al.*, 1998).

Control strategies for the strawberry root rot complex

Because several factors are involved in the black root or root rot complex of strawberry, including a range of infectious agents and various abiotic factors (Wing, Pritts & Wilcox, 1994), the disease control is complicated and no general control measure is totally effective. To reduce incidence of strawberry root rot complex following measures have been recommended: use of healthy and certified plants for planting establishment; crop rotation excluding strawberries for at least 2-3 years before replanting; minimization of soil compaction and incorporation of organic matter; avoidance of heavy, wet soils and improved drainage in marginal soils by tiling or planting on raised beds, and pre-plant fumigation of soil (Wilhelm, 1984; Himerlick & Dozier, 1991; Yean *et al.*, 1991; Maas & Galletta, 1997; Martin & Bull, 2002). Differences in cultivar tolerance to black root rot have also been observed. However, as found during the screening of wide range of strawberry cultivars in several fields with problems of black root rot, this has no practical value due to the complexity of the disease and can be effective only if the cause of root rot for a particular location is identified (Wing, Pritts & Wilcox, 1995).

In several major strawberry production regions of the world management of strawberry root diseases, including root rot complex, has been build on and still relies mostly on chemicals, particularly on fumigation of soil despite its short term effect (Himerlick & Dozier, 1991; Sutton, 1994; Maas & Galletta, 1997; http://www.epa.gov/ozone/mbr/FactSheet_2007Request.html, 29-July-2006.). The increased emphasis on reducing or restricting the use of pesticides, especially phaseout of methyl bromide, has forced development of alternative means for strawberry disease management and practices for the control of soil-borne pathogens in general (Easterbrook *et al.*, 1997). One of the alternative approaches is the use of microbial antagonists that are naturally present in the soil. The research on biological control of strawberry diseases has been mainly focused on fruit rots and foliar pathogens (Sutton, 1994; Maas & Galletta, 1997), although some promising effective biocontrol agents have been also found for root infecting pathogens. *Streptomyces anulatus* and non-pathogenic *Fusarium oxysporum* was shown to suppress wilt of strawberry caused by *F. oxysporum* f.sp. *fragariae* in soil (Tezuka & Makino, 1991; Sutton, 1994; Maas & Galletta, 1997). Another

Streptomyces sp. strain isolated from nematode suppressive soils was demonstrated to be effective against black root rot of strawberry (Maas & Galletta, 1997). A strain of non-pathogenic *Rhizoctonia fragariae* was demonstrated to suppress strawberry root rot caused by pathogenic *R. fragariae*, one of the most damaging pathogens involved in root rot complex of strawberry (D'Ercole, 1993). In Israel, for example, *Trichoderma harzianum* is applied into the fumigated soils to prevent outbreaks of soil-borne diseases afterwards (Cook & Baker, 1996). In conclusion, biological control by employing various beneficial microorganisms in combination with cultural practises could be a sustainable option for managing the root rot complex in strawberry production.

Aims of the study

The overall aim of the work described in this thesis was to characterize *Gnomonia fragariae* as a pathogen involved in the root rot complex of strawberry and to investigate biological control possibilities of the disease caused by *G. fragariae*.

More specifically, the objectives were:

- To investigate on the strawberry root rot complex in Latvia and Sweden and to identify pathogenic fungi involved (**Paper I**).
- To evaluate pathogenicity of *Gnomonia fragariae* on strawberry and to describe the disease it causes (**Paper I**).
- To characterize *Gnomonia fragariae* strains obtained from different geographical regions by analyses of nucleotide sequences of ribosomal RNA genes and to infer genetic relationships with other *Gnomonia* species and members of *Diaporthales* (**Paper II**).
- To transform *Gnomonia fragariae* with green fluorescent protein (GFP) gene and to use the GFP-tagged strain to analyze different stages of strawberry infection and colonization process (**Paper III**).
- To evaluate biocontrol effect of non-pathogenic *Fusarium* spp. strains originated from strawberry on root rot caused by *Gnomonia fragariae* (**Paper IV**).

Materials and Methods

Collection sites and plant sampling

During the survey on possible fungal causes of strawberry decline twenty one field in Latvia and four fields in Sweden with severely stunted and wilting strawberry plants were identified, and diseased plants showing root and crown rot symptoms were sampled from May to November in the years 2001 to 2004 (Paper I). The age of strawberry plantings varied from one to four years. Whole plants were collected and taken to laboratory for examination. Figure 2 shows the sampling locations where diseased strawberry plants were collected (See Results and Discussion section).

Isolation of fungi

In order to obtain a maximum range of possible pathogenic fungi involved in strawberry decline several agar media and different isolation strategies were performed (Paper I). Plants were carefully washed under running tap water. Segments of stem bases, crowns and roots showing disease symptoms were surface sterilized in 1.25 % sodium hypochlorite for 2 min (younger roots and petioles) and 3 min (crowns), washed 3 times in sterile distilled water, and blot-dried. Tissue fragments, ca. 0.5 cm long, were plated on potato-dextrose agar (PDA; Oxoid, Basingstoke, UK), half strength PDA, and water-agar (WA; Oxoid, Basingstoke, UK) media. Crowns were cut in slices and plated in the same way. To detect a possible *Phytophthora* infection, non surface sterilised tissues from diseased roots and crowns were plated either on *Phytophthora* selective medium (Tsao & Ocana, 1969) or placed in water to induce sporangia formation. The growing fungal colonies were transferred on PDA and pure cultures maintained at 4 °C both as test-tube slants on PDA, synthetic nutrient-poor agar (SNA; Nirenberg, 1976), oatmeal agar (OM; Difco, Detroit, USA), and as 4 mm diam. mycelial plugs in sterile distilled water.

Freshly collected petioles and roots of diseased plants collected from several locations were examined for the presence of fruiting bodies. Further, the infected petioles and roots were also incubated in moist chambers and the possible formation of fruiting bodies was monitored up to one month.

Morphological examination and identification

For morphological identification (Paper I) isolated fungi were grown on several agar media including PDA, potato carrot agar (PCA; Dhingra & Sinclair, 1995), corn meal agar (CMA; Oxoid, Basingstoke, UK), OA, and WA and incubated at room temperature. To stimulate sporulation isolates were also inoculated on PCA plates containing autoclaved toothpicks or incubated under black light-blue lamp (Sylvania F15W/BLB-T8, Japan). Obtained isolates were separated into groups based on colony morphology and identification at genus and species level was

carried out based on morphological characters by using light microscopy. Obtained fungi, where appropriate, were compared with authentic herbarium specimens (Paper I).

In the study described in Paper II, 19 isolates belonging to six *Gnomonia* species obtained in this work and from culture collection at Centraalbureau voor Schimmelcultures (CBS), The Netherlands were used for morphological examination and comparisons. To induce sporulation the isolates were grown on OA, potato carrot agar (PCA) and water agar (Difco, Detroit, USA) plates to which autoclaved toothpicks or pieces of leaves and stems of *Fragaria*, *Rubus* or *Rosae* (host plants from which fungi were originally isolated), were added. Plates were incubated at 19 °C with 12 h daily illumination of black light-blue (Sylvania F15W/BLB-T8, Japan) and cool-white (Osram L 15W/840, Germany) lamp combination.

Pathogenicity tests

The isolation on agar media followed by morphological identification and evaluation of pathogenicity is a basic and the most reliable approach to determine the unknown plant diseases caused by fungi. Evaluation of pathogenicity of fungi of interest was performed in detached-leaf assays and subsequently on strawberry plants in several greenhouse and growth chamber experiments.

The detached-leaf assay is a widely used method for pilot screening of pathogenicity for different fungi and host plants. In this study 17 isolates of *Gnomonia fragariae* (Paper I), 48 isolates of *Cylindrocarpon* spp., 10 isolates of *Discohainesia oenotherae* (Cooke & Ellis) Nannf. (conidial state *Hainesia lythri* (Desm.) Hoehn.); 7 isolates of *Verticillium* spp., and 17 isolates of *Fusarium* spp. were tested in the detached-leaf assays. Leaflets of strawberry cultivar ‘Honeoye’ were inoculated by mycelial plugs (5 mm diam.) taken from young fungal colonies and incubated on moist filter paper in Petri plates at room temperature for two weeks. After two weeks the re-isolation was performed from surface-sterilized tissues on PDA to recover the fungi.

Pathogenicity of *G. fragariae* isolates on strawberry plants was evaluated in three separate bioassays (Paper I). Runner plants or micro-propagated plants of strawberry cultivars ‘Honeoye’, ‘Syriuz’, and ‘Zefyr’ obtained either from Stiftelsen Trädgårdsodlingens Elitplantstation, Sweden or Pure Horticultural Research Station, Latvia were used in the experiments. Fungal inoculum was prepared and applied in three ways depending on experiment performed: 1) the fungus was grown on autoclaved perennial ryegrass (*Lolium perenne*) seeds and applied into the soil at different concentrations; 2) mycelial plugs from young cultures (5 mm diam.) were placed near the roots; 3) roots were dipped in the ascospore suspension for 2 h. Non-inoculated plants were used as a controls. The steam treated potting soil mixture was used in all the experiments. To monitor the disease development symptoms and dead plants were recorded once a week. The length of experiments varied from six to eleven weeks. Disease severity (DS) was recorded at the end of experiments according to original scale developed to evaluate particular disease: 0 - plant well developed, no disease symptoms; 1 - no

visible symptoms on above ground parts, ≤ 25 % of roots discoloured; 2 - plant slightly stunted, black necrosis on petiole bases, 26–50 % of roots discoloured; 3 - plant stunted, black necrosis on petiole bases, yellowing and death of outer leaves, 51–75 % of roots discoloured; 4 - plant severely stunted, outer leaves collapsed, younger leaves bluish green and wilting, > 75 % of roots discoloured; 5 - plant dead. The re-isolation was done from root and crown tissues on PDA and WA following the same procedure as for the initial isolation.

Three isolates of *Discohainesia oenotherae* (*Hainesia lythri*) were also evaluated for their pathogenicity on strawberry. The experimental set up was identical as described in Paper I for pathogenicity test No. 1.

Molecular characterization of *Gnomonia fragariae*

DNA-based techniques in combination with morphological traits have been proved to be of great value for characterization of species and populations, as well as for inferring phylogenetic relationships in fungi. In this thesis (Paper II) the nucleotide sequences from two loci of the ribosomal rDNA array were used to characterize *G. fragariae* isolates and to infer phylogenetic relationships of *G. fragariae* with other *Gnomonia* species and members of *Diaporthales*. In addition to molecular methods, morphological comparisons were also made. The methods used for this purpose are shortly described below.

For the characterization, 13 isolates of *G. fragariae* and one isolate of *G. comari* isolated from strawberry during the study described in Paper I were used in this study. In addition, cultures of *G. fragariae* and of three other *Gnomonia* species pathogenic on rosaceous hosts were obtained from CBS. The culture of type species of the genus, *G. gnomon*, was obtained from Uppsala University Culture Collection of Fungi (UPS).

DNA extraction, PCR amplification and sequencing

To obtain mycelium for DNA extraction, potato carrot broth (PCB) was inoculated with several mycelial plugs taken from 7-11 day-old fungal colonies grown on PDA or PCA. Plates were incubated at room temperature for 8-13 days in stationary condition. To harvest mycelia, the medium was decanted and agar plugs were removed. Mycelial mats were rinsed three times with sterile distilled water and blot dried. Harvested mycelia were freeze-dried prior to DNA extraction. DNA was extracted with DNeasy Plant Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

The amplification of nuLSU rDNA was performed with primers LR0R and LR5 (Vilgalys & Hester, 1990). The total ITS region and 5.8S subunit of the rRNA gene cluster was amplified with primers ITS1 and ITS4 or with ITS 1-F and ITS4 (Gardes & Bruns, 1993; White *et al.*, 1990). The amplification of DNA fragments, in general, was followed to the standard procedures (White *et al.*, 1990; Newton & Graham, 1994) and it was carried out in Perkin Elmer PCR thermocycler (Perkin Elmer Cetus, Norwalk, USA). The amplified fragments were separated by

electrophoresis in 1 % agarose gels, stained with ethidium bromide and visualised under UV light.

The PCR products of ITS/5.8S region were purified with QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and direct sequencing was performed in both directions with ITS1, ITS2, ITS3 and ITS4 primers (White *et al.* 1990). Amplified fragments of partial LSU were ligated into the pCR4-TOPO vector using TOPO TA Cloning Kit (Invitrogen, UK) according to the manufacture's instruction and positive clones were screened directly by PCR with the same primer set used for initial amplification. Plasmid preparation for sequencing was done by QIAprep Spin Miniprep columns (Qiagen, Crawley, UK) and sequencing of plasmid DNA was performed with primer pairs T7/M13R and LR3R/LR3 (Vilgalys & Hester 1990). All the sequencing reactions were carried out on a Mega BACE 1000 capillary sequencer (Amersham Pharmacia Biotech, Piscataway, USA) at the Department of Animal Breeding & Genetics at the Swedish University of Agricultural Sciences in Uppsala, Sweden.

Sequence analyses

Sequences were manually edited using SeqMan in the DNASTar computer package (Lasergene, Madison, US). The multiple sequence alignments were undertaken using Clustal W method available in MagAlign program in DNASTar and sequence pair distances were determined between aligned sequences using the same program.

Phylogenetic analyses of sequences were performed on three alignment sets. Alignment 1 of nuLSU rDNA sequences was created by incorporating taxa sequenced in this study into an alignment file containing 71 diaphorthean taxa available in TreeBase (accession number S815) deposited by Castlebury *et al.* (2002). To this data set, sequences of the homologous region from *Hapalocystis occidentalis* (AY616231), *H. berkleyi* (AY616231) and *Gnomonia petiolorum* (AY818963) available in GenBank were also added, comprising 88 taxa in total. The second alignment set of nuLSU rDNA consisted of sequences from five *Gnomonia* species sequenced in this study as well as of those from *G. petiolorum* (AY818963) and *H. occidentalis* (AY616231), incorporated in an alignment file originally containing 47 taxa from seven ascomycetous orders (TreeBase accession number M2046; Réblová & Seifert, 2004). The third alignment was created of nucleotide sequences of the total ITS/5.8S region from six newly sequenced *Gnomonia* species and from the sequences of homologous region from 14 diaphorthean species representing nine genera available in GenBank.

To infer phylogenetic relationships among *Gnomonia* species and *Diaphortheales* the alignments were subjected to Maximum Parsimony (MP) and to Bayesian analysis. MP analysis was performed using PAUP (version 4.0b10; Swofford 2002) computer software in general followed the settings as described by Castlebury *et al.* (2002) and Réblová & Seifert (2004). Bayesian analysis was performed in general following to the steps as described by Voglmayr, Fatehi & Constantinescu (2006).

Transformation of *G. fragariae* and study of plant infection

Gnomonia fragariae was transformed with GFP gene in order to obtain a tool to investigate the infection and colonization of strawberry roots and petioles (Paper **III**).

Transformation with GFP gene

Protoplasts of strain UN22 were transformed by a polyethylene glycol/CaCl₂ mediated transformation procedure with two vectors (pIGPAPA and pCT74) containing the hygromycin resistance gene (hph) as a selective marker and the synthetic green fluorescence protein gene (sGFP – TYG) as a reporter marker. The transformation in general was followed to the protocol of Kistler & Benny (1988), modified by Mes *et al.* (1999) with some additional modifications (Paper **III**). Hygromycin B resistant colonies were screened in a selective medium containing hygromycin B and the expression of GFP gene was verified under an epifluorescent microscope. Clonies expressing bright GFP were selected for further evaluation and comparisons with wild type. Before plant inoculation experiments pathogenicity of the transformants was tested and compared with the wild type strain in detached leaf assay as described in Paper **I**.

Plant inoculation and microscopic examinations

Micropropagated strawberry plants of cultivar ‘Korona’ (MTT, Agrifood Research, Finland) were inoculated with GFP-transformant and wild type strain by root dipping in ascospore suspension (10⁵ spores per ml) for 2 h. Experimental procedure and conditions were in general followed as described in Paper **I**.

Development of symptoms was evaluated during each time point and final disease assessment was performed as described in Paper **I**. 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 two weeks until eighth week. Until appearance of obvious symptoms whole roots were placed on glass slides, covered with a cover glass and examined. Crowns first were examined from the surface under the microscope, then cut lengthwise in thin slices and middle slices were examined. Later, symptomatic parts of roots, crowns and petioles were selected for microscopy. Microscopic observations were performed with Leitz Aristoplan fluorescence microscope equipped with appropriate filter blocks and Canon E4500 digital camera.

Screening of *Fusarium* spp. for biocontrol

During the initial survey for possible fungal causes of strawberry decline in Latvia (Paper **I**) about 50 *Fusarium* spp. strains were originated from diseased and healthy strawberry roots and crowns. It has been suggested (Cooke, 1993) that microbial strains originally isolated from roots or rhizosphere of specific plants can

be effective antagonists against their diseases because they are already adapted to the host and environmental conditions where they occur naturally. Thus, the *Fusarium* spp. strains originated from strawberry roots were subjected to initial *in vitro* screening for their antifungal properties against several plant pathogens, including *G. fragariae*. Tests were performed in dual cultures on PDA plates and growth inhibition was evaluated after one week of incubation at room temperature. Strains showing clear inhibition of pathogen growth were selected for further studies to evaluate their pathogenicity on strawberry and possible ability to suppress strawberry root rot caused by *G. fragariae* (Paper **IV**).

The pathogenicity to strawberry and ability of *Fusarium* sp. strains to reduce strawberry root rot was tested by the use of non-sterile or semi-sterile experimental set up in four separate experiments in greenhouse conditions. Cultivars 'Zefyr' or 'Korona' were used in this study depending on experiment. In the non-sterile experimental set up, runner plants rooted in peat were inoculated with *Fusarium* spore suspension applied into the soil around roots and the plants were grown in non-sterile potting mixture. In the semi-sterile experimental set up, aseptically grown micropropagated plants were inoculated by root dipping in *Fusarium* spore suspensions and grown in steam-treated potting mixture. The isolate UN22 of *G. fragariae* was used in biocontrol experiments as pathogen. Pathogen inoculum was prepared on ryegrass seeds as described in Paper **I** and mixed into the soil prior planting. In all experiments appearance of disease symptoms was monitored once a week and disease severity evaluated at the end of experiments (Paper **IV**). In order to evaluate general plant development runners were counted and fresh and dry weights of roots and shoots were measured at the end of experiments.

Results and Discussion

Disease symptoms in the surveyed fields

During the survey on strawberry decline (Paper I) twenty one field in Latvia and four fields in Sweden with root and crown rot problems were identified in several locations (Fig. 1). Strawberry plants in these fields showed wide range of symptoms which could be assigned to the disorders typical for black root rot or root rot complex (Wing, Pritts & Wilcox, 1994) and other pathogens, such as *Verticillium* spp. and *Phytophthora* spp. (Maas, 1998). However, the particular combination of symptoms took our attention and they were as follows: severe stunt and collapse of plants from one side; discolouration and collapse of outer leaves often in combination with upwards developing black rot on petiole bases, black lesions on roots and discolouration in crown (Fig. 1).

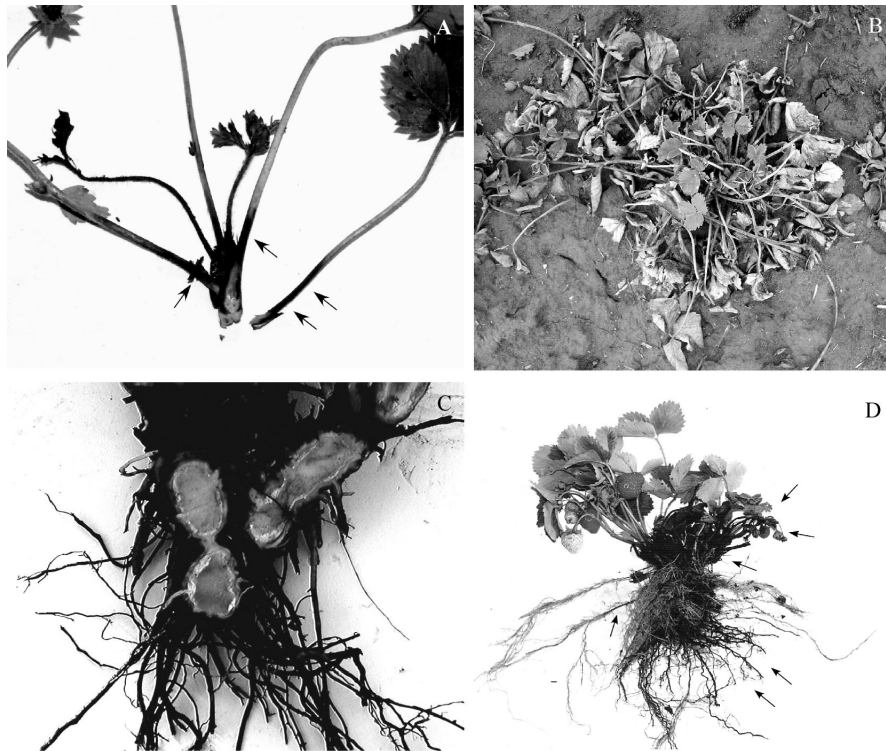


Fig. 1. Typical disease symptoms of strawberry plants infected with *G. fragariae* in the field: (A) black rot on petiole bases; (B) collapsed outer leaves; (C) black root rot and discoloration in crown; (D) severe stunt, black rot on roots and collapse of plant from one side.

Fungi associated with strawberry root rot complex

A range of fungi were recovered from diseased strawberry plants during the screenings and collections performed in the years 2001-2004 and they are listed in Table 1. The range of fungi obtained in our collections was similar to the fungal spectrum isolated from strawberries in other studies (Strong & Strong, 1931; Parikka, 1981; Parikka & Kukkonen, 2002).

Table 1. Fungi isolated from strawberry roots, crown and petioles collected in Latvia and Sweden in the years 2001 to 2004.

Fungi	Strains	Plant tissues which isolated from		
		roots	crowns	petioles
<i>Fusarium</i> spp.	80	√	√	√
<i>Cylindrocarpon</i> spp.	76	√	√	-
<i>Gnomonia fragariae</i>	68	√	√	√
<i>Verticillium</i> spp.	37	√	√	-
<i>Phoma</i> spp.	22	√	√	-
<i>Rhizoctonia</i> spp.	14	√	√	-
<i>Botrytis</i> sp.	18	√	√	-
<i>Hainesia lythri</i>	10	√	√	-
<i>Gnomonia comari</i>	9	-	-	√
<i>Pythium</i> sp.	7	√	√	-
Other	222	√	√	√

During the initial pathogenicity tests in detached-leaf assays 41 *Cylindrocarpon*, 17 *G. fragariae* (Paper I), 10 *H. lythri*, 7 *Verticillium* and 2 *Fusarium* isolates caused necrosis on strawberry leaves. Tested fungi were re-isolated from surface sterilised symptomatic tissues. Necrosis on leaves inoculated with *G. fragariae* and *H. lythri* appeared 2-3 days after inoculation and leaflets became completely rotten within 1-2 weeks followed by pathogen sporulation (Paper I). Based on aggressive behaviour in detached leaf assays *H. lythri* and *G. fragariae* (Paper I) were subjected for pathogenicity test on strawberry plants. In the greenhouse test *H. lythri* caused severe root and crown rot and was re-isolated from diseased tissues. *Hainesia lythri* is well known as a cause of tan-brown rot on strawberry fruits which can be very destructive (Maas, 1998). However, this fungus was isolated only from one field in Latvia and its role as a root infecting pathogen on strawberry still needs to be evaluated.

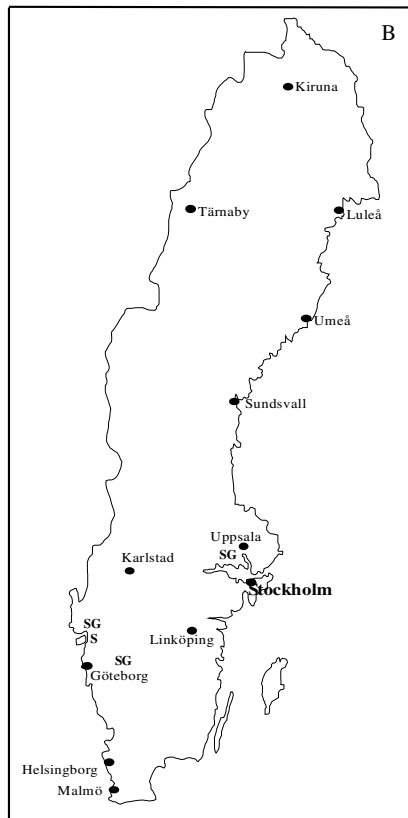
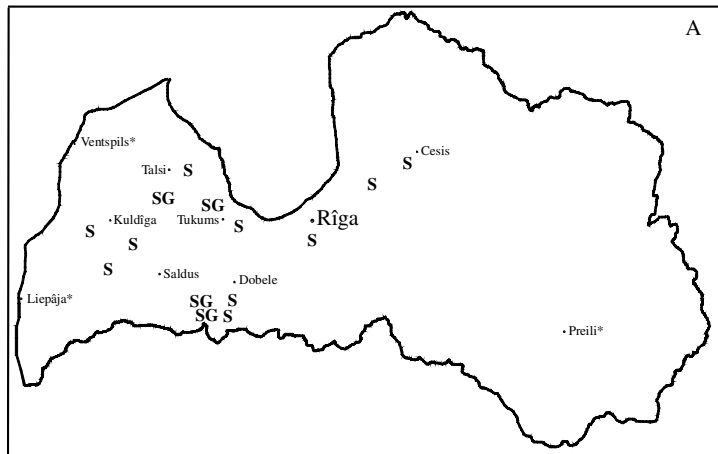


Fig. 2. Map of Latvia (A) and Sweden (B) showing locations of strawberry fields (Paper I) where diseased plants were sampled. **S** shows location of sampling sites; **SG** refers to the locations where *G. fragariae* was isolated or collected on petioles. Places marked with asterix (*) refer to district in Latvia where *G. fragariae* was isolated or collected on petioles after year 2004.

Contrary to expectations based on strong symptom indications (Paper I) for possible *Phytophthora* presence in diseased strawberry fields, no *Phytophthora* was isolated on selective medium or any sporangia were formed when diseased tissues of roots and crowns were incubated in moist chambers or in water. Moreover, *Phytophthora* oospores were not observed in severely diseased root and crown tissues when they were examined microscopically, thus indicating other causes for these types of symptoms (Paper I).

Since *G. fragariae* was repeatedly recovered from severely diseased plants showing certain symptom combinations in several locations, further studies were carried out on its pathogenicity on strawberry (Paper I).

***Gnomonia fragariae*, a cause of strawberry root rot and petiole blight (Paper I)**

Thirty six isolates of *G. fragariae* were obtained from eight strawberry fields in Latvia and 32 isolates from three fields in central and southern part of Sweden (Fig. 2). The fungus was isolated alone or associated with other known pathogenic fungi such as *Cylindrocarpon*, *Fusarium*, *Verticillium* and *Rhizoctonia* from strawberry roots, crowns and petioles from 2-4 year-old plants mostly in farms with long history of strawberry production. The recovery of *G. fragariae* from diseased tissues highly correlated with symptoms shown in Figure 1. Moreover, numerous ascomata of *G. fragariae* were found on the necrotic bases of petioles on such plants collected in May, June, July, September and November both in Latvia and Sweden.

The morphology of ascomata, asci and ascospores of *G. fragariae* isolates on agar media and those found on naturally infected plant tissues was in agreement with the characteristics of *G. fragariae* described by Klebahn (1918), Bolay (1972) and Monod (1983). When our collection was compared with herbarium specimens collected by Bolay (1972), and also examined by Monod (1983), no considerable morphological differences were observed. The only exception was the ascospore appendages that were longer and showed greater variation in the length among our specimens (Paper I). Attempts to find any specimens of *G. fragariae* deposited or identified by Kleban were not successful.

The pathogenicity tests of *G. fragariae* on strawberry plants performed in greenhouse experiments clearly showed that all the isolates tested were pathogenic and capable of causing severe root rot and petiole blight on strawberry. Disease symptoms developed in the greenhouse experiments (Fig. 3) strongly resembled those observed on infected plants in the field. In the greenhouse experiments *G. fragariae* was able to infect and cause the disease by both mycelium when applied into the soil or around roots and with ascospores when roots were dipped in suspension for 2 h. The fungus did not cause rapid plant death, but growth and development of inoculated strawberry plants was severely affected (Fig. 3). This also corresponded to our observations from infected fields that rapid death was not noticed but plants became severely stunted in longer period of time. No fruiting bodies of *G. fragariae* were found on roots or petioles of inoculated plants in the

greenhouse, but they were formed within two months when infected tissues were incubated in the moist chambers on a laboratory bench. *Gnomonia fragariae* was recovered from roots and petioles of all the plants that developed disease symptoms.

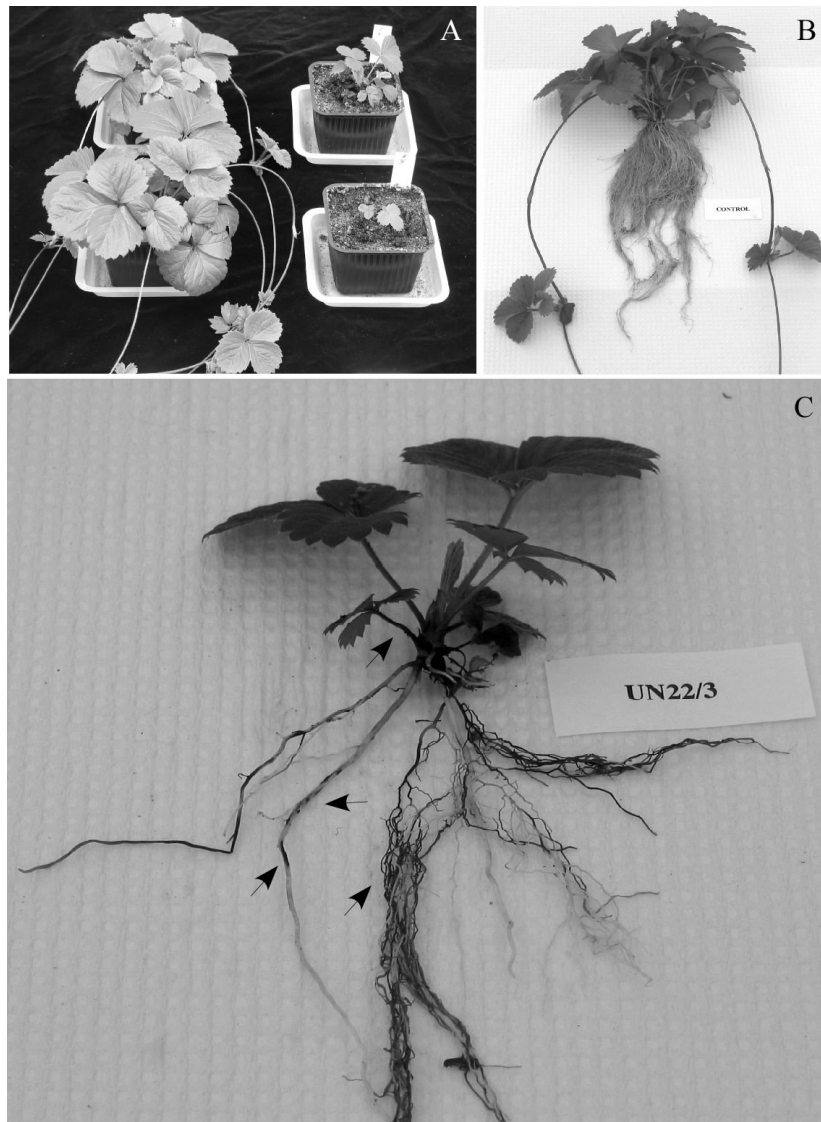


Fig. 3. Symptoms on strawberry plants cv. 'Zefyr' caused by *Gnomonia fragariae* in the greenhouse 11 weeks after inoculation: (A) Severe stunt of plants inoculated by root dipping in ascospore suspension (left) in comparison to control plants (right); (B) Healthy control plant with well developed root system and runners; (C) Typical appearance of black rot on roots and on petiole bases.

A severe strawberry petiole blight caused by *G. comari* was reported by van Adriechem & Bosher (1958). Symptoms described in that study particularly yellowing and collapse of outer leaves and black discoloration girdling the basis of petioles are very similar to those caused by *G. fragariae* and observed during this work. Numerous ascomata were also found on affected petiole bases in the field. However, in that study inoculation of *G. comari* on roots did not cause any disease symptoms. During the survey *G. comari* was found only twice on diseased petioles together with *G. fragariae* on the same plants in Latvia. This indicates that in the field both *Gnomonia* species can attack strawberry petioles and cause similar symptoms, but *G. fragariae* is also capable to cause root rot.

Obtained results of this study showed that *G. fragariae* is a serious pathogen involved in root rot complex of strawberry in Latvia and Sweden. To my knowledge this was the first time when pathogenicity of *G. fragariae* on strawberry has been evidently demonstrated.

Molecular characterization of *Gnomonia fragariae* (Paper II)

The nucleotide sequence analyses of the ribosomal RNA genes have been proved to be of great value to characterize relationships of fungi at both species and genus level as well as to infer phylogeny of different taxonomic ranks in fungi (Bruns *et al.* 1992; Bruns, White & Taylor 1991; James *et al.*, 2001; Moncalvo *et al.* 2002). Initial aim of the study presented in Paper II was to characterize *G. fragariae* isolates originating from strawberry at different geographical localities in Latvia and Sweden by using molecular analyses of nucleotide sequences of ITS/5.8S rDNA and to compare it with few other *Gnomonia* species. However, as found out during the first analyses, there was more extensive work needed, which is described in Paper II and shortly below.

The isolates of *G. fragariae* examined in this study appeared to be genetically uniform in nuLSU rDNA sequences, but more divergent based on nucleotide sequences of ITS region. The variation of the ITS region was mostly due to the presence of the variable GC rich, short fragments located at ITS1 and ITS2. However, the analysis of this region did not group the isolates based on their geographic origins or host tissues from which they were isolated.

When LSU sequences of *G. fragariae* were subjected to BLAST search in the GenBank for homologous sequences, it revealed very high similarity to homologous region of *Hapalocystis occidentalis* W.M. Jaklitsch & H. Voglmayr and *H. berkeleyi* Auersw. ex. Fuckel, but not to any members of *Gnomoniaceae*. Moreover, the BLAST search for homologous sequences of ITS region of *G. fragariae* did not find similarity to any members of *Diaporthales* available in the GenBank. The subsequent phylogenetic analyses of partial LSU and ITS/5.8S regions performed with both MP and Bayesian analyses clearly showed that *G. fragariae* was genetically distinct from *G. gnomon*, the type species of *Gnomonia* and several other species of this genus, and other members of *Gnomoniaceae* studied so far. The results of initial blast search and further phylogenetic analysis clearly demonstrated that *G. fragariae* was closely related to *Hapalocystis*

occidentalis and *H. berkleyi*. *Gnomonia fragariae*, *G. rubi*, and *G. rosae* together with *H. occidentalis* and *H. berkeleyi* formed a separate, well-supported clade as a sister group to other *Diaporthales* while *G. comari*, *G. rostellata*, and *G. petiolorum* fell within *Gnomoniaceae* clade as it would be expected (Fig. 4).

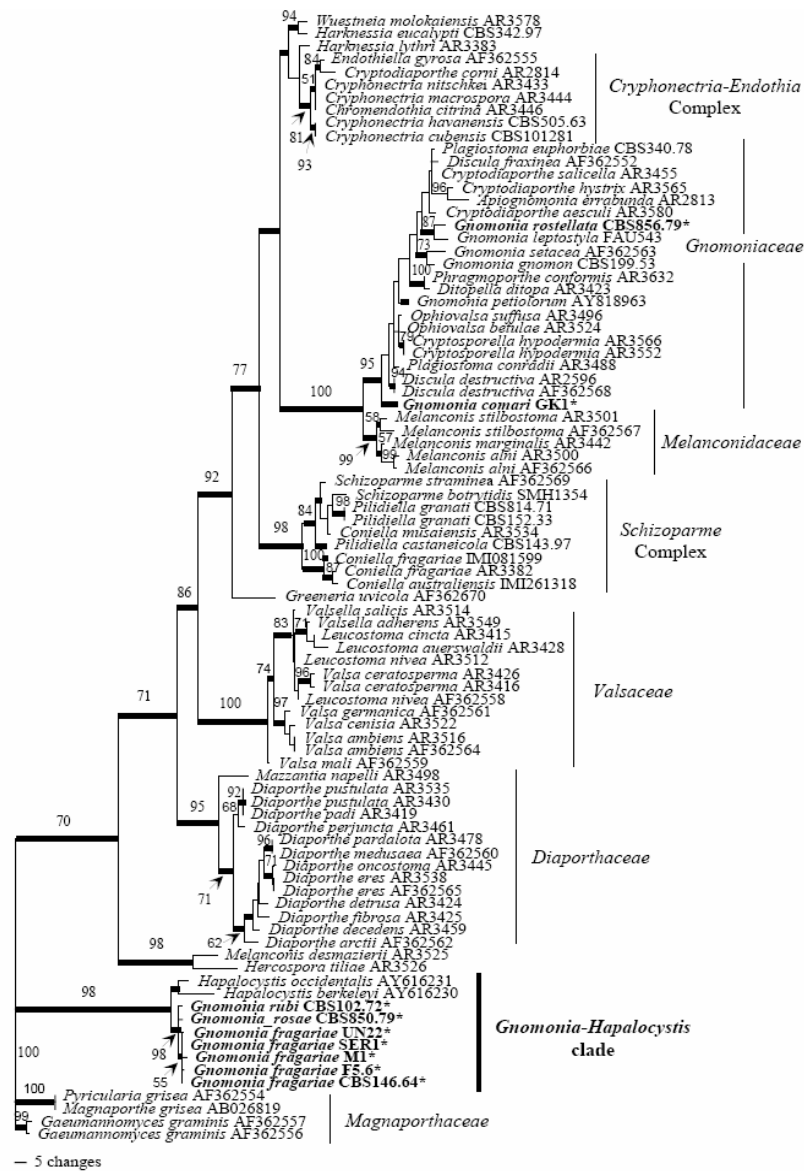


Fig. 4. One of 280 most parsimonious trees (length = 634) from MP analysis based on partial nuLSU rDNA sequences of 82 diaphorthalean taxa. Taxa in bold and marked with asterisk (*) represent sequences obtained during the current study. Numbers above lines represent bootstrap values higher than 50 %. Thickened lines indicate branches that appeared in strict consensus of the 280 trees (Paper II).

Morphological characters of the *Gnomonia* isolates examined in this study were in concordance with the descriptions given by Monod (1983) and evidences which could support results obtained in molecular analyses were not found. The only exception was *G. rosae* isolate (CBS 850.79) which morphology was completely different from Monod's (1983) description, although the isolate has been originally identified and deposited to CBS by Monod. The sequence analyses and morphological characters suggested that it might belong to *G. rubi*.

Hapalocystis occidentalis is a recently described species and it has been found on dead twigs of *Platanus occidentalis* in Tennessee, North America (Jaklitsch & Voglmayr 2004). The genus *Hapalocystis* has been characterized by having fused beaks in ascospores, forming a reduced stromatic disk and having ascospores with elongated and strap like appendages (Jaklitsch & Voglmayr 2004). The relatively long, gelatinous appendages are the only apparent morphological characters, which are shared by *G. fragariae* and *H. occidentalis*. Jaklitsch & Voglmayr (2004) stained the appendages and gelatinous outer integument of *Hapalocystis* to deep pink colour by using toluidine blue and they suggested that this might be a key characteristic feature for this genus. *Gnomonia fragariae* ascospores were also stained with similar dye in this study, but such a colour was not observed. Moreover, a very thin sheet-like coat was present around these ascospores as well as those of *G. comari*. Therefore the presence of such gelatinous outer integuments around ascospores might not to be specific only to *Hapalocystis* or *G. fragariae*, but might be a more common feature in other members of *Gnomoniaceae*.

On the basis of analyses performed it can be suggested, that the *Gnomonia/Hapalocystis* clade represents a genetically distinct group, possibly representing a new family within *Diaporthales*. The study also demonstrates that *Gnomonia* isolates of this clade are genetically distant from the other *Gnomonia* species within *Gnomoniaceae*. However, so far no evident morphological features have been found to support the separation of these two *Gnomonia* groups. A number of other *Gnomonia* species originating from rosaceous hosts included in this study, supports the polyphyly of the genus *Gnomonia* and suggests that the delineation of these taxa need to be reconsidered. Moreover, this study also supports a need for the re-evaluation of the current generic concepts in the *Diaporthales*, as it has been pointed out previously by Castlebury *et al.* (2002, 2003) and Zhang & Blackwell (2001).

The genetic deviation in *Gnomonia* and the presence of genetically distinct groups within this taxon, possibly, may be explained by the differences in their biology and nature of the groups. Typically, *Gnomonia* species do not attack plant roots. They are known as pathogens of aerial constituents of their hosts (Barr 1978). However, as recently shown, there are *Gnomonia* species, that severely attack host plant roots (Amsing 1995; Paper I). Schneider, Paetzholdt & Willer (1969) also showed that, besides cane canker of roses, *G. rubi* is capable to cause crown rot as well. The pathogens belonging to genus *Gnomonia* and infecting rosaceous hosts, in general, are poorly studied and little is known about their biology and pathogenic properties. The biological features in combination with morphological and molecular information from more *Gnomonia* may help to understand the generic and species concepts in these taxa.

As it was mentioned before, Barr (1978) in the study on *Diaporthales* from North America questioned the identity of *G. rubi* as a separate species or as a four-spored form of *G. rostellata*. Monod (1983) and later on Barr (1991) considered *G. rubi* as a synonym of *G. rostellata* by having asci with four ascospores or eight spores of which four are degenerated. The results of molecular analyses in this study clearly showed, that the two isolates received as *G. rubi* and *G. rostellata* were genetically different and they grouped in two phylogenetically distinct clades, despite high morphological similarities. However, the colony appearance and the growth rate of these two isolates were different. High variation in colony characters such as colour and growth rate among isolates of *G. rubi* has been reported by Schneider, Paetzholdt & Willer (1969) in a study on a serious dieback of brambles and roses. In that study the colonies of *G. rubi* from roses and brambles in Pinneberg were dark olive-green to dark brown, but those obtained in South Germany were pale yellow on PDA. The same differences in colony appearance on PDA were also noticed between the *G. rubi* and *G. rostellata* isolates in our work. This suggests that Schneider *et al.* (1969) might have been dealing with two different species in their work. Though only one isolate of each species was examined, it can still be suggested that there are two genetically distinct, but morphologically very similar species occurring on same hosts in nature. This has to be further clarified by examining more specimens and by the use of both morphological and molecular means.

Transformation of *Gnomonia fragariae* with GFP gene and the study of plant infection (Paper III)

The aim of the study described in Paper III was to transform strawberry pathogen *G. fragariae* with the GFP gene in order to investigate the process how the pathogen invades and colonizes the roots and petioles of its host. The use of GFP-tagged strains for plant inoculation serves a tool to monitor the pathogen in the host plant tissues by fluorescence microscopy without disturbance by staining and fixation of the sample.

Transformation and selection of a stable transformant

Gnomonia fragariae was successfully transformed by polyethylene glycol/CaCl₂ procedure with two reporter gene constructs pCT74 and pIGPAPA. These vectors previously have been successfully applied for constitutive expression of GFP in several other plant pathogenic ascomycetes (Jansen *et al.*, 2005, Lorang *et al.*, 2001; Horwitz *et al.*, 1999). Although transformation was successful with both vectors, transformants with pCT74, that expresses sGFP under the control of the ToxA gene promoter from *Pyrenophora tritici-repentis*, revealed slightly weaker fluorescence intensity in comparison to transformants with pIGPAPA in which sGFP – TYG is flanked with the constitutive expression promoter of the isocitrate lyase gene (IL) of *Neurospora crassa*. Thus, a transformant with pIGPAPA was chosen for further inoculation studies.

The GFP expression by modified fungi generally does not affect phenotype and pathogenicity of the modified fungi as shown previously in several studies (Sarocco *et al.*, 2006; Visser *et al.* 2004; Nahalkova & Fatehi, 2003; Horowitz, Freeman & Sharon, 2002; Lagopodi *et al.*, 2002; Lorang *et al.*, 2001; Nonomura *et al.*, 2001; van West *et al.*, 1999; Maor *et al.*, 1998; Spelling, Bottin & Kahmann, 1996). Similarly, the comparisons of transformants with wild type strain in this work revealed that the transformation did not affect colony morphology, growth rate or sporulation of the fungus and transformants showed stable GFP expression after several transfers on agar media with or without antibiotic pressure. Moreover, *G. fragariae* retained its pathogenicity when initially tested in detached-leaf assay and subsequently on strawberry plants. One of stable transformants, pIG-2, was selected on the basis of brightest fluorescence, no differences in growth rate and morphology in comparison to the wild type, the ability to cause symptoms on strawberry leaves in detached-leaf assay and typical appearance after the re-isolation from host plant tissues.

Early stages of the disease and microscopic observations

As early stages of the disease development the first 10 days were considered. During this time microscopic examinations were mostly focused on roots. Plant sampling and microscopic observations started 24 h after inoculation with ascospores. Ascospores of *G. fragariae* was possible to detected by fluorescence microscopy 24 h after inoculation all along the primary and lateral roots attached in random places of all the roots examined. Although, ascospores of *G. fragariae* were attached all along the primary and lateral roots in random places, they were more concentrated to the middle and younger regions of primary roots which later correlated with appearance of symptoms on the roots.

Microscopic observations of whole roots 24 h after inoculation showed that penetration occurs very quickly. Majority of the ascospores were germinated at this stage and invaded roots directly penetrating at the junctions of epidermal cells or continued to elongate superficially on the root surface. Hyphae often appeared swollen at infection sites and penetration peg originating from swollen hyphal tips penetrated the cells. During the first 48 h, developing hyphae sensed and grew along the junctions of epidermal cells and showed preference to penetrate root tissues at the junctions. The specific infection structures such as appressoria were not formed, however hyphal swellings at infection sites and subsequent formation of penetration pegs showed similarities to infection structures characteristic for some root infecting pathogens (Sesma & Osbourn, 2004; Mendgen, Hahn & Deising, 1996). Attachment of spores and penetration was also observed at lateral root tips, but it seems to be rather coincidence than clear preference of fungus for infection site since it was observed only in few cases.

The penetration and further development of *G. fragariae* on the root surface took place during the first week dramatically decreasing after day tree. Within three days the fungus was inside cells and started to proliferate longitudinally in the epidermal cells of strawberry roots. The growth and proliferation of hyphae on root surface discontinued when the fungus was inside the root tissues. Both intra- and

intercellular growth in plant tissues was observed. The intracellular hyphae were swollen at the cell walls with constrictions in places where they crossed the wall. These hyphae were thinner and less fluorescent than intercellular, but they revealed intensive fluorescence in swollen structures indicating high activity of the fungus in places where it penetrated adjacent cells. Similarly, the indication of high enzymatic activity of the fungus during its spread within host plant tissues was observed also in later stages of infection. The walls of cortex cells were completely disrupted at places where hyphae of the fungus crossed the cell walls.

During the first 10 days the infection of strawberry plants appeared to be symptomless despite the invasive growth of the fungus. During this stage of infection only slightly brown discoloration on roots was observed, but such discoloration was present also on control plants and the pathogen infection was detected only in 50 % on discoloured tissues, indicating that discolouration on roots was caused by other factors (e.g. mechanical root damaging during planting).

Advanced stages of the disease and microscopic observations

Ten to 12 days after inoculation plants treated with both, wild type and GFP transformed strain, exhibited initial signs of the disease. First symptoms appeared as small, dark brown and black lesions on primary and lateral roots, blackening of root tips and delay of root and shoot development when compared with control plants. Microscopic observations of discoloured root segments and healthy looking tissues around them confirmed that symptomatic roots were extensively colonized by the fungus. The hyphae were deep inside in cortical tissues close to the stele and spreading longitudinally. At the places, where hyphae spread from one cell to another, lysing of cell walls was also observed. Invasion of vascular tissues was not observed at this stage.

As revealed by fluorescence microscopy examination of longitudinal and cross sections of symptomatic segments of roots and petioles, the pathogen intensively colonized and destroyed cortex of roots and petioles, and rapidly spread longitudinally within intercellular spaces. Contrary to root infection pattern, intracellular growth was predominant in the petioles while intracellular growth in the roots was apparent only in the late stages of the disease. The fungus was capable to infect also vascular tissues of petioles at later stages of infection and spread quickly into main veins of leaflets. However, these did not seem to be the primary tissue of colonization. In late stages of plant infection the fungal hyphae were not detectable in dead tissues, but abundant fluorescent hyphae were present in adjacent layers suggesting, that the fungus had withdrawn cytoplasm from hyphae in the dead areas.

Observations of the pathogen development in root and petiole tissues suggests, that the petioles are more sensitive to the pathogen infection than roots resulting in more severe damages in shorter period of time. Once the fungus had infected the petioles, the colonization of the cortex was rapid and massive. The fungus rapidly degraded the cortex, hollowing out the petiole and invaded also vascular tissues which all together resulted in collapse of leaves. Longitudinal sections of crowns showed that the places where petioles are attached to the crown are points where

the petiole infection starts. According to the observations on root infection pattern one of the hypotheses could be that the fungus reached the crown through intercellular spaces of epidermal tissues from upper portions of infected roots and invaded the crown and petioles. However, this needs to be further clarified.

The lesion development places on roots were in agreement with microscopic observations of initial infection and colonization sites which were predominately the middle portions of primary roots. Although in early stages fungal spores were observed mostly freely in the root hair zone, the obvious appearance of necrosis as first symptoms also on upper parts of the roots indicates, that infection of root hairs may play more significant role during the initial infection of the root. After the appearance of first typical symptoms the disease developed rapidly resulting in severe damages to both root system and shoots. The symptoms caused to strawberry plants did not differ between GFP transformed and wild type strain and the disease developed in similar pattern. However, the sporulation on diseased petiole bases of wild type was observed one week earlier and in a greater extent than on plants infected with transformant. This could be explained also by not completely even conditions in the growth chamber, which might have been influenced the sporulation of the fungus since other considerable differences were not observed. Perithecia, asci and ascospores which were formed on infected plants with GFP transformed *G. fragariae* revealed strong fluorescence demonstrating that GFP could be stably transmitted through fungal progenies confirming the results obtained *in vitro*.

Effect of non-pathogenic *Fusarium* spp. on root rot (Paper IV)

Two strains named A37 and A38 were selected for further biocontrol experiments among the range of *Fusarium* spp. strains screened in pilot *in vitro* tests for possible biocontrol properties and evaluation for pathogenicity on strawberry plants. Based on no pathogenicity observed and a constant re-isolation from strawberry roots they were considered as non-pathogenic colonizers of strawberry roots. It has been hypothesized that disease-suppressing microbial strains originally isolated from roots or rhizosphere of specific plants can be effective antagonists to the diseases on a particular plant because they are already adapted to the host and environmental conditions where they occur naturally (Cooke, 1993). Therefore two *Fusarium* sp. strains were further evaluated for their potential to reduce strawberry root rot caused by *G. fragariae* (Paper IV).

Pre-inoculation of plants with both *Fusarium* sp. strains A37 and A38 considerably reduced disease severity in two experiments performed in non-sterile experimental system. However, the disease suppression effect of the isolates was reduced when semi-sterile experimental set up was used. Reduction of disease severity by A37 and A38 ranged from 26 – 77 % relative to water control in non-sterile experimental system and from 9 – 24 % in semi-sterile system. The strain A38 reduced disease severity more effectively than A37 in both non-sterile and semi-sterile system. The effect of A38 on strawberry plants planted in the soil either mixed with pathogen or no pathogen inoculum applied is shown in Figure 5. The variation of disease suppression obtained in two experimental setups indicated

that other interactions are involved than those between host and antagonist. Since soil inoculation, as used in the non-sterile experimental system, proved to be more efficient than root inoculation, it could be speculated that disease suppression was due to the competition in the soil or on the roots rather than mediated through the plant. However, this indication may not be conclusive since in addition to the inoculation method used, several other factors such as type of soil, strawberry cultivars as well as age of the planting material were different between these two experimental setups.

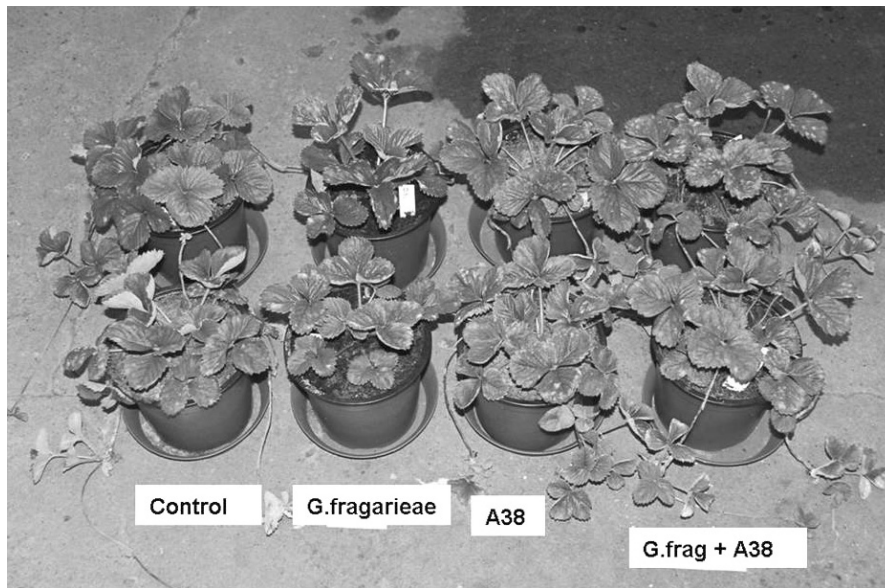


Fig. 5. Effect of *Fusarium* sp. strain A38 on strawberry plants cv. 'Zefyr' in non-sterile experimental system 8 weeks after planting in soil mixed with *G. fragariae* inoculum and with no pathogen inoculum applied.

The unexpected high disease severity and appearance of black root rot symptoms on roots of control plants in the non-sterile system experiments indicated that the potting mixture used already contained pathogens or, more likely, that the test plants used in these experiments were already infected with minor pathogens in the nursery. Furthermore, the finding that application of *Fusarium* strains greatly reduced disease severity in the control plants with no *G. fragariae* inoculum, make it probable that the tested *Fusarium* strains had effect also on other pathogens than *G. fragariae* or on plant performance in general. Such a conclusion is strengthened by the results of the pilot *in vitro* screening performed. The isolates A37 and A38 showed strong antifungal activity both against *G. fragariae* and several other pathogens tested.

Besides the disease suppression ability of the beneficial root-associated microorganisms, a stimulation of plant growth has also been observed. Plant growth promotion to a great extent is assigned to different bacteria (Kloepper, Ryu & Zhang, 2004; Whipps, 2001; Chiarini *et al.*, 1997), but it has also been demonstrated for several biocontrol fungi. For example, some species of *Trichoderma*, binucleate *Rhizoctonia* isolates and *Pythium oligandrum* besides their biocontrol properties have also a great ability to enhance plant growth in absence of any of major pathogens (Elad, Kohl & Shtienberg, 2002; Whipps, 2001; Altomare *et al.*, 1999). The non-pathogenic *Fusarium* sp. strains used in this study, in addition to root rot suppression, also showed ability to enhance plant growth and development in the absence of the pathogen.

Conclusions

The major problem of strawberry decline in the surveyed fields in Latvia and Sweden is a root rot complex, in which several pathogenic fungi are involved. Among fungi associated with root rot complex in Latvia and Sweden, *Cylindrocarpon* spp., *Fusarium* spp., *Phoma* spp., *Rhizoctonia* spp. and *Pythium* spp. are the usual causes of these types of disorders world-wide.

Gnomonia fragariae is found as serious pathogen involved in the root rot complex of strawberry in Latvia and Sweden, as a result of this study. The field infected plants with this pathogen present a particular combination of symptoms such as severe stunt and collapse of plants from one side, discoloration and collapse of outer leaves in combination with upwards developing black rot on petiole bases, black lesions on roots and discoloration in crown. In this study for the first time pathogenicity of *G. fragariae* is established and the disease caused by this pathogen is designated as strawberry root rot and petiole blight (Paper I).

Gnomonia fragariae is genetically distinct from the type species of the genus *Gnomonia*, *G. gnomon*, and other members of *Gnomoniaceae*, and the *Gnomonia/Hapalocystis* clade represents a genetically distinct group, possibly a new family, within *Diaporthales*. The re-evaluation of the current generic concepts in the *Diaporthales* is needed as strongly supported by the results obtained in this study (Paper II).

Gnomonia fragariae is an aggressive invader of both strawberry roots and petioles as revealed by studies with GFP tagged strain (Paper III). The cortex of roots and petioles are the most severely affected tissues. The petioles are more sensitive to the pathogen infection than roots resulting in more severe damages in shorter period of time. The pathogen is also able to infect vascular tissues of petioles in the later stages of disease development. Ascospores of the pathogen are highly infective and could be one of major means for disease spread in the field. However the inoculation method by root dipping in ascospore suspension does not represent other possible means of spread of the pathogen, and therefore these aspects need to be further clarified.

Results in Paper III showed that GFP is an efficient marker to visualize *G. fragariae* in strawberry tissues and the use of GFP-tagged fungus is an informative approach to study plant infection and colonization. Moreover, the transformation of the pathogen with GFP gene provides great openings to elucidate other aspects concerning the disease caused to strawberry and the pathogen itself.

The study carried out on two non-pathogenic *Fusarium* sp. strains in greenhouse condition proves that they have beneficial effect on strawberry development and can reduce root rot on strawberry caused by *G. fragariae*. However, the evaluation of disease suppression efficiency and effect on strawberry productivity in actual culturing systems and elucidation of modes of action of these *Fusarium* sp. strains need to be further studied.

References

- van Adriechem, M.C.Y. & Bosher, J.E. 1958. Leaf blotch and petiole blight of strawberry caused by *Gnomonia fructicola*. *Plant Disease Reporter* 42, 772-775.
- Agrios, G. N. 1997. *Plant Pathology*. 4th edition. Academic Press, San Diego, California. 635 pp.
- Alexopoulos, C.J. & Cation, D. 1948. Stem-end rot of strawberries. *Phytopathology* 38, 698-706.
- Alexopoulos, C.J. & Cation, D. 1952. *Gnomonia fragariae* in Michigan. *Mycologia* 44, 221-223.
- Altomare, C., Norwell, W.A., Björkman, T. & Harman G.E. 1999. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Applied and Environmental Microbiology* 65, 2926-2933.
- Amsing, J.J. 1995. *Gnomonia radicola* and *Phytophthora* species as causal agents of root rot on roses in artificial substrates. *Acta Horticulturae* 382, 203-211.
- Anonymous. 1996. Guideline on good plant protection practice. *Bulletin OEPP/EPPO Bulletin* 26, 369-390.
- Armstrong, M.J. & Armstrong, J.K. 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. In: Nelson, P.E., Tousson, T.A., Cook, R.J., (eds.) *Fusarium: diseases, biology and taxonomy*. Pennsylvania State University Press, 391-399.
- Arsenijević, M. & Veselić, M. 1995. *Gnomonia rostellata* (Fr.) Wehm., the pathogen of cultivated blackberry plants (*Rubus fruticosus* L., agg.). *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* 102, 366-374.
- Baker, B., Zambryski, P., Staskawicz, B. & Dinesh-Kumar, S.P. 1997. Signalling in plant-microbe interactions. *Science* 276, 726-733.
- Bao, J.R. & Lazarovits, G. 2001. Differential colonization of tomato roots by nonpathogenic and pathogenic *Fusarium oxysporum* strains may influence Fusarium wilt control. *Phytopathology* 91, 449-456.
- Barr, M.E. 1978. The *Diaporthales* in North America with emphasis on *Gnomonia* and its segregates. *Mycologia Memoir No.7*. J. Cramer Publisher, Lehre
- Barr, M.E. 1991. Revisions and additions to the *Diaporthales*. *Mycotaxon* 41, 287-305.
- 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., Bloembergen, 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.
- Bruns, T.D., Vilgalys, R., Barnes, S.M., Gonzales, D., Hibbet, D.S., Lane, D.J., Simon, L., Stickel, S., Szaro, T.M., Weisburg, G.W. & Sogin, M.L. 1992. Evolutionary relationships within the fungi: analyses of nuclear small subunit rRNA sequences. *Molecular Phylogenetics and Evolution* 1, 231-241.
- Bruns, T.D., White, T.J. & Taylor, J.W. 1991. Fungal molecular systematics. *Annual Review of Ecology and Systematics* 22, 525-564.
- Bushnell, W.R. 1972. Physiology of fungal haustoria. *Annual Review of Phytopathology* 10, 151-176.
- Castlebury, L.A., Farr, D.F., Rossman, A.Y. & Jaklitsch, W.M. 2003. *Diaporthe angelicae* comb. nov., a modern description and placement of *Diaportheopsis* in *Diaporthe*. *Mycoscience* 44, 203-208.
- Castlebury, L.A., Rossman, A.Y., Jaklitsch, W.M. & Vasilyeva, L.N., 2002. A preliminary overview of the *Diaporthales* based on large subunit nuclear ribosomal DNA sequences. *Mycologia* 94, 1017-1031.
- Chen, T.A. & Rich, A.E. 1962. The role of *Pratylenchus penetrans* in the development of strawberry black root rot. *Plant Disease Reporter* 46, 839-843

- Chiarini, L., Bevivino, A., Tabacchioni, S. & Dalmastri C. 1997. Inoculation of *Burkholderia cepacia*, *Pseudomonas fluorescens* and *Enterobacter* sp. on *Sorghum bicolor*: root colonization and plant growth promotion of dual strain inocula. *Soil Biology and Biochemistry*, 30, 81-87.
- Cooke, R.J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. *Annual Review of Plant Pathology* 31, 53-80.
- Cooke, R.J. & Baker, K.F. 1996. *The nature and practice of biological control of plant pathogens*. APS Press, Minesota, USA. 539 pp.
- Dean, R. 1997. Signal pathways and appressorium morphogenesis. *Annual Reviews of Phytopathology* 35, 211-234.
- D'Ercole, N. 1993. Biological control of strawberry root rot through non-pathogenic isolate of *Rhizoctonia fragariae* Hussain and McKeen. *Acta Horticulturae* 348, 515.
- Dhingra, O.D. & Sinclair, J.B. 1995. *Basic Plant Pathological Methods*. CRC Press, US
- Domsch, K.H., Gams, W. & Anderson, T.H. 1993. *Compendium of soil fungi*. IHW-Verlag, Germany. 306 pp.
- Dufresne, M. & Osbourn, A.E. 2001. Definition of tissue-specific and general requirements for plant infection in a phytopathogenic fungus. *Molecular Plant Microbe Interactions* 14, 300-307.
- Duncan, J.M. 2002. Prospects for integrated control of Phytophthora diseases of strawberry. *Acta Horticulturae* 567, 603-610.
- Easterbrook, M.A., Crook, A.M.E., Cross, J.V. & Simpson, D.W. 1997. Progress towards integrated pest management on strawberry in the United Kingdom. *Acta Horticulturae* 439, 899-904.
- Elad, Y., Kohl, J. & Shtienberg, W.J. 2002. The effect of cultural and environmental conditions on the performance of *Trichoderma harzianum* strain T-22. *Bulletin OILB/SROP* 25, 49-52.
- Ellis, M.A., Kuter, G.A. & Wilson, L.L. 1984. Fungi that cause cane cankers on thornless blackberry in Ohio. *Plant Disease* 68, 812-815.
- Eriksson, O.E., Baral, H-O., Currah, R.S, Hansen, K., Kurtzman, C.P., Rambold, G. & Laessle, T. 2001. Outline of Ascomycota-2001. *Myconet* 7, 1-88.
- van Etten, H.D., Sandrock R.W., Wasmann, C.C., Soby, S.D., McCluskey, K. & Wang, P. 1995. Detoxification of phytoanticipins and phytoalexins by phytopathogenic fungi. *Canadian Journal of Botany* 73 (Suppl), 518-525.
- Farr, D.F., Bills, G.F., Chamuris, G.P. & Rossman, A.Y. 1989. *Fungi on Plants and Plant Products in the United States*. APS Press, St. Paul, Minesota
- Farr, D.F., Castlebury, L.A., Rossman, A.Y. & Erincik, O. 2001. *Greeneria uvicola*, cause of bitter rot of grapes, belongs in the *Diaporthales*. *Sydowia* 53, 185-199.
- Freeman, S., Katan, T. & Shabi, E. 1998. Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Disease*, 82, 596-605.
- Freeman, S., Nizani, Y., Dotan, S. & Sando T. 1997. Control of *Colletotrichum acutatum* in strawberry under laboratory, greenhouse and field conditions. *Plant Disease* 81, 749-752.
- Gardes, M. & Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2, 113-118.
- Gubler, W.D. & Feliciano, A. 1999. Occurrence of leaf blotch and stem-end rot of strawberry in the Central Coast California. *Plant Disease* 83, 199.
- Guilleroux, M. & Osbourn, A. 2004. Gene expression during infection of wheat roots by the 'take-all' fungus *Gaeumannomyces graminis*. *Molecular Plant Pathology* 5, 203-216.
- Himerlick, D.G. & Dozier, W.A.J. 1991. Soil fumigation and solarization in strawberry production. *Advances in Strawberry Production* 10, 12-27.
- 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.
- Howard, R.J. 2001. Cytology of fungal pathogens and plant-host interactions. *Current Opinion in Microbiology* 4, 365-373.

- Horwitz, B.A., Sharon, A., Lu, S.-W., Ritter, V., Sandrock, T.M., Yoder, O.C. & Turgeon, B.G. 1999. A G protein alpha subunit from *Cochliobolus heterostrophus* involved in mating and appressorium formation. *Fungal Genetics and Biology* 26, 19–32.
- Jaklitsch, W.M. & Voglmayr, H. 2004. *Hapalocystis occidentalis* – a new species of *Diaporthales* from North America and key to the species of *Hapalocystis*. *Studies in mycology* 50, 229-234.
- James, T.Y., Moncalvo, J.-M., Li, S. & Vilgalys R. 2001. Polymorphism at the ribosomal DNA spacers and its relation to breeding structure of the widespread mushroom *Schizophyllum commune*. *Genetics* 157, 149-161.
- 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*. *PNAS* 102, 16892-16897.
- Kahmann, R. & Basse, C. 2001. Fungal gene expression during pathogenesis-related development and host plant colonization. *Current Opinion in Microbiology* 4, 374-380.
- 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 Askomycyeten*. Verlag von Gebrüder Borntraeger, Leipzig.
- Kloepper, J.W., Ryu, C.M. & Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94, 1259-1266.
- Kurppa, S. & Vrain, T.C. 1989. Effect of *Pratylenchus penetrans* on the infection of strawberry roots by *Gnomonia comari*. *Journal of Nematology* 21, 511-516.
- Liu, C.H., Shang, H.S. & Tan, R.X. 2000. Infection process of take-all causing fungus (*Gaeumannomyces graminis* var. *tritici*) on wheat (*Triticum aestivum*) and oat (*Avena sativa*) roots. *Indian Journal of Agricultural Sciences* 70, 23-27.
- 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. & Bloemberg, G.V. 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., Wolpert, T. J., Johnson, K. B., Rodriguez, R. J., Dickman, M. B. & Ciuffetti, L. M. 2001. Green fluorescent protein is lighting up fungal biology. *Applied and Environmental Microbiology* 67, 1987-1994.
- la Mondia, J.A. & Martin, S.B. 1988. The influence of *Pratylenchus penetrans* and temperature on black root rot of strawberry by binucleate *Rhizoctonia* spp. *Plant Disease* 73, 107-110.
- Maas, J.L. 1998. *Compendium of Strawberry Diseases*, 2nd edn. APS Press, St. Paul, Minnesota, 38-39.
- Maas, J.L. & Galletta, G.J. 1997. Recent progress in strawberry disease research. *Acta Horticulturae* 439, 769-779.
- Maas, J.L., Galletta, G.J. & Ellis, M.A. 1989. Cane canker diseases of thornless blackberry in Eastern United States. *Acta Horticulturae* 262, 205-208.
- 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.
- Martin, F.N. & Bull, C.T. 2002. Biological approaches for control of root pathogens of strawberry. *Phytopathology* 92, 1356-1362.
- Mendgen, K., Hahn, M. & Deising, H. 1996. Morphogenesis and mechanisms of penetration by plant pathogenic fungi. *Annual Review of Plant Pathology* 34, 367-386.
- Mendgen, K. & Hahn, M. 2002. Plant infection and the establishment of fungal biotrophy. *Trends in Plant Science* 7, 352-356.
- Mes, J.J., Wit, R., Testerink, C.S., de Groot, F., Haring, M.A. & Cornelissen, B.J.C. 1999. Loss of avirulence and reduced pathogenicity of a gamma-irradiated mutant of *Fusarium oxysporum* f. sp. *lycopersici*. *Phytopathology* 89, 1131-1137.

- Moncalvo, J.-M., Lutzoni, F.M., Rehner, S.A., Johnson, J. & Vilgalys, R. 2002. Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Systematic Biology* 49, 278-305.
- Monod, M. 1983. Monographie taxonomique des *Gnomoniaceae* (Ascomycètes de l'ordre des Diaporthales I.). *Beihefte zur Sydowia* 9, 1-315.
- Montgomerie, I.G. 1984. Red stele root rot. In: Maas JL (ed.) *Compendium of Strawberry Diseases*. APS Press, St. Paul, Minesota., 79-83.
- 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.
- Newton, C.R. & Graham, A. 1994. *PCR*. BIOS Scientific Publishers Lt., Alden Press Ltd., Oxford, UK.
- Nirenberg, H. 1976. Untersuchungen über die morphologische und biologische Differenzierung der *Fusarium*-Section *Liseola*. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem* 169, 1-117.
- Nonomura, T., Matsuda, Y., Takasugi, M., Ootani, T., Hasegawa, T., Miyajima, K., Hatasa, T. & Toyoda, H. 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., Maeda, K., Matsuda, Y. & Toyoda H. 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 radiculicola*, spec. nov., a new pathogen on roses. *Persoonia* 14, 47-49.
- Nordskog, B., Stensvand, A. & Heiberg, N. 2003. Fungi occurring on aerial constituents of cultivated blackberry (*Rubus fruticosus* L.) in Norway. *Acta Agriculturae Scandinavica. Section B, Soil and plant science* 53, 21-28.
- Olivain, C. & Alabouvette, C. 1999. Process of tomato root colonization by a pathogenic strain of *Fusarium oxysporum* f. sp. *lycopersici* in comparison with a non-pathogenic strain. *New Phytologist* 141, 497-510.
- 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.
- Oliver, R. & Osbourn A. 1995. Molecular dissection of fungal phytopathogenicity. *Microbiology* 141, 1-9.
- Parikka, P. 1981. Strawberry root rot in Finland. *Annales Agriculturae Fenniae/ Seria Phytopathologia* N.85., 20, 192-197.
- Parikka, P. & Kukkonen, S. 2002. Root damage – a very common problem in strawberry production in Finland. *Acta Horticulturae* 567, 643-646.
- Paulus, A.O. 1990. Fungal diseases of strawberry. *HortScience*, 25, 885-888.
- Réblová, M. & Seifert, K.A. 2004. *Conioscyphascus*, a new ascomycetous genus for holomorphs with *Conioscypha* anamorphs. *Studies in Mycology* 50, 95-108.
- Recorbet, G., Steinberg, C., Olivain, C., Edel, V., Trouvelot, S., Dumas-Gaudot, E., Gianinazzi, S. & Alabouvette, C. 2003. Wanted: pathogenesis-related marker molecules for *Fusarium oxysporum*. *New Phytologist* 159, 73-92.
- Salerno, M.I., Gianinazzi, S. & Gianinazzi-Pearson, V. 2000. Effects on growth and comparison of root tissue colonization patterns of *Eucalyptus viminalis* by pathogenic and nonpathogenic strains of *Fusarium oxysporum*. *New Phytologist* 146, 317-324.
- Sarrocco, S., Mikkelsen, L., Vergara, M., Jensen, D.F., Lübeck, M. & Vannacci, G. 2006. Histopathological studies of sclerotia of phytopathogenic fungi by GFP transformed *Trichoderma virens* antagonistic strain. *Mycological Research* 110, 179-187.
- Schneider, R., Paetzholdt, M. & Willer, K.H. 1969. *Gnomonia rubi* als Krankheitserreger an Kühlhausrosen und Brombeeren. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 21, 17-21.

- Seemüller, E. 1984. Crown rot (vascular collapse). In: Maas JL (ed.) *Compendium of Strawberry Diseases*. APS Press, St. Paul, Minnesota., 79-83.
- Sesma, A. & Osbourn, A.E. 2004. The rice blast pathogen undergoes developmental processes typical of root-infecting fungi. *Nature* 431, 582-586.
- Shipton, P.J. 1967. A fruit rot of strawberries caused by *Zythia fragariae*. *Plant Pathology* 16, 123-125.
- Spelling, T., Bottin, A., & Kahmann, R. 1996. Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. *Molecular and General Genetics* 252, 503-509.
- Strong, F.C. & Strong, M.C. 1931. Investigations on the black root rot of strawberries. *Phytopathology* 21, 1041-1060.
- Sutton, J.C. 1994. Biological control of strawberry diseases. *Advances in Strawberry Research* 13, 1-11.
- Swofford, D.L. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*And Other Methods). Version 4.0b10. *Sinauer Associates*, Sunderland, Massachusetts.
- Tezuka, N. & Makino, T. 1991. Biological control of fusarium wilt of strawberry by nonpathogenic *Fusarium oxysporum* isolated from strawberry. *Annals of the Phytopathological Society of Japan* 57, 506-511.
- Thines, E., Anke, H. & Weber, R.W.S. 2004. Fungal secondary metabolites as inhibitors of infection-related morphogenesis in phytopathogenic fungi. *Mycological Research* 108, 14-25.
- Tjamos, E.C. 1981. Virulence of *Verticillium dahliae* and *V. albo-atrum* isolates in tomato seedlings in relation to their host of origin and the applied cropping system. *Phytopathology* 71, 98-100.
- Tsao, P.H. & Ocana, G. 1969. Selective isolation of species of *Phytophthora* from natural soils on an improved antibiotic medium. *Nature* 223, 636-638.
- The Methyl Bromide Phaseout Fact Sheet. Ozone Depletion Rules & Regulations. U.S. Environmental Protection Agency.
http://www.epa.gov/ozone/mbr/FactSheet_2007Request.html (accessed 29-July-2006).
- Vajna, L. 1968. [The occurrence of two fungal pathogens of strawberry in Hungary] In: *Review of Applied Mycology* 47, 317-318.
- Veselić, M. & Arsenijević, M. 1998. Host range of *Gnomonia rostellata* (Fr.) Wehm., the pathogen of blackberry plants (*Rubus fruticosus* L.). *Acta Phytopathologica et Entomologica Hungarica* 33, 291-296.
- Vilgalys, R. & Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* 172, 4238-4246.
- Vinnere, O. 2004. Approaches to species delineation in anamorphic (mitosporic) fungi: A study on two extreme cases. *Acta Universitatis Upsaliensis. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 917. 72 pp. ISBN 91-554-5832-7. ISSN 1104-232X.
- 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.
- Voglmayr, H., Fatehi, J. & Constantinescu, O. 2006. Revision of *Plasmopara* (Cromista, Peronosporales) parasitic on Geraniaceae. *Mycological Research* 110, 633-645.
- Walton, D.J. 1994. Deconstructing the cell wall. *Plant Physiology* 104, 1113-1118.
- Wan, J., Dunning, F.M. & Bent, A.F. 2002. Probing plant-pathogen interactions and downstream defence signalling using DNA microarrays. *Functional & Integrative Genomics* 2, 259-273.
- Waugh, M.V., Ferrin, D.M. & Stanghellini, M.E. 2005. Colonization of cantaloupe roots by *Monosporascus cannonballus*. *Mycological Research* 109, 1297-1301.
- 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.

- van West, P., Reid, B., Cambell, T.A., Sandrock, R.W., Fry, W., Kamoun, S. & Gow, N.A.R. 1999. Green fluorescent protein (GFP) as a reporter gene for the plant pathogenic oomycete *Phytophthora palmivora*. *FEMS Microbiology Letters* 178, 71-80.
- Whipps, J.M. 2001. Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany* 52, 487-511.
- White, T.J., Bruns, T.D., Lee, S. & Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, Y.J. (eds). *PCR protocols: A guide to methods and application*. Academic Press, San Diego, California, pp. 315-322.
- Wilhelm, S. 1984. Black root rot. In: Maas, J.L. (ed.) *Compendium of Strawberry Diseases*. APS Press, St. Paul, Minnesota, pp. 90.
- Wilhelm, S. 1984. *Ceratobasidium (Rhizoctonia fragariae)* root rot. In: Maas, J.L. (ed.) *Compendium of Strawberry Diseases*. APS Press, St. Paul, Minnesota, pp. 90-93.
- Wilhelm, S., Nelson, P.E., Thomas, H.E. & Johnson, H. 1972. Pathology of strawberry root rot caused by *Ceratobasidium* species. *Phytopathology* 62, 700-705.
- Wing, K.B., Pritts, M.P. & Wilcox, W.F. 1994. Strawberry black root rot: a review. *Advances in Strawberry Research* 13, 13-19.
- Wing, K.B., Pritts, M.P. & Wilcox, W.F. 1995. Field resistance of 20 strawberry cultivars to black root rot. *Fruit Varieties Journal* 49, 94-98.
- Yuen, G.Y., Schroth, M.N., Weinhold, A.R. & Hancock, J.G. 1991. Effects of soil fumigation with methyl bromide and chloropicrin on root health and yield of strawberry. *Plant Disease* 75, 416-420.
- Zhang, N. & Blackwell, M. 2001. Molecular phylogeny of dogwood anthracnose fungus (*Discula destructiva*) and the *Diaporthales*. *Mycologia* 93, 355-365.

Acknowledgements

Hereby I would like to express my gratitude to all those who have supported me during my PhD studies and have contributed to this work in one way or another.

First, I would like to thank my supervisor, Dr. **Jamshid Fatehi**, for teaching, guiding and putting me on right tracks during my PhD studies, and constructive critics on my work. Without you this work would never be in the way it is. My deepest respect to you!

Deepest thanks to my supervisor, asoc. professor **Biruta Bankina**, for guiding me in plant pathology starting from MSc studies, and your endless support through my PhD studies.

My supervisors, Professors **Berndt Gerhardson** and **Jan Stenlid**, for support, valuable discussions and constructive comments on my work.

My deepest gratitude to **Swedish Institute, Latvian Council of Science** (Grant D34) and **Strategic Foundation for Environmental Research** (MISTRA) for financial support.

My deepest gratitude to Dr. **Ovidiu Constantinescu**, for help to identify my strains, valuable consultations in mycology, supplying isolates and constructive comments on my manuscripts.

Thanks to Botaniska Analys gruppen and Dr. Jens Levenfors for help in arranging the field trips in Sweden.

I am grateful to Dr. Lennart Jonsson for help in statistical analyses of the data.

Thanks to MASE and former Plant Pathology and Biocontrol Unit people for sharing the lab and great multi-cultural environment.

Thanks to my former Latvian colleagues, Valda, Līga P., Līga L., Sanita, Agrita, Vita, Inese D., Inese B., Jānis and Andris from Pure Horticultural Research Station, for being good colleagues and for kind support during my MSc studies and in the initial stage of this work. Thank you, Valda, for guiding my first steps into the strawberry research.

My deepest thanks to colleagues from former Department of Plant Biology and Protection, Latvia University of Agriculture, especially to Tania and Dzintra for keeping bureaucracy away from me and technical help during my experiments.

My deepest gratitude to Mykopat people who have helped me in one way or another, especially to Karin Backström for kind support in practical and administrative matters.

Thanks to Anda Liniņa, Latvia University of Agriculture, for kindly provided technical facilities during some part of my experiments.

Thanks to my newly gained friends in Sweden, **Olga, Daiga** and **Māris**, for wild parties and for reminding me that there are other things on this earth than just

research. Thank you, Olga, for not just being a great friend, but also for being a good and helpful colleague. My warmest memories to all of you!

My warmest and deepest thanks to my family and friends for always supporting me. Thank you, **Reinis**, for your endless support, patience to listen to my monologs about fungi and research, and supporting me during my long absences. Thank you, **Ilze**, for always willing to hep me with plant sampling in early mornings, sharing creasy ideas and just being a great friend.