RHIZOCTONIA SOLANI AS A POTATO PATHOGEN

- VARIATION OF ISOLATES IN FINLAND AND HOST RESPONSE

MARI J LEHTONEN

Department of Applied Biology
Faculty of Agriculture and Forestry

Viikki Graduate School in Molecular Biosciences

University of Helsinki
Finland

ACADEMIC DISSERTATION IN PLANT PATHOLOGY

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Supervisor: Academy Professor
Jari PT Valkonen
Department of Applied Biology
Plant Pathology Laboratory
University of Helsinki
Finland

Reviewers: Professor (Emer.)
Marjatta Raudaskoski
Laboratory of Plant Physiology and Molecular Biology
University of Turku
Finland

Senior Scientist, PhD
Annemarie Fejer Justesen
Department of Integrated Pest Management
Research Centre Flakkebjerg
Slagelse
Denmark

Opponent: Associate Professor, Associate Dean
Suha Jabaji
Faculty of Agricultural & Environmental Sciences
Plant Science
McGill University
Montreal, Quebec
Canada

Custos: Professor
Minna Pirhonen
Department of Applied Biology
Plant Pathology Laboratory
University of Helsinki
Finland

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals (I – IV).

I. **Lehtonen MJ, Ahvenniemi PM, Wilson PS, German-Kinnari M, Valkonen JPT (2008)** Biological diversity of *Rhizoctonia solani* (AG-3) in a northern potato-cultivation environment in Finland. Plant Pathology 57:141-151.

II. **Ahvenniemi PM, Wolf M, Lehtonen MJ, Wilson PS, German-Kinnari M, Valkonen JPT (2008)** Evolutionary diversification indicated by compensatory base changes in ITS2 secondary structures and incomplete concerted evolution revealed by heterogeneity of ITS sequences in complex fungal species, *Rhizoctonia solani*. (Manuscript, submitted).

III. **Lehtonen MJ, Somervuo P, Valkonen JPT (2008)** Infection with *Rhizoctonia solani* induces defence genes and systemic resistance in potato sprouts grown without light. Phytopathology 98:1190-1198.

IV. **Lehtonen MJ, Wilson PS, Ahvenniemi PM, Valkonen JPT (2008)** Formation of canker lesions on stems and black scurf on tubers in experimentally inoculated potato plants by isolates of AG2-1, AG3 and AG5 of *Rhizoctonia solani* occurring on potatoes in Finland. (Manuscript, submitted)

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ABSTRACT

This thesis describes the results of an extensive survey of *Rhizoctonia solani*, a fungal pathogen of potato, in Finland. Over 500 isolates were collected from potato fields. Nearly all characterized isolates belonged to anastomosis group 3 (AG-3), the most typical strain of *R. solani* infecting potato. Additionally, three other AGs (AG-2-1, AG-4 and AG-5) were found associated with potato plants, as also reported previously in many other countries.

Comparison and phylogenetic analysis of the internal transcribed spacer sequences (ITS-1 and ITS-2) indicated that all Finnish AG-3 strains are closely related, despite the wide variability of their other biological features, e.g., virulence, growth rate and fungicide tolerance. Detailed analysis of AG-3 isolates revealed polymorphism within the ITS sequences. The novel type of ITS-2 sequence heterogeneity in *R. solani* was detected as compensatory base changes (CBC). These changes are considered to be important markers of evolutionary distance. Results indicated that further research is needed to clarify whether AG-3 strains should be separated from other *R. solani* strains into their own species.

Additionally, this thesis contains the first report on detection of molecular defence reactions of potato sprouts against virulent *R. solani*. Extensive systemic resistance signalling, exploiting several known defence pathways, was activated as soon as *R. solani* came into close contact with the sprout. The defence response was strong enough to protect vulnerable sprout tips from new attacks by the pathogen. These results at least partly explain why potato emergence is eventually successful even under heavy infection pressure of *R. solani*.

Besides its evidently high virulence on potato, one of the reasons why AG-3 is so common in potato is its ability to form sclerotia (black scurf) on tubers. According to the results of this study, AG-3 is much more efficient in producing sclerotia on the tubers than are other AGs such as AG-2-1 and AG-5. This also reflects the highly specialized nature of AG-3 in infecting potato, whereas other AGs more commonly infect other crop plants.

To those I Ihold dear and near to my heart

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AG	anastomosis group
a.i.	active ingredient (mm^{-1})
AM	arbuscular mycorrhizal fungi
ATP	adenosine triphosphate
avr	microbial avirulence factor gene loci
BCA	biocontrol agent
BI	bridging isolates of *R. solani*
BNR	binucleate *Rhizoctonia* species
bp	base pairs
CBC	compensatory base changes
CC	coil-coil, N-terminus of R protein
cv	cultivar
DAPI	4´-6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dpp	days post planting
dsRNA	double stranded RNA
Flutolanil	3'-isopropoxy-2-(trifluoromethyl) benzanilide
GC-content	Guanine + Cytosine content in DNA
GTP	guanosine triphosphate
hpi	hours post inoculation
HR	hypersensitive reaction
IL	interleukin
ISG	intraspecific groups (*R. solani*)
ISR	induced systemic resistance
ITS	internal transcribed spacer region
JA	jasmonic acid
LRR	leucine rich repeat domain in R proteins and PRRs
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NB	nucleotide-binding domain in R proteins
np-BNR	non-pathogenic binucleate *Rhizoctonia* spp.
NPR1	key molecule for SAR development together with SA
NSAI	non-self anastomosing isolates
PAMP	pathogen-associated molecular pattern
PAL	phenylalanine ammonia lyase
PCD	programmed cell death
PCR	polymerase chain reaction
PDA	potato dextrose agar
PR	pathogenesis related proteins
PRR	pathogen recognition receptor
PT	potato infecting subgroup of AG-3
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
R	resistance protein
RAPD	random amplified polymorphic DNA
RDD	Rhizoctonia disease decline

rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RLK	receptor like kinases
RLP	receptor like proteins
RNA	ribonucleic acid
rRNA	ribosomal RNA
ROS	reactive oxygen species
SA	salicylic acid
SAI	self anastomosing isolates
SAR	systemic acquired resistance
SDC	succinate dehydrogenase complex
SGA	steroidal glycoalkaloids
SNP	single nucleotide polymorphism
SSV	symptom severity value
TB	tobacco infecting subgroup of AG-3
TGA	transcription factor in SAR
TIR	Toll/Interleucin receptor
VCP	vegetatively compatible populations (*R. solani*)

1. INTRODUCTION

1.1 Plant pathogens and diseases

1.1.1 Plant pathogenic organisms

Besides the damage by herbivores and pest animals, many different types of micro-organisms can cause yield losses by inducing diseases in plants (Agrios 1997). These plant pathogens include bacteria, moulds (Oomycetes), fungi and viruses. Successful pathogens have developed strategies that enable them to efficiently invade, live and reproduce inside their host plants. In the most severe disease situations, the plant dies soon after pathogen infection, but in many cases the pathogen interferes with the metabolism of the host plant leading to retarded growth and development, which contributes to the yield losses even when there are no clear signs of microbial infection.

Pathogenic micro-organisms infecting plants can be classified according to their dispersal mechanism (e.g., via soil, air, animal vector), the tissue or plant organ they infect (e.g., roots, leaves, flowers) or their infectious lifestyle and mode of disease outcome. Based on the strength of the host – pathogen relationship, parasitizing organisms are divided into obligate parasites (biotrophs) and nonobligate parasites (hemi-biotrophs and necrotrophs) (Agrios 1997). Biotrophic pathogens are obligatory parasites that need living plant tissue in order to survive. All steps in their lifecycle are dependent on finding a suitable host. Besides viruses, biotrophic parasites can be fungi (e.g., rusts, smuts, and powdery and downy mildews), bacteria (e.g., 'hairy root' disease-inducing *Agrobacterium rhizogenes* and *Pseudomonas syringae*), animals (e.g., potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida*) or even plants (*Nuytsia floribunda*). Hemi-biotrophs e.g., the fungal pathogen *Monilia fructigena*, are dependent on their living host only until necessary growth and reproduction has occurred. After successful dispersal of the pathogen, infected plant tissue will die, as happens already at early stages of disease development in plants infected with necrotrophic pathogens. Necrotrophic pathogens infect and destroy plant tissues, eventually killing the plant. Necrotrophs are only optionally parasitic, meaning that they can remain saprophytic, living and proliferating on wilting or decaying plant material. Biotrophic pathogens are strictly specialized to infect certain host plant species or a small group of alternative host plants, whereas necrotrophic pathogens are more flexible and can infect several different host plants (Bolton et al. 2006; van Kan 2006), stay as saprophytic organisms, or form special survival structures e.g., fungal sclerotia, to survive until better times.

In order to cause diseases, pathogens must come into contact with the potential host plant and enter its tissues. Viruses enter the plants with help of vectors such as aphids and thrips that feed on the plants or via other mechanical damage caused by for example that adjacent virus-infected plants thrashing in the wind. Other pathogenic micro-organisms enter through wounds or natural openings, such as lenticels and stomata. Bacteria colonize plants passively through these openings whereas fungal pathogens also enter the plants actively, either by exploiting mechanical pressure of the special entering structure, appressorium, or by creating the passage with plant surface-degrading hydrolytic enzymes (Agrios 1997). Necrotrophic fungal pathogens in particular are able to produce a huge arsenal of hydrolytic enzymes, including cutinases, pectinases, cellulases, xylanases and polygalacturonases that actively degrade polymers of the plant cell wall (Hammond-Kosack and Jones 1996). Fragments of the disturbed cell wall can initiate host defence reactions that arrest pathogen invasion. Unlike fungi, bacteria rely on their number prior to a successful attack. When the population density of bacteria is sufficient, gene expression patterns change and synchronized infection is initiated. This phenomenon is called 'quorum sensing' (Fuqua et al. 2001) and besides pathogenic interactions, it is commonly used by animals that live in large colonies, such as ants and bees.

1.1.2 Pathogenic fungi

Fungi are eukaryotic, normally filamentous, microscopic organisms that lack chlorophyll and often reproduce via sexual (or asexual) spores able to spread via air or water (Agrios 1997). However, many members of a large group of fungi, called the 'Fungi Imperfecti' (Deuteromycetes) do not form spores, or the sexual phase is rare or still unknown. These fungi spread by hyphal growth and fragments. Like plants, fungi have cell walls but the wall is built from chitin and glucan elements, sugars and glycoproteins. Most of the known fungi (over 100 000 species) are strict saprophytes, that live on dead organic material, but over 10 000 fungal species are known to cause diseases on plants (Agrios 1997). The majority of plant pathogenic fungi spend at least part of their life outside host, but in biotrophs, only inactive, ungerminated spores are able to survive on soil.

Many airborne pathogens are biotrophic. These pathogens are spread by wind-carried asexual spores, conidia, which infect above ground parts of the plant. Production of many important crop plants is annually jeopardised due to the infection pressure of foliar biotrophic pathogens causing rusts, and powdery and downy mildews (Sillero et al. 2006; Hovmøller and Henriksen 2008).

Throughout history, one of the most devastating airborne plant disease outbreaks in Europe was potato famine caused by late blight (oomycete *Phytophthora infestans*) in the mid 19th century (Fry and Goodwin 1997). Besides appropriate cultivation practices, fungicide sprays and computer-based modelling of the infection pressure (Beest et al. 2008), intensive conventional and molecular breeding programmes have been employed in order to improve crop resistances against various obligate and non-obligate parasitic fungi, with variable degrees of success (Hammond-Kosack and Parker 2003).

The host range of soilborne necrotrophic fungi is considerably wider than that of airborne bio- or hemi-biotrophs, which are more dependent for proliferation on a suitable host plant (Knogge 1996). For example, soilborne necrotrophic *Sclerotinia sclerotiorum* can infect hundreds of hosts (Bolton et al. 2006). Fortunately, specific host parasitism also occurs among necrotrophs, and then the population levels of the necrotrophic fungus tend to decline in the absence of the proper hosts (Carling and Leiner 1990a; Agrios 1997).

In order to infect a host plant, a pathogenic fungus needs to grow into close proximity of the host and enter the plant tissue to feed. Some fungi grow only on the surface of the host, whereas some enter the plant via natural openings (Agrios 1997). Depending on the fungus, its feeding structures, haustoria, can grow inter- or intracellularly after breakdown of the cell wall (Hardham and Mitchell 1998). To obtain nutrients, the fungus has to pass through the rigid cell wall and this is accomplished by producing hydrolytic enzymes (Aro et al. 2005) or by using mechanical force (biotrophs and hemi-biotrophs) (Genre and Bonfante 2007). In general, fungi that need a living host rarely produce enzymes, in order to prevent cell death, whereas for necrotrophs, the fate of the host is unimportant (Agrios 1997).

1.1.3 Symptoms, signs and management of the fungal diseases

On plants, disease symptoms caused by fungal infection are numerous. The most common symptoms of fungal infection are local necrosis or death of the host, and stunting of certain organs or the entire plant (Agrios 1997). Depending of the infection site and appearance, diseases caused by fungi are called leaf spots (lesions on leaves), blights (browning/death of the leaves, shoots and flowers), cankers (lesions on stems), diebacks (necrosis of the shoots), scabs (localized raised or sunken lesions on leaves, fruits or tubers), anthracnose (ulcerlike lesions on above ground organs), decline (stunted and poor growth) and rots that can occur on roots, stems, bulbs, fruits and fleshy

leaves. Visible fungal structures on the plant, i.e., mycelium, spore-bearing structures or sclerotia are termed signs of the disease.

Control of pathogenic fungi can be challenging due to the complexity of the diseases. The most effective methods to prevent disease outbreak are good hygiene (clean tools and planting material), destruction of the infected plant / plant parts, elimination of the alternative hosts, proper watering and aeration of the plantations, and application of biocontrol organisms that either kill or compete with the pathogen. As a rule, organisms that are used for biocontrol applications are not pathogenic to the plants but are able to parasitize pathogenic fungi (mycoparasitism) or lyse their mycelia (mycolysis) (Agrios 1997). For example, many *Trichoderma* species have been exploited successfully for controlling soilborne pathogenic fungi on decaying wood (Verma et al. 2007; Schubert et al. 2008), vegetables (Grosch et al. 2007; Verma et al. 2007; Coşkuntuna and Özel 2008; Wilson et al. 2008a,b) and cereals (Cardo et al. 2007; Verma et al. 2007). However, application of fungicides by spraying or dipping is sometimes necessary. Both air- and soilborne pathogenic fungi can be controlled by following these basic guidelines.

1.1.4 Soilborne plant diseases

Soilborne pathogenic fungi cause diseases on roots and other underground plant organs such as stolons, tubers and basal parts of the stems (Agrios 1997). Compared to airborne pathogenic fungi, soilborne pathogens have wider host range and they persist longer without suitable hosts (Agrios 1997). Typical symptoms of the soilborne diseases are damping-off of seedlings, wilting of adult plants, and death of the aboveground plant parts due to the decay of the root system and breakdown of the flow of water and nutrients (Agrios 1997). The incidence of plant infection with soilborne pathogens increases during relatively cool and wet weather that, at the same time, slows infection by many airborne pathogens that spread better during dry conditions. Control of soilborne diseases is relatively more laborious than that of airborne ones, since the pathogen starts to do the damage while hidden in the soil and the disease outbreak may become visible too late for effective plant protection. For example, *Pythium* spp., *Fusarium* spp., *Sclerotinia sclerotiorum* and *Rhizoctonia solani* cause significant losses on crop quantity and quality of many crop species annually (Weinhold et al. 1982; Banville 1989; Martin and Loper 1999; Green and Jenson 2000; Bolton et al. 2006; Wagacha and Muthomi 2007).

1.2 *Rhizoctonia solani* Kühn

Genus *Rhizoctonia* is a highly heterogeneous group of filamentous fungi that share similarities in their anamorphic, sterile state. For example, they do not produce asexual spores and the teleomorphic, sexual state occurs only rarely. The group contains several economically important and global plant pathogens like *Rhizoctonia solani* Kühn [anamorph, telemorph *Thanatephorus cucumeris* (Frank) Donk]. As an exception, species forming mychorrhiza on orchids (usually from genus *Ceratobasidion*) are also known (Gonzáles García et al. 2006). Because of the absence of the teleomorphic state, the name of the anamorph is usually used in the literature.

R. solani is the most widely known and most studied species of genus *Rhizoctonia*. It was originally described by Julius Kühn from potato in 1858 (Kühn 1858). *R. solani* is soilborne Basidiomycete occurring world-wide, with complex biology. Its highly destructive lifestyle as a non-obligate parasite causes necrosis and damping-off on numerous host plant species. Because of the lack of conidia and the scarcity of the sexual spores, *R. solani* exists as vegetative hyphae and sclerotia in nature. Sclerotia are an encapsulated, tight hyphal clump that protects and preserves the fungus over non-optimal times. The fungus is dispersed mainly via sclerotia, contaminated plant material or soil spread by wind, water or during agricultural practices such as tillage and seed transportation. The fungus can stay in the soil as a saprophyte for long periods. Nutrients leaking from actively growing plant cells or decaying plant material attract the fungus. Nutrient status is important also for the pathogenic lifestyle since virulence of the isolates has been reported to decrease when the supply of nutrients available from decaying plant material becomes insufficient, and this is probably the sum effect of all physiochemical changes in the local environment (Doornik 1980). Rhizoctonia disease decline (RDD) has been reported to occur in monocultural cropping systems as a result of possible in soil physiochemical and climatic factors or in soil biota such as population changes of the pathogen or the appearance of an antagonistic organism (Hyakumachi 1996). Nutrient usage by *R. solani* differs since it has been reported to be suppressed in saprophytic systems rich in organic nutrients. However, *R. solani* seems to have an ability to utilize other carbon sources, such as cellulose, that are only rarely used by other micro-organisms which makes it efficient competitor when resources are limited (Deacon 1996).

The host range of *R. solani* is wide and it causes various diseases on important crop plants of the world (Table 1) including species in the *Solanaceae*, *Fabaceae*, *Asteraceae*, *Poaceae* and

Braccicaceae as well as ornamental plants and forest trees (Ogoshi 1996). Disease symptoms include leaf blights, leaf spots, damping-off, rots on roots, shoots and fruits, canker lesions on sprouts and stolons, and sclerotial diseases. However, some *R. solani* strains form symbiotic mycorrhizal relationships with orchid plants (Carling et al. 1999; Chang and Chou 2007).

Rhizoctonia solani infection initiates when mycelia or hyphae from a germinating sclerotium starts to grow towards a suitable host as a result of attracting chemical exudates, e.g., amino acids, sugars, organic acids and phenols, from the plants (Keijer 1996). After the first contact, loose and still unattached hypha starts to grow over the plant and within a few hours the hypha flattens and directional growth over the epidermal cells is initiated. Before actual active penetration of the host, T-shaped hyphal branches form thick infection cushions that attach strongly to the host epidermis (Keijer 1996). Topological signalling, namely identification of the appropriate host by its surface structure, seems to be important for establishment of the infection. The fungus enters the plant actively by finding a weak spot on the surface where it can break down the protecting layer (Weinhold and Sinclair 1996). Passive entry by the fungus into the plant is rare and limited to leaf pathogenic isolates (Weinhold and Sinclair 1996) and it is not the usual infection mechanism for soilborne *R. solani* (Keijer 1996). Swollen hyphal tips on infection cushions concurrently form infection pegs that penetrate the cuticle and epidermal cell walls into the host epidermal tissue and outer layer of the cortex (Demirci and Döken 1998). Penetration is established by using hydrostatic pressure, even though degrading enzymes such as cutinases (Baker and Bateman 1978), pectinases (Bertagnolli et al. 1996; Jayasinghe et al. 2004) and xylanases (Peltonen 1995), are most probably also involved in infection and penetration. When inside the host, the fungus starts to grow inter- and intracellularly degrading the tissue, which can be seen as necrotic lesions on epidermal tissue of shoots, roots and stolons or as damping-off of the young seedlings (Demirci and Döken 1998).

Table 1. Disease symptoms and host range of different anastomosis (AG) groups of *Rhizoctonia solani*.

Anastomosis group	Symptoms[a]	Host	Reference
AG1	sheath blight	rice	Sayler and Yang 2007
	leaf blight	corn	Tomaso-Peterson and Trevathan 2007
	leaf blight	common bean	Muyolo et al. 1993
	brown patch	turfgrass	Herr and Fulton 1995
	web blight	common bean	Muyolo et al. 1993
	web blight	rice	Hashiba and Kobayashi 1996
	rot	soybean	Yang et al. 1990
	bottom rot	cabbage	Tu et al. 1996
	damping-off	lettuce	Herr 1993
	damping-off	buckwheat	Herr and Fulton 1995
	damping-off	carrot	Grisham and Anderson 1983
	bud rot	soybean	Hwang et al. 1996
AG2	root rot	flower bulbs	Dijst and Scneider 1996
	root rot	radish	Grisham and Anderson 1983
	root rot	clover	Wong and Sivasithamparam 1985
	root rot	sugarbeet	Herr 1996
	root rot	common bean	Muyolo et al. 1993
	stem canker	potato	Chand and Logan 1983
	damping-off	tree seedlings	Hietala and Sen 1996
	damping-off	oilseed rape	Kataria et al. 1991a
	damping-off	clover	Wong and Sivasithamparam 1985
	damping-off	sugarbeet	Herr 1996
	damping-off	soybean	Nelson et al. 1996
	brown patch	turfgrass	Herr and Fulton 1995
	large patch	turfgrass	Burbee and Martin 1996
	sheath blight	rice	Hashiba and Kobayashi 1996
	leaf blight	sugarbeet	Herr 1996
AG3	stem canker	potato	Bundy et al. 1988
	black scurf	potato	Bundy et al. 1988
	target spot	tobacco	Ogoshi 1987
	brown spot	eggplant	Kodama et al. 1982
	leaf blight	tomato	Date et al. 1984
AG4	stem canker	potato	Anguiz and Martin 1989
	damping-off	onion	Erper et al. 2005
	fruit rot	tomato	Strahnov et al. 1985
	stem rot	pea	Hwang et al. 2007
	root rot	soybean	Liu and Sinclair 1991
	root rot	tree seedlings	Hietala and Sen 1996
	root rot	pea	Hwang et al. 2007
	root rot	common bean	Muyolo et al. 1993
	root rot	cotton	Rothrock 1996
	root rot	corn	Mazzola et al. 1996
	basal rot	oilseed rape	Verma 1996
AG5	stem canker	potato	Bandy et al. 1984
	black scurf	potato	Bandy et al. 1984
	root rot	soybean	Nelson et al. 1996
	root rot	wheat	Rush et al. 1994
	root rot	barley	Rush et al. 1994
	root rot	tree seedlings	Hietala and Sen 1996
	late emergence	lupin	Valkonen et al. 1993
	reduced growth	common bean	Valkonen et al. 1993
	reduced growth	broan bean	Valkonen et al. 1993
AG6	mycorrhizal		Carling et al. 1999
AG7	root canker	cotton	Baird and Carling 1997
AG8	bare patch	cereals	Mazzola et al. 1996
AG9	minor pathogen	potato	Carling et al. 1994
AG10	minor pathogen	lupin	MacNish et al. 1995
AG11	not pathogenic		Eken and Demirci 2004
AG12	mycorrhizal		Carling et al. 1999
AG13	minor pathogen	cotton	Carling et al. 2002a

[a] A huge range of disease symptoms is caused by *R. solani* and only a limited number of host – pathogen combinations has been presented here.

1.2.1 Grouping of R. solani isolates

The teleomorphic, sexual state of the *R. solani* is *Thanatephorus cucumeris* (Banville et al. 1996). Since the teleomorphic state is rare, identification of the fungus is based on characteristics of vegetative hyphae as with other *Rhizoctonia* spp. On artificial nutrient media *R. solani* is identified from the colour of the mycelium which can vary from near white to dark brown or almost black, with irregularly shaped, undifferentiated, light brown to dark brown sclerotia. The diameter of vegetative hyphae is more than 7 μm and the hyphal branches occur at 90^{o} angles. There are more than two nuclei in apical compartments of hyphae (Parmeter and Whitney 1970; Ogoshi 1987).

Grouping of *R. solani* isolates is accomplished by evaluation of the anastomosis reactions of the vegetative hyphae of the individual fungal strains. Anastomosis is defined as a vegetative compatibility of different, closely related isolates (Anderson 1982). Earliest reports about anastomosis reactions in *R. solani* by T. Matsumoto date to the early 20^{th} century (Carling 1996). During anastomosis, hyphae of the two different but still related strains fuse or anastomose. Hyphal fusion has been proved to be a reliable method for grouping *R. solani* strains into anastomosis groups (AGs) (Ogoshi 1987). The concept has given rise to currently 13 AGs (Carling et al. 2002a) that vary in morphology, host range and ability to infect plants at different ages. Binucleate *Rhizoctonia* (BNR) species are sometimes considered as an additional AG since they are able to anastomose with some of the existing AGs (Kuninaga et al. 1979). AGs 1, 2, 3 and 4 are the biggest pathogen groups, characterized individually in three different geographical locations, in Europe (reviewed by Carling et al. 1996), in the North America (Parmeter et al. 1969), and in Asia (Watanabe and Matsuda 1966) whereas groups 5 to 13 are less important as plant disease agents. BNR species have been reported to be weakly pathogenic on sugar beet (Olaya and Abawi 1994), common bean, soybean, pea (Yang et al. 2005) and strawberry (Martin 1988).

Since individual isolates belonging clearly to the same AG still differed in morphological features and host range, hypotheses about smaller groups within AGs have been tested by several biochemical, serological and molecular biological methods. Hyphal anastomosis reactions, pathogenicity on certain host plants, morphology, thiamine utilization, fatty acid composition, protein zymograms and deoxyribonucleic acid (DNA) markers have been used to group isolates into smaller, more homogeneous intraspecific groups (ISG) within AGs (Ogoshi 1987) and further as vegetatively compatible populations (VCP), comprising highly similar isolates of *R. solani* (MacNish et al. 1995). Isolates belonging to same VCP can be considered almost as clones,

representing identical fungal strains, whereas ISGs within specific AGs are considered as subspecies. Numerous subgroups have been identified in AGs 1, 2, 3, 4, 6, 8 and 9 (Carling and Kuninaga 1990; Liu and Sinclair 1993; Schneider et al. 1997b; Carling et al. 2002b; Godoy-Lutz et al. 2008) and their members can have very different hosts or climatic preferences. For example, in AG-2, AG-2-1 infects mainly species from *Braccicaceae*, AG-2-2 those of *Chenopodiaceae*, and AG-2-3 is more highly specialized to soybean (Salazar et al. 2000). Sometimes subgroups can be further divided into ecological types based on their host preference, which can be the result of altered ability to produce virulence factors such as hydrolytic enzymes (Schneider et al. 1997a,b). Biochemical and serological investigations of *R. solani* have included analysis of soluble proteins (Reynolds et al. 1983; Liu and Sinclair 1992), pectic isozymes (zymograms) (Schneider et al. 1997a; Balali et al. 2007; Stodart et al. 2007), lectins (Kellens and Peumans 1991), profiles of whole-cell fatty acid composition (Johnk and Jones 2001; Priyatmojo et al. 2002) and mono- and polyclonal antibodies against hyphal or soluble proteins (Adams and Butler 1979; Matthew and Brooker 1991).

In addition to biochemical studies, more and more *R. solani* investigations are conducted using molecular methods dealing with differences in DNA base composition, homology and sequence variability. Guanine-cytosine (GC) -content analysis and DNA homology measured by the strength of DNA / DNA hybridization have been used for separating AGs and even their subgroups from each other (Vilgalys 1988). Additionally, several authors have collected information about genetic variation and relationships of AGs and their subgroups by using amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) methods (Ceresini et al. 2002a,b; Ceresini et al. 2003; MacNish and O´Brian 2005; Balali et al. 2007; Guleria et al. 2007; Taheri et al. 2007). To detect variation in highly conserved genome areas, genes coding for ribosomal proteins (rRNA) and internal transcribed spacer regions (ITS) separating ribosomal protein encoding parts have been analyzed (Boysen et al. 1996; Kuninaga et al. 1997; Salazar et al. 2000; Lees et al. 2002; Carling et al. 2002b; Elbakali et al. 2003; Guillemaut et al. 2003; Grönberg et al. 2003; Justesen et al. 2003; Woodhall et al. 2007; Godoy-Lutz et al. 2008). The approaches have clarified the taxonomy, genetic relationships and population structures of this complex pathogen.

1.2.2 R. solani as a potato pathogen

Rhizoctonia solani is the causal organism of Rhizoctonia disease complex in potato (Wilson et al. 2008b) resulting in two different appearances of the disease, namely stem canker and black scurf. These are recognized as necrotic lesions on underground plant parts, and sclerotia covering progeny tubers, respectively (Carling and Leiner 1986). Early in the growing season, the fungus causes necrosis in emerging sprouts, killing the sprout tip and causing stem canker disease symptoms. Later in the season, *R. solani* attacks the developing stolons, preventing them from growing to their full length (Banville 1989). In the field, stem canker infection causes late emergence of the potato plants, lowers the stem number, shortens the length of the stolons resulting in misshapen progeny tubers with high size distribution, and lowers the processing quality of the potato (Hide and Horrocks 1994). Extensive greening of the tubers is also found since the short stolons do not allow tubers to form deep enough in the soil. Incidence of the disease is higher in cool and wet growing seasons (Anderson 1982). The second form of the disease can be seen later in the season close to the harvest, when dark sclerotia start to cover maturing daughter tubers (Banville et al. 1996). Volatile organic chemical exudates have been reported to protect new tubers from fungus infestation until senescence of the aboveground plant parts late in the growing season (Dijst 1990). *R. solani* spreads to new growing areas on sclerotia-covered seed tubers (Tsror and Peretz-Alon 2005). Seedborne (i.e., tuberborne) inoculum is the main source of primary infection leading to stem canker symptoms on the underground plant parts. Secondary inoculum of the Rhizoctonia disease is soilborne and accomplished by *R. solani* mycelia and sclerotia already inhabiting soil where the potato crop is planted (Balali et al. 1995). Soilborne infection emerges later in the season since the fungus needs some time to grow into proximity with its potato host (Carling and Leiner 1986). Extensive yield losses, from 10% to 30% on marketable size tubers as a result of Rhizoctonia disease have been reported by Carling et al. (1989) and Read et al. (1989). In Finland, estimated annual loss of potato crop quantity due to *R. solani* infection can be 15 - 20% (http://www.tarkkelysperuna.info/site?node_id=480).

Protecting potato from *R. solani* is done by combining good agricultural practices with appropriate chemical and/or biological plant protection methods. Several cultural methods for protecting potato from Rhizoctonia disease have been proposed (Anderson 1982). First of all, use of sclerotia-covered seed tubers should be avoided (Banville 1978; Frank and Leach 1980). Planting should be done into relatively dry and warm soil to achieve rapid emergence (Anderson 1982; Bandy et al. 1988; Carling and Leiner 1990b) and an appropriate crop rotation programme should be followed

(Anderson 1982). In potato production, cereals are good rotation crops since their AGs differ from those of potato (Anderson 1982). Appropriate crop rotation leads to decline of the population levels of the *R. solani* AGs that are potential potato pathogens and reduces the infection incidence when potato is grown again. Crop rotation is an effective protection method against soilborne secondary *R. solani* infection (Carling et al. 1989). Further protection of the sprouting plant from the primary seed-borne infection is accomplished by dressing seed tubers with fungitoxic chemicals or biocontrol agents (BCA) that either compete with the pathogen or kill it before development of the stem canker symptoms (Anderson 1982). Good results for controlling *R. solani* in greenhouse or field conditions have been achieved using *Trichoderma* spp. such as *Trichoderma harzianum* along with *Gliocladium virens* that act as antagonists of the pathogen and are able to prevent or lower the incidence of infection (Wilson et al. 2008a). *Trichoderma* spp. are well documented for their ability to protect plants from *R. solani* infection by production of antibiotics, antifungal chemicals and hydrolytic enzymes. Some *Trichoderma* spp. have been reported to provide plant growth enhancement as well as crop protection (Verma et al. 2007). Several fungicides have been used for controlling Rhizoctonia diseases. In some cases BCA-based plant protection seems to be more efficient (Verma et al. 2007), but recent results of Wilson et al. (2008b) suggest that combining chemical control with antagonist treatment can protect potato during the whole growing season.

Anastomosis group 3 (AG-3) is the main *R. solani* group infecting potato worldwide (Abe and Tsuboki 1978; Chand and Logan 1983; Otrysko et al. 1985; Carling and Leiner 1986; Carling et al. 1986; Bandy et al. 1988; Castro et al. 1988; Carling and Leiner 1990a; Bains and Bisht 1995; Balali et al. 1995; Cedeño et al. 2001; Campion et al. 2003; Justesen et al. 2003; Truter and Wehner 2004; Woodhall et al. 2007). AG-2-1, AG-4 and AG-5 are frequently found associated with potato, and they have been reported to be virulent and able to cause Rhizoctonia disease symptoms on potato (Abe and Tsuboki 1978; Chang and Tu 1980; Chand and Logan 1983; Carling and Leiner 1986; Anguiz and Martin 1989; Carling and Leiner 1990a; Bains and Bisht 1995; Balali et al. 1995; Cadeño et al. 2001; Truter and Wehner 2004; Woodhall et al. 2007). AG-2-2 from Japan and AG-7 collected from sclerotia on potato from Mexico have been reported to be pathogenic to potato by Abe and Tsuboki (1978) and Carling and Brainard (1998), respectively, although AG-7 is not normally able to infect potato. Rarely other AGs have been found associated with potato and even then the evidence regarding their virulence against potato is conflicting or damage is limited to certain plant organs. For example, AG-1 was isolated from potato roots by Bandy et al. (1988) and AG-8 has been reported to infrequently infect and prune potato roots, leading to damping-off and reduced foliage although no classical signs of Rhizoctonia disease were apparent (Carling and

Leiner 1990a; Hide and Firmager 1990; Truter and Wehner 2004). AG-9 has been found associated with potato and potato-growing soils, but its relationship with this crop seem to be only loose and limited to infection of the young seedlings (Carling et al. 1986, 1987). The same phenomenon has been reported from binucleate *Rhizoctonia* spp. (BNR) (Bandy et al. 1984). Artificial inoculation of potato with AG-13 led to formation of small stem canker lesions on shoots and roots (Carling et al. 2002a), indicating that in experimental situations some AGs not normally infecting potato are able to initiate limited necrosis.

The severity of the symptoms of Rhizoctonia disease in potato depends on inoculum potential, i.e., the amount of pathogen in the soil and on seed tubers along with local climatic conditions. AG-3 is most virulent in cool growing conditions such as in some parts of the USA, Canada, and Northern and Central Europe (Otrysko et al. 1985; Bandy et al. 1988; Carling and Leiner 1990b; Bains and Bisth 1995; Campion et al. 2003; Justesen et al. 2003) whereas virulence of AG-2-1, AG-5 and AG-4 increases with increasing temperature (Anguiz and Martin 1989; Carling and Leiner 1990b; Woodhall et al. 2007). According to Anguiz and Martin (1989) AG-4 is dominant in warm areas at low altitudes in Peru. Additionally, the recovery of a single pathogenic AG-7 isolate from sclerotia attached to Mexican potato from high altitudes indicates that this AG might have an optimum in relatively cool climates, as does AG-3 (Carling and Brainard 1998).

1.3 Plant resistance to pathogens

Even though plants and animals do not have the same kinds of immunity systems (Nürnberger at al. 2004), inductions of the resistance and defence reactions in plants have similarities with the ones found in animals. In animals, defence is accomplished by motile defender cells and an adaptive immune system, lacking in plants. In plants, recognition and defence are organized independently in each plant cell, and then systemic signalling may emanates from the infected areas (Jones and Dangl 2006). The immobile, innate immunity system allows plants to resist most micro-organisms as a result of defence mechanisms induced by previous invasion of either the pathogen or a non-pathogenic micro-organism. The phenomenon is termed 'priming', which is considered as constant activation of the basal level of defence is swiftly boosted when the host plant is under attack for a second time. Priming is exploited in some biocontrol applications where host defence is primed with a compatible, non-pathogenic micro-organism (e.g., Cardinale et al. 2006).

Recognition of the pathogen is a key feature of the onset of the defence response activation and for a long time, it has been largely attributed to the gene-for gene hypothesis (Flor 1955), where the gene product of the pathogen is recognised by the gene product of the host in order to establish the defence reaction. The theory behind the gene-for-gene hypothesis is analogous to that of the receptor - ligand relationship of mammal antibodies and antigens (McDowell and Simon 2006). The outcome can contribute to broad-spectrum defence against a wide array of species as in non-host resistance or against certain pathogen subspecies as in specialized resistance, it can also be restricted to certain race- or plant cultivar-specific resistance (Nürnberger and Scheel 2001).

1.3.1 Preformed defence systems

Plants have assembled many strategies that help them either to avoid infection or to restrain the invasive growth and feeding of the pathogen, and finally destroy the intruder (Figure 1; Nürnberger and Lipka 2005). Mechanical barriers on the plant surfaces are the first host protection system that pathogens have to overcome in order to start infection. These include hairs, spikes, resins, waxes on the cuticle, and finally the strongly built cell wall (Agrios 1997; Nürnberger et al. 2004). The mechanical barriers impede the entry of the pathogen and when they turn out to be insufficient, chemical defence is initiated (Nürnberger and Lipka 2005).

A wide array of secondary chemical compounds, protecting the host against herbivores, pests and pathogens, is constitutively expressed in healthy plants, either in their active forms or as inactive precursors ready to react when the integrity of the tissue is damaged (Figure 1) (Bennett and Wallsgrove 1994; Osbourn 1996a; Morissey and Osbourn 1999). The inhibitory effect of some of these plant compounds, such as chatecols (Osbourn 1996a), can be so strong that inhibitory effect reaches from the original point of synthesis to the host surface, preventing the growth of the pathogen or repelling the pathogens and other pests (Osbourn 1996a). The paradox is that in many cases, specialized pathogens can circumvent these toxic chemicals, or even use them as a guide to the host or as a source of nutrients (Bennett and Wallsgrove 1994, Osbourn 1996b). Preformed inhibitors ('phytoanticipins' of van Etten et al. 1994) are normally located in cells directly under the plant surface, inside plant cell vacuoles and other organelles and they are often tissue-specific (Bennett and Wallsgrove 1994). Therefore these substances can be avoided by pathogens that do not cause extensive tissue damage, such as biotrophic pathogenic fungi (Osbourn 1996a). Many of these chemicals have been reported to have antibiotic properties, such as saponins (Osbourn 1996b), terpenoids (Cheng et al. 2007), phenols, sulphur-containing compounds, sugar-containing phenolic and cyanogenic glucosides and glucosinolates (Bennett and Wallsgrove 1994). For example, steroidal glycoalkaloids (SGA) are major saponins found from plants belonging to the *Solanaceae* (Friedman 2004; Morissey and Osbourn 1999; Osbourn 1996a).

Biologically active secondary metabolites are common in the *Solanaceae*, including pepper, tomato, eggplant and tobacco, and they are produced in plants during growth and after harvest. These sometimes toxic metabolites protect the plant from minor pathogens and pests such as herbivorous insects, and in high doses cause medical problems also to mammals, including humans. The two most abundant potato glycoalkaloids, α-chaconine and α-solanidine, synergistically inhibit growth and germination of several important pathogenic fungi, including *R. solani*, in *in vitro* conditions (Fewell and Roddick 1997). However, there are inconsistent reports about their antimicrobial effects against pathogenic fungi and bacteria (Rokka et al. 2005; Valkonen et al. 1996). For example *Phytophthora infestans* is able to inhibit production of SGA (Choi et al. 1994). The long-term aim in potato breeding and biotechnology has been to lower the amounts of the major SGAs, α-solanine and α-chaconine derived from solanidine (Stobiecki et al. 2003, Rokka et al. 2005).

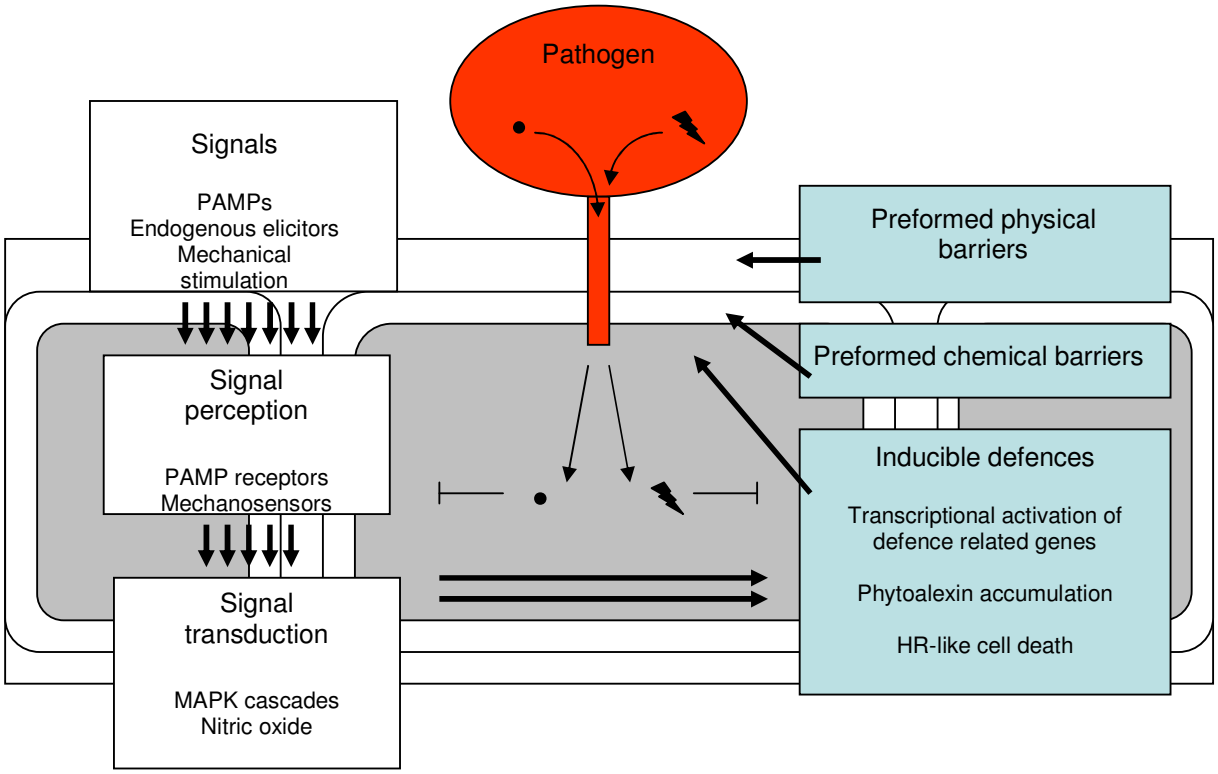


Figure 1. Plant response to pathogen invasion. Preformed physical and chemical barriers protect the plant from microbial attack. If the pathogen is able to cross this first line of defence, inducible defence is activated. The plant recognizes the pathogen from its molecular pattern and activates signalling cascades leading to transcriptional activation of defence-associated genes. HR – hypersensitive reaction, PAMP – pathogen associated molecular pattern, MAPK – mitogen activated protein kinase. Adapted from Nürnberger and Lipka (2005).

1.3.2 Induced defence systems

A broad spectrum of induced defence systems is brought into action immediately after recognition of pathogen-derived molecules (Figure 2a). These elicitors, pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively) are recognized by the host pattern recognition receptors (PRRs) (Iriti and Faoro 2007) with similarities to those also found in animals (Nürnberger and Scheel 2001, Nürnberger and Lipka 2005). Plant PRRs are single membrane-spanning receptor-like kinases (RLKs) or receptor-like proteins (RLPs) with multiple leucine-rich repeats (LRR) that recognize microbe-derived molecules once they have breached the physical barrier of the host protective surface. PAMPs are synonymously called general or exogenous elicitors (Montesano et al. 2003; Nürnberger at al. 2004) in contrast to the endogenous elicitors comprising the breakdown products of the plant cell wall. The endogenous elicitors also initiate the recognition pathway

(Nürnberger and Lipka 2005), raising defensive reactions against a wide array of pathogens, especially tissue-damaging necrotrophic fungi and bacteria (Nürnberger at al. 2004). Several pathogen-originated molecules, e.g., bacterial flagellin, harpin and necrosis-inducing proteins as well as fungal xylanase, β-glucans and chitin have been demonstrated to act as exogenous elicitors for defence establishment (Wei et al. 1992; Felix et al. 1999; Ito et al. 1997; Enkerli et al. 1999; Fliegmann et al. 2004; Mattinen et al. 2004).

Recognition of the pathogen by the plant leads to transcriptional activation of genes, causing a series of modifications that strengthen the barriers and attack the invader. Cell walls are reinforced by deposition of lignin-like material, antimicrobial phytoalexins are synthesized and activities of hydrolytic enzymes such as chitinases and proteinase inhibitors are increased (Lawton and Lamb 1987). Phytoalexins are synthesised from their precursors in response to the stress or pathogen attack after host recognition, and are one form of induced defence response associated with disease resistance (Morissey and Osbourn 1999). Phytoalexins are neither particularly strong antimicrobial compounds nor specifically targeted against certain pathogens, but their accumulation is faster and to a higher level in resistant plants (Morissey and Osbourn 1999).

Compared to the pathogen recognition accomplished by PRRs, faster and more specialized pathogen recognition is contributed by disease resistance (R) proteins, especially against obligatory biotrophic micro-organisms (Jones and Dangl 2006) (Figure 2b). Resistance proteins in plants can be divided into five main classes based on their structure (Dangl and Jones 2001). The biggest group of R gene products in plants is cytoplasmic, polymorphic R proteins, characterised by their conserved nucleotide-binding site (NB) and leucine-rich repeat (LRR) domains. NB-LRR proteins can be further divided into two groups based on their N-terminus structure that can resemble *Drosophila* Toll and mammalian interleukin (IL)-1 receptors (TIR-NB-LRR) or contain coil-coil domains (CC-NB-LRR). The specificity of the R proteins arises from the highly variable LRR part of the protein which is the key to pathogen recognition, whereas the NB domain serves as a binding site for ATP or GTP. The role of the R proteins is to recognize products of the avirulence genes (*avr*) produced by the pathogen and to activate the signal cascades that lead to host protection and arrest of pathogen growth. If the plant is unable to recognise these avirulence factors due to the lack of a suitable R protein or absence of the avirulence factor itself, defence fails leading to disease development (Dangl and Jones 2001) (Figure 2).

One of the most studied R gene-mediated plant responses to pathogen invasion is programmed cell death (PCD) of host tissues as a result of the hypersensitive reaction (HR) (Iriti and Faoro 2007; Mur et al. 2008). Typically HR does not exceed further from the infected cell (Jones and Dangl 2006). HR is well studied in plant – biotrophic pathogen –interactions. However, its role in host interactions with necrosis inducing pathogens is unclear since these pathogens consume nutrients from the dead plant tissue (Hammond-Kosack and Jones 1996). Reactive oxygen species (ROS) are generated and establish the HR (Mur et al. 2008). Superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroperoxyl radicals (HO_2) are toxic to microbes and to the host cells as well (Hammond-Kosack and Jones 1996). These enzymes are also induced in lignification, promoting and reinforcing cell wall synthesis (Blokhina et al. 2003). Some studies indicate that ROS have important roles in activation of short distance defence responses (Levine et al. 1994) as well as reactions that spread from the original point of infection, such as systemic acquired resistance (SAR) (Mur et al. 2006). Besides arrest of the pathogen via PCD, HR can thus act as a trigger for inducible defence reactions (Hammond-Kosack and Jones 1996) via salicylic acid (SA) signalling.

SAR is established parallel to HR and PCD of the host and is characterized by production of SA that activates nuclear localized NPR1, a central positive regulator of the SAR (Pieterse et al. 1998). Interaction of NPR1 with a TGA transcription factor leads to synthesis of pathogenesis-related (PR) proteins (Beckers and Spoel 2006; Sanz-Alférez et al. 2008), of which some are antimicrobial (Klessig and Melamy 1994). PR proteins are defined as extra- or intracellular proteins that increase in concentration after challenges by a pathogen or treatment with an elicitor (Bowles 1990). Once initiated, SAR gives plants a long-lasting and broad resistance against mainly biotrophic pathogens (Sanz-Alvérez et al. 2008). Induced systemic resistance (ISR), however, is accomplished via signals mediated by jasmonic acid (JA) and it protects plants from necrotrophic pathogens and insect feeding (Pieterse et al. 1998). Synergistic as well as antagonist interactions have been observed between SA- and JA-dependent defence signalling systems (Pieterse et al. 1998; Beckers and Spoel 2006) (Figure 2b). For example, cytosolic NPR1 monomers inhibit synthesis of JA and the transcription of JA-activated downstream genes (Figure 2b).

There is evidence that SA- and JA-mediated defence pathways are connected to the upstream signalling system by protein phosphorylation reactions conducted by serine/threonine kinases named mitogen-activated protein kinases (MAPK) (Mishra and Tuteja 2006; Shoresh et al. 2006). Involvement of the MAPKs in plant defence reactions has been intensively studied and, besides pathogen attack, abiotic stress and mechanical wounding also activate them (Zhang and Klessig

2001; Shoresh et al. 2006). Reaction pathway involving MAPKs have been identified also from fungi. In yeast (*Saccharomyces cerevisiae*) MAPK signalling is involved in mating response, filamentous growth, cell wall integrity, osmoregulation and responses to stress (Zhao et al. 2007). MAPK signalling is activated in many phytopathogenic fungi during invasive hyphal growth, appressorium formation and penetration initiation (Xu 2000), conidiation (Moriwaki et al. 2007) and sclerotia production (Harel et al. 2005; Rauyaree et al. 2005) as well as in full virulence establishment of pathogens that do not form an appressorium (Ganem et al 2004; Zhao et al. 2007).

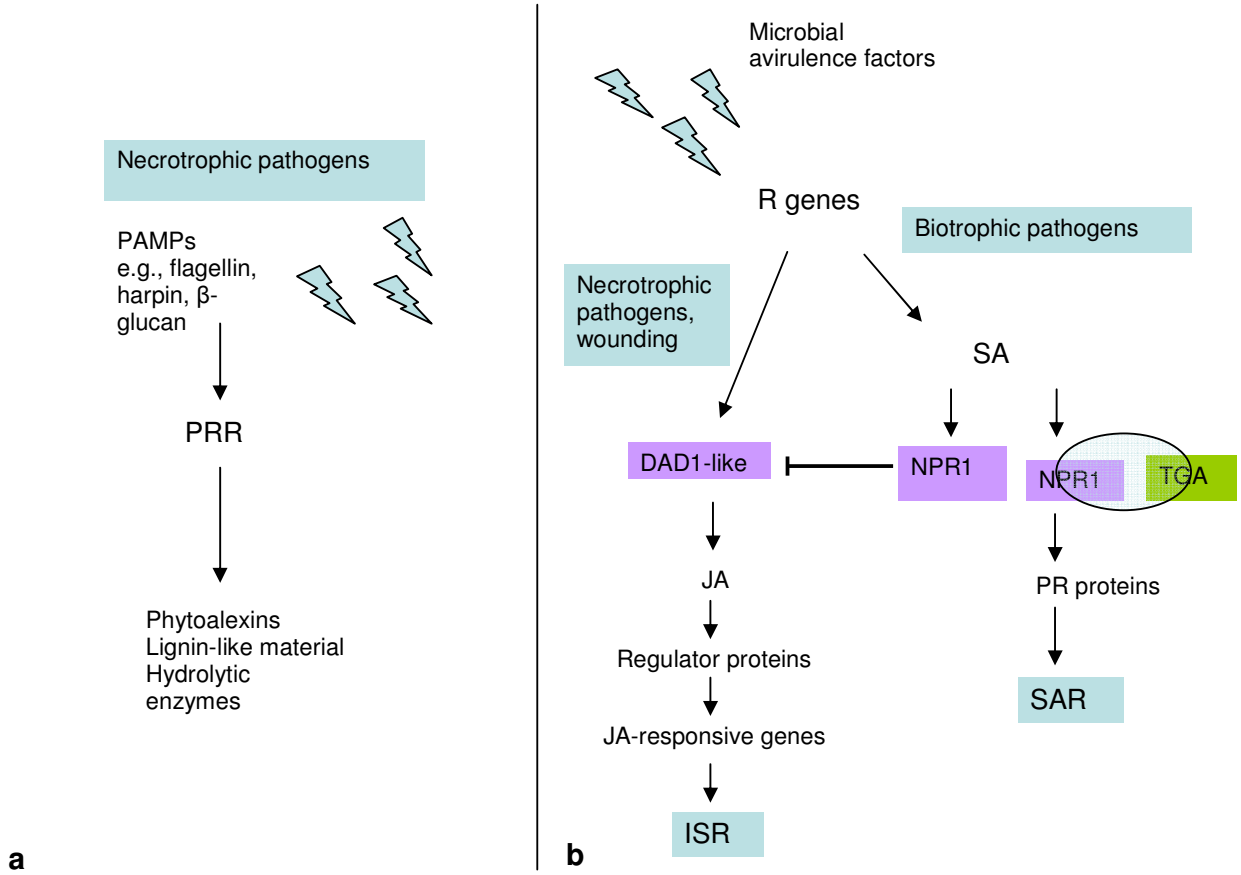


Figure 2. Activation of different types of plant resistance. Establishment of broad-scale, non-host resistance through recognition of PAMPs with (a) PRRs and (b) ISR via JA activated genes. Race- or cultivar-specific SAR is activated through SA production (b). PAMP – pathogen associated molecular pattern, PRR – pathogen recognition receptor, R – resistance genes, DAD1-like – octadecanoid enzyme, JA – jasmonic acid, ISR – induced systemic resistance, SA – salicylic acid, NPR1 – key molecule in SA signalling, TGA – transcription factor, SAR – systemic acquired resistance. Adapted from Beckers and Spoel (2006).

1.4 Potato production and value for human nutrition

The year 2008 was designated The International Year of the Potato by the Food and Agricultural Organization of the United Nations. The importance of the potato as a food of the world is increasing. The failure of cereal surpluses to meet international demands together with rapidly increasing food prices has increased the requirements for potato as an easily grown and nutritious food source. Since the potato was first established in Europe from South America in the 16th Century, it was at first mistreated, then loved and finally feared because of its poor disease resistance. The potato famine in the mid-19th Century resulted in over one million deaths, leading to the recognition of the vulnerability of the potato crop and establishment of effective breeding programmes. Intensive breeding has helped to protect potato from its pathogenic viruses, bacteria and fungi and potato is now one of the most important crop plants of the world.

Potato can be grown in all parts of Finland whereas more temperature and day length sensitive crops such as wheat and oilseeds are largely restricted to the southern parts of the country. In these northern latitudes, the warming effect of the Gulf Stream makes intensive and successful agriculture possible, but the short growing season and extreme climatic conditions reduce potential crop yields below those achieved in Central Europe and North America. According to FAOSTAT, in 2006 Finnish potato yield was close to 20 600 kg/ha, whereas in Germany and in United States, 36 600 and 43 700 kg/ha were obtained, respectively. Potato cultivation area in Finland was 28 000 ha in 2006 (FAOSTAT, http://faostat.fao.org).

Seed potato production in Finland is concentrated in the north-western region, Northern Ostrobothnia. Because of the Nordic climatic conditions, the area is relatively free of viruses due to the low populations of virus-transmitting aphids, and incidences of other devastating plant pathogens are also low. The area is designated as a 'High Grade' seed potato production zone by European Union (Hömmö 1996). For food consumption, potato is cultivated all around the country but according to the Information Centre of Agriculture and Forestry in Finland (TIKE), the main region for potato production is located on Western coastline of the country (http://www.mmmtike.fi).

Nutritionally, potato is ideal for human consumption. It contains energy (starch), high quality proteins, vitamins and dietary fibres but only little fat. Additionally, numerous plant secondary metabolites in the tubers may be beneficial for the human diet (Friedman 2006).

2. AIMS OF THE STUDY

The first objective of this study was to characterize field strains of *Rhizoctonia solani* isolated from infected potato plants and to comprehensively survey the key molecular and biological properties and variation of the isolates **(Papers I, II)**. Characterization included testing virulence in terms of the ability to cause stem canker lesions on potato sprouts, tolerance against flutolanil, and sequence variation of the ITS-2 region.

Host-pathogen interactions between a highly virulent *R. solani* AG-3 strain and potato were monitored during the early phase of the infection process using potato sprouts as a model system. The aim was to investigate possible defence reactions at the molecular level and hence find the means by which some potato sprouts emerge under heavy infection pressure from *R. solani* AG-3 **(Paper III).** Additionally, the ability of strains belonging to different anastomosis groups to form sclerotia on potato tubers under controlled conditions were investigated **(Paper IV).**

3. MATERIALS & METHODS

3.1 Fungal strains

In total 503 isolates of *Rhizoctonia* spp. associated with symptoms of Rhizoctonia disease complex on potato were characterized and of these 499 originated from important potato growing areas in Finland, one was from Russian Karelia and three were from Holland.

3.2 Plant material

All experiments were carried out with potato (*Solanum tuberosum* L.). Disease free, certified potato seed tubers were obtained from Seed Potato Centre Ltd, Tyrnävä Finland. The tubers had been grown in controlled greenhouse facilities from disease free, tested *in vitro* plant material and are termed 'minitubers'. In paper I, cultivar (cv.) Nicola was used for virulence testing of 99 Finnish *Rhizoctonia* spp. Additionally, cv. Nicola was used in greenhouse experiments for testing development of black scurf on progeny tubers as described in paper IV. Host response to *R. solani* invasion was investigated in minitubers of cv. Saturna (Paper III).

3.3 Nucleic acid analysis

For AG determination, total DNA from pure fungal cultures growing on potato dextrose agar (PDA) was isolated as described in papers I and II. For verification of AG from inoculated plants and progeny tubers, DNA was extracted with Qiagen Plant Mini Kit (Qiagen, Hilden Germany) as reported in paper IV.

Anastomosis groups of the *Rhizoctonia* strains were determined from the sequence of the polymerase chain reaction (PCR) products of fungal ITS region amplified with universal primers ITS-1 and ITS-4 (White et al. 1990). The amplification product contains ITS-1 and ITS-2 regions on flanking sites of 18S and 28S rRNA genes (Figure 3) (Papers I, IV). For closer evaluation of detected single nucleotide polymorphisms (SNP) within the amplified area, PCR products of three *R. solani* AG-3 isolates were cloned into a pGEM-T vector (Promega, Madison, WI, USA) (Paper II).

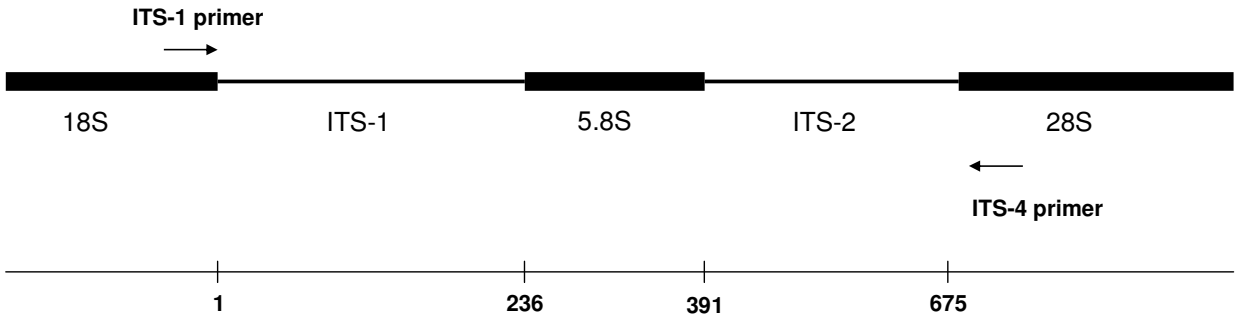


Figure 3. Arrangement of ITS region. Schematic positioning of Internal Transcribed Spacer region (ITS) 1 and 2 inside genes coding ribosomal subunits; 18S, 5.8S and 28S. Primer ITS-1 is located on the DNA sense strand (3' – 5') of the 18S rRNA gene and the ITS-4 on DNA antisense strand (5' – 3') of the 28S rRNA gene as explained by White et al. (1990). Nucleotide numbering follows the sequence of *R. solani* AG-2-2 (DQ452128) (Godoy-Lutz et al. 2008).

Activation of systemic defence reactions on potato sprouts as a response to infection was studied by way of detection of changes in levels of messenger ribonucleic acid (mRNA) with potato microarrays obtained from The Institute for Genomic Research (TIGR) (Paper III). Total RNA from the sprouts challenged by *R. solani* AG-3 and by mock inoculation was isolated using Trizol reagent as reported by Caldo et al. (2004). For microarray analysis, mRNA from the total RNA extraction was enriched and manipulated as described in paper III. Activation was verified with quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) according the instructions given by the LightCycler 480 manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

3.4 Microscopy

Microscopy was used during characterization of the collected *Rhizoctonia* strains. For testing the reliability of the ITS region based AG determination, AGs of seven selected strains were determined according to their ability to anastomose with tester strains obtained from Japan. Hyphae were examined without staining as reported in papers I and II.

Numbers of nuclei in young hyphal cells of 11 *Rhizoctonia* spp. were determined. Hyphae grown on clean microscope slides were fixed and stained with 4´-6-diamidino-2-phenylindole (DAPI) and finally viewed as explained in paper II.

Development of stem canker symptoms and growth of the fungi on inoculated potato sprouts and uninoculated sprouts were inspected stereomicroscopically on stained plant organs as reported in paper III.

3.5 Various methods used in the study

The methods are listed in Table 2. and are described in the respective original papers.

Table 2. Methods used in this study

Method	Reference[a]
Culture of bacteria	II
Culture of fungi	I, II, III, IV
DNA electrophoresis	I, II, III, IV
Fungicide testing	I
Inoculations of fungi into plants	I, II, III, IV
Microarray data analysis	(III)
Microarray hybridizations	III
Microscopy	I, II, III
Photography of the plants/fungi	I, II, III, IV
Phylogenetics	(II)
Plant culture	I, II, III, IV
Polymerase chain reaction (PCR)	I, II, IV
Primer design	III
Quantitative PCR	III
RNA extraction	III
Sequence analysis	(I, II), III, IV
Standard cloning and DNA manipulation	II, III
Statistical analysis	(I, II, III, IV)
Virulence testing of the fungi	I

[a] Reference number in parenthesis indicates that the method was applied entirely by a co-author of the published/submitted article.

4. RESULTS & DISCUSSION

4.1 Genetic variability of *R. solani* on potato in Finland

4.1.1 Anastomosis groups of R. solani associated with potato in Finland

In this study, an extensive national survey of *Rhizoctonia* spp. associated with Rhizoctonia disease symptoms on potato in Finland was conducted by isolating field strains from important potato growing areas covering 268 field plots from mainland and 13 from Ahvenanmaa. The majority of the samples were taken from areas practicing intensive potato cultivation and production of high quality seed tubers (Figure 1 in Paper I). In total 499 Finnish *Rhizoctonia* samples associated with potato were isolated, of which 52.7% originated from canker lesions on stems (244 isolates), stolons (7) or roots (12). Almost as many isolates were assembled from sclerotia (42.7%) either from tubers (212) or from roots (1). Furthermore, 6 mycelial and 17 hymenial (teleomorphic state; *Thanatephorus cucumeris*) isolates were recovered. Additionally, investigation included one stem canker lesion sample from Russian Karelia and three sclerotia originated from tubers imported to Finland from the Netherlands.

Determination of anastomosis group was conducted on all 503 isolates by sequence analysis of ITS-1 and ITS-2 regions (Papers I and II) and by microscopically evaluating hyphal contact and anastomosis of seven randomly selected isolates. Recently, most identification of *Rhizoctonia* spp., their AGs and intraspecific groups (ISG , AG subgroups) as well as phylogenetic relationship of the strains has been accomplished by investigation of the ribosomal genes encoding region and especially ITS-1 and ITS-2 areas (e.g., Ceresini et al. 2007; Kuramae et al. 2007; Godoy-Lutz et al. 2008; O'Brian et al. 2008). Nevertheless, the time-consuming identification based on anastomosis is still in broad use (Aiello et al. 2008). Both methods have limitations, especially in ISG identification which relies heavily on biological features of the fungi, including hyphal morphology and appearance, DNA relatedness, pathogenicity and zymogram patterns (Carling 1996). In general, ISGs cannot be distinguished exclusively according to their microscopic anastomosis reactions, except for AG-2-1 and AG-2-2 (Carling 1996).

4.1.2 Phylogenetic analysis of AGs

Phylogenetic analysis of amplification products (ITS-1 and ITS-2 region, including the 5.8S rRNA gene, see Figure 3. in Materials & Methods) verified that 98.8% (497) of isolates tested belonged to anastomosis group 3 (AG-3) of *R. solani*, and were closely related to reference tester isolate AG-3 PT ST-11-6 in the AG-3 subgroup able to infect potato. Recovered isolates R25 (Papers I, II), 131 L-7 (Paper II) and 173-4 (Papers II, IV) were members of AG-2-1 and isolates 239-3 (Paper II) and R96 (Papers I, II, IV) were members of AG-4 and AG-5, respectively. In contrast, isolate R92 recovered from a stem canker lesion (Papers I, II) was not related to *R. solani*, but had the highest identity (99%) with an unknown *Rhizoctonia* spp. recovered from hops (sequence accession no. AY842491) and with ectomycorrhizal fungi (Ceratobasidiaceae) from orchids (sequence accession AY634128). According to the conducted phylogenetic analysis, the 497 sequenced AG-3 isolates were divided into 67 genotypes that were clearly separated from the tobacco infecting AG-3 subgroup, AG-3 TB 1600 (accession no. AB000004), and were closely related to the potato infecting AG-3 PT subgroup. Representative ITS sequences from the isolate collection are presented in Table 1 of Paper II.

4.1.3 Anastomosis reactions between strains of R. solani

Hyphal anastomosis reactions have been classified by several authors during the last 70 years, but the contradictory and confusing terminology and focus of observations sometimes make them difficult to compare. The clearest and most widely applied terminology is that presented by Carling et al. (1988) and MacNish et al. (1993). According to this system, anastomosis reactions are vegetatively compatible reactions of the hyphae as described by Anderson (1982). They can be divided into four groups, from C0 to C3 (Table 3). No visible reaction between confronting hyphae of the isolates (C0 reaction) indicates that the isolates are not related, whereas total hyphal fusions (C3 reaction) between isolates indicates that they are closely related, either clones or members of the same vegetatively compatible population (VCP) within an AG. C3 reaction also occurs when exactly identical isolates are contacting. In the 1960s C3 reaction was therefore called ‘perfect’ or S (self) fusion (Carling 1996). An isolate’s ability to anastomose with itself is common and these strains can also be called self-anastomosing isolates (SAI) (Hyacumachi and Ui 1987). Rarely identified non-self anastomosing isolates (NSAI) of AG-2-2 have been found associated with sugar

beet (Hyakumachi and Ui 1987). They apparently contain some genetic factors missing from isolates that are able to accomplish self anastomosis.

Table 3. Classification of anastomosis reactions in *Rhizoctonia solani* according to Carling et al. (1988)

Category	Relatedness	Anastomosis group relationship	Description of the hyphal interaction
C3	Closely related	Same AG[a] and VCP[b] or clonal, i.e., somatically compatible isolates	Cell walls and membranes fuse, anastomosing and adjacent cells do not die
C2	Related	Same AG but different VCP	Cell walls join but membrane connection is uncertain, anastomosing and adjacent cells always die
C1	Distantly related	Different or same AG	Some contact between hyphae, apparently cell walls join, either one or both anastomosing cells and adjacent cells occasionally die
C0	Not related	Different AG	No interaction between confronting hypha

[a] Anastomosis group
[b] Vegetatively compatible population

The term VCP was introduced by MacNish et al. (1997) to replace the term 'clonal' relationship and to indicate close relatedness between the isolates. Morphological features seen macroscopically can be exploited for predicting anastomosis reaction when two isolates meet on artificial nutrient media (MacNish et al. 1997). The authors stated that macroscopical features, such as a 'tuft' type of border region formed during confrontation of related but vegetatively incompatible *R. solani* strains, predicts a C2 type of microscopical anastomosis reaction. The reaction was described from pairing of AG-8 isolates from different zymogram groups which led to semi-compatible, C2 hyphal interaction (MacNish et al. 1997).

To verify sequence-based AG identification, seven randomly selected isolates of Finnish AG-3, two of AG-2-1 (R25, 173-4) and each isolate of AG-4 (239-3) and AG-5 (R96), were tested for their ability to anastomose (Papers I, II). Reference testers AG-2-1 (PS-4 from pea), AG-3 (PT ST 11-6 from potato), AG-4 HG-II (Rh165 from sugar beet) and AG-5 (Rh184 from sugar beet) were obtained from Japan. Perfect hyphal fusions, showing a compatible (C3) anastomosis reaction,

indicated that all seven AG-3 isolates collected from Finland were closely related to PT ST 11-6. A compatible hyphal C3 reaction was obtained also when the Finnish AG-4 was paired with the tester isolate AG-4 HG-II (Rh165). However, AG-2-1 and AG-5 isolates collected from Finland were able to anastomose only semi-compatibly (C2) with their counterpart reference testers (Table 2 of Paper II). The C2 reaction verified that isolates were members of the nominated AGs but were not closely related to the reference testers. The C2 anastomosis reactions between Japanese tester AGs and the new Finnish AG-2-1 and AG-5 isolates indicate the same kind of distant relationship and variation of biological features as reported in AG-8 by MacNish et al. (1997). Thus the Finnish AG-2-1 and AG-5 isolates are apparently members of a different VCP than the testers.

Observed C3 reactions between the seven Finnish AG-3 isolates indicate that they belong to same VCP and are highly similar even though not identical. From the ITS region sequence and C3 hyphal interactions with the tester isolate, it is clear that all Finnish AG-3 strains are members of the potato infecting subgroup of AG-3, AG-3 PT. Following the same criterion, the Finnish AG-4 isolate was identified as highly similar to Japanese tester isolates AG-4 HG-II (Rh165). Since all tested AG-3 isolates gave strong C3 reaction during confrontation with the tester AG-3 and were members of AG-3 PT according to ITS sequence, it was concluded that the rest of the sequencing results of AG-3 isolates are reliable.

4.1.4 AGs associated with potato globally

According to ITS region sequence data, 98.8% of the Finnish strains were AG-3 and 0.6% AG-2-1. Additionally, single isolates from AGs 4 and 5, and one *Rhizoctonia* spp. were detected, each representing 0.2% of the total number of strains studied. These results correspond to global reports and confirm that AG-3 is the predominant causal agent of the Rhizoctonia disease complex in cool growing conditions around the world. Other AGs, mostly from anastomosis groups 2-1, 4 and 5, are occasionally found infecting potato and their role in infection becomes clearer at elevated temperatures (Table 4). Results indicate that in Finland AG-3 might be even more commonly associated with potato than in other countries in the Northern hemisphere, possibly excluding Denmark. Justesen et al. (2003) reported isolation of 60 Danish *R. solani* strains from stem canker and black scurf symptoms on potato and all were members of AG-3. In the present study, more isolates were collected and analysed than in Denmark but the result was almost the same (98.8%). It seems likely that the proportion of AG-3 associated with potato in Denmark is the same as in Finland.

The temperature range for AG-3 virulence establishment is wide (Carling and Leiner 1990a) and the pathogen has been noted to infect potato efficiently in low temperatures where the virulence of other potato-associated AGs is diminished (Carling and Leiner 1990b). In Finland where potato monoculture is common and crop rotation species if used are usually cereals, AG-3 is probably recovered from the soils, since cereals are not main host plants of the other potato infecting AGs such as AG-2-1, AG-4 and AG-5 (Table 1 in Introduction). Seed dressing is not always used, so seed-borne infections also play a role.

Table 4. Relative abundance (%) of anastomosis groups of *Rhizoctonia solani* and binucleate *Rhizoctonia* spp. isolates associated with potato in different geographical locations and climate conditions. Number of identified isolates is presented in the parenthesis after % amount.

Location	Climate	% of the total number of inspected isolates										Reference
		AG-1	AG-2-1	AG-2-2	AG-3	AG-4	AG-5	AG-7	AG-8	BN[a]	AG-NO[b]	
Japan	cool	0.7 (2)		0.4 (1)	96 (262)		3 (8)					Abe and Tsuboki 1978
Peru	warm					67 (40)					33 (20)	Anguiz and Martin 1989
	cool				40 (4)						60 (6)	Anguiz and Martin 1989
Canada (West)	cool				76.6 (49)	10.9 (7)	10.9 (7)				1.6 (1)	Bains and Bisth 1995
Australia (South)	cool				87 (272)	6.7 (20)	2.2 (7)		0.3 (1)	3.8 (12)		Balali et al. 1995
USA Maine	cool	1.4 (1)			79.8 (55)		13 (9)			2.9 (2)	2.9 (2)	Bandy et al. 1988
France	cool			4 (10)	94 (226)		2 (5)					Campion et al. 2003
USA Alaska	cool		20.1 (45)		73.7 (165)					2.7 (6)	3.6 (8)	Carling and Leiner 1986
Venezuela	warm		5.7 (10)		92.6 (163)					1.7 (3)		Cedeño et al. 2001
UK Northern Ireland	cool		4.4 (8)		95.6 (174)							Chand and Logan 1983
China	NA[c]	9.3 (10)	11.1 (12)		39.8 (43)	38.9 (42)					0.9 (1)	Chang and Tu 1980
Denmark	cool				100 (60)							Justesen et al. 2003
South Africa	warm				91.2 (526)	5.6 (32)	2.2 (13)	0.5 (3)	0.5 (3)			Truter and Wehner 2004
Mexico	warm				73.5	26.5						Virgen-Calleros et al. 2000[d]
UK Norfolk	cool		6.7 (9)		92.6 (125)		0.7 (1)					Woodhall et al. 2007

[a] Binucleate *Rhizoctonia* spp.
[b] Anastomosis group not designated
[c] Not designated
[d] Number of isolates unknown

4.1.5 Variation of ITS-1 and ITS-2 sequences

ITS regions are exceedingly variable and rapidly evolving sequences within an area containing highly conserved ribosomal coding sequences, so they are extensively exploited in studies dealing with fungal identification and phylogenetic relationships (Bruns et al. 1992; Boysen et al. 1996; Pope and Carter 2001; Grosch et al. 2007; Moreno et al. 2008). In nuclei, ribosomal DNA (rDNA) is organized in possibly hundreds of tandem copies and during transcription, ITS regions are also transcribed into the immature pre-rRNA and then spliced. A recent report by Anderson and Parkin (2007) showed that ITS regions can be detected in the RNA pool, indicating that removal of the regions is indeed post-transcriptional. For phylogenetic uses, ITS regions are known to be more informative and contain more variation than conserved rRNA genes.

During the sequencing of Finnish *R. solani* strains associated with potato, the existence of single nucleotide polymorphisms (SNPs) inside ITS-1 and ITS-2 regions became apparent as double signals, i.e., peaks of two alternative bases at a single nucleotide site. These observations led to a hypothesis that nuclei in multinucleate cells of an individual *R. solani* isolate may not be identical. The 497 AG-3 isolates exhibited polymorphism in 10 and 4 nucleotide positions in ITS-1 and ITS-2, respectively. Manual re-examination of the sequencing data revealed 42 individual sequences showing polymorphism (Table 5 within Paper II). Out of the examined AG-3 isolates, 86% showed this kind of ITS region heterogeneity while 34% (72) contained only one type of ITS region sequence, a haplotype of ITS region termed as ‘unisequence’ (Table 5 in Paper II). The haplotype nature of one unisequence isolate was confirmed by ligating the PCR amplification products into expression vector pGEM-T (Promega Corporation); all analysed sequence clones were identical. In this study, 80% of detected heterogeneity inside *R. solani* AG-3 ITS regions was attributable to pairwise combinations of the 9 novel unisequences detected (Table 5 in Paper II).

One AG-3 isolate, 201-3, showing a high level of polymorphism with direct sequencing was further analysed. According to direct sequencing, isolate 201-3 had one of the most frequently found heterogeneity-showing ITS sequence types, b12 (Table 5 in Paper II). Heterogeneity of the isolate 201-3 was further investigated by sequencing of 47 clones containing pGEM-T plasmid with PCR fragments of ITS1-5.8S-ITS2 region of isolate 201-3. Since not all detected polymorphism in isolate 201-3 could be assembled by combining previously identified unisequences, more unisequence types are expected to be found in future in *R. solani* AG-3.

Present research is the first one to identify extensive polymorphism in *R. solani* ITS regions. Without examination of the primary sequencing data obtained by direct sequencing of the PCR products, polymorphism remains easily unnoticed. The only preceding investigation presenting polymorphism of the ITS-1-5.8S-ITS-2 region within an isolate was that of Justesen et al. (2003) which concentrated on heterogeneity of ITS-1. Justesen et al. (2003) detected polymorphism in the ITS-1 region from 75 % of the 60 studied AG-3 isolates, and further verified that heterogeneity was a result of combinations of two different forms of ITS-1. Comparison of sequence data from Finland and Denmark showed that heterogeneity of Finnish AG-3 isolates was localized at the same 13 nucleotide positions in ITS-1 and ITS-2 as noted by Justesen et al. (2003). Additionally, in the present study, one new SNP location in ITS-2 region was identified.

4.1.6 Evolutionary predictions based on compensatory base changes in ITS-2

The ITS region database is currently the largest sequence data collection for identification of fungi (Anderson and Parkin 2007). Additionally, for fungi, ITS regions may be important for rRNA transcript processing and therefore proper folding of the area is extremely important (Mai and Coleman 1997). Folding also gives information about evolutionary processes in general, since ITS-2 structural features allow distinguishing species from each other (Selig et al. 2008). The secondary structure of the ITS-2 region has been proposed as an additional important character in fungal taxonomy (Krüger and Gargas 2008) since nucleotide positions involved in correct folding of the area are highly conserved even though the sequence of ITS-2 region evolves rapidly (Selig et al. 2008). A similar, conserved centre of the ITS-2 structure can be found even in organisms related only very distantly (Mai and Coleman 1997; Joseph et al. 1999).

In the present study, SNPs inside the ITS-2 region in Finnish AG-3 were studied for their effect on ITS-2 folding and secondary structure. Detected SNPs on ITS-2 were situated in four different nucleotide positions as presented in Tables 5 and 6 of Paper II. During preparation of ITS-2 sequences for analysis, extra information about errors in junction placement between *R. solani* AG-3 ITS-2 and 28S regions was addressed. It seemed that ITS-2 region, as annotated in GeneBank, contained nucleotides that belong to the coding region of the 28S rRNA gene. Seilig et al. (2008) reported about the same kind of GeneBank annotation errors. Accurate placement of the ITS-2 and 28S junction was re-evaluated by comparing the complete AG-3 ITS-1-5.8S-ITS-2 sequence to the corresponding sequence from the well defined biocontrol fungus *Metarhizium anisophilae* (Pantou

et al. 2003). Accordingly, the ITS-2 region of *R. solani* AG-3 was determined to contain 235 base pairs (bp) instead of 270 bp as previously reported in GeneBank. Since there is no further information about ITS-1-5.8S-ITS-2 junction placement the borders of ITS regions and adjacent rRNA genes were refined as presented in Figure 1 in Paper II. Since errors in junction placements can easily lead to wrong phylogenetic classification, proper sequence evaluation prior to ITS-2 structure modelling is crucial (Selig et al. 2008).

Structural analysis of Finnish *R. solani* AG-3 ITS-2, showed that the detected SNPs at locations 15, 45, 62 and 128, did not have any influence on the formation of accurate secondary structure. The structure of the ITS-2 was shown to be the universal four-helix containing domain found in numerous other organisms (Shultz et al. 2006). However, compensatory base changes (CBC), i.e., mutated nucleotide pairs on highly conserved paired regions of ITS-2 that do not have any influence on self pairing (Müller et al. 2007), were detected from one or two nucleotide locations in potato infecting AG-3 when compared to other AGs of *R. solani*. Additionally, potato- and tobacco-infecting subsets of AG-3 were found to differ by two CBC. According to Müller et al. (2007) and Selig et al. (2008), CBCs are specific structural features in ITS-2 and they can be used to separate species from each other. Our finding may indicate that potato-infecting AG-3 should be regarded as a separate species from other AGs as well as from the tobacco-infecting AG-3 subset.

Species are considered as independent taxonomic units in which group members are able to reproduce with each other. According to Coleman and Vacquier (2002), in eukaryotes, evolutionary distance able to produce even one CBC in ITS-2, is able to prevent hybridization, so it may be concluded that more intensive research about *R. solani* ITS-2 regions is needed in order to decide whether AG-3 should be considered as its own species. Different techniques are needed because at the time of writing, classical methods used for genetic studies of fungi have been mostly unsuccessful when applied to *R. solani*. The concept of species in genus *Rhizoctonia* might be even more complex, because of the existence of bridging isolates (BI), and some AGs of *R. solani* are able to produce a C1 anastomosis reaction with more than one AG (Carling et al. 1996). Based on host range, vegetative incompatibility and genetic variation, it has been suggested by McCabe et al (1999a) that all individual AGs were originally separate species.

4.1.7 Number of nuclei and molecular variability

Besides colony colour and hyphal diameter, number of nuclei per vegetative hyphal compartment has been one of the key morphological parameters evaluated during study of *Rhizoctonia solani* multinucleate isolates, considered to be imperfect stage of the Basidiomycete fungus *Thanatephorus cucumeris* (Parmeter et al. 1967; Kronland and Stanghellini 1988). For counting, nuclei have been stained with numerous methods. More or less effective results for staining *Rhizoctonia* spp. nuclei have been obtained with acid fuchsin (Sanford and Skoropad 1955), trypan blue (Burpee et al. 1978), aniline blue (Tu and Kimbrough 1973), acridine orange (Yamamoto and Uchida 1982), safranin O (Kranland and Stanghellini 1988), HCl-Giemsa (Herr 1979), ethidium bromide (Burpee *et al.* 1980) and 4´-6-diamidino-2-phenylindole (DAPI) (Martin 1987; Kulik and Dery 1995). An advantage of DAPI is that it reacts only with intact nuclei and can be used simultaneously during anastomosis group determination (Kulik and Dery 1995) even though in the present study anastomosis verification and nucleus counts were done independently. DAPI was used on 11 isolates representing the four detected Finnish anastomosis group, AG-2-1, AG-3, AG-4 and AG-5, and unknown *Rhizoctonia* spp. R92 (Paper II). All isolates previously identified as AGs of *R. solani* were multinucleate containing 3 – 21 nuclei per cell. Members of AG-2-1 and AG-4 contained significantly more nuclei per cell than members of AG-3 or AG-5 (Table 4 in Paper II). In previous DNA complementarity (sequencing) and hyphal compatibility (anastomosis) tests, isolate R92 showed no relatedness to any *R. solani* AGs, and according to its nuclear count, R92 was a binucleate *Rhizoctonia* spp. (BNR) (Paper I; Table 3 in Paper II).

Nuclear distribution and behaviour during the lifecycle of *R. solani* has been studied by several authors. Young vegetative compartments of hyphae contain 4 – 12 nuclei per cell and during cell division uneven numbers of nuclei migrate to the new cells, while in older hyphae, nuclear numbers per cell are reduced since branch or septa can form without nuclear division (Flentje et al. 1963). In *R. solani*, mitotic division of the nuclei is asynchronous and dividing cells can contain nuclei at several different mitotic stages (Shatla and Sinclair 1966), which explains some of the variation of nucleus numbers between hyphal cells. Nuclei also may be able to migrate occasionally from cell to cell through septal pores (Sanford and Skoropad 1955), although this was not confirmed by Flentje et al. (1963).

Even though multinucleate hyphal compartments have been one of the key features inspected during analysis of *R. solani*, few studies have reported differences in relative nuclei numbers

between AGs. To our knowledge, experiments presented in Paper II are the first to report variation in nucleus number in AGs associated with potato. Mpofu and Julian (1994) concluded that tobacco-infecting AG-3 isolates contained significantly more nuclei per cell than AG-4 strains infecting tobacco. Hyphal tips cells in a single *R. solani* isolate commonly contained 4 – 8 nuclei, whereas in side branch forming cells the number of nuclei could be as high as 25, and in straight growing vegetative hyphal cells, nucleus number was normally varying from 6 – 11 (Sanford and Skoropad 1955).

A compatible C3 anastomosis reaction between isolates leads to formation of a heterokaryotic *R. solani* strain (Cubeta et al. 1993, McCabe et al. 1999a). Nevertheless, migration of nuclei or other organelles with genetic information from one anastomosing strain to another has not been verified (McCabe et al. et al. 1999b). However, the heterokaryons and genetic variation revealed during present investigation must have their origin in the perfect hyphal fusions formed during anastomosis. Multinucleate hyphal compartments of Finnish AG-3 PT probably contain mosaics of nuclei with variable ITS regions, and genetically different mitochondrial types as well, as proposed by McCabe et al. (1999a). This molecular heterogeneity of AG-3 subgroups (Kuninaga et al. 1997; Ceresini et al. 2002a), subdivided by their host specialization into those able to infect potato (PT) or tobacco (TB), was studied by Ceresini et al. (2002a, 2002b; 2003). Population structures of AG-3 PT isolates in North Carolina implied the existence of intensive gene flow between local populations due to anastomosis between hyphae and recombination between nuclei, which both increase the diversity of isolates (Ceresini et al. 2002b). Further gene flow is accomplished by seed tuber transfer and this has led to relative uniformity of the AG-3 PT populations even over long distances (Ceresini et al. 2003). The present results verify the lack of differences between distant AG-3 PT populations, since Finnish AG-3 PT strains did not form separate clusters from strains collected elsewhere in the world (Figure 2 in Paper II).

4.2 Variation of biological traits in *Rhizoctonia solani* isolated from potato

4.2.1 Variation of morphological and biological features between isolates

In this study there was an opportunity to investigate *R. solani* strains collected from a geographically restricted area, and with a short and well recorded history in laboratory conditions. As a morphological feature specific to *R. solani*, numbers of nuclei in young hyphal cells were counted. Additionally, measured biological variables were i) virulence in terms of the ability to induce stem canker symptoms on potato, ii) tolerance against the fungicide, flutolanil ([3'-isopropoxy-2-(trifluoromethyl) benzanilide], Moncut 40 SC, Berner Ltd, Finland) commonly used for preventing diseases caused by *R. solani* in greenhouse and field conditions, and iii) growth rate on artificial solid growing media (PDA). All variables are key features in biological fitness and important for successful lifestyle for a fungus as a pathogen and plant colonizer.

4.2.2 Variation of virulence

Variation in isolate virulence, i.e., the ability to cause disease symptoms indicative of stem canker on underground parts of the developing potato sprouts prior to emergence was tested with 98 selected Finnish *R. solani* isolates, including AG-2-1 (R25), AG-5 (R96), and binucleate *Rhizoctonia* spp. R92. AG-3 has been noted to be the main and most aggressive pathogen of potato (Abe and Tsuboki 1978; Chand and Logan 1983; Otrysko et al. 1985; Carling and Leiner 1986; Carling and Leiner 1990b; Bains and Bisht 1995; Cedenõ et al. 2001; Truter and Wehner 2004), but virulence of individual isolates can fluctuate greatly, or damage can develop only on certain organs or appear at a certain time in the season. Woodhall et al. (2007), for example, reported that variation of virulence is largest among the AG-2-1 isolates collected from potato, and Abe and Tsuboki (1978) reported that incidence of AG-5 infecting potato becomes greater later in the growing season. In the present experiments, isolates were ranked according to their ability to damage potato sprouts with discolouration, necrotic lesions or death of the emerging stem. The results were presented as a mean symptom severity value (SSV); and virulence was found to vary greatly between isolates (SSV 1.6 – 59.2). Three arbitrary classes were then defined, low (SSV <25), moderate (SSV 25 – 50) and high (SSV >50). As expected, all eleven isolates highly pathogenic to potato were members of AG-3. Nineteen AG-3 isolates and members of AG-5 (R96) and binucleate *Rhizoctonia* spp. (R92) were weak pathogens with low SSV (<25). SSV values for R96 and R92 were 12.3 and 1.7, respectively.

AG-5 is commonly found associated with symptoms of stem canker or black scurf (Abe and Tsuboki 1978; Bandy et al. 1988; Bains and Bisht 1995; Balali et al. 1995; Woodhall et al. 2007). The single AG-2-1 (R25) isolate in the pathogenicity tests was not able to cause stem canker symptoms on potato sprouts and was clearly not pathogenic. Non-pathogenic isolates of AG-2-1 associated with stem canker or black scurf on potato have been reported by Woodhall et al. (2007). This result clearly indicated that AG-2-1 (R25) collected from Finland is rather a minor pathogen (Valkonen et al. 1993) than a true potato pathogen. To induce symptoms associated with the Rhizoctonia disease complex on potato, isolates do not have to be virulent since non-pathogenic strains are frequently found inhabiting sclerotia. This, together with the present findings, supports the statement of Chand and Logan (1983) that the ability of an isolate to cause sclerotia on tubers does not correlate with the ability to induce stem canker, i.e., virulence. The vast majority (74) of the isolates that were tested in the present study were members of AG-3 and moderately pathogenic to potato. Similar variation in virulence was found when the experiment was repeated a year later with seven isolates including non-pathogenic AG-2-1 and mildly virulent AG-5 and AG-BI (Figure 5a and b in Paper I). The present findings are in agreement with other reports stating that AG-3 is the predominant pathogen of potato, even though other AGs have a minor value in different climatic conditions.

The ability to infect a host plant efficiently is an important feature for a pathogen. Several reports about wide differences in the ability of *R. solani* strains to infect potato, even under favourable conditions, clearly show that genetic factors are involved in variable virulence (Rubio et al. 1996). For some *R. solani* strains, the pathogenic lifestyle is important for establishing full aggressiveness when the isolate has to infect the preferred host, whereas other *R. solani* strains persist for long periods of time in soil as saprophytes or minor pathogens of their alternative host plants (Carling and Leiner 1986; 1990a). The production of sclerotia as a seed-borne or soilborne inoculum and survival as mycelium is vital for new establishment of the disease (Mordue 1996) and it surely benefits even those isolates with relatively low virulence.

The genetic basis of *R. solani* virulence has been studied, with conflicting results. Studies have been focused on extrachromosomal element such as linear DNA plasmids and the double stranded RNA (dsRNA) found frequently in fungal hyphae. Hypovirulence has been positively associated with the presence of dsRNA (Liu et al. 2003), but in stark contrast, viral origin dsRNA was found only in virulent isolates and loss of the dsRNA in full or in part was correlated with loss of virulence (Finkler et al. 1985). The influence of extrachromosomal elements has not been verified (reviewed

by Rubio et al. 1996). The degree of virulence may be affected by the diversity of dsRNA occurring commonly in natural *R. solani* populations. The large dsRNA (M1) is associated with increased virulence whereas the small particle (M2) is suppressive of virulence in isolates infecting potato (Jian et al. 1997; Liu et al. 2003). The link between dsRNA and isolate virulence is likely to be the suppressive or enhancing effect of dsRNA on metabolic pathways involved in the synthesis of compounds critical for infection. Since these dsRNAs move from one strain to another (Charlton and Cubeta 2007), their value as biocontrol tools against virulent strains of *R. solani* is meaningful (Bandy and Tavantzis 1990; Charlton and Cubeta 2007). The virulence of AG-1, causing sheath blight on rice, was lost after disruption of the gene Rga1 encoding Gα protein, and this protein has been reported to be crucial for pathogenicity as well as for growth and development in many filamentous fungi (Charoensopharat et al. 2008).

4.2.3 Variation of fungicide tolerance and growth rate

Soilborne diseases, like the Rhizoctonia disease complex, are usually difficult to control. Protecting potato from seed-borne *R. solani* infection is accomplished with fungicide applications and by following a sufficiently long crop rotation plan. Seed-borne disease inoculum, seen as fungal sclerotia on the seed tuber surface, is controlled by seed tuber dressings. In some countries, soilborne inoculum (mycelia/sclerotia in soil) can be managed with soil fumigation (soil sterilization by hot steam or chemicals) but in Finland this is prohibited. Consequently, soilborne *R. solani* infestation can be controlled mainly by good agricultural practices and crop rotation which together can shift the AG collection in soil towards the groups that are not the main pathogens of the next crop (Herr 1993). Weed control is necessary since removal of the alternative host plants also decreases populations of the unwanted AG. A fresh crop should not be planted too soon after herbicide application, since decaying plant debris is easily colonized by the pathogen, which temporally increases the inoculum potential (van Bruggen et al. 1996). Additionally, biocontrol agents (BCA) can be applied with chemical disease control or without (Duffy 2000; Wilson et al. 2008b).

In the studies presented here, a collection of 118 isolates of *R. solani* and one AG-BI (R92) were tested for their sensitivity to flutolanil as well as for their growth rate on un-amended PDA *in vitro*. All isolates tested were relatively sensitive to flutolanil, the mean EC_{50} ranging from 0.14 to 0.75 µg active ingredient (a.i.) mL^{-1}. This range overlaps with that inhibiting growth of AG-3, AG-2-1

and AG-5 isolates collected from potato in France (EC_{50} 0.05 – 0.50 µg mL^{-1}) (Campion et al. 2003), while a wider tolerance range of AG-3 isolates from tobacco in the USA (ED_{50} 0.09 – 2.63 µg mL^{-1}) has been reported (Csinos and Stephenson 1999). When three classes of flutolanil response were defined as 'sensitive' (EC_{50}<0.30 µg a.i. mL^{-1}), 'intermediate' (EC_{50} 0.30 – 0.50 µg a.i. mL^{-1}) and 'insensitive' (EC_{50}>0.50 µg a.i. mL^{-1}) most of the isolates (67) were in the intermediate category (Figure 3a in Paper I) and 27 were insensitive. These results show that evolution of more tolerance is possible. However, the manufacturers instructions for effective use of Moncut® 40 SC (15% v/v solution for potato seed dressing containing 67.35 µg mL^{-1} flutolanil) result in concentrations nearly 100 times over the tolerance levels of the most insensitive Finnish isolates. This clearly shows that flutolanil is an effective fungicide for controlling Rhizoctonia disease in Finland and if more tolerant strains start to emerge it can be used in combinations with other effective fungicides.

Protective chemicals are widely used during the crop growing season. Besides substances targeted at pathogen inhibition, several other chemicals such as herbicides, have influence on disease outbreaks of soilborne pathogens. For example, herbicide application for potato haulm destruction prior to harvest makes plants more vulnerable to *R. solani* and other soilborne fungi, since volatile organic chemicals that attract fungi leak from the damaged tissues (Mulder et al. 1992). A wide selection of fungicidal chemicals can be used for protecting plants from *R. solani* and binucleate *Rhizoctonia* spp.; for example, phenylurea pencycuron (Monceren) is highly selective against *R. solani* and binucleate *Rhizoctonia* spp. (Jeger et al. 1996; Duffy 2000). Nevertheless, no fungicide has been found that can inhibit invasion of all *Rhizoctonia* species (Kataria et al. 1991b). In the present studies, Finnish potato-associated AGs were challenged with flutolanil (Moncut), which is used for as a dressing to control damage caused by *R. solani* hyphae germinating from the sclerotia affected seed material. Flutolanil is a systemic benzanilide fungicide (Araki and Yabutani 1993). Fungicides containing flutolanil (or benzanilide derivatives) have been used successfully to prevent diseases caused by *R. solani* on other crops including rice (Hirooka et al. 1989, Groth and Bond 2007), tall fescue turf (Green et al. 1999), snap bean (Sumner et al. 1992), tomato (Kondoh et al. 2001) and peanut (Johnson et al. 2001). Tolerance for fungicide concentrations has been demonstrated to vary between collected strains *in vitro* (Csinos and Stephenson 1999; Campion *et al*. 2003). Flutolanil inhibits mycelial consumption of O_2 and blocks the succinate dehydrogenase complex (SDC) in the mitochondrial respiratory chain that is important for Basidiomycete fungi (Motoba et al. 1988; Araki and Yabutani 1993).

Often a pathogen greatly benefits from an ability to grow rapidly in the soil, since this enables quick colonization of the potential host plant and access to nutrients. Consequently, a high growth rate can be seen as a key feature that can make a fungus highly hazardous, particularly if rapid growth is combined with high virulence and high tolerance to fungicides. During flutolanil testing, the growth rate of the same 119 Rhizoctonia disease-associated isolates, on PDA without fungicide varied from 5.1 to 14.8 mm day^{-1} (Paper I, Figure 3b). Three growth-rate groups were defined, consisting of 31 isolates with 'low' (<10 mm d^{-1}), 62 isolates with 'intermediate' (10 – 12 mm d^{-1}) and 26 isolates with 'high' (>12 mm d^{-1}) daily growth. The source of the isolate (stem canker lesion or sclerotia) was not associated with other distinctly different biological features. Only two isolates with high virulence, rapid growth and insensitivity to flutolanil were found, along with one isolate with high virulence and high fungicide tolerance, and five fast growing fungicide-insensitive isolates with intermediate or low virulence. While there were few isolates with highly expressed levels of two or three analysed traits, no correlation between variables was statistically significant (Figure 6 in Paper I). Extended storage slightly reduced virulence, growth rate and fungicide tolerance of selected *R. solani* strains when tested 12 months after first experiment.

4.3 Defence responses in potato against the early phase of infection with *R. solani* AG-3

Infection of potato by *R. solani* takes place underground prior to sprout emergence (Carling et al. 1989). Hyphae germinating from sclerotia or from the soil reservoir are infesting seed tubers swiftly and grow up to the vulnerable young sprout (Banville 1989). The outcome of the infection is called stem canker, and its symptoms are necrotic, dark lesions on sprouts and on developing stolons or roots, and extensive damage of sprout tips that may lead to death of the whole organ (Carling and Leiner 1986). Since no potato breeding programmes against Rhizoctonia diseases exist, and the knowledge about controlling soilborne fungal diseases is still very limited, basically all available potato cultivars are more or less susceptible to this pathogen. More progress with improving the resistance or tolerance to *R. solani* has been accomplished with other crop plants than with potato. In the USA, there is an intensive breeding programme for improving rice resistance against the most important rice disease in the world, sheath blight caused by AG-1-1A (Groth 2008). Intensive germplasm screening of tobacco, bean, rice and peanut has identified some sources of resistance or tolerance to the diseases caused by *R. solani* (Montoya et al. 1997; Franke et al. 1999; Pan et al. 1999; Scholten et al. 2001; Elliot et al. 2008). These reports have not, however, provided details of host molecular responses so the benefits have been limited. Franke et al. (1999) found that even though some peanut lines showed some resistant to hypocotyl rot in the greenhouse, in the field they were susceptible to limb rot caused by the same pathogen. Similarly Sholten et al. (2001) found that leaf symptoms on sugar beet were not reliable for predicting disease severity on roots, but greenhouse testing could still be used for rapid screening of available germplasm.

4.3.1 Initiation of defence responses in sprouts upon infection

Host plant responses to invasion of pathogenic *R. solani* and resistance mechanisms have not been intensively studied mostly due to the complexity of the resistance and the lack of reliable inoculation methods (Venu et al 2007). There has been some progress in the understanding of natural defence reactions in rice as a response to attack of *R. solani* (Lee et al. 2006; Venu et al. 2007; Zhao et al. 2008). Zhao et al. (2008) concluded that in rice, a response to the necrotrophic rice sheath blight pathogen is a combination of defence responses occurring during infection by different fungal and bacterial pathogens. There are clear indications of simultaneous activation of multiple defence pathways, but the details remain obscure (Lee et al. 2006) and no reports about

potato responses to *R. solani* infection are available. Therefore, Paper III presents a novel molecular study on systemic defence responses on potato sprouts after they were challenged by the pathogenic isolate of *R. solani* AG-3.

4.3.2 Experimental set-up and hypothesis

Secondary potato sprouts clearly shows improved *R. solani* resistance after first ones are infected and killed by aggressive *R. solani* (Sanford 1938). This finding indicates that in potato, some kind of dynamic defence system is activated after the first contact with the pathogen. We hypothesised that some resistance responses occur in underground parts of potato during sprouting, which could perhaps be analyzed by microarrays. The first use of microarray technique in host – *R. solani* interaction studies was in the response of rice to *R. solani* (Venu et al. 2007). Previous to that, systemic defence responses, measured as changes in levels of chitinase, glucanase and PR1 protein, were reported to be activated in tomato after plants were treated with hypovirulent *R. solani* AG-4 (Cardinale et al. 2006). Additionally, a report about changed peroxidase levels in bean hypocotyls caused by *R. solani* infection (Wasfy et al. 1984) led us to propose that peroxidases might also have an important role in potato responses to *Rhizoctonia*.

In order to study systemic defence signalling in potato sprouts, a novel experimental set-up for plant inoculations was developed, in which pathogen-inoculated basal parts of sprouts were isolated from the apices with collars made from soft plastic (Figure 4). Since in natural systems, both sprouting and infection initiation occur underground in darkness, attention was given to mimicking natural conditions. All plant handling was dome under photosynthetically inactive light. The Finnish *R. solani* AG-3, isolate R11 used, had already been characterized as highly pathogenic to potato (Paper I). The experiment was designed to measure the induction of systemic defence response in reaction to pathogen progress at two time points, 48 and 120 hours post inoculation (hpi), and changes in gene expression patterns in apices were analysed by microarrays. The two time points were chosen according to the preliminary experiments as being indicative of different phases of the infection process.

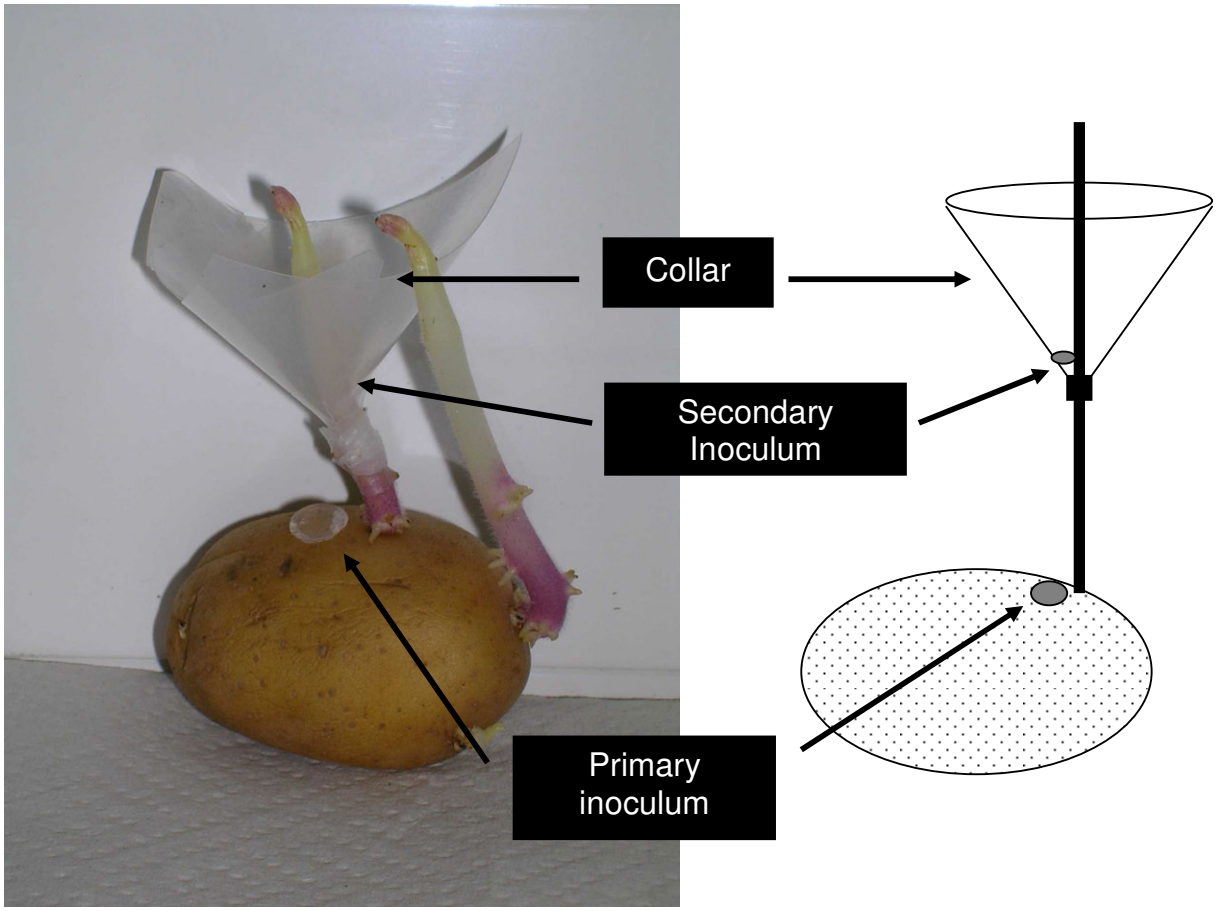


Figure 4. Experimental set-up to study induction of systemic defence responses in sprout apices of potato in response to challenged by highly pathogenic *R. solani* AG-3. The apical part of the sprout was isolated from the basal part by a soft plastic collar. Primary inoculum (lower arrowhead) was placed close to the sprout and the fungus was allowed to initiate infection. In array experiments, gene expression changes in the apical zone (area above collar) were analysed 48 and 120 hours post inoculation (hpi) and compared to non-inoculated control plants. In addition, strength of the defence response was studied by microscopy when secondary pathogen inoculum (middle arrowhead) was placed 120 hpi after inoculation of the sprout base.

No significant alterations on gene expressions were detected on mock inoculated samples between 48 and 120 hpi, so all observed molecular changes were considered to be induced by pathogen. Results of the microarray analysis were further verified with qRT-PCR analysis.

4.3.3 Early molecular events in infection of sprouts with virulent Rhizoctonia solani

The microarray analysis showed significant changes in potato gene expression patterns at 48 hpi when 77 and 45 genes were found to be up-regulated or down-regulated, respectively (Table 1 in Paper III). At 120 hpi 453 genes were up-regulated and 326 genes down-regulated (Table 1 in Paper III). The genes with changed expression at 48 and 120 hpi were grouped into nine subjective classes according to their known or predicted biological function (Figure 5). The greatest amount of alteration in expression was found in the groups associated with 'Primary and secondary metabolism' and 'Disease defence and cell rescue'. The number of up-regulated genes was greatest in the defence related class, in which 27 and 79 were noted to be up-regulated at 48 and 120 hpi, respectively.

All resistance associated genes active in potato sprouts at 48 hpi, except for the one encoding phenylalanine ammonia lyase (PAL), were still highly expressed at 120 hpi. Early accumulation of PAL indicates rapid activation of phenylpropanoid pathway leading to production of fungitoxic phenols and phytoalexins (Sareena et al. 2006). In potato, PAL also catalyses the first step of SA production and lignin synthesis (Nakane et al. 2003) which leads to establishment of SAR and strengthening of cell walls with barriers that protect the host from the invading pathogen. Similarly, increased PAL and peroxidase levels by SA enhanced resistance to *R. solani* in cowpea (Chandra et al. 2007).

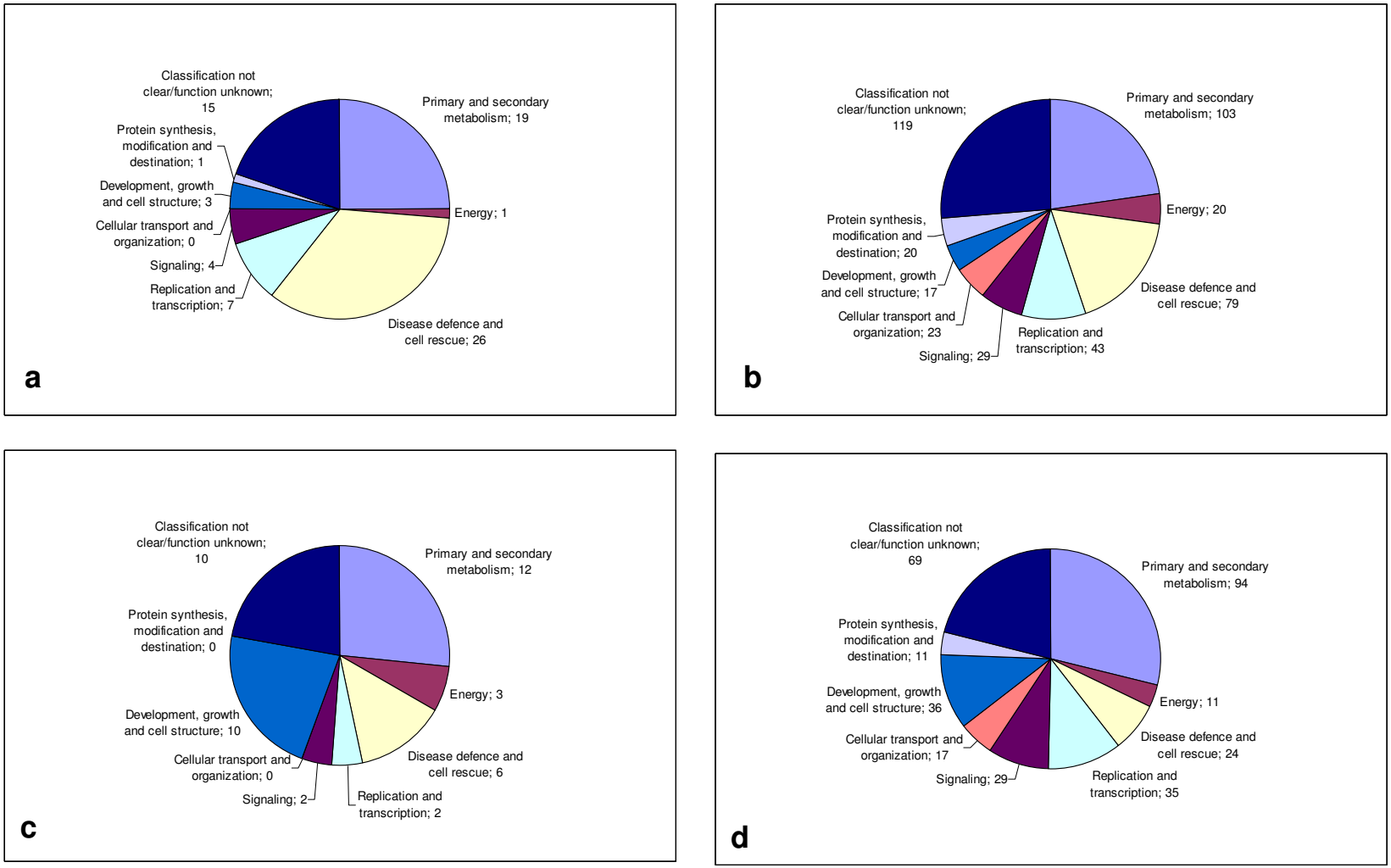


Figure 5. Changes in potato gene expression in the uninoculated apical part of the sprout due to inoculation of the base of the sprout. In total 76 genes were noted to be induced at 48 hours post inoculation (hpi) (a) whereas at 120 hpi 453 genes were induced (b). In addition, 45 and 326 genes were noted to be suppressed at 48 hpi (c) and 120 hpi (d), respectively.

Phytoalexin accumulation in soybean roots as a result of *R. solani* infection was reported by Wyss et al. (1991). Similar types of host responses, effective against virulent *R. solani*, can be initiated in host by treating host plants with hypovirulent *R. solani* strains (Cardinale et al. 2006) or with non-pathogenic binucleate *Rhizoctonia* spp. (np-BNR) (Jabaji-Hare et al. 1999; Wen et al. 2005) or with other BCAs, e.g., *Pseudomonas fluorescens* (Radjacommare et al. 2004). Successful activation of defence response has also been accomplished by treating plants with eliciting chemicals (inducers) such as chitin or SA (Rajkumar et al. 2008), α-1,3-glucan (Wolski et al. 2006), and acibenzolar-S-methyl (Faessel et al. 2008). These applications have been used effectively for biocontrol purposes to induce defence response, i.e., to prime resistance before contact with the real pathogen (Wen et al. 2005; Wolski et al. 2006). For example, glucanase and chitinase activity in potato sprouts was greatly enhanced after treatment with α-1,3-glucan isolated from np-BNR (Wolski et al. 2006). In all these examples, breakdown products of cell walls as a result of hydrolytic action of the fungal/bacterial enzymes apparently acted as elicitors of defence response (Jabaji-Hare et al. 1999) and the induced defence enzymes were able to make plants more resistant to pathogen invasion (van Loon et al. 1998).

Nevertheless, defence responses initiated in bean by the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* did not significantly reduce the disease symptoms when plants were later challenged by virulent *R. solani* (Guillon et al. 2002). This may indicate that after defence initiation, AM fungi are able to suppress resistance in order to form mycorrhiza. This was verified by Guenoune et al. (2001) who reported that defence responses to pathogenic *R. solani* on alfalfa roots were significantly reduced when plants were inoculated with AM fungus, *G. intraradices*. Similar results have been reported on soybean (Wyss et al. 1991). In addition to priming the resistance with BCAs or chemical elicitors, constitutive expression of antifungal proteins has been shown to increase tolerance of transgenic plants against pathogenic *R. solani* (reviewed by Punja 2001). Several positive results have been obtained by transforming plants with genes encoding PR proteins (e.g., Punja and Rahardo 1996; Datta et al. 1999; Sareena et al. 2006) which are normally expressed in the host plant under pathogen attack or when stressed physiologically (van Loon and van Strien 1999). A recent report by Almasia et al. (2008) describes successful enhancement of resistance against *R. solani* and *Erwinia carotovora* in potato plants that overexpress the *snakin-1* gene, a cystein-rich peptide with antimicrobial activity.

In the present study, SA induced basic PR1 was found to be activated as reported in rice when challenged to *R. solani* (Zhao et al. 2008). In addition, numerous defence genes coding known antimicrobial PR proteins (van Loon and van Strien 1999) such as different classes of chitinases, 1,3-β-glucanases, peroxidases and their precursors were activated in sprout tops after the base was challenged with pathogenic *R. solani*. Previous studies has shown that several PR proteins are 1,3-β-glucanases and chitinases (Kombrink et al. 1988). Furthermore, a number of genes encoding defence-associated kinases, host protein protecting substances, and genes leading to phytoalexin accumulation were detected in the present study. In rice, expression level of chitinases I and II correlate positively to resistance against sheath blight inducing *R. solani* (Shrestha et al. 2008). SA-independent basic Class I chitinases expressed rapidly and strongly in the present studies have been reported to accumulate in potato as a result of mould infection, elicitor treatment and during plant wounding (Ancillo et al. 1999). Furthermore, WRKY-like transcription factors were up-regulated in the present study. These transcription factors have been previously reported to be co-expressed with class I chitinase in potato as a result of infection with the soft-rot causing bacterium (*Erwinia carotovora* subsp. *atroseptica*) or late blight inducing mould (*Phytophthora infestans*) (Dellagi et al. 2000). Thus in potato, activation of defence pathways other than that mediated by SA seems to occur in a synchronised manner establishing improved tolerance as also found in rice (Zhao et al. 2008)

Not only basic chitinases, but also acidic chitinases were induced, demonstrating the close connection and orchestrated action of these enzymes. In the present study, one of the most up-regulated acidic chitinases was a member of class IV. This class has been reported to be activated in Norway spruce after infection with *Heterobasidion parviporum* (Karlsson et al. 2007) and in tobacco after eliciting the plant with β-1,3,-1,6-glucan from *Alternaria alternata* (Shinya et al. 2007). There are strong indications that IV class chitinase is important for establishment of early and general defence responses after fungal and bacterial infection of tobacco (Ott et al. 2006; Shinya et al. 2007). Expression of class IV chitinases was stronger and levels of PAL and peroxidases were lower in the susceptible than in resistant spruce trees (Karlsson et al. 2007). In our studies, no comparisons between resistant and susceptible plants were made as no resistant cultivars were available.

Many genes activated in potato after *R. solani* infection were those induced by JA. Similarly, in grapevine, chitinase IV, serine protease inhibitor and 1,3-β-glucanase were induced by JA (Belhadj et al. 2006). Regulation of basal defence by chitinase IV may however, be more complex since in

tobacco, SA or JA merely suppressed the elicitor-activated chitinase IV expression, suggesting its role in early defence reactions (Shinya et al. 2007). In the present studies, at 120 hpi, JA-induced proteinase inhibitor II was found to be activated, as reported also from potato leaves only four hours post infection with *Erwinia carotovora* ssp. *carotovora* (Montesano et al. 2005).

The results of these analysis show that defence responses of potato to highly virulent *R. solani* AG-3 isolates are complex, and co-activation/suppression of both SA- and JA-mediated defence signalling pathways are indicated, as found also in rice (Lee et al. 2006; Zhao et al. 2008). Similar complexity of signalling has been reported to occur in numerous situations during biotic and abiotic stresses that induce defence responses (reviewed by Reymond 2001). Here, potato defence responses were demonstrated to be rapidly induced even before the fungus initiates the infection structures needed for penetrating the host. Since there were clear markers for both SA and JA pathways, we cannot determine their relative importance without further experiments. The results demonstrate the existence of a complex defence signalling network activated in potato sprout tips when the sprout bases were infected with the pathogenic *R. solani* AG-3 isolate. The spread of defence reactions from the original point of infection demonstrates the systemic nature of the response.

4.4 Development of infection structures on potato sprouts

Sanford's (1938) claim that secondary potato sprouts showed improved tolerance to *R. solani* infection together with the present demonstration of systemic defence response in pathogen-inoculated sprouts, prompted the evaluation of the strength of the induced resistance. In additional experiments plants were treated as before (Figure 4) but the apical parts of the symptomless sprouts from the previously inoculated and mock-inoculated experiments were challenged later by the same virulent strain R11 of *R. solani* AG-3. Infection was left to develop for three weeks, long enough for emergence of fully mature fungal infection structures and host necrotic lesions, and then symptoms were viewed by microscopy using lactophenol-cottonblue as a stain (Figure 2 c, d, e, f in Paper III).

The results of both experiments consistently demonstrated high levels of systemically induced resistance to secondary infection by *R. solani*. Hyphal growth was strongly inhibited and the formation of infection structures was negligible on 14 out of the 16 sprouts tested (87.5 %) (Figure 2 c, d in Paper III). In contrast, hyphal growth was substantial and numerous infection cushions

were formed on the tips of sprout that had not been basally inoculated (Figure 2 e, f in Paper III). The most extensive growth and damage was found on emerging lateral stolons and on a zone 3 – 7 mm under the sprout tip which are consistent with the preferred infection points identified by Richards (1921) and Sanford (1938). In this thesis, primary inoculation with the virulent *R. solani* isolate led to enhanced tolerance to further infections with the same pathogen and this finding helps to elucidate how, in field conditions, total failure of potato emergence due to *R. solani* does not happen even under heavy disease pressure, as noted already decades ago by Sanford (1938).

4.5 Development of black scurf and survival of the *R. solani*

R. solani survives between growing seasons as sclerotia, which are melanised, tight hyphal clumps formed on surfaces of the tubers or in soil, and as saprophytic mycelia in soil or on decaying plant material (Herr 1993). In old potato growing areas, infection is established from both inoculum sources whereas in new areas, the infecting pathogen is transferred as sclerotia on the seed tuber as discussed previously. Both infection routes are important for the development of black scurf on progeny tubers in greenhouse conditions (Tsror and Peretz-Alon 2005), and co-existence of the inoculum types increases black scurf symptoms of the field crop (Tsror and Peretz-Alon 2005). It seems likely that the ability to develop sclerotia is a survival mechanism that initiates promptly after host resistance and inhibitory actions decrease or the concentration of attracting substances leaking from the new tubers is increasing.

In the present study, the majority of the *R. solani* isolates collected from sclerotia on potato were members of AG-3, even though AG-2-1, AG-4 and AG-5 have also been isolated from sclerotia all over the world (Abe and Tsuboki 1978; Chand and Logan 1983; Carling and Leiner 1986; Anguiz and Martin 1989; Balali et al. 1995; Truter and Wehner 2004; Woodhall et al. 2007). The ability to form sclerotia is not dependent on the virulence of the isolate towards the host (Chand and Logan 1983) but seems to be a combination of environmental factors and host preference of the AG in question (Paper IV).

In greenhouse experiments, disease-free seed tubers were grown in clean sand inoculated with Finnish *R. solani* virulent isolates representing AGs 2-1, 3 and 5, and also Japanese tester isolates for AG-2-1 and AG-5 (Paper IV). In these favourable conditions, all the tested Finnish *R. solani* isolates from different AGs were able to induce at least some stem canker symptoms as determined

48 days post planting (dpp). Interestingly, Finnish AG-5 was the most virulent, which might be due to the growing temperature that was close to optimal for establishment of full virulence of AG-5 (Carling and Leiner 1990b). According to Dijst (1985), production of sclerotia is dependent on the maturation stage of the developing tubers. During the growing season, CO_2 produced by respiration of the potato plants seems to be an important chemical for preventing the formation of sclerotia (Dijst 1990). However, as soon as plant senescence starts or haulms are destroyed, the tuber surface strengthens and the connection between tubers and parent plants weakens, the amount of inhibitory volatile chemicals leaking from the underground plant parts decrease and they no longer prevent the formation of sclerotia (Dijst 1985; 1990). After maturation, other, still unidentified volatile and ephemeral chemicals promoting sclerotial development including those produced by stress and decomposition of the plant parts become more abundant (Dijst 1990). As proposed by Dijst (1990), the best techniques to prevent the formation of sclerotia by *R. solani* after haulm killing are to quickly cut the connection between tubers and plant, and loosen the soil in order to hasten the evaporation of stimulant volatile chemicals.

In the present studies, in order to maximize development of black scurf on progeny tubers, haulms of the potato plants were removed two weeks before harvest at 120 dpp and soil was left undisturbed. All Finnish AG-3 isolates were able to induce sclerotia on progeny tubers but only one other isolate, AG-2-1, caused black scurf, but the signs of the disease were minor in comparison with those from extensive AG-3 infestation. Infection with AG-3 caused mild to moderate black scurf signs on progeny tubers, of which 5 – 10% were free from sclerotia, whereas infection with AG-2-1 caused only very mild sclerotia formation and over 90% of tubers were healthy with no signs of black scurf. Only infection with AG-3 had a negative effect on yield of marketable size tubers as well as tuber number in total, in agreement with previous findings (Carling et al. 1989; Read et al. 1989).

It is concluded that AG-3 is major pathogen of potato, capable of causing extensive damage to the crop prior to and after tuber formation, especially in cool and moist climatic conditions. Continuous potato production and use of seed tubers covered with black scurf builds up the inoculum potential in the soil. One reason for the success of AG-3 seems to be its close and highly specialized relationship with potato, since the other AGs able to infect potato, do not necessarily do so, even in controlled systems favouring infection development. It is likely that other AGs occasionally associated with potato are merely “stowaways”, taking some benefit from potato in the absence of more suitable host plants.

CONCLUDING REMARKS & FUTURE PROSPECTS

In this thesis, the variability of the fungal pathogen of potato, *Rhizoctonia solani*, was surveyed in Finland, in one of the northernmost countries with intensive potato production (Paper I). The vast majority of the *R. solani* isolates were closely related, even though some genetic heterogeneity was detected. AG-3 is the main anastomosis group infecting potato in Finland, as it is all over the world. Variability of the biological features obviously important for the pathogenic lifestyle of the fungus was wide, but fortunately for users of the crop, 'super-isolates' that are fast growing, highly virulent and fungicide tolerant were not detected. This indicates that for the foreseeable future, chemical control will remain one of the effective methods for plant protection. However, plant protection applications exploiting the natural defence reaction occurring in potato might be worth for investigating as a part of crop protection scheme, since together with chemical control, crop rotation and good agricultural practises it might give a new, more effective means to prevent annual yield losses caused by Rhizoctonia disease complex.

R. solani is most studied species of the intricate genus of *Rhizoctonia*. For years, classification of the isolates has been done by microscopical examination together or separately with biochemical tests. Since development of DNA based assays, research of *R. solani* is moving more and more towards using molecular analysis methods. New assays have speed up research and a huge amount of important information has been gathered about the individual anastomosis groups and diversity and heterogeneity of the isolates. In the work presented above, new information about the ITS-2 region heterogeneity between and within potato infecting AG-3 isolates and ITS region compensatory base changes is presented for the first time. Detected base changes confirm that *R. solani* AG-3 PT isolates differ from other *R. solani* AGs and also from tobacco infecting AG-3 subgroup TB as presented in Paper II. In the future more detailed analysis is needed to investigate if AG-3 PT should consider as a separate species and not just an AG of *R. solani*.

The Rhizoctonia disease complex mainly affects quality and quantity of the crop, rather than destroying it, so existing breeding programmes have concentrated on protecting potato from other, more severe pathogens and there are no available cultivars resistant to stem canker and black scurf. Existence of the active defence mechanism against *R. solani* in potato has not been studied before. The decades ago reported information that potato plants are still able to emerge even under heavy

disease pressure made us assume that some defence reactions have to occur. A novel study (Paper III) showed that potato sprouts are able to start systemic defence responses against *R. solani* when the fungus comes into contact with the host plant. The defence reaction is initiated soon after confrontation, and once established, the pathogen resistance of sprouts continues to increase. At last, sprout tip becomes resistant to *R. solani*. At molecular level, occurred defence responses are complex and signalling happens via both SA and JA dependent reaction pathways. Comprehensive research of the defence process is needed in order to understand its mechanism. Hopefully in the future we are able to develop methods to protect potato against diseases caused by *R. solani*.

Formation of sclerotia was studied (Paper IV) since in normal crop rotation systems, sclerotia on tubers are the main source of long-distance dispersal of disease inoculum. The results indicated that AG-3 isolates typically infecting potato were also efficient in forming sclerotia on progeny tubers. Hence, the probability of survival of these strains is better than those of other *R. solani* AGs poor in sclerotia-formation when associated with potato. However, saprophytic survival of the AG-3 is poor. It seems likely that AG-3 PT is highly specialized to its host plant potato and without it population levels decline fast until totally fading. If innovative methods to protect new tubers from the sclerotia formation are developed and resistance against *R. solani* is improved, we might be able to reduce significantly the incidence of the Rhizoctonia disease complex, and finally have a valuable way to prevent spread and long term survival of this pathogen.

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Helsinki, April 2009

Mari

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