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Development of Alternative Strategies for the Control of the Important Phytopathogens *Phytophthora infestans* (Mont.) and *Erwinia amylovora* (Burrill)

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ABBREVIATIONS

Avr	Avirulence
BAP	6-Benzylaminoburine
bp	base pair
BTh	Break-Thru® S 240
BTH	Benzothiadiazole
BVL	Federal Office of consumer protection and food safety
ºC	Celsius grade
Ca^{+2}	Calcium Ions
$CaCl_2$	Calcium Chloride
$CaCO_3$	Calcium Carbonate
CF	Culture Filtrate
CGG	Centrum Grüne Gentechnik
cm	centimetre
$CoCl_2$	Carbonyl dichloride
$CuSO_4$	Copper sulphate
cv	cultivar
d	day
DBU	Deutsche Bundesstiftung Umwelt
DCINA	Dichloroisonicotinic Acid
ddH_2O	Double Deionised Water
DLR	Dienstleistungszentrum Ländlicher Raum´Rheinpfalz
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
E	Elicitor
EDTA	Ethylene-diamine-tetraacetic Acid
Em	Emission
EtOH	Ethanol
Ex	Excitation
$FeSO_4$	Ferrous Sulphate
g	gram
GA	Gibberelic Acid
GFP	Green Fluorescent Protein
GmbH	Gesellschaft mit beschränkter Haftung
GUS	β-glucuronidase
H_3BO_3	Boric Acid
HCl	Hydrochloric Acid
HPLC	High-Performance Liquid Chromatography
HR	Hypersensitive Reaction
HrpN	Harpin
IBA	Indol Butyric Acid
IAA	Indole Acetic Acid
INA	Iso Nicotinic Acid
ISR	Induced Systemic Resistance
KNO_3	Potassium Nitrate
L	Litre
LR	Local Resistance
µg	Micro gram
µl	Micro litre
Mg	Magnesium
mg	milligram
$MgSO_4$	Magnesium Sulphate
MGA	Mycelial Growth Area
min	minute
ml	Millilitre
mm	millimetre
mM	millimole
$MnSO_4$	Manganese Sulphate

mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrophotometer
MS-medium	Murashige and Skoog medium
MyEx	Mycelium Extract
N&N-Vitamin	Nitsch&Nitsch-Vitamin
NAA	1-Naphthalin Acetic Acid
NaClO	Sodium hypochlorite
Na_2EDTA	EDTA Disodium Salt
$NaH2PO_4$	Sodium Monobasic Phosphate
NaOH	Sodium hydroxide
Na_2MoO_4	Sodium Molybdate Crystal
NB	Nutrient Broth
ng	nano gram
$(NH_4)2SO_4$	Ammonium Sulphate
NMR	Nuclear Magnetic Resonance
Nr	Number
OD	Optical Density
Pc	Parsley cells test
Pc+	Positive in parsley cells test
PCR	Pathogen Chain Reaction
PDA	Potato Dextrose Agar
pH	potential hydrogen
Pi	*Phytophthora in vitro* test
Pi+	Positive in *Phytophthora in vitro* test
ProInno	Programm zur Förderung der Erhörung der Innovationskompetenz mittelständischer Unternehmen
PR-Proteins	Pathogenesis Related Proteins
PTGS	Post-transcriptional Gene Silencing
RF	Relative Fluorescence
ROS	Reactive Oxygen Species
RM	Rooting Medium
RNA	Ribonucleic Acid
RNAi	RNA Interference
rpm	rotation per minute
RT	Room Temperature
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SD	Standard Deviation
SDS	Natrium-dodecyl-sulphate
Sec	Second
siRNA	small interfering RNA
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
UV	Ultra Violet-light
Vol	Volume
WGA	Weight of Growth Area
WT	Wild Type
Xg	Acceleration of Gravity
YEP-medium	Yeast Extract Peptone-medium
$ZnSO_4$	Zinc Sulphate

General introduction

1 INTRODUCTION

1.1 Tomato late blight disease

Tomato (*Lycopersicon esculentum* Mill.) is one of the most economically important crops in several production countries, and it is grown worldwide. Tomato is highly susceptible to environmental stress, to numerous arthropod pests and most importantly, to a number of fungal, bacterial and viral pathogens. Up to 95% of losses of tomato yield is due to the incidence of biotic diseases (Kalloo, 1993).

Several fungal diseases cause severe losses of tomato crop like late blight caused by *Phytophthora infestans*, damping-off and wilt diseases caused by *Pythium* spp., *Rhizoctonia solani*, *Verticillium* and *Fusarium* wilts as well as to powdery and downy mildews (Agrios, 1997; Larkin and Fravel, 1988; Sumner and Phatak, 1988).

Pathogen description and life cycle: Late blight, caused by *P. infestans* (Mont.) de Bary, is the most devastating disease for tomato as well as for potato plants worldwide unlike most *Phytophthora* species. More than 150 years have been elapsed since *P. infestans* caused the Irish potato famine, but strategies for managing potato and tomato late blight often remain unsustainable and costly (Kamoun, 2003).

P. infestans (Kingdom: Chromista (Stramenopiles), Phylum: Oomycota, Class: Oomycetes, Order: Peronosporales, Family: Pythiaceae, Genus: *Phytophthora*) is a specialized pathogen, primarily causing disease on the foliage, stems, potato tubers and tomato fruits, with most infection spread by airborne asexual sporangia during the growing season (Shattock, 2002; Erwin and Ribeiro, 1996; Dowley *et al.*, 1995). *P. infestans* belongs to the class of Oomycetes. The Oomycetes comprise a diverse group of eukaryotic microorganisms that include a wide range of plant pathogens such as water molds, white rusts and downy mildews. The position of the Oomycetes as a unique lineage of the eukaryotes, unrelated to true fungi but closely related to heterokont (brown) algae and diatoms is now well established based on molecular phylogenetic and biochemical studies (Kamoun, 2003; Baldauf *et al.*, 2000; Sogin and Silberman, 1998). It is evident from these analyses that Oomycetes evolved the ability to infect plant independently from other eukaryotic plant pathogens.

For a successful infection and colonisation of *P. infestans* in its host plant, a series of pathogenic processes are needed (Huitema *et al.*, 2004; Kamoun, 2003). The infection starts with the adhesion to the plant surfaces, followed by penetration and colonisation of host tissues. The pathogen secretes proteins and some other molecules which

participate and help the pathogen to attach the plant surfaces, while extracellular enzymes are able to degrade physical barriers of cell walls and other macromolecules of host cells. Other molecules influence the physiology of the host by suppressing or inducing host defence responses (Torto *et al.*, 2003).

The fungus appears as a whitish mycelium on the surface of infected potato and tomato leaves and stems when it emerges through the stomata in humid conditions. Microscopic treelike hyphae grow away from the plant tissue, producing lemon-shaped sporangia at their tips (Figure 1). A sporangium is a structure that develops zoospores, the reproductive units of a fungus. The sporangia are usually dispersed by air currents to neighbouring plants and can be transported easily to nearby fields when the air is moist. They dry out and die at high temperatures. At cool temperatures, a change occurs in sporangia when they land on a wet potato or tomato leaf or stem. After a few hours, the cellular material inside the sporangium is converted into about eight wiggling zoospores, each with two flagella. The zoospores are released from the sporangium and after swimming they form cysts which attach to the leaf surface. A germ tube formed penetrates the host tissues. From the tiny infection sites, extensive networks of threadlike hyphae develop intercellularly and intracellularly by producing an organ called haustorium which absorbs nutrients to feed the growing fungus. Brown lesions of dead plant cells and newly produced sporangia begin to appear about five days after infection. The fungus can produce many generations of sporangia in a short time and rapidly colonize all available tissues. Beside asexual sporulation, the late blight fungus is also able to perform sexual propagation by the meeting between the oogonium (female organ) and the antheridium (male organ) resulting in a thick walled oospore which also serves as the survival and sexual stage of the fungus. The sexual life cycle may affect the epidemiology and increase the development of virulent races which overcome resistance genes in crop cultivars and also the selection of fungicide resistant strains in the population of the late blight fungus (Agrios, 1997; Fry and Goodwin, 1995).

A combination of high inoculum pressure and humid conditions that favour pathogen growth and frequent fungicides applications have resulted in emergence of pathogenic strains resistant to specific acting fungicides in several countries (Gisi and Cohen, 1996). *P. infestans* causes losses of billions of dollars annually in potato and tomato production (Ristanio, 2002; Smart and Fry, 2001; Duncan, 1999). In the United States and some other developed countries, chronic use of chemical fungicides to manage

late blight reduces the profit margins of farmers and is not always successful (when fungicides are not used properly). Some populations of the pathogen are resistant to metalaxyl (and the active enantiomer mefenoxam), which previously had been shown to be very successful for controlling *P. infestans* (Fry and Goodwin, 1997).

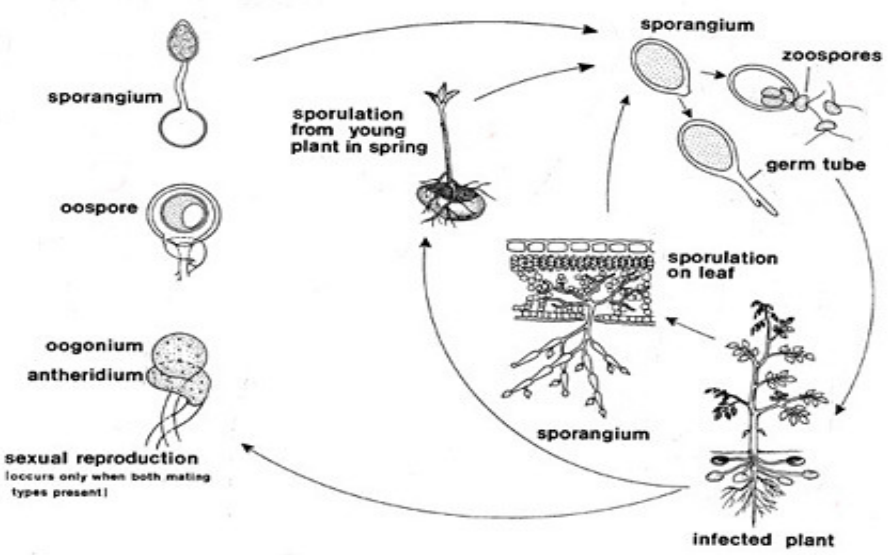


Figure 1: Sexual and a sexual life cycle diagram of *Phytophthora infestans*, the causal agent of late blight of potato and tomato.

Disease control: To control this disease, it was found that integrated management practices are necessary for successful suppression of late blight. In the absence of sexual reproduction, *P. infestans* requires a living host to survive between seasons. Therefore, sanitation (elimination or exclusion of infected plant parts from a farm) is important in the overall management strategy. Ideally, no infected potatoes should be present in the vicinity of the crop. Volunteer plants that might be infected should be destroyed. Only tubers that are free of *P. infestans* should be planted. The certified grade for seed potatoes and tomatoes allows up to 1% late blight infection. Growers should request information from potato seed producers as to whether late blight was observed during field or harvest inspections. After planting, additional precautions will reduce the chances of successful inoculations and can suppress development and reproduction of the pathogen. Using resistant cultivars will reduce the chances of infection and slow the pathogen growth rate if some infections develop. Early in the season, treatment with protecting fungicides will provide protection and thus prevent a rapid epidemic. Fungicide may be applied either at an appropriate regular interval or adjusted on the basis of weather conditions. Several forecasting systems that identify

favourable weather conditions are available (e.g., Blitecast, Tomcast) and can be used to adjust the disease development as well as the frequency of fungicide applications. Hilling of potatoes increases the amount of soil between tubers and the soil surface and thus helps to protect tubers from sporangia that land on the soil surface (Bouma and Schepers, 1997; Fry and Goodwin, 1997; Inglis *et al.*, 1996; Ingram and Williams, 1991; Fry and Shtienberg, 1990).

In developing countries, late blight also affected subsistence in the 1990s. In Eastern Europe, the disease has caused significant losses in yield (Garelik, 2002; Schiermeier, 2001). In 2003, potato production was nearly eliminated in Papua New Guinea, one of the few countries in the world that was previously free of the disease (Anonymous, 2004). Remarkably, the disease spread through the entire country within two months of first incidence. Reports predict that potato late blight will continue to cause food shortages and hunger in several parts of the world (Garelik, 2002; Schiermeier, 2001).

The use of some synthetic chemicals to control fungal disease is restricted due to their possible carcinogenicity, high and acute toxicity, long degradation periods, and environmental pollution. So, the use of biologically based compounds can be an alternative to currently applied chemical fungicides to control phytopathogenic fungi and bacteria (Burt, 2004; Isman, 2000). The biological compounds which are active against fungal and bacterial pathogens have to be biodegradable to non-toxic products, and potentially suitable for use in integrated pest management programs; they may represent a new class of safer disease control agents (Soylu *et al.*, 2006; Schmutterer, 1990). Some of these biologically based compounds are produced by kinds of bacteria called Actinomycetes.

1.2 Apple fire blight disease

Disease discovery: Fire blight is a disease of *Maloideae* (apple, pear, ornamentals) caused by the bacterium *Erwinia amylovora* (Burril). Fire blight was first described in North America about 200 years ago (Van Der Zwet and Beer, 1992). The first discovery of this bacterium in Europe was in 1957 (Lelliot, 1959) through imported contaminated fruits and bud wood from North America. Since that time it has been started to distribute in most of the European countries (Van Der Zwet and Bonn, 1999). Figure 2 shows the distribution of the fire blight disease in Germany.

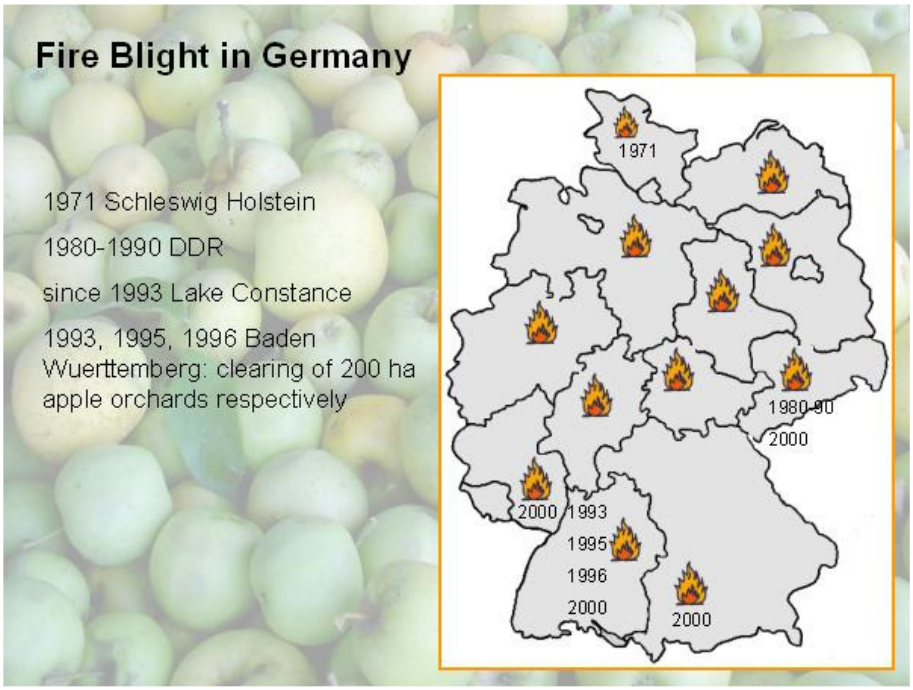


Figure 2: Distribution of fire blight disease in the different states in Germany, the first appearance in some states is indicated.

Disease description and life cycle: *E. amylovora* is a Gram-negative bacterium which invades the plants via natural openings in flowers (nectarthods) or through wounds on young aerial vegetative parts. The bacterial pathogen causing fire blight overwinters almost exclusively in cankers on limbs infected the previous season. The largest number of cankers and, hence, most important in contributing to the inoculum, occur on limbs smaller than 38 mm in diameter, especially around cuts made the previous year to remove blighted limbs. During the early spring, in response to warmer temperatures and rapid bud development, the bacteria at canker margins begin multiplying rapidly and produce a thick yellowish to white ooze that appears onto the bark surface up to several weeks before the bloom period. Many insect species (predominantly flies) are attracted by the ooze, and subsequently disperse the bacteria throughout the orchard. Once the first few open blossoms are colonized by the bacteria, pollinating insects rapidly spread the pathogen to other flowers, initiating more blossom blight. These colonized flowers are subject to infection after wetting events caused by rain or heavy dew when the average daily temperatures are equal to or greater than 16 ºC, while the flower petals are intact (flower receptacles and young fruits are resistant after petal fall). Once blossom infections occur, early symptoms can be expected (Figure 3) (Brisset *et al.*, 2000; Agrios, 1997; Thomson, 1992).

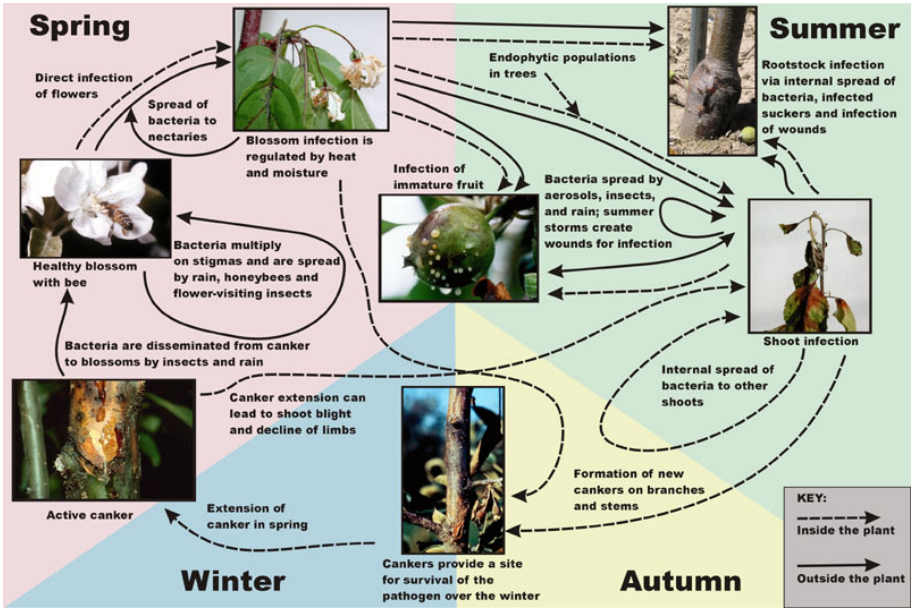


Figure 3: Disease cycle of *Erwinia amylovora* the causal agent of fire blight disease in apple trees.

Disease control: The control of this disease is focused on many strategies:

1. Chemical and biological control: A copper spray applied at the 1/4-inch green tip stage may reduce the amount of inoculum on the outer surfaces of infected trees. At bloom, antibiotic sprays are highly effective against the blossom blight phase of the disease. These sprays are critical because effective early season disease control often prevents the disease from becoming established in an orchard. Predictive models, particularly Maryblyt, help to identify potential infection periods and improve the timing of antibiotic treatments, as well as to avoid unnecessary treatments. Strains of the pathogen that are resistant to streptomycin are present in some orchards in USA and Europe, and are widespread in most apple and pear regions. Copper compounds applied during flowering period usually cause phytotoxicity to flowers and fruits. Biological control agents, although not widely used, have provided partial control of blossom infections. More effective biological agents are required (Brisset *et al.,* 2000*;* Ortega *et al.,* 1998).

2. Removing sources of infection: Dormant pruning by removing overwintering infections helps to reduce inoculum for the next season. On young trees, and those on dwarfing rootstocks, early infections in the tops of the trees often provide inoculum for later infections of shoots and sprouts on lower limbs near the trunk, which may result in tree losses. These early strikes in the tops of trees should have a high priority for removal (Paulin*,* 1996).

3. Insect control: The role of insects in the transmission of fire blight bacterium is under investigation. It is likely that insects that cause wounds (leafhoppers, plant bugs, pear psylla) may create sites for bacterial cells to enter the tree, and some summer infections (shoot blight) are probably facilitated by insects. Where fire blight is a problem, and until more is known about their specific role in the spread of the disease, controlling these insects at levels below their economic injury threshold is advised (Paulin, 1996).

4. Resistant cultivars: When establishing new orchards, susceptibilities of the scion and rootstock to fire blight should be considered. Although non immune plant material is available, there is considerable variation among apple cultivars (and pear cultivars) in susceptibility to fire blight. Some cultivar-rootstock combinations are so susceptible to fire blight that investments in these are an extremely high risk (Ortega *et al*., 1998; Kessmann *et al*., 1994).

5. Plant induced resistance: This method represents also one of the disease control possibilities, but, only few studies have reported on induced resistance in fruit trees. One of the effective chemicals inducing resistance against fire blight was 2,6-dichloroisonicotinic acid (INA) which provided a good level of protection against pear fire blight, against apple scab, and against apple fire blight disease (Brisset *et al*., 2000).

6. Plant growth regulators: Prohexadione-calcium (Phd-Ca) is a plant growth regulator that suppresses shoot growth and fire blight in apple. Phd-Ca reduces longitudinal shoot growth by inhibiting gibberellin biosynthesis (Rademacher, 2000). The compound does not have any activity against *E. amylovora*, but increases host resistance by reducing plant vigor. In addition, treatments of apple with Phd-Ca results in alteration of phenylpropanoid biosynthesis pathways that may also enhance resistance (Halbwirth *et al*., 2002; Rademacher, 2000). However, in young apple orchards where there is a need to control fire blight and allow sufficient tree growth for tree establishment, the utility of Phd-Ca was unclear (Norelli and Miller, 2006 and 2004).

1.3 Actinomycetes

These organisms are a heterogenic group of very diverse Gram-positive bacteria and they are found in a filamentous form with a branching growth shape (like mycelium) resulting in an extensive colony in the soils to break down tough substances like cellulose (which makes up the cell walls of plants) and chitin (which makes up the cell

walls of fungi) even under harsh conditions such as high soil pH. Actinomycetes are the source of over ten thousand bio-compounds (including 60% of naturally occurring antibiotics like streptomycin, erythromycin and tetracycline) and of valuable enzymes and vitamins (Fenical and Jensen, 2005; Pfefferle *et al.*, 2000; Kennedy and Papendick, 1995; Holt, 1994; Vollmer *et al.*, 1977).

1.4 Plant strengthen (protection) products (Pflanzenstärkungsmittel)

Plant protection products are substances intended to protect plants from animals, parasitic plants, micro-organisms and diseases. Plant protection products may only be distributed and used if they have been authorised.
The competent authority in Germany is the BVL (Federal Office of Consumer Protection and Food Safety) which collaborates with three evaluation authorities; firstly, the Federal Biological Research Centre for Agriculture and Forestry, secondly, the Federal Institute for Risk Assessment and thirdly, the Federal Environmental Agency. Important aims in authorization of plant protection agents are a sufficient protection of crops, avoiding harmful effects on human health and avoiding unacceptable effects on the environment. After the authorisation of a plant protection product, its application is surveyed by means of control and monitoring programmes.
All member states in the EU apply the same evaluation procedures and authorisation criteria. A joint positive list of active substances permitted in plant protection products is also part of the harmonisation process. Active substances are evaluated in a joint procedure and a decision on their inclusion in the positive list is made. Besides plant protection products, adjuvant substances are sometimes provided as plant resistance improvers, like plant leaf surface wetting agents. (http://www.bvl.bund.de/cln_007/nn_496812/EN/07__TheFederalOffice/federal__office__node.html__nnn=true).

1.5 Parsley cells culture

Generally, plant cell cultures are useful tools for studying several aspects of plant biology including plant defence mechanisms (Dicosmo and Misawa, 1985). The response of parsley cells (*Petroselinum crispum*) ((MILL.) NYM. ex A.W. HILL) to components derived from fungal pathogens is one way to study the interaction between plants and microbes. Furthermore, parsley cell cultures have been studied for

several years for signal perception and transduction as well as coordination of plant defence responses (Fellbrich *et al.*, 2002; Katz *et al.*, 1998; Siegrist *et al.*, 1998; Hahlbrock *et al.*, 1995; Kauss *et al.*, 1995).

Cells trigger a cascade of events when induced by different micro-organisms, elicitors, or by certain chemical substances leading to gene activation. One result of gene activation in parsley cells is the secretion of furanocoumarin phytoalexins into the medium of the cells which can be measured by a fluorescence reader (Ebel and Mithofer, 1998; Katz *et al.*, 1998; Hahlbrock *et al.*, 1995; Dixon *et al.,* 1994). Preparations from cell walls of different pathogens either fungi (like the Oomycetes *Phytophthora sojae*, *Phytophthora megasperma*, *Phytophthora parasitica*) or bacteria (like harpin from *Erwinia amylovora*) are called elicitors, which trigger a cascade of events in the cells leading to gene activation and resulting in the synthesis and secretion of furanocoumarin phytoalexins and in the incorporation of phenolic compounds into the cell walls (Brunner, *et al.*, 2002; Ligterink *et al.*, 1997; Wirtz *et al.*, 1996; Nürnberger *et al.*, 1994; Renelt *et al.*, 1993; Kombrink and Hahlbrock, 1986).

In addition to elicitor-mediated responses, some chemicals inducing systemic acquired resistance (SAR) were studied also in parsley cell cultures. Such compounds (benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester (BTH), salicylic acid, and 2,6-dichloroisonicotinic acid) sensitise parsley cells to elicitors in a process called conditioning (priming) (Katz *et al.*, 1998; Kauss and Jeblick, 1995; Kauss *et al.*, 1993; Kauss *et al.*, 1992).

The success of a plant to combat microbial pathogens depends on speed and intensity of response. The sooner a plant recognises an attacking pathogen, the more efficient will be its defence response. If the plant cell fails to recognise the attacking pathogen at an early stage, the appropriate defences are activated too late and the pathogen starts invading the plant. Upon tissue damage, the plant activates defence mechanisms around the sites of pathogen invasion. This so-called basal resistance contributes to slowing down the growth of the pathogen, but is often too weak to prevent disease. Nevertheless, plants are very well capable of enhancing their defensive capacity against future pathogen attack once they are appropriately stimulated. This phenomenon is called induced resistance and brings the plant into an alarmed state. Recently, it became clear that this broad-spectrum protection is not so much based on direct defence activation after induction treatment, but rather on a faster and stronger activation of basal defence mechanisms after the induced plant has been exposed to

pathogen attack. By analogy with a phenotypically similar phenomenon in animals and humans, the enhanced capacity to express basal defence mechanisms during the alarmed state is called priming (Conrath *et al.,* 2002).

1.6 Strategy of resistance in plants

In many plant species, resistance against infections by pathogenic bacteria, viruses, and fungi can be induced by a variety of biotic and abiotic elicitors (Bol *et al*., 1990; Ouchi, 1983; Sequeria, 1983; Jens and Kuk, 1980; McIntry and Dodds, 1979). After the plant recognises the pathogen, a local induced resistance (LR) appears and that may lead to: (1) cell wall reinforcement by deposition of and cross linking of polysaccharides, proteins, glycoproteins and insoluble phenolics; (2) stimulation of secondary metabolic pathways, some of which yield small compounds with antibiotic activity (e.g. phytoalexins) but also defence regulators such as SA, ethylene and lipid-derived metabolites and (3) accumulation of a broad range of defence-related proteins and peptides (Fritig *et al*., 1998; Hammond-Kosack and Jones, 1996; Kuc, 1995). Defence related proteins are soluble proteins related to pathogenesis related (PR) proteins (PR-1 to PR-11) which accumulate in plants infected by viruses, viroids, fungi or bacteria (Camacho and Sänger, 1982; Gianinazzi *et al*., 1970; Van Loon and Van; Kammen, 1970) as a marker of resistance induction in the plant (induced systemic resistance (ISR) or systemic acquired resistance (SAR)).

If a plant detects an invasion, then a set of inducible defence responses is deployed; these include programmed cell death (referred to as the hypersensitive response or HR), tissue reinforcement at the infection site, and production of anti-microbial metabolites and induction of defence-associated gene expression (Hammond-Kosack and Jones, 1996). HR is a rapid death of plant cells which is associated with restriction of pathogen growth (Heath, 2000). Signalling cascades leading to the HR and disease resistance, particularly following *R*-gene-mediated pathogen recognition, trigger earlier cellular responses including changes in ion flux, generation of reactive oxygen species (ROS) which lead to a rapid development of cell death (HR) (Bin Nasir *et al*., 2005; Torres *et al*., 2002) which result in the presence of brown and dead cells at the infection site (Heath, 2000).

Various inorganic and organic compounds have been described as inducers of resistance in different crops, like BTH which is commercially called Bion® or Actigard®

(Brisset *et al.*, 2000; Narusaka *et al.*, 1999; Siegrist *et al.*, 1997; Friedrich *et al.*, 1996; Görlach *et al.*, 1996; Lawton *et al.*, 1996).

Furthermore, specific interaction models describing induced defence responses in plants have been greatly influenced in the recent years by the gene-for-gene interactions. In these specific host-pathogen interactions, resistance to a particular pathogen is conditional on the presence of a specific *Avr* (avirulence) gene of the pathogen and a specific *R* (resistance) gene in the plant host (Veronese *et al.*, 2003; Staskawicz *et al.*, 1984; Flor, 1956).

The quantification of induced disease resistance is often evaluated with classical or traditional methods such as estimation of disease area or determination of sporulation or other parameters, but these measurements can be rather subjective and time-consuming. The availability of visual molecular markers has led to a new approach in the quantification of disease resistance. The transformation of *Cladosporium fulvum* with the *uidA* gene permitted to pursue the fungal infection at the microscopic level as well as by photometric quantification of GUS activity as a measure of fungal biomass (Wubben *et al.*, 1994). *Phytophthora* species were also transformed using the GUS method (van West *et al.*, 1998; Baily *et al.*, 1993; Judelson *et al.*, 1992, 1993) but it was rarely used in monitoring the disease progression (Kamoun *et al.*, 1998) or in analysing of promoter activity (van West *et al.*, 1998). With the availability of the green fluorescent protein (GFP) as a vital marker, several plant pathogenic fungi and bacteria were successfully transformed with this reporter gene (Si-Ammour *et al.*, 2003; Fakhouri, 2001; Dumas *et al.*, 1999; Vanden Wymelenberg *et al.*, 1997). In Oomycetes, *Phytophthora palmivora*, *Phytophthora infestans*, *Phytophthora brassicae* and *Phytophthora parasitica* var. *nicotianae* were transformed with GFP as a reporter gene (Hamiduzzaman *et al.,* 2005; Si-Ammour *et al.*, 2003; Bottin *et al.*, 1999; van West *et al.*, 1999).

1.7 Green fluorescent protein (GFP)

The gene encoding the green fluorescent protein (GFP) was cloned from the jellyfish *Aequoria vicitoria* (Chalfie *et al.*, 1994) which is being used as a marker for gene expression as well as for monitoring and visualisation of cell structures in studies of both eukaryotic and prokaryotic organisms (Suarez *et al.*, 1997; Matthysse *et al.*, 1996; Niswender *et al.*, 1995). GFP is a 27 kDa polypeptide and its natural function is

converting the blue chemiluminescence of the Ca^{+2}-sensitive photoprotein Aequorin (*Aequorea forskalea*) into green light emission (Cody *et al*., 1993).

This gene is being used frequently in transgenic researches due to the following reasons: (1) it does not need cofactors or exogenous substrates for expressing its activity (Inouye and Tsuji, 1994). (2) GFP can be visualised in living cells without destruction or fixation of organisms by using 395/509 excitation/emission (Ward *et al*., 1980). (3) GFP mutants with shifted wavelength of excitation and emission have been also developed which permit simultaneous use and detection of multiple marker genes (Delagrave *et al*., 1995). (4) Some of GFP mutants or codon alterations exhibited more rapid formation and higher expression of the chromophore than the wild type GFP protein (Pang *et al*., 1996).

1.8 Plant transformation

With the advent of recombinant DNA technology in the 1970s, the genetic manipulation of plants entered a new age. Genes and traits previously unavailable through traditional breeding became available through DNA recombination, and with greater specificity than ever before. Genes from sexually incompatible plants, or from animals, bacteria or insects can now be introduced into plants. Products of modern plant genetic engineering are already on the market. Examples include slow-softening tomato fruits and cotton plants resistant to herbicides and insects. With many more products in the pipeline, the genetic engineering of plants will have a profound impact on the future of agriculture.

Modern plant genetic engineering involves the transfer of the desired genes into the plant genome, and then regeneration of a whole plant from the transformed tissue. Currently, the most widely used method for transferring genes into plants is *Agrobacterium*-mediated transformation. *Agrobacterium* is a naturally occurring pathogenic bacterium in the soil that has the ability to transfer its DNA into a plant's genome. *Agrobacterium* infection and gene transfer normally occurs at the site of a wound in the plant, and causes a characteristic growth referred to as a crown gall tumor. Scientists have taken advantage of this naturally occurring transfer mechanism, and have designed DNA vectors from the tumor-inducing plasmid DNA found in the bacteria that are capable of carrying desired genes into the plant. The engineered or constructed genes are inserted into the *Agrobacterium* vectors and enter the plant by

the bacteria's own internal transfer mechanisms. Transformation is typically done on a small excised portion of a plant known as an explant. This small piece of transformed plant tissue is then regenerated into a mature plant through tissue culture techniques (Hooykaas and Shilperoort, 1992; Arencibia *et al.*, 1998).

1.9 Gene silencing in plants

Gene silencing is a general term describing epigenetic processes of gene regulation. It is generally used to describe the "switching off" of a gene by a mechanism other than genetic mutation. That is, a gene which would be expressed (turned on) under normal circumstances is switched off by machinery in the cell. Genes are regulated at either the transcriptional or post-transcriptional level. Transcriptional gene silencing is the result of histone modifications, creating an environment of heterochromatin around a gene that makes it inaccessible to transcriptional machinery (RNA polymerase, transcription factors, etc.). Post-transcriptional gene silencing (PTGS) is the result of mRNA of a particular gene being destroyed. The destruction of the mRNA prevents translation to form an active gene product (in most cases, a protein). A common mechanism of post-transcriptional gene silencing is RNAi. Both transcriptional and post-transcriptional gene silencing are used to regulate endogenous genes. Mechanisms of gene silencing also protect the organism's genome from transposons and viruses. Gene silencing thus may be part of an ancient immune system protecting from such infectious DNA elements (Goto *et al.*, 2003; Hamilton et al., 2002; Hamilton and Baulcombe, 1999).

1.10 Plant tissue culture technique

In vitro cultures could be an effective alternative method to avoid soil or environmental stress factors when studying plant response (Shibli *et al.*, 1992; Murashige and Skoog, 1962). *In vitro* cultures have the advantage of small scale and treatment’s control, with clear visibility for monitoring shoot and root responses in the presence of the imposed stress (Shibli and Smith, 1999; Pieper and Smith, 1988). Such systems can provide reasonable information concerning physiological and biochemical responses (Shibli *et al.*, 2001; Shibli *et al.*, 2000).

Different applications can benefit from this technique; (1) micropropagation using meristem and shoot culture to produce large numbers of identical individuals. (2) screening programmes of cells of advantageous characters, rather than plants. (3)

large-scale growth of plant cells in liquid culture as a source of secondary products. (4) crossing distantly related species by protoplast fusion and regeneration of the novel hybrid. (5) production of di-haploid plants from haploid cultures to achieve homozygous lines more rapidly for breeding programmes. (6) for transformation, which may be used either for short-term testing of genetic constructs or regeneration of transgenic plants. (7) removal of viruses by propagation from meristematic tissues (Cooper *et al*., 2006; Zare *et al*., 2002; Muse *et al*., 1996; Ibrahim *et al*., 1992).

Eventually, plant tissue cultures have played an increasingly critical role in the development of gene modification and modern plant biotechnology research (Durante *et al*., 2002; Collin and Edwards, 1998). Additionally, tissue culture technique is not a theoretical prerequisite for plant transformation, but it is employed in practical information systems to achieve an efficient gene transfer, selection and regeneration of transformants (Birch, 1997). Furthermore, plant genetic transformation is a core research tool in modern plant biology and agricultural biotechnology (Bevan *et al.,* 1983).

Aims of this study:

In the present study, two projects were performed; ProInno 'PROgramm zur Förderung der Erhöhung der INNOvationskompetenz mittelständischer Unternehmen' (*E. amylovora* as a target pathogen) and DBU 'Deutsche Bundesstiftung Umwelt' (*P. infestans* as a target pathogen). Aim of the ProInno project was to identify and isolate alternative antibiotics for the control of *E. amylovora* and/or to find substances with a plant resistance inducing action using the parsley cells culture test. The objective of the DBU project was to find substances that have an indirect (resistance inducing) effect on plants against *P. infestans* 'Plant Strengthen Substances' (Pflanzenstärkungsmittel). Identifying such substances is commercially more preferable for medium sized enterprises because of the legal regulation of 'Plant Strengthen Substances', where compounds should not act directly against the pathogen but indirectly e.g. by induction of defence or resistance. Otherwise, they may have to be classified as plant protecting agents requiring cost intensive toxicological and eco-toxicological studies, for a legal admission and for their application (Pflanzenschutzgesetz **§**2 Nr. 10). To achieve the major aims, the following items were investigated:

1. Screening of phytosanitary compounds based on microbial extracts.
2. Establishment and optimization of rapid *in vitro* screening systems to test the biological activity of Actinomycetes extracts against *P. infestans* and *E. amylovora*.
3. Establishment and development of rapid *in vitro* screening system to test Actinomycetes extracts for their ability to induce defence reactions in a parsley cell culture producing furanocoumarin phytoalexins.
4. Screening for substances directly effective against *P. infestans* and *E. amylovora* using *in vitro* plants.
5. Screening of substances for an indirect action (local resistance-inducers) using detached tomato leaves against *P. infestans*.
6. Testing for an indirect action (induced resistance) of substances using intact plants against *P. infestans* and against *E. amylovora* visually and microscopically.
7. Silencing the GFP-fluorescence of transgenic *P. infestans* (208m2) as a model for silencing fungal genes responsible for plant infection.

Chapter I

Screening and identification of phytosanitary compounds derived from Actinomycetes extracts against *growth of Phytophthora infestans*

2 MATERIALS AND METHODS

2.1 Establishing of an *in vitro* screening test system

2.1.1 Preparation of the media

V8 medium (contents / L)

- 300 ml vegetable juice
- 2 g $CaCO_3$
- 6 g Gelrite or 10 g Micro Agar
- 25 mg/ml kanamycin (stock solution)

The vegetable juice was filtrated through three layers of cheesecloth. The collected supernatant was added to the other medium components and the volume was filled up to 1 L with distilled water. After autoclaving at 125 °C for 15 min, the medium was cooled to 50 - 60 °C in order to add kanamycin (1 ml/l). Then, the medium was poured directly into 9 cm Petri-dishes (25 ml/plate). The Petri-dishes were kept at 4 °C for maximum four weeks.

Rye-glucose medium (contents / L)

- 200 g Rye seeds
- 10 g Glucose
- 10 g Gelrite or 18 g Micro Agar
- 25 mg/ml kanamycin (stock solution)

Rye seeds were boiled with distilled water on a hot plate for 40 min. The extract was filtrated using three layers of cheesecloth. The filtrate was centrifuged at 14000 rpm for 15 min. The supernatant was added to the rest the components of the medium and then the volume was completed to 1 L with distilled water. Medium was divided into portions of each 100 ml and then autoclaved at 115 °C for 25 min. Kanamycin (1 ml/l) was added to the medium at 50 – 60 °C. The medium was pipetted into sterile multi well plates.

Malt agar medium (contents / L)

- 20 g Malt extract
- 9 g Gelrite or 18 g Micro Agar
- 25 mg/ml kanamycin (stock solution)

The components of medium were mixed together in 1 L distilled water and then autoclaved at 115 ℃ for 25 min. The medium was poured into 9 cm Ø Petri dishes or in multi well plates depending on the purpose of using.

Malt peptone medium (contents/ L)

- 20 g Malt extract
- 2 g Peptone
- 9 g Gelrite or 18 g Micro Agar
- 25 mg/ml kanamycin (stock solution)

Medium components were mixed together in 1 L distilled water and then autoclaved at 115 ℃ for 25 min. Then the medium was poured into 9 cm Ø Petri dishes or in multi well plates depending on the purpose of using.

Potato dextrose agar (PDA) medium (contents / L)

- 39 g PDA
- 2.5 g Gelrite or 5 g Micro Agar
- 25 mg/ml kanamycin (stock solution)

After preparation of the medium in 1 L distilled water and autoclaving at 115 ℃ for 25 min, the medium was poured into 9 cm Ø Petri dishes or in multi well plates depending on the purpose of utilization.

When liquid media were used, gelrite or micro agar was omitted.

2.1.2 Subculturing of the fungus

Wild type of *Phytophthora infestans* (WT) was kindly supplied by Dr. Jürgen Siegrist (CGG, DLR Institute, Neustadt/WS, Germany). Transformed *P. infestans* (208m2) isolate CRA 208 (Si-Ammour *et al.*, 2003) was kindly obtained from Dr. Felix Mauch (University of Fribourg, Switzerland). 208m2 has a transformation construct vector p34GFN containing a selectable marker cassette (nptII) and a reporter gene cassette (gfp) (Figure 1 and 2). Both types of *P. infestans* were sub-cultured on V-8 medium. Mycelium plugs of 8 mm Ø were taken from 10 – 12 d old agar cultures, and transferred in the centre of the new medium (Petri-dish). The new plates were closed by parafilm to avoid water evaporation and then kept at 18 ℃ under dark conditions

(Xu, 1982; Judelson and Roberts, 2002). Subculturing of both *P. infestans* isolates was repeated each 10 d.

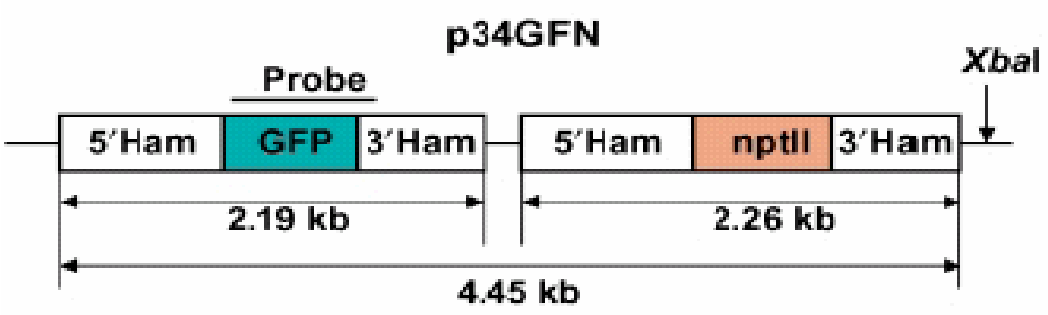


Figure 1: *Phytophthora infestans* 208m2 transformant containing the construct vector p34GFN currying a selectable marker cassette (nptII) and a reporter gene cassette (gfp). 5' Ham: constitutive promoter from *Bremia lactuca*, 3' Ham: Terminator

2.1.3 Preparation of Actinomycetes extracts

This following part was done by Sourcon-Padena GmbH (Tübingen, Germany): (Not mentioned details are confidential information)

Isolation of Actinomycetes: Strains were isolated from soil. Soil samples were collected from all European countries and were conform with the Rio-convention. The isolated bacteria were stored on HA-agar medium (contents/l: 4 g yeast extract, 4 g malt extract, 4 g glucose, at pH 7.3).

Cultivation of Actinomycetes: Strains were cultivated in three different liquid media at 27 °C on incubation shakers (Fa. Noctua, Mössingen) for 5 to 14 d depending on the biomass produced. Then, the resulted culture broth was run through an extraction-roboter (constructed by Sourcon-Padena Company).

Fractionation of positive extracts: This step was executed by using HPLC and HPLC-MS techniques, where the promising extracts from the different test systems were sub-fractionated to be tested again in the bio-test systems in order to be identified, optimized, and manufactured later as a biological pesticide.

The following part was done by AlPlanta - RLP AgroScience GmbH: The extracts were obtained in 96-well plates as dry crude extracts (50 µl/well). The crude extracts were dissolved in dimethyl sulfoxide (DMSO) (200 µl/well). Dissolved extracts were shaken at 175 rpm for 1 h at 28 °C, and then they were kept at 4 °C until used in the tests.

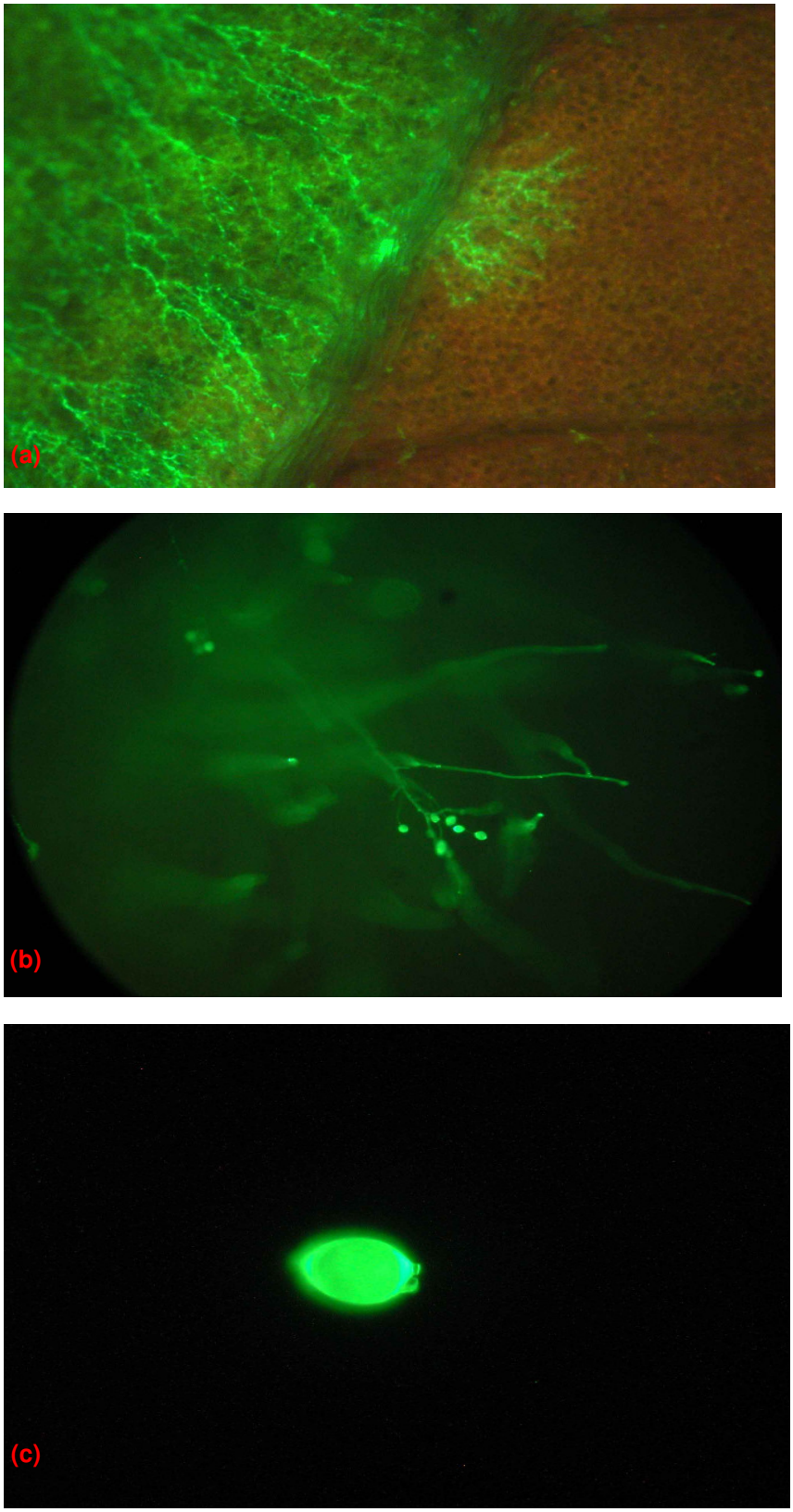


Figure 2: Different structures of the fluorescent *P. infestans* transformant. (a) Mycelia grow in plant tissues, (b) Sporangiophores carrying sporangia at their tips and (c) a fluorescent sporangium.

2.1.4 Collection of fungal sporangia

20 ml sterilised distilled water were added to plates containing 10 - 12 d old *P. infestans* mycelium. The mycelium was gently pressed to remove the air bubbles, and shaken carefully. Then the resulting suspension was filtrated through a layer of Mira cloth to remove the mycelium from the sporangia suspension.

2.1.5 Measurement of sporangial concentration

Using a haemocytometer (Fuchs-Rosenthal), 5 µl of the suspension were applied. Sporangia in the large square were counted and the sporangial suspension adjusted to the concentrations needed for the tests.

2.1.6 Investigation of mycelial growth of *P. infestans* using 9 cm Ø Petri-dishes

In 9 cm Ø plates, mycelial growth area (MGA) and weight of growth area (WGA) were used to determine the mycelial growth of both *P. infestans* types. Five different media (V-8, rye-glucose, malt-agar, malt-peptone, and PDA) were selected to investigate the mycelium growth at two different temperatures (18 and 23 °C):

(i) Mycelia growth area (MGA)

On a transparent sheet of a diameter of 9 cm, a grid was plotted with a permanent marker with squares 1 cm^2 each. The sheet was placed on the cover of the Petri-dish. Only squares covering the mycelia growth area were counted and registrated each 2 d. The total number of squares represented the area of the mycelium growth (Figure 4b).

(ii) Weight of a copy of the growth area (WGA)

This was done by photocopying the mycelium growth using a photostat or a Scanner. Photocopied sheets were cut around the periphery of the growth area and then weighed. The increase of weight of the paper piece represents the increase of size of the mycelium areas (Figure 4a).

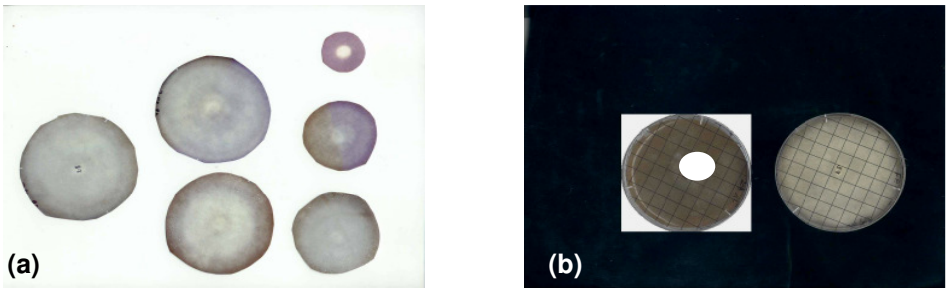


Figure 4: Photocopied sheets of mycelial growth on 9 cm Ø plates (a) and grids containing squares of each 1 cm^2 area for determining mycelium growth on agar medium (b).

2.1.7 Screening extracts using plate diffusion test (9 cm Ø Petri-dishes)

The plate diffusion test was used in order to test the effectiveness of extracts on growth inhibition of the wild type of *P. infestans*. Sterilised 9 mm Ø filter papers (antibiotic disks) were placed obverse at the edges of Petri dishes containing V-8 medium. 15 µl of each extract were pipetted onto these filter papers. Control filter paper dishes were treated with the appropriate solvent (50 - 60% EtOH). At the same time, a mycelium plug was inserted into the centre of the medium. Then, plates were kept at 18 °C under dark conditions. Mycelium growth was measured using the previously described methods (MGA and WGA). Results were collected at 7, 14, and 21 d after extract application. About 56 extracts were tested in this system for their effectiveness against *P. infestans*.

2.1.8 Establishing a screening test system for extracts by using 24-well multiplates

2.1.8.1 Time course of sporangia formation in V-8 medium at 18 °C

This experiment was performed in order to study the time course of sporangia formation for *P. infestans*. Mycelium plugs from 10 - 12 d old cultures were transferred on V-8 agar plates (9 cm Ø) and incubated at 18 °C in darkness. Sporangia collected from the colonies were filtered through Mira cloth. The sporangia concentration in the suspension was measured using two methods: firstly, by using a haemocytometer (for both *Phytophthora* types), secondly, by measuring GFP-fluorescence of 1 ml of a sporangial suspension in a fluorescence reader (only for the transformant 208m2). Finally, growth curves for the sporangia formation from both procedures were compared. The experiments were repeated twice and five plates were used for each experiment. The aim of this experiment was to find the time point where the highest

amount of sporangia was produced to be harvested as starting propagules for further optimisations.

2.1.8.2 Growth of the transformant 208m2 in liquid media at 18 and 23 ºC in 24-well multiplates

Using 24-well multiplates, 500 ml each of the two liquid media (V-8 and rye-glucose) were pipetted into the wells. 5 µl of sporangial suspension ($1x10^5$ sporangia ml^{-1}) were added to the centre of each well and incubated at two different temperatures (18 and 23 ºC) under dark conditions. Samples were taken immediately after inoculation and after each 24 h incubation for 6 d, and growth was determined by using different Ex/Em combinations (485/528 and 360/528) of the fluorescence reader (FLx 800). This experiment was executed to check the ability of mycelium growth in a liquid medium at different temperatures.

2.1.8.3 Growth of the transformant 208m2 on solid media at 18 and 23 ºC in 24-well multiplates

Two different solid media were used in this experiment; V-8 and rye-glucose medium at different temperatures (18 and 23 ºC). 500 µl of each medium were pipetted into the wells and left for half an hour until it is cooled. 5 µl of sporangial suspension ($1x10^5$ sporangia ml^{-1}) were added to the centre of each well. The fluorescence (485/528 nm Ex/Em) was red immediately after inoculation and each 24 h of incubation for 7 d. This experiment was performed to find the most suitable growth medium and transparence medium which can help the beam of the fluorescence reader to penetrate the medium and to find the best temperature which induces in the fungus a fast production of mycelium with low production of sporangia.

2.1.8.4 Correlation between production and fluorescence of sporangia

Dilutions of 50, 25 and 12.5% from a suspension of $1x10^5$ sporangia ml^{-1} were used. 500 µl of each dilution were measured as described above. In parallel, the number of sporangia in each dilution was counted using a haemocytometer. Fluorescence and sporangia concentration were depicted as a standard curve and a regression line was prepared (Figure 5).

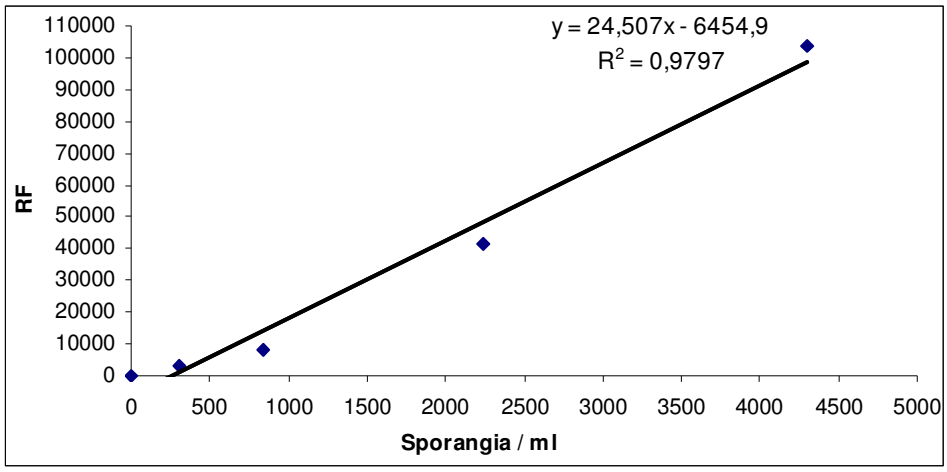


Figure 5: Correlation between the sporangia concentration and sporangia fluorescence. RF = Relative Fluorescence.

2.1.8.5 Optimization of the volume of standard medium

Rye-glucose medium was chosen as standard medium for screening at 23 °C. Different volumes (250, 300, 400, 500, 750, and 1000 µl/well) of rye-glucose medium were pipetted into 24-well plates. 5 µl of sporangia suspension ($1x10^5$ sporangia ml^{-1}) were added onto the centre of each well. The fluorescence (485/528 nm Ex/Em) was read immediately after inoculation and each 24 h after incubation for 7 d. The volume that allowed a consistent mycelium growth and resulted in the highest fluorescence was selected as standard volume for the test system.

2.1.8.6 Optimization of the concentration of sporangia suspension

Sporangia were harvested after 10 - 12 d as mentioned before, adjusted to $1x10^5$ sporangia ml^{-1}, and then diluted to different concentrations ($5x10^4$, $2x10^4$, $1x10^4$ and $1x10^3$ sporangia ml^{-1}). Then, a 24-well plate was inoculated with 5 µl of the different sporangia concentrations. Finally, data were taken immediately after inoculation and every 24 h after incubation for 10 d. The concentration showing the highest fluorescence was considered as a standard starting concentration for the screening test.

2.1.8.7 Optimization of the incubation temperature

In high throughput screening, length of time is an important parameter, therefore, to determine the temperature for optimization of mycelium growth was necessary. Plates were inoculated with sporangia and incubated at 15, 18 and 23 °C, respectively. The

fluorescence was measured as mentioned above for 11 d. The temperature allowing the highest fluorescence in the shortest time was chosen for the screening. Additionally, the lowest sporulation level was examined microscopically which did not affect the fluorescence and represented only mycelium growth determined by fluorescence.

2.1.8.8 Screening the extracts using 24-well plate method

Selected extracts from the plate diffusion test were re-tested in this experiment to confirm the results and the efficiency of the 24-well plate test system. Dilutions of the extracts of 10%, 1% and 0.1% were made from crude extracts with high, moderate, and weak effectiveness. 15 µl from each dilution were pipetted at the centre of the well, the plates were kept for 24 h at 23 °C allowing the substances to diffuse in the medium. 10 µl of the sporangia suspension ($1x10^5$ sporangia ml^{-1}) were added to the centre of each well, and then the plates were kept for 6 d at 23 °C in darkness. Mycelium growth was monitored by measuring the fluorescence after inoculation (0 d) and after each 24 h of incubation for 5 d.

2.1.9 Establishing a screening test system of extracts using 96-well multiplates

For a more effective handling of the extracts delivered by the corporation partner (Sourcon-Padena GmbH), it was necessary to check whether the test could be performed in 96 well plates. Therefore, further optimizations should be carried out in addition to the 24-well plate system.

2.1.9.1 Optimization of standard medium volume

Different volumes (75, 80, 90, 100, 110, 120, 140, 160, 180 and 200 µl/well) of solid rye-glucose medium were pipetted into 96-well plates. 5 µl of sporangial suspension ($1x10^5$ sporangia ml^{-1}) were added to the centre of each well. The fluorescence was read after inoculation (0 d) and after each 24 h of incubation for 8 d. The volume allowing consistent mycelium growth and resulting in the highest fluorescence was selected and considered as standard volume for the screening.

2.1.9.2 Optimization of the starting concentration of sporangia

Different concentrations of sporangia ($1x10^5$, $5x10^4$, $2x10^4$, $1x10^4$, and $1x10^3$ sporangia ml^{-1}) were prepared. Rye-glucose medium was inoculated with 5 µl of sporangia incubated at the above mentioned conditions. Fluorescence was measured as described in 2.1.9.1. The concentration resulting in the highest fluorescence and in a consistent mycelium growth was considered as standard concentration for the 96-well plate screening.

2.1.9.3 Optimization of the kanamycin concentration

The construct of the transformant 208m2 which has a kanamycin resistance gene, the optimum antibiotic concentration was determined. Appropriate kanamycin concentrations (100, 75, 50, and 25 mg ml^{-1}) were prepared and added to the rye-glucose cooled medium; the medium was shaken and poured into the 96-well plates. 5 µl of $1x10^5$ sporangia ml^{-1} of the transformant 208m2 were pipetted onto the medium and the plates were measured using the fluorescence reader at 23 °C to be tested each 24 h for 8 d to monitor the effect of kanamycin on the mycelia growth. Kanamycin should prevent the microbial contamination of the test but should have no effect on the mycelium growth compared to the control.

2.1.9.4 Mycelium growth in 96-well plates

200 µl of autoclaved rye-glucose medium were pipetted into the wells and left for half an hour for solidification. 5 µl of sporangia suspension ($1x10^5$ sporangia ml^{-1}) were added to the centre of each well. The fluorescence was read as described above at 24 h intervals for 7 d. The time course of mycelium growth was recorded to find the mycelium saturation level.

2.1.9.5 Screening of extracts using 96-well plate method

200 µl rye-glucose medium were pipetted into each well. Plates were left for half an hour under sterile conditions until the medium was cooled, and then a dilution of 1:1000 from the extracts to be tested was prepared. Four plates were used for each screening test; three plates (as replications) were inoculated with sporangia. The fourth plate contained only extracts without sporangia as a background (control) plate in order to subtract the fluorescence obtained from the extracts. 10 µl from the extract dilution

were pipetted at the centre of the well and the plates were kept for 24 h at 23 °C. 5 µl of the sporangia suspension ($1x10^5$ sporangia ml^{-1}) were added to the medium. The fluorescence was measured using the fluorescence reader (485/528 nm Ex/Em) at the 1st, 3rd, and the 6th d post extract application. Then, results were exported to the appropriate program (Microsoft Excel) to calculate the changes in growth. After that, the effective extracts against the transformant 208m2 reducing or inhibiting the mycelium growth, were fractionated and re-tested to find the effective substance in the extract.

2.1.9.6 Statistical analysing (assessment) of *P. infestans* screening results

Generally, the generated data varied in a wide range due to the fact that we have worked with Actinomycetes (the major source of the extracts), hence a biological system. The variation of the effect had been taken into consideration and to be corrected.

No mycelium growth frontier (mycelium growth inhibition):

Minimum average – standard deviation = 535 RF

Maximum average – standard deviation = 902 RF

Inhibition uncertain frontier (no mycelium growth inhibition):

Minimum average – standard deviation = 2021 RF

Maximum average – standard deviation = 4860 RF

These values included RF of pure medium but excluded RF of the extracts.

2.2 Assessment of the potential of Actinomycetes extracts for direct action and for resistance induction against *P. infestans* in greenhouse tomatoes

Tomato plants (*Lycopersicon esculentum* Mill) of cv. 'Hellfrucht' were used in all experiments. The extracts or extract fractions that had a direct effect on growth of *P. infestans* from *in vitro* screening (Pi^+), and those that had an inducing effect in parsley cells to produce furanocoumarin phytoalexins (Pc^+) were selected to be tested on tomato plants. This test system was set up in three parts:

(i) Testing the phytotoxicity of active extracts and extract fractions on detached leaves and on intact plants to find the suitable concentration not to be toxic to the tissue.

(ii) Testing the extracts and extract fractions on detached leaves for a direct effect to the fungus and/or an induction of local resistance against the disease.
(iii) Testing the extracts and extract fractions for an indirect effect (induced resistance) in intact tomato plants against the late blight disease.

2.2.1 Preparation of tomato plants

Seeds of tomato (cv. Hellfrucht) were sown in small pots (10 cm Ø) containing a mixture of Perlite and Peatmoss (1:5). Plants were grown in the greenhouse in a 16/8 h light/dark cycle at 25/18 °C, respectively. Seedlings were irrigated each 2 d and fertilized twice a week with Hakaphos blau 20 g/l and Fetrilon 0.2 g/l solution.

2.2.2 Phytotoxicity tests

Pi+ and Pc+ extracts were tested for phytotoxicity either on intact plants or detached leaves. Furthermore, ethanol which was used as a solvent for the extracts and other additionally solvents (DMSO and BreakThru® S 240) were tested for their phytotoxicity in order to find a non phytotoxic solvent and that facilitates the penetration into leaf tissues (Figure 6).

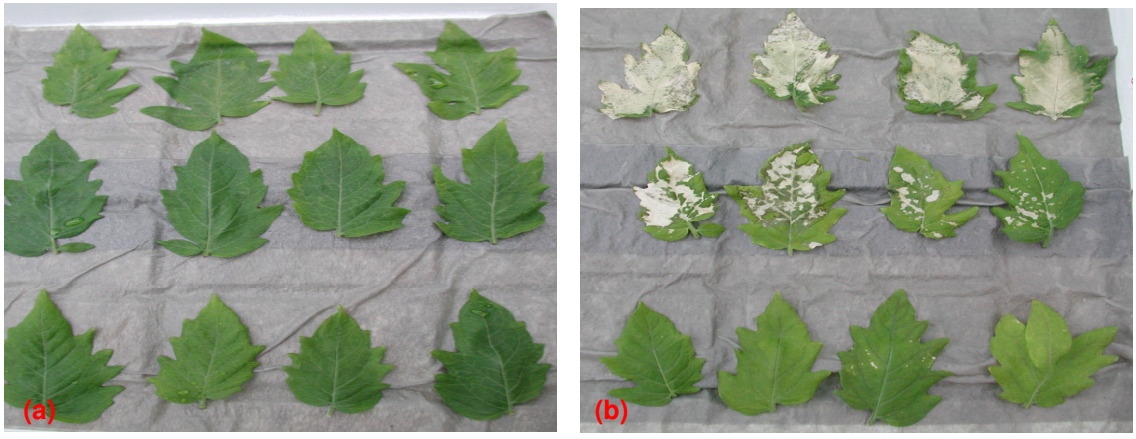


Figure 6: Different extracts causing no phytotoxic (a) and phytotoxic (b) effects on tomato leaves.

2.2.2.1 Phytotoxicity of the promising substances on detached leaves and intact plants

A random selection of leaves (of different ages) of tomato plants were cut with a fine blade and were put either in normal position or upside down position (depending on the purpose of the experiment) on three layers of wet paper towel inside plastic plates.

From the substances and the solvents (EtOH, DMSO, and BreakThru® S 240), different dilutions were prepared and applied either by using a fine paint-brush or using a fine pressure container sprayer (ecological sprayer; Roth company). Then, the leaves were left for 5 - 7 d and the phytotoxic symptoms were monitored using the following index:

Phytotoxicity index	Description
0	healthy tissues
1	necroses on less than 25% of the treated part
2	necroses on 25-50% of the treated part
3	necroses on 50-75% of the treated part
4	necroses on 75-90% of the treated part
5	death of the treated leaf

In case of testing the phytotoxicity of the solvents on intact tomato plants, the solvents were applied to three weeks old tomato seedlings as mentioned above on the upper or on the lower leaf surfaces or on both leaf surfaces. Data were collected and the suitable solvent was selected for the next experiments.

2.2.3 Assessment of the potential of extracts to induce local resistance in detached leaves

This test was carried out in order to find a direct (toxic) or indirect (local resistance) effect for Pi^+ and Pc^+ extracts. Extracts dissolved in EtOH were dried using a vacuum centrifuge (Speed vacuum) and re-dissolved again in BreakThru® S 240 (0.06%), an organo-silicone surfactant. Dilutions were made from the dissolved substances before starting the test. Plastic plates and leaves were prepared as mentioned above. The leaves were put in the upside down position over the wet towel papers.
In these experiments, 4 - 5 detached leaves were used for each dilution (Figure 7).

Water and BreakThru® S 240 (0.06%) as solvents were used in control treatments. Detached leaves were treated with the substances either by brushing them with a fine painting brush or by spraying them with a fine spray pump. Then, the leaves were covered with a transparent cover for 24 h to keep the leaves enough moist. The sporangial suspension of *P. infestans* ($0.7x10^5$ – $1x10^5$ sporangia ml^{-1}) was prepared as mentioned before and then the leaves were inoculated using a small fine sprayer (1

ml/leaf) (2 leaves in each treatment were left without infection as a control). The experiments were conducted in a greenhouse chamber under defined conditions (16 ℃, 60% humidity, 16 h/day light, and 8 h/day dark). After inoculation, the leaves were kept in darkness for 12 h.

The formation of greyish leaf areas representing the disease symptoms and the sporulation level were monitored and registrated for 5 - 7 d after the infection date, the following disease evaluation scale was used:

Infected area index (a)	Description
0	no infected leaf area
1	infection of less than 10% of the leaf area
2	infection between 10-25% of the leaf area
3	infection between 25-50% of the leaf area
4	infection between 50-75% of the leaf area
5	infection between 75-100% of the leaf area

Sporulation index (b)	Description
0	no sporulation
1	sporulation on less than 10% of the leaf area
2	sporulation on 10-25% of the leaf area
3	sporulation on 25-50% of the leaf area
4	sporulation on 50-75% of the leaf area
5	sporulation on 75-100% of the leaf area

2.2.4 Assessment of the potential of the active extracts to induce systemic resistance in plants

The Pi+ and Pc+ extracts and their fractions were tested for their systemic induced resistance effect. Three weeks old plantlets having a primary and secondary leaf developed were selected for this test. Different dilutions of the substances were prepared as mentioned before. Water and BreakThru® S 240 (0.06%) were used as control treatments.

Plants were treated with the substances either by brushing them with a fine painting brush or by spraying them with a fine spray pump. Then, the plants were left for 48 h (72 h in some cases) in the greenhouse chamber under special conditions (16 ℃, 60% humidity, 16 h/day light, and 8 h/day dark). The fungal suspension of *P. infestans*

($0.7x10^5$ – $1x10^5$ sporangia ml^{-1}) was prepared as mentioned before, and then the plants were inoculated (the 1st infection) by spraying the whole plant with the fungal suspension using a small fine sprayer (2 ml/plant) (two plants in each treatment were left without infection as a control). After that, the plants were covered overnight with black plastic sheets to keep the moisture for an effective infection. The black plastic sheets were removed after 12 h.

The disease symptoms were monitored for 5 - 7 d after inoculation depending on the infected area index. Seven d after inoculation, sporulation was induced by spraying the infected plants with water and covering them overnight with wetted plastic bags. After 24 h, sporulation was viewed on the leaves under the fluorescent microscope (ZEISS Fluorescence Microscope: GFP BP filter; BP 470/40 BP 525/50 and/or FITC filter; BP 450-490 LP 515), to monitor the production and the morphological shape of the sporangia and sporangiophores. The newly grown (systemic) leaves of these plants were also inoculated (the 2nd challenging infection) with *P. infestans* sporangia suspension ($0.7x10^5$ – $1x10^5$ sporangia ml^{-1}) in order to test an effect of the applied substances on the plants regarding systemic induced resistance.

Four - five d after the 2nd inoculation, the disease symptoms on the systemic leaves were evaluated using the infected leaf area index. Five d later from the symptoms appearance in the infected systemic leaves, sporulation was induced. The sporulation was viewed under the Fluorescence Microscope.

3 RESULTS

3.1 Investigation of growth of the transformant 208m2 and wild type of *P. infestans* using 9 cm Ø plates

3.1.1 Growth of both strains in V-8 medium at 18 °C

The mycelium growth of both strains was recorded 3 - 4 d after subculturing in new V-8 medium. It was found that the transformant grew faster at 18 °C compared to the non-transformed strain after 14 d of incubation (Figure 8).

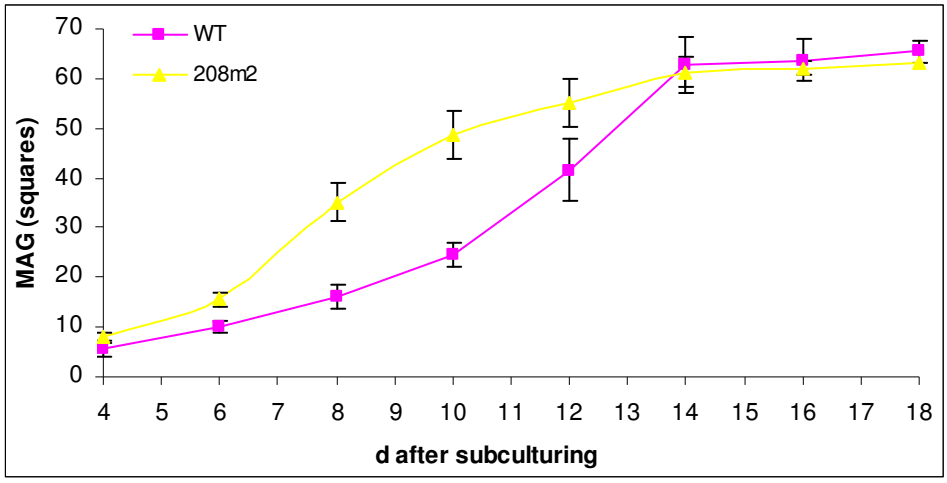


Figure 8: Growth area for both *P. infestans* strains (WT and 208m2) in V-8 medium at 18 °C in Petri dishes. d = days; error bars are standard deviations of MGA of three replications.

3.1.2 Growth of the 208m2 transformant in different media at 18 and 23 °C

Mycelium growth of the 208m2 transformant varied among 5 media tested (V-8, rye-glucose, malt, malt-peptone, and potato dextrose agar). This experiment was conducted at two different temperatures (18 and 23 °C) under dark conditions. It was found that the transformant 208m2 grew faster on V-8 and rye-glucose media at both temperatures, so these media were selected for further optimization tests.

A slow growth of the transformant 208m2 was recorded on malt, malt-peptone and PDA media (Figure 9). The isolate grew faster on rye-glucose medium than on V-8 medium, and the plate was completely grown with mycelium within 7 d in rye-glucose medium compared to about 12 d in V-8 medium both at 18 and 23 °C.

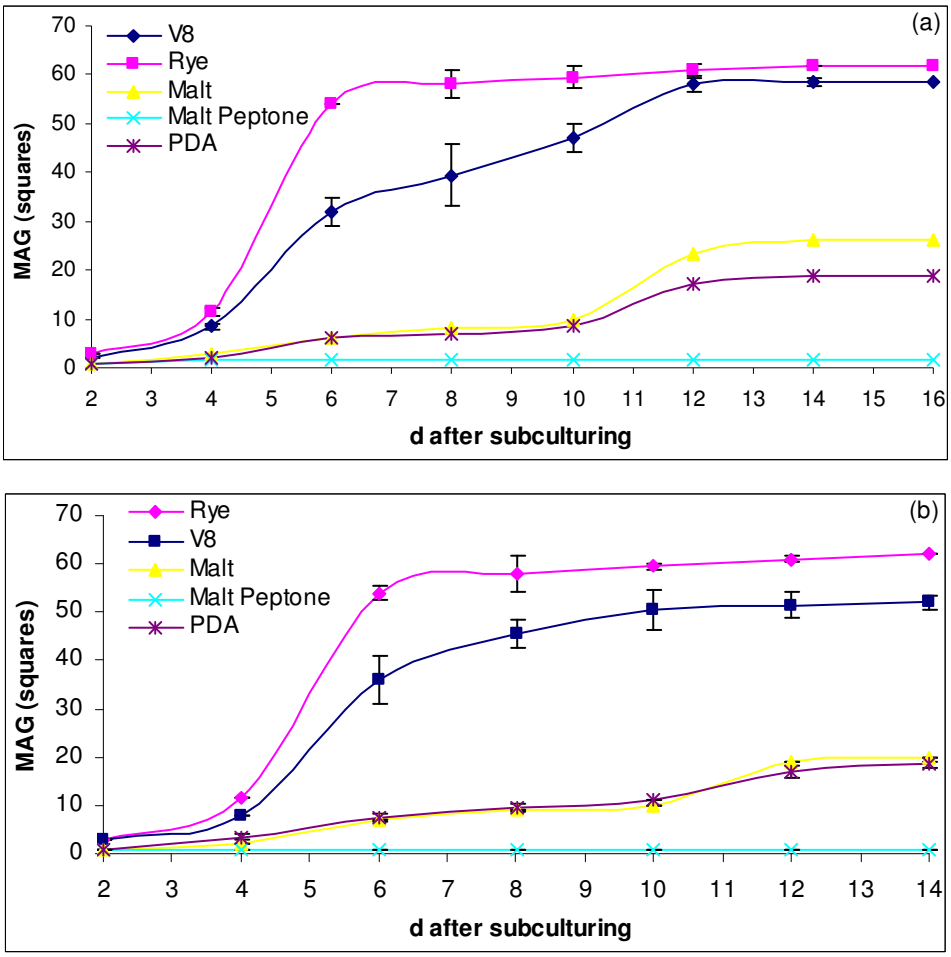


Figure 9: Mycelial growth area (MGA) for the transformant 208m2 in different media at 18 °C (a) and at 23 °C (b) in plates; d = days; error bars are standard deviations of MGA of three replications.

3.1.3 Time course of sporangia formation in V-8 medium at 18 °C

Figure 10 shows the sporangia formation of the transformant 208m2 expressed as relative fluorescence of the sporangia measured at different incubation periods. The formation of sporangia could be observed 4 d after incubation and increased gradually until the 9th d after cultivation, reaching its highest level. Then, the amount of fluorescing sporangia harvested, decreased gradually.

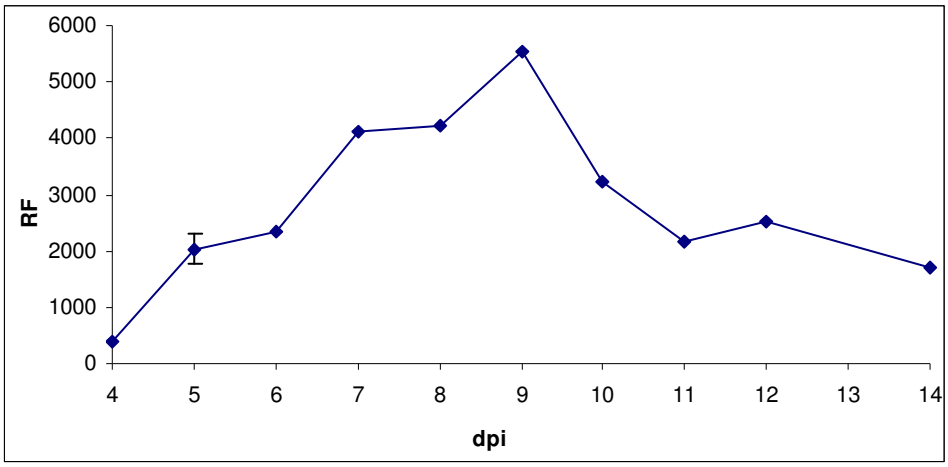
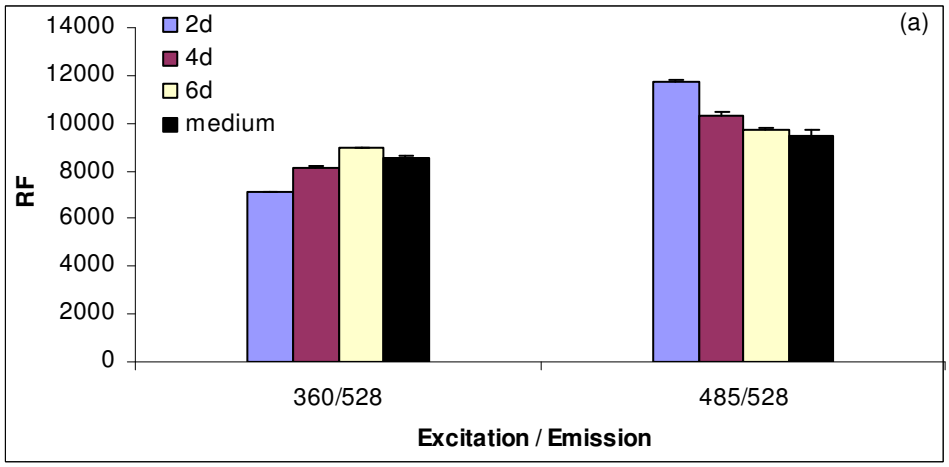


Figure 10: Relative fluorescence of sporangia of the transformant 208m2 obtained from V-8 medium in Petri dishes at 18 °C. RF = Relative Fluorescence at 485/528 nm Ex/Em; dpi = days post inoculation; error bars represent standard deviations of sporangia fluorescence of three replications.

3.2 Establishment and optimization of screening system of extracts using the transformant 208m2 in 24-well plates

3.2.1 Liquid media

Inconsistent growth was observed in both liquid media tested throughout the experimental period (Figure 11). Mycelium aggregations accumulated at the bottom of the wells in both rye-glucose and V-8 medium. Due to these results, liquid medium was not used in the further experiments.



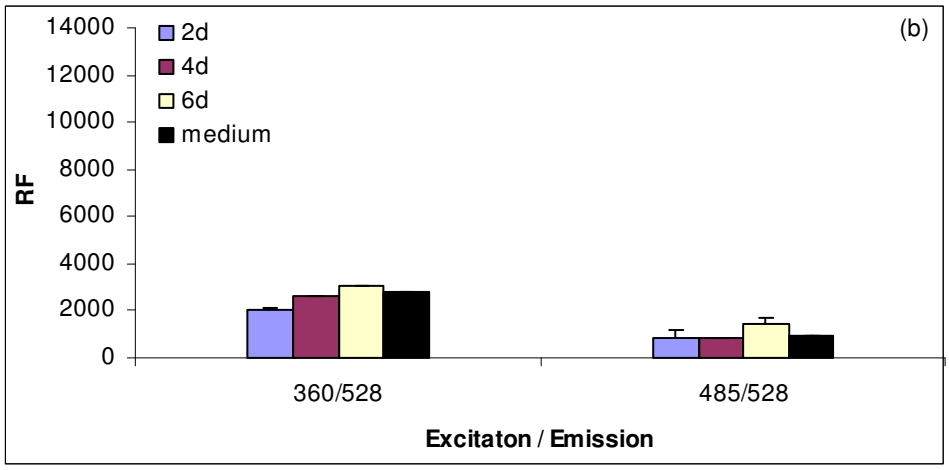
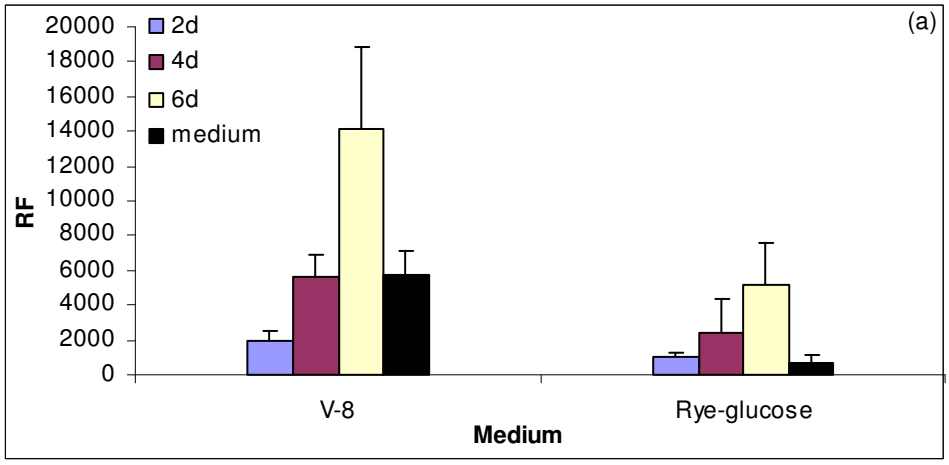


Figure 11: Relative fluorescence of mycelium of the transformant 208m2 grown in V-8 liquid medium (a); and in rye-glucose liquid medium (b) at 18 °C under different excitation/emission filter combinations using 24-well plates. RF = relative fluorescence; d = days post inoculation; error bars are standard deviations of RF of four replications.

3.2.2 Solid media

Mycelium grew more rapidly in solid rye-glucose medium than in solid V-8 medium at both temperatures chosen as shown in Figure 12. V-8 medium showed a higher fluorescence compared to solid rye-glucose medium. A more intense mycelium growth (relative fluorescence) was observed at 23 °C than at 18 °C; so, solid rye-glucose medium at 23 °C was used for further optimisations.



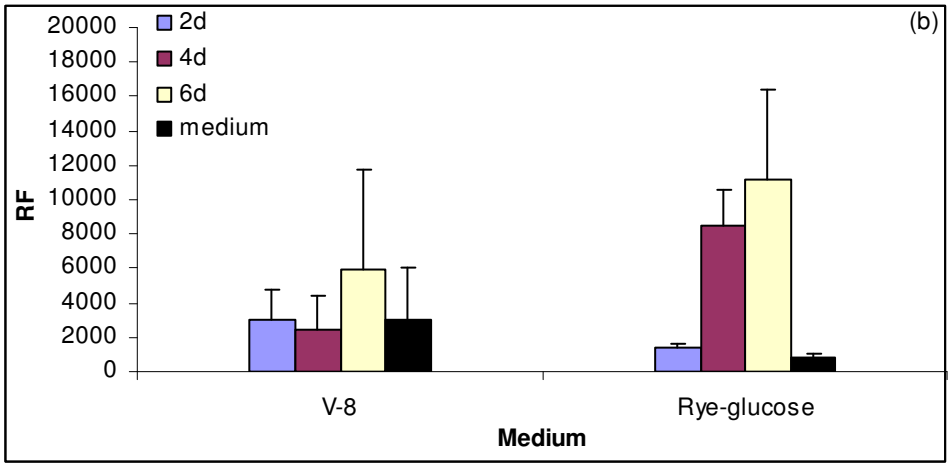


Figure 12: The relative fluorescence of mycelium of the transformant 208m2 grew in V-8 solid and rye-glucose media at 18 °C (a) and at 23 °C (b) at 485/528 nm Ex/Em in 24-well plates; RF = relative fluorescence; d = days post inoculation; error bars are standard deviations of RF of four replications.

3.2.3 Optimization of standard medium volume

An important parameter, that could have a disadvantageous effect on the performance of the test, might be the volume of the medium in the 24-well plate test. This could be of importance for the optical properties of the fluorescence measurement (too high amount of medium) and for the mycelium growth due to dehydration and/or deprivation of nutrients (too less medium). Therefore, different volumes were tested (1000, 750, 500, 400, 300, and 250 µl/well). Figure 13 shows that high fluorescence was registrated when medium volumes of 500 to 1000 µl/well were used, affecting a higher growth compared to the smaller medium volumes (250 to 400 µl/well). No differences were observed in the growth when 500 to 1000 µl of medium/well were used. The highest fluorescence was obtained at the 7th d after inoculation in all examined medium volumes, and the fluorescence reduced gradually afterwards.

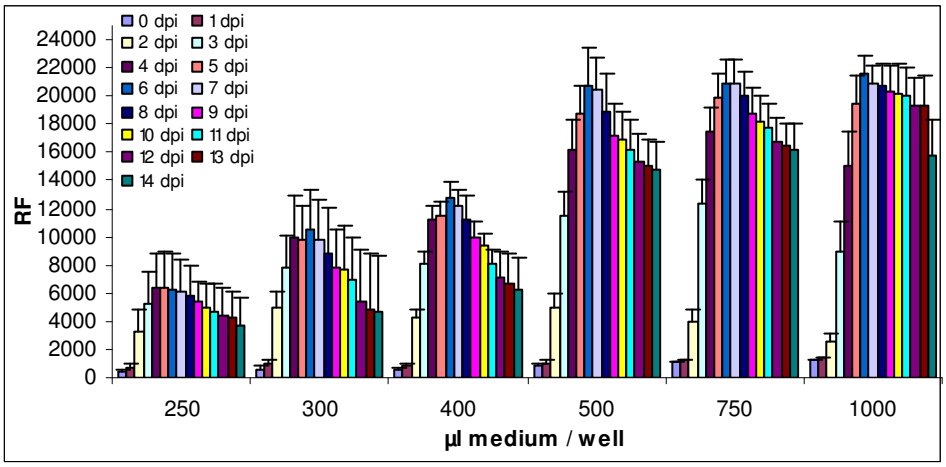


Figure 13: Relative fluorescence of the growth time course of the transformant 208m2 depending on different volumes of rye-glucose solid medium at 23 °C in a 24-well plate. dpi = days post infection; RF = relative fluorescence at 485/528 nm Ex/Em; error bars represent standard deviations of RF of four replications

3.2.4 Optimization of the concentration of sporangia inoculum

In order to find a suitable inoculum concentration for a reproductive and temporally convenient screening method, 10 µl of different sporangia concentrations (100 000, 50 000, 25 000, 12 500 and 6 250 sporangia ml^{-1}) were tested in this experiment (Figure 14). Results indicated that the highest fluorescence was obtained when wells were inoculated with 100 000 and 50 000 sporangia ml^{-1} and incubated about 6 d. Thereafter, the fluorescence began to decrease with increasing mycelium age. Inoculation with low concentrations of sporangia, growth and fluorescence were lower. The concentration of 100 000 sporangia ml^{-1} was chosen for the screening test system.

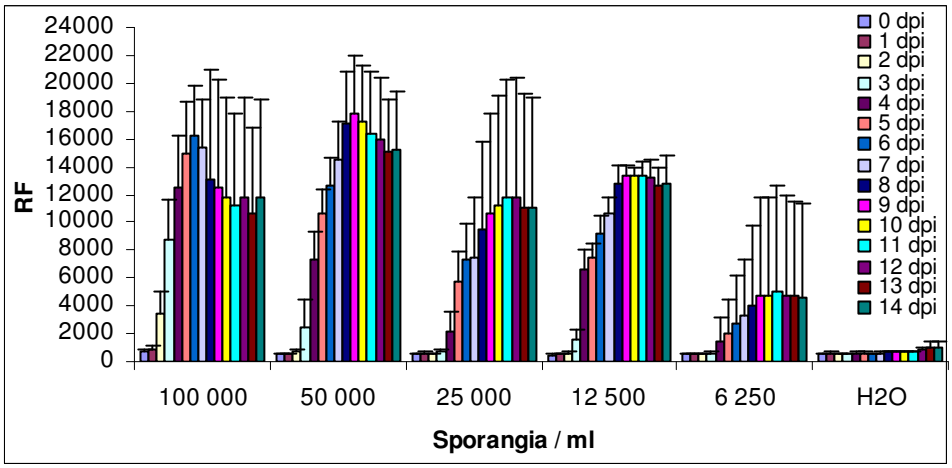


Figure 14: Rye-glucose solid medium inoculated with different amounts of sporangia of the transformant 208m2 using 24-well plates. RF = relative fluorescence at 485/528 nm Ex/Em; dpi = days post inoculation; error bars are standard deviations of sporangia RF of three replications.

3.2.5 Optimization of the incubation temperature

The *P. infestans* transformant 208m2 grew faster at 23 °C than at 15 and 18 °C which corresponded with a faster increase of fluorescence. The results showed that optimal growth of the transformant was obtained after 7 d of incubation, whereas, incubation at 15 and 18 °C increase in fluorescence was delayed and at a significantly lower level (Figure 15). After the 6th d of incubation, only a very low development of sporangia on the sporangiophores in the mycelia grown at 23 °C was recognized. The number of the sporangia on the sporangiophores was clearly increased after incubation at 18 and 15 °C, respectively (Figure 16).

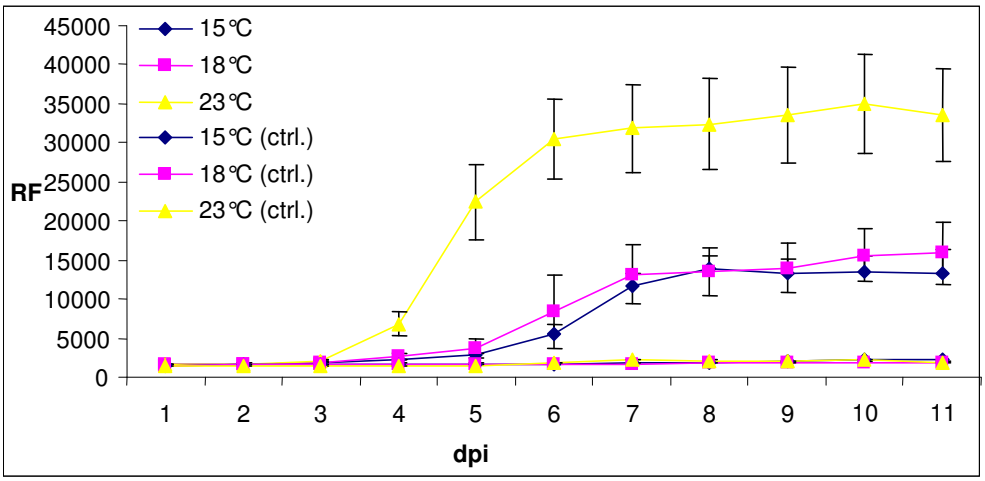


Figure 15: Mycelium growth of the transformant 208m2 on rye-glucose medium at different temperatures in 24-well plates. RF = relative fluorescence at 485/528 nm Ex/Em; ctrl. = control medium; dpi = days post inoculation; error bars are standard deviations of sporangia RF of three replications.

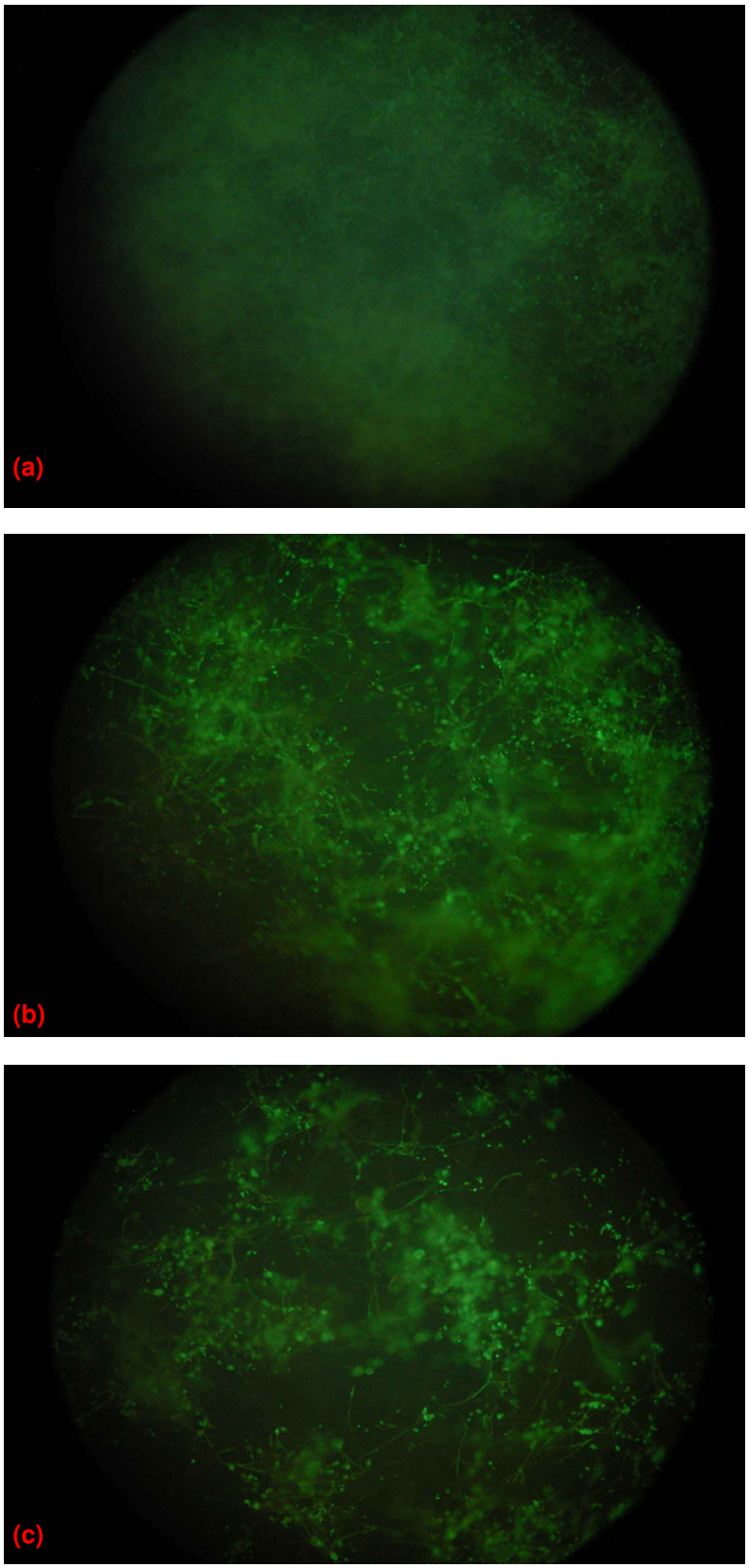


Figure 16: Sporangia formation on the tips of sporangiophores of the transformant 208m2 grown in rye-glucose medium in 24-well plates and incubated at 23 °C (a), at 18 °C (b), and at 15 °C (c).

3.3 Optimization of a screening system of extracts in 96-well plates for the transformant 208m2

Some modifications were carried out in the 24-well multiplate system to achieve a screening test system for the 96-well multi plate. To achieve an easier handling for screening the plates in the screening procedure, multi-channel pipettes were used in order to dissolve the sterile extracts, to pipette the samples into the test plates and thereby to reduce the possibility of pipetting mistakes, the screening test was performed in the 96-well multiplates format.

3.3.1 Optimization of standard medium volume

In principle, the 96-well format led to better results the more medium had been applied before inoculation. Maximal fluorescence could be clearly obtained 4 d post incubation (dpi) using 10 µl of a concentration of 100 000 sporangia ml^{-1} (Figure 17). After reaching the saturation level, the fluorescence values decreased slightly or remained on a constant level.

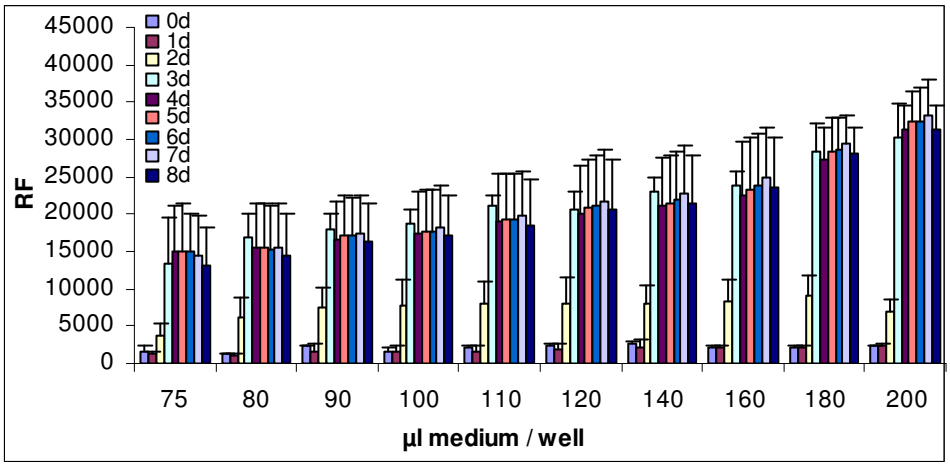


Figure 17: Relative fluorescence of the transformant 208m2 grown in different volumes of rye-glucose solid medium at 23 °C in a 96-well plate. RF = relative fluorescence at 485/528 nm Ex/Em; d = days post inoculation; error bars represent standard deviations of RF of four replications.

3.3.2 Optimization of inoculum concentration of sporangia in the 96 well assay

Results in Figure 18 indicate that after 5 d the highest fluorescence was recorded when 100 000 sporangia ml^{-1} were used as inoculum, thereafter, the fluorescence decreased gradually. Lower concentrations (50 000, 25 000, 12 500 and 6 250 sporangia ml^{-1}) resulted in a lower growth and consequently in a lower fluorescence. 100 000 sporangia ml^{-1} were chosen for the screening in the 96-well plates.

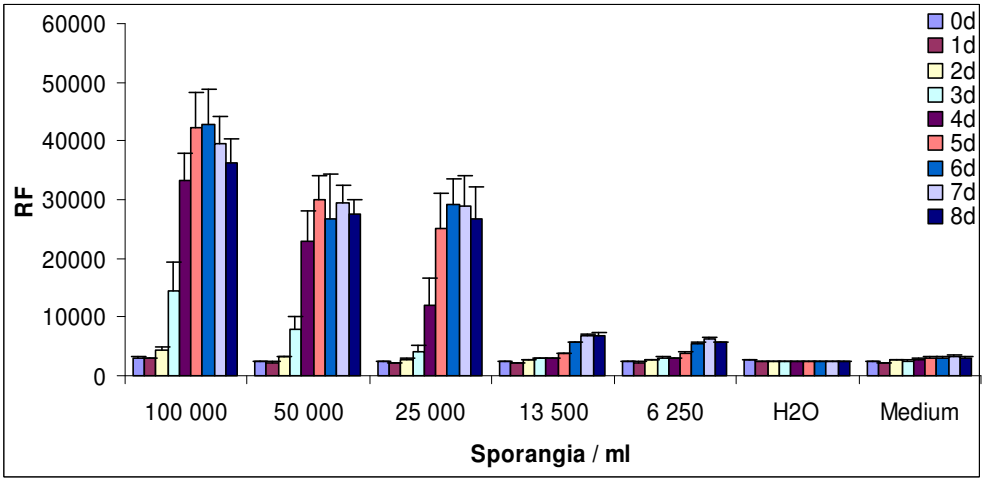


Figure 18: Rye-glucose solid medium inoculated with different sporangia concentrations of the transformant 208m2in 96-well plates. RF = relative fluorescence at 485/528 nm Ex/Em; d = days post inoculation; error bars represent standard deviations of sporangia RF of three replications.

3.3.3 Optimization of the kanamycin concentration

The kanamycin resistance of the transformant 208m2 usually used as selection marker permits the use of kanamycin in the medium to prevent microbial contaminations during the experiment. Kanamycin concentrations up to 100 mg/ml showed no detrimental effects of the growth of the strain 208m2 (Figure 19). It was noticed that no new contaminations appeared in the medium when kanamycin was added to rye-glucose medium. 25 mg/ml kanamycin was considered as a standard in the screening test.

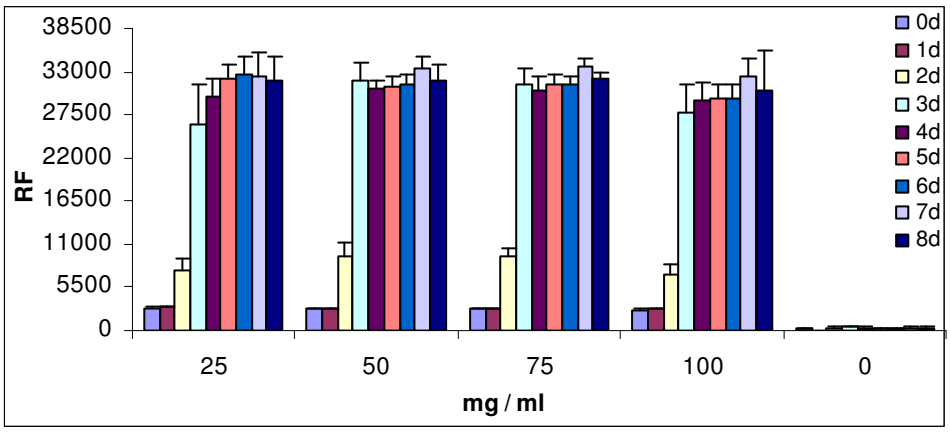


Figure 19: Relative fluorescence of the transformant 208m2 (100 000 sporangia/ml) grown in rye-glucose solid medium with different kanamycin concentrations in 96-well plates. RF = relative fluorescence at 485/528 nm Ex/Em; d = days post inoculation; error bars are standard deviations of RF of four replications.

3.4 Screening antifungal effects of extracts using plate diffusion test

Eight of the effective extracts resulting from the *E. amylovora* test were tested also for growth inhibition against the Oomycetes fungus *P. infestans*. Despite of the strong or the moderate effects of the eight extracts on *E. amylovora*, it was found that these extracts did not have the same effect when they were tested against *P. infestans* WT (Table 1).

Table 1: Different extracts tested were classified according to their effectiveness against *P. infestans* WT using the agar diffusion test.

Strain number	**Effect on *E. amylovora***	**Effect on *P. infestans***
000092	Strong	Moderate
000072	Strong	Moderate
012347	Moderate	Weak
012583	Moderate	Moderate
012601	Strong	Moderate
012357	Strong	Weak
012228	Strong	Weak
000159	Strong	Weak

Testing further extracts against *P. infestans* WT in the same test system showed that there were clear variations in the effectiveness against *P. infestans* WT (Table 2). Depending on their activity, extracts were sorted into three groups (Figure 20):

(i) Extracts preventing mycelium to grow towards the treated filter paper were classified as strong effective extracts.

(ii) Extracts preventing growth, and mycelium did not reach the treated filter paper were classified as moderate effective extracts.

(iii) Extracts slightly or not affecting mycelium growth were classified as weak or not effective extracts.

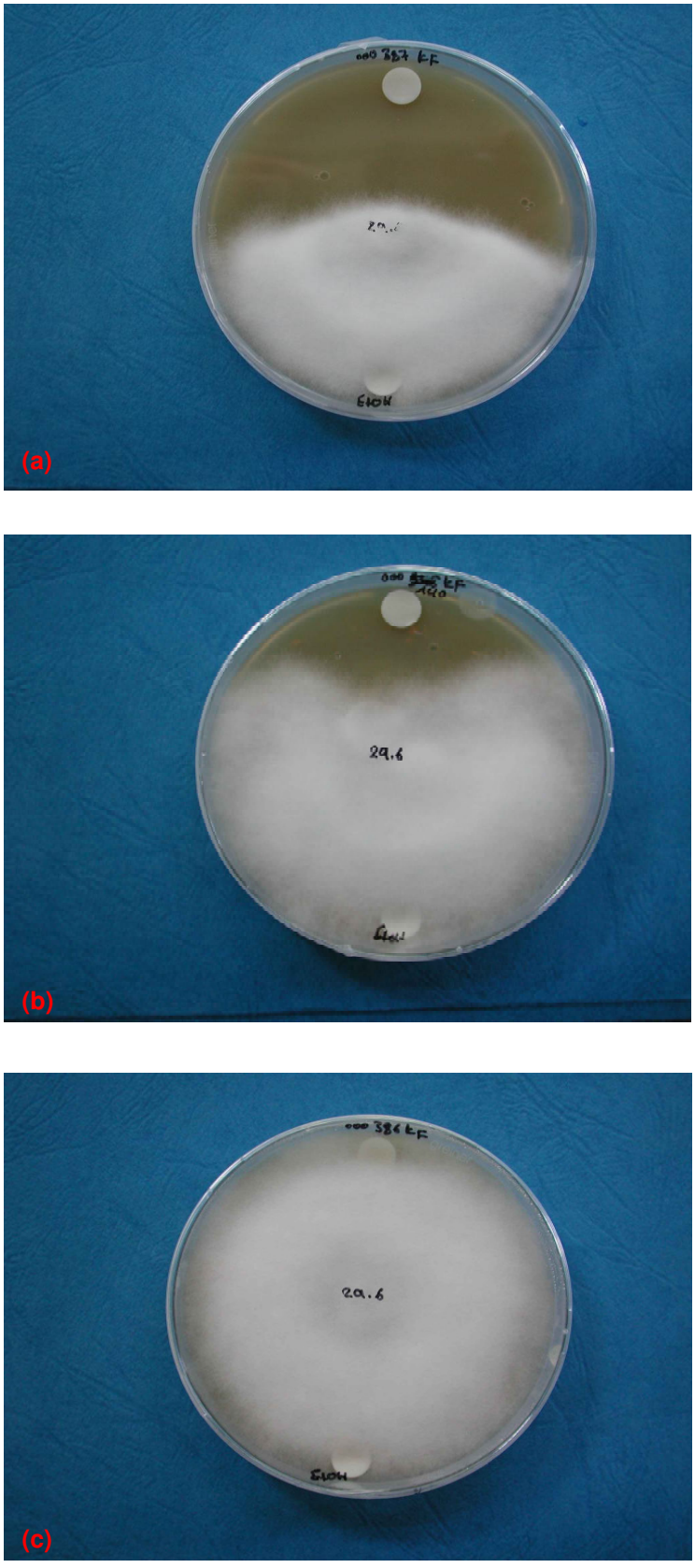


Figure 20: Strong effective extract (a), Moderate effective extract (b), and weak or not effective extract (c).

Table 2: Different extracts tested against *P. infestans* WT in the agar plate diffusion test (9 cm Ø plates). CF = culture filtrate, MyEx = mycelium extract.

Extract number	Effect on *P. infestans*
010413 CF	Moderate
010413 MyEx	Weak
000140 CF	Moderate
000140 MyEx	Moderate
000545 CF	Weak
000387 CF	Strong
000387 MyEx	Moderate
000386 CF	Weak
000065 CF	Weak
000305 MyEx	Moderate
000389 CF	Moderate
000389 MyEx	Weak

3.5 Screening extracts using 24-well multi plates

Selected extracts showing strong (000 387 CF), moderate (000 140 CF) and weak (000 386 CF) activity in the plate diffusion test system were re-tested against *P. infestans* in a 24-well plate test system. Different concentrations (10%, 1% and 0.1%) were prepared from each crude extract (Figure 21). Results indicated that the extracts themselves recorded certain fluorescence when they were used in high concentration levels at the wavelengths used. Only the lowest concentrations of the strongly active extracts allowed the fungal mycelium to grow, but, high concentrations prevented the growth. The moderate effective extracts were strongly effective and prevented the mycelium growth when 100% and 10% of the extract concentrations were used, but allowed the mycelia to grow at lower concentrations (1% and 0.1%). There were no or slight effects on mycelium growth when the weak active extracts were tested at all concentrations; higher growth was, however, observed in the control (sporangia-untreated extract).

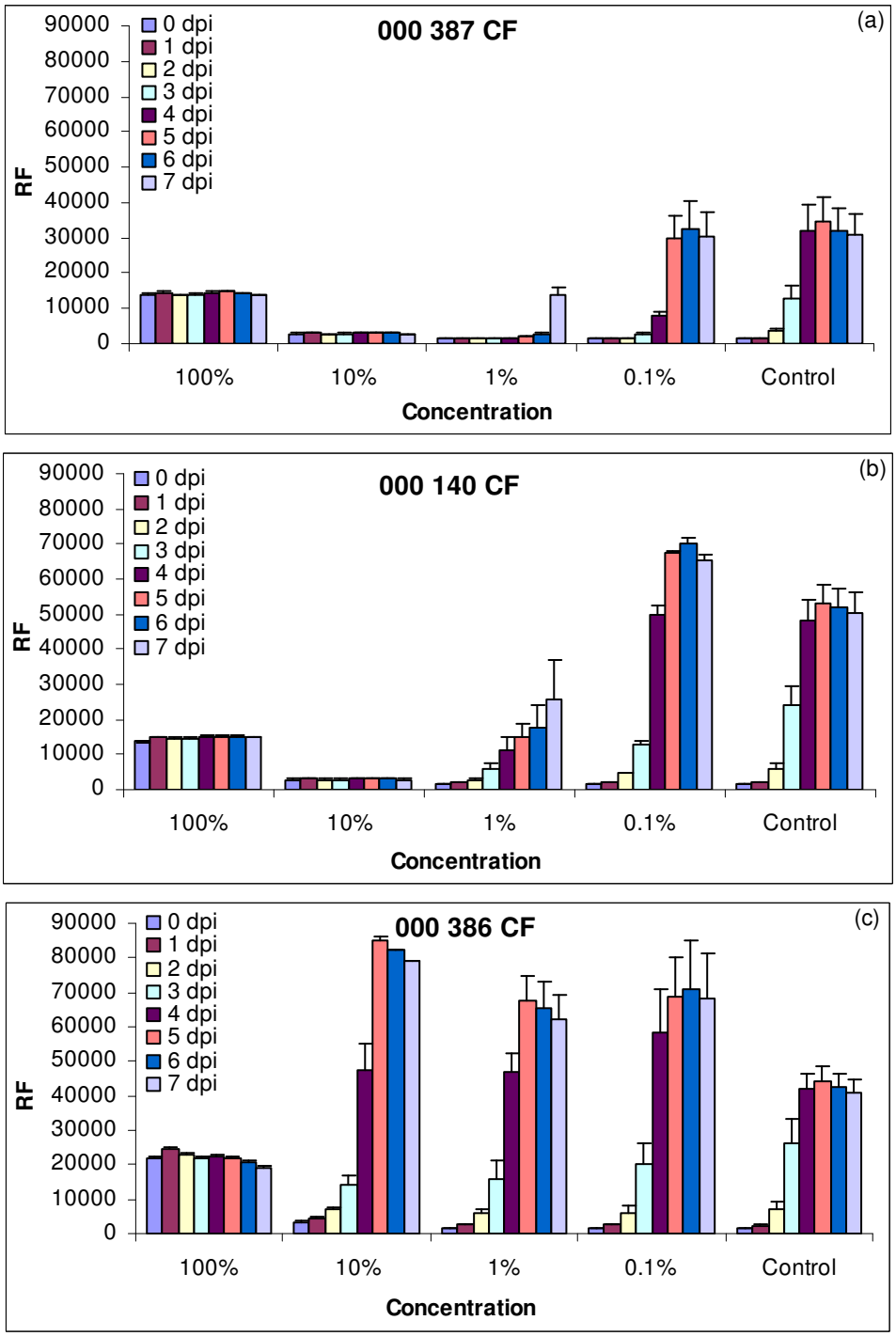


Figure 21: Effect of different extracts on the growth of the transformant 208m2 in 24-well plates at 23 °C, strong (a), moderate (b), and weak (c) extract. RF = relative fluorescence at 485/528 nm Ex/Em; dpi = days post inoculation; error bars are standard deviations of RF of four replications.

3.6 Screening the effects of extracts using 96-well multi plates

The 96-well plate assay was chosen as standard test to determine the antifungal activity of the extracts in an *in vitro* screening. About 8335 extracts were tested on their effect on the growth of *P. infestans* (Pi test system). Some extracts showed a strong fluorescence by their own, this non-inoculated reference plate was used in all screening tests to subtract this basic fluorescence value from that of the GFP. Table 3 shows that the extracts tested differentiated into three groups according to their activity against *P. infestans* (Pi+). These extracts needed to be fractionated later by our partner (Sourcon-Padena GmbH). For each group, a different positive relative fluorescence frontier value was set, those extracts having the same relative fluorescence or lower than this value were selected as positive (effective) extracts concerning *P. infestans* growth inhibition (Pi+). If the extracts in the same group had higher relative fluorescence values than the border fluorescence they were selected as negative (not effective) extracts. It was found that there was only 0.62% from all tested extracts inhibiting the growth of *P. infestans* effectively.

The relative fluorescence of the test backgrounds (medium and the extracts) was another important factor which was also determined in order to correct the absolute values (effect) of the extracts. The scattered (fluctuating) data of extracts from the Pi test system were corrected by subtracting the standard deviation from the absolute value to obtain at the end the real effect of the extract on mycelium growth. Generally, the results of relative fluorescence values were divided into three major groups depending on their antifungal activity (Table 3):

(i) 1st group represented extracts preventing growth of *P. infestans*.

(ii) 2nd group represented extracts affecting growth of *P. infestans*.

(iii) 3rd group represented extracts that did not effect growth of *P. infestans*.

Table 3: RF frontier area of different extracts tested against *P. infestans* WT using 96-well multi plates. RF = relative fluorescence; * = the values contained medium background.

Group	RF Value*
No mycelium growth (mycelium growth inhibition)	< 902
Inhibition uncertain	903 – 4860
No growth inhibition	> 4860

3.7 Establishment of a test system for extracts using detached tomato leaves

3.7.1 Phytotoxicity tests

In order to find solvents dissolving the extracts to be tested satisfyingly and to facilitate the penetration into the plant tissue, a set of solvents was tested for their phytotoxicity. The solvents used in the *in vitro* test system were found to be toxic for the plant tissues depending on the concentrations. DMSO was found to be phytotoxic at high concentrations (Figure 22a). EtOH was also phytotoxic to the plant tissues but only if applied concentrated (Figure 22b). EtOH proved to be a suitable solvent for the application of extracts when it was used at concentrations between 50 and 60%; at these concentrations no phytotoxicity was observed.

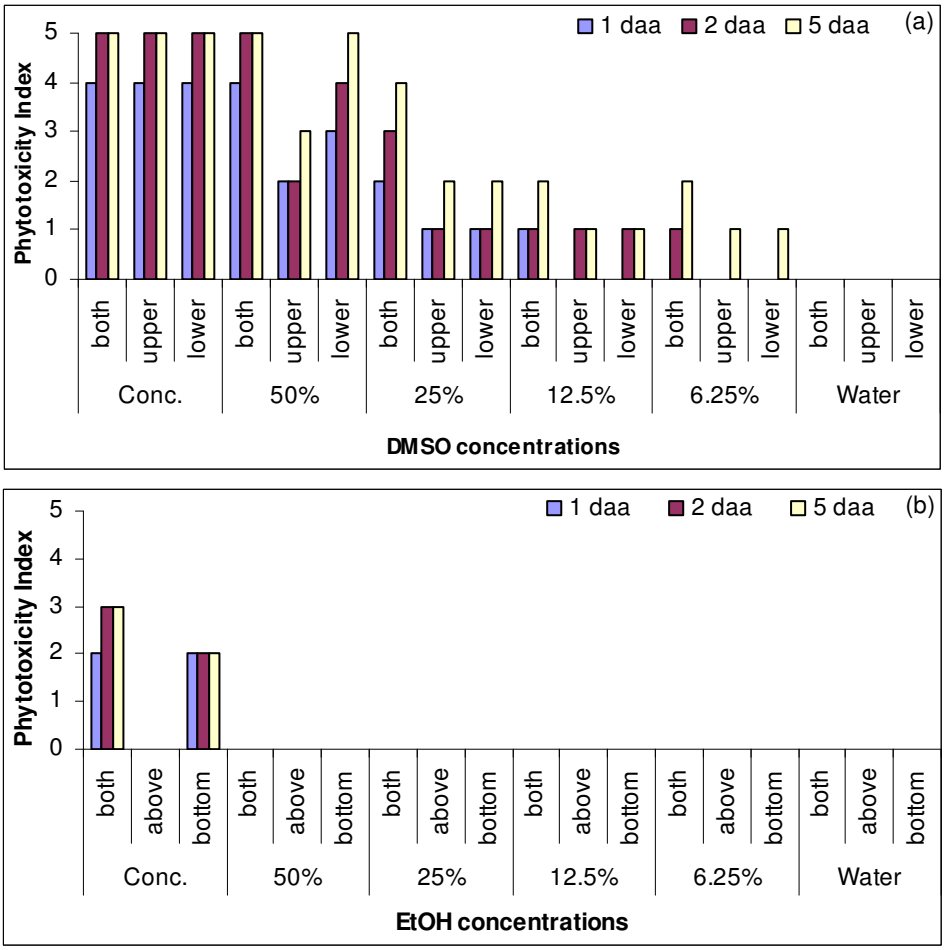


Figure 22: Phytotoxicity of DMSO (a) and EtOH (b) in different concentrations on tomato leaves. Experiments were repeated at least twice (2 replications for each treatment). 0 = non affected tissues, 1 = less than 25% of treated area necrotic, 2 = 25-50% of treated area necrotic, 3 = 50-75% of treated area necrotic, 4 = 75-100 of treated area necrotic, 5 = death of treated leaf area, EtOH = Ethanol. daa = days after application; Conc. = concentrated solvent; both = both leaf sides; upper = upper leaf side; lower = lower leaf side.

However due to the fast evaporation of ethanol in which the tested compounds were dissolved, resulted in a short solution period of the extracts; therefore, it was assumed, that the active agents could not sufficiently enter the leaf tissues. It was necessary to find an alternative solvent that facilitated entrance of the active compounds into the leaf tissues (Figure 23). BreakThru® S 240 known to enter the leaf tissues effectively, was found to be phytotoxic above a concentration of 0.03%, so, the extracts originally dissolved in 60% EtOH were re-dissolved in this solvent at the concentration of 0.03%.

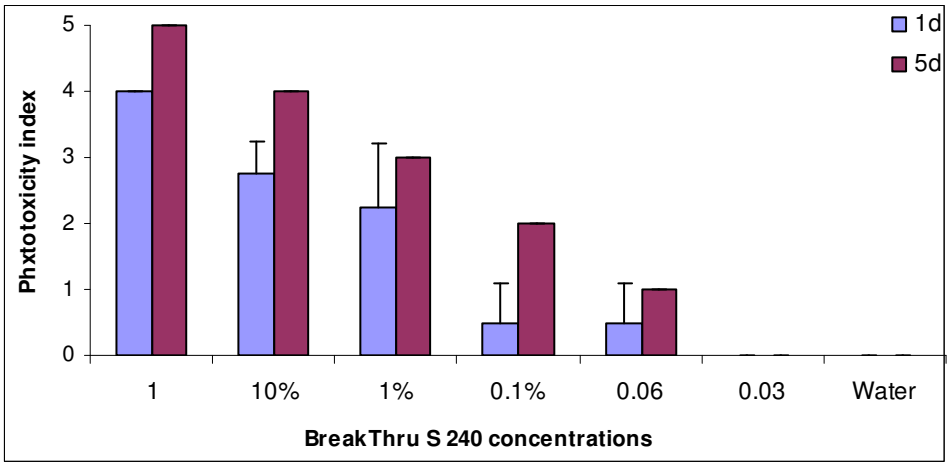


Figure 23: Phytotoxicity of BreakThru® S 240 in different concentrations on tomato leaves. 0 = non affected tissues, 1 = less than 25% of treated area necrotic, 2 = 25-50% of treated area necrotic, 3 = 50-75% of treated area necrotic, 4 = 75-100% of treated area necrotic, 5 = death of treated area; error bars are standard deviations of three replications.

During testing the extracts for their phytotoxicity on tomato leaves, it was found that the extracts varied in their phytotoxicity. While some extracts were highly toxic, others were moderate and some were not phytotoxic to the leaf tissues.

3.7.2 Detached leaves test system (local resistance test)

A visual estimation method was applied for quantifying the symptoms caused by the disease. Three important factors were estimated; firstly, the infected area representing the spread of mycelium in the leaf. Secondly, the sporulation representing the completion of the asexual development and thus the first step for a new infections. Thirdly, necrosis production representing local resistance in the leaves due to the hypersensitive reaction (HR). Disruption of any of these developmental steps of *P. infestans* might either be due to local resistance induction through the extract applied or due to its direct action on the life cycle would present an important possibly of a

highly specific property of the active agent. Extracts that were dissolved in EtOH 60% and showed a slight positive effect in detached tomato leaves against *P. infestans* (small infected area and/or low sporulation rate), were dried up and re-dissolved in BreakThru® S 240 (0.03%) and then retested again in the detached leaves assay to compare their efficiency using these two kinds of solvents. All positive extracts from *in vitro* growth inhibition assays (Pi+) and from the furanocoumarin inducing assays (Pc+) were re-dissolved in BreakThru® S 240 (0.03%) and were tested on detached leaves. Table 4 shows the positive extracts against *P. infestans* in detached tomato leaves. Some of the active extracts of Pi+ and Pc+ showing an effect in detached leaves tests were selected and tested on intact plants.

Table 4: Positive extracts from detached tomato leaves test showing an effect on *P. infestans*. Results present the average of three replications, B.Thr. = BreakThru® S 240, Pi = *P. infestans in vitro* test, Pc = Parsley cells test.

Extract	Positive from	Infected area (% / leaf)		Sporulation (% / leaf)		B.Thr. (0.03%)	*P. infestans* 10^5 sporangia ml^{-1}
		1:4 (25%)	1:10 (10%)	1:4 (25%)	1:10 (10%)		
010 290-1	**Pi**	10	35	0	5	65	75
014 008-2	**Pi**	25	25	0	5	50	75
014 568-2	**Pi**	50	50	0	10	50	65
014 458-1	**Pc**	35	35	25	35	75	75
010 678-3	**Pc**	15	-	15	-	75	75

Extract of 014 008-2 (014 008 represents Actinomycetes strain number, 2 represents the medium culture of Actinomycetes) classified as a Pi+ extract, was effective in the detached leaves assay against *P. infestans* when dissolved in both solvents (EtOH 60% and BreakThru® S 240 (0.03 - 0.06%). However, when dissolved in EtOH (60%), there was a positive effect only at the high extract concentration, with infected areas and sporulation levels lower than in the controls (EtOH 60% and water treatments) (Figure 24a). In BreakThru® S 240 (0.1%), the positive effect of the extract (reduction of infected area and sporulation) could be reproduced. Even at high dilutions, the extract effectively reduced the infected leaf area and sporulation (Figure 24b). At lower concentrations of BreakThru® S 240 (0.06%), the results from the previous experiments were confirmed and showed a stronger effect on growth of *P. infestans* in detached leaves compared to the controls (Figure 24c). Due to low available amount from the positive extracts, only two replications were used in the experiments.

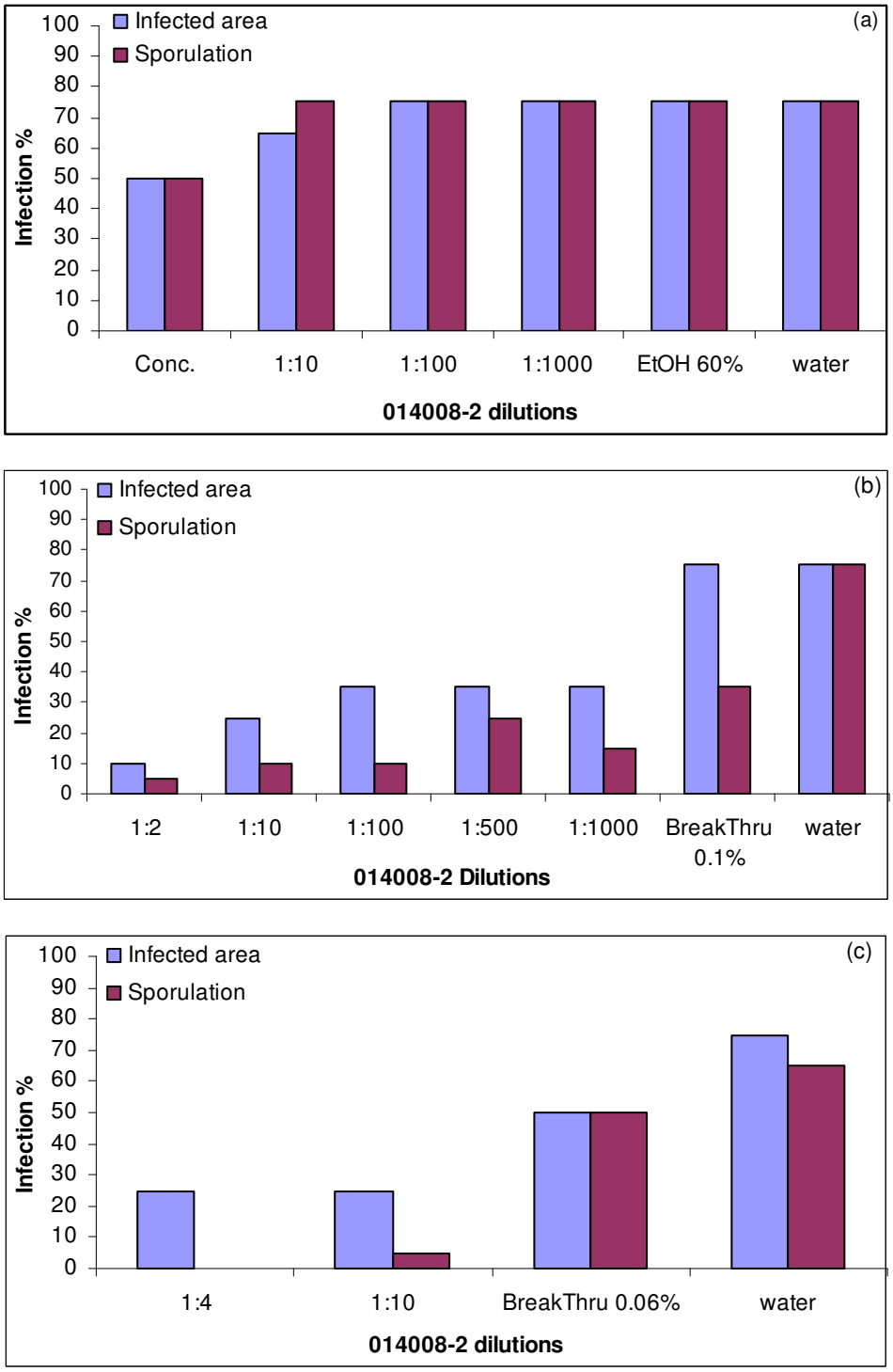
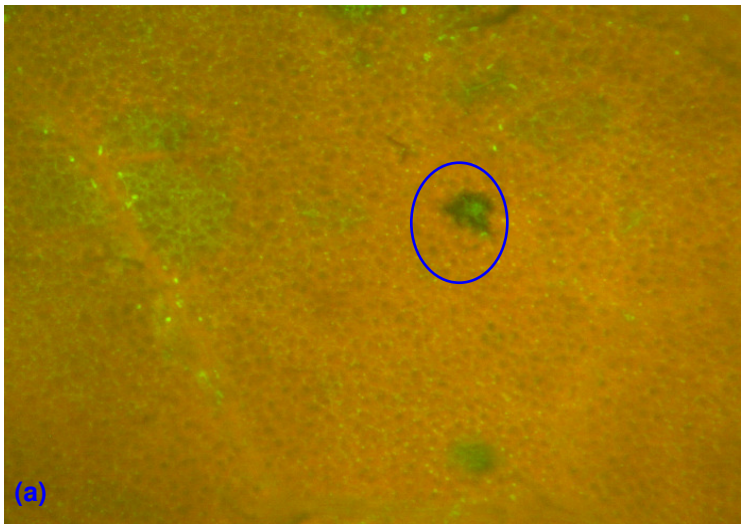
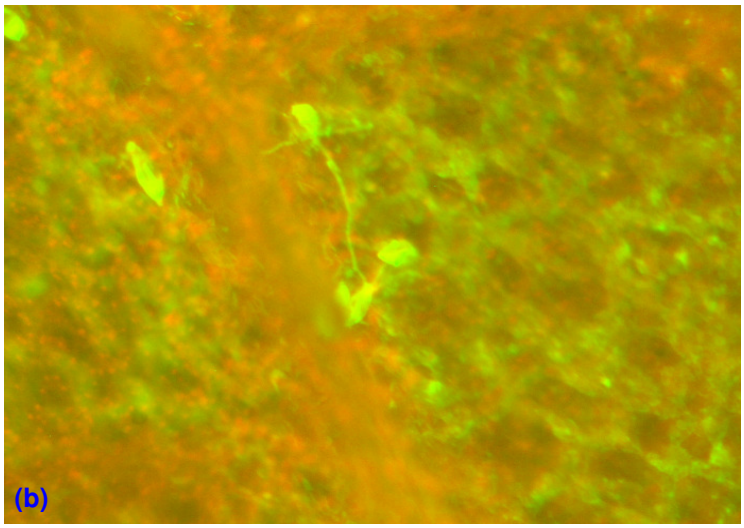


Figure 24: Effect of different dilutions of extract 014 008-2 against *P. infestans* in detached tomato leaves. The extract was dissolved with EtOH 60% (a), BreakThru S® 240 0.1% (b), and BreakThru® S 240 0.06% (c); Experiments were repeated at least twice (2 replications for each treatment).

Another effective extract was 010 290-1. In detached leaves treated with this extract, it was found that most of the sporangia did not release zoospores on the surface of the treated leaves (Figure 25). Only few sporangia succeeded to germinate, but, the germ tubes differed morphologically from those of the control treatments. It was recognised also that there were some limited necroses in the treated-infected detached leaves, possibly representing HR.





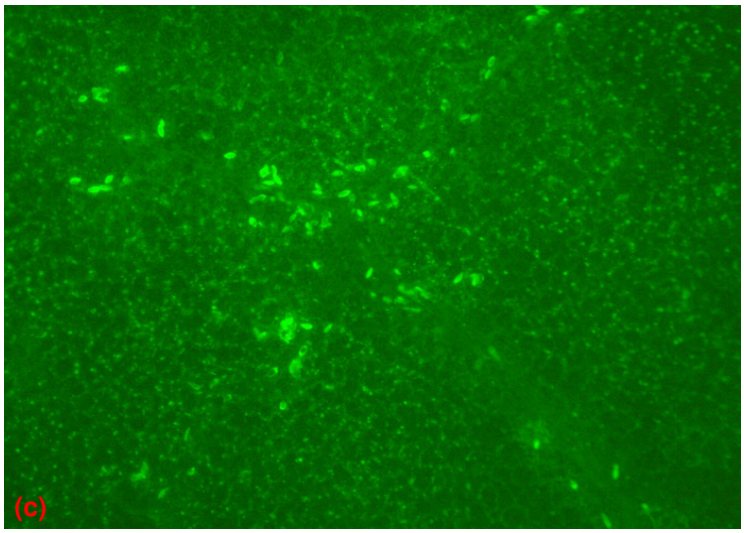


Figure 25: Selected pictures from tomato detached leaves treated with the extract 010 290-1 and inoculated with *P. infestans*. A limited number of dead cells (necroses) within the circle (a), abnormal growth of sporangiophores, abnormal developed sporangia in treated-infected leaves (b), Sporangia on leaf surface did not germinate directly or indirectly; thus, no mycelia was developed (c).

The same phenomenon was found also in parsley cells with one of the positive extracts (Pc+). This extract (010 678-1) was fractioned into three fractions (F1, F2 and M). F1 and F2 fractions did not show an effect on the fungal growth, but, M fraction (mycelium extract) induced formation of a dark brown limited necrotic area in leaves treated with the concentration of 25% compared to control plants, suggesting a resistance reaction (HR) against the fungal infection (Figure 26).

Figure 26: Detached leaves of tomato treated with the extract fraction 010 678-M and inoculated with *P. infestans*. Limited black brown lesions appeared in the leaves.

It was may be assumed that these promising extracts had a specific effect on a certain fungal life stages, it reduced the infected area, the sporulation levels caused limited

necrotic lesions. These extracts were selected to be fractionated in order to be tested further on an intact tomato plant.

3.7.3 Intact tomato plants test system (systemic induced resistance)

The test system will be exemplarily described for the Pi+ extract 000 387 CF in detail. The extract was found to be effective against *P. infestans* even when dissolved in EtOH (50 - 60%). This extract was phytotoxic for the leaves only at high concentrations (Figure 27).

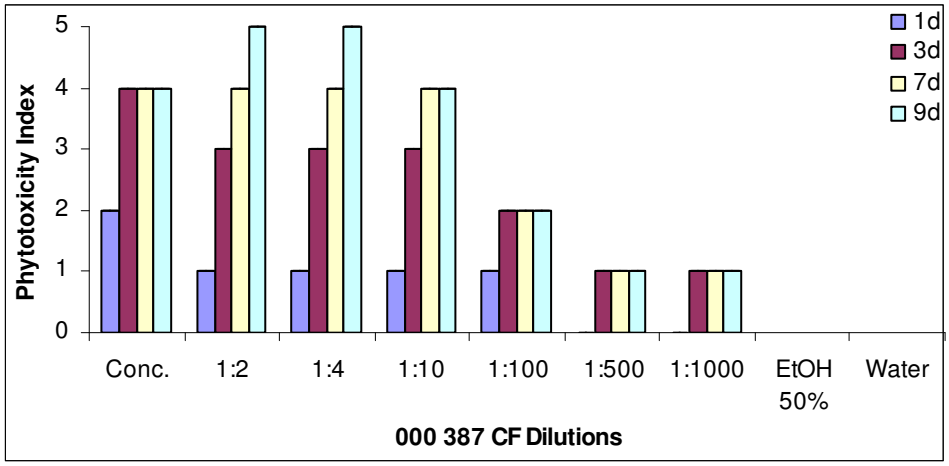


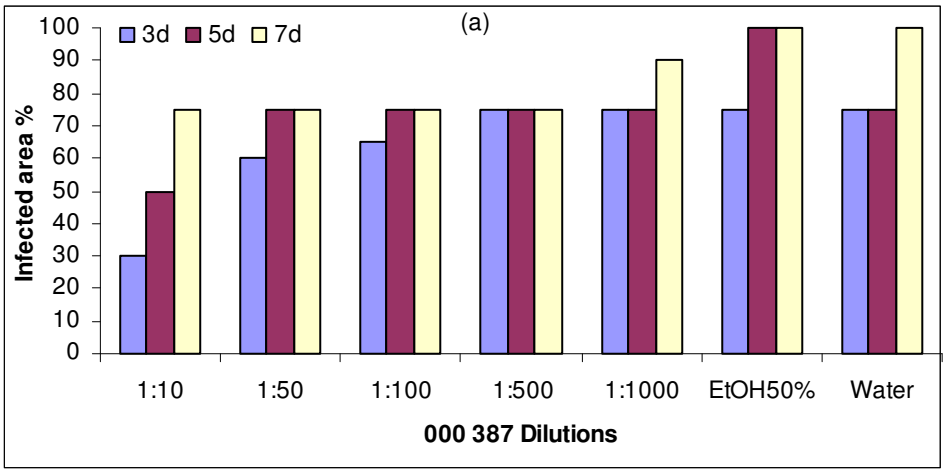
Figure 27: Phytotoxicity of different dilutions of the 000 387 CF extract on intact tomato plants. CF = culture filtrate; d = days post application; Conc. = concentrated extract; experiments repeated at least twice (2 replications for each treatment).

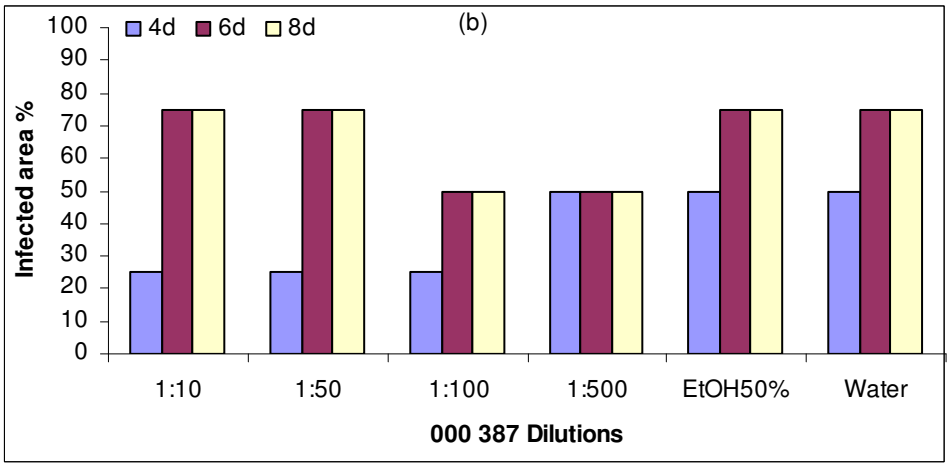
On the intact plant system, not only the direct effect of the extracts on mycelial growth could be investigated, but also the plant systemic response eventually based on induced resistance (hypersensitive reaction and systemic induced resistance).

In the case of the extract 000 387 CF, it was applied to the true leaves of 2-true leaves plant stage. 48 h later, plants were inoculated with the sporangial suspension of *P. infestans* ($1x10^5$ sporangia ml^{-1}). Extract dilutions (1:2 to 1:4) were avoided due to high toxicity to the tomato leaflets. Four days after inoculation, the results showed that the extract did not affect the symptom development (1^{st} infection) in the local leaves, and the symptoms spread as in control plants (Figure (28a). The visual estimation of the systemic (new) leaves from 1^{st} infection, showed that the infected area was reduced to 50% when plants were treated with a highly diluted extract (more than 1:500) compared to control treatments (Figure 28b). Two weeks after the 1^{st} infection, only the systemic (new) leaves were inoculated (2^{nd} infection) to check if a resistance effect has been acquired in the systemic leaves. A stronger inhibition of mycelium growth was

observed in the infected leaves, where slight and no symptoms were observed on plants treated with 1:100 and 1:500 dilutions, respectively (Figure 28c).

A control experiment (Figure 28d) was performed in parallel with the original mentioned experiment, in order to determine whether an induction of systemic resistance could be activated by the 1st infection on the local leaves, or the presence of the extract in the plant tissues would induced (primed) a kind of resistance in the treated plants. The plants in this experiment were infected at the time of the 2nd infection of the original experiment. It was found that the results of the control experiment confirmed the results of the 2nd infection of the original experiment; symptoms varied from no to very slight brown lesions in the systemic leaves of plants treated with 1:10 to 1:1000 dilutions. It was assumed that a plant resistance response (reduction of infected area) might be due to the extract treatment itself and not due to the fungal infection.





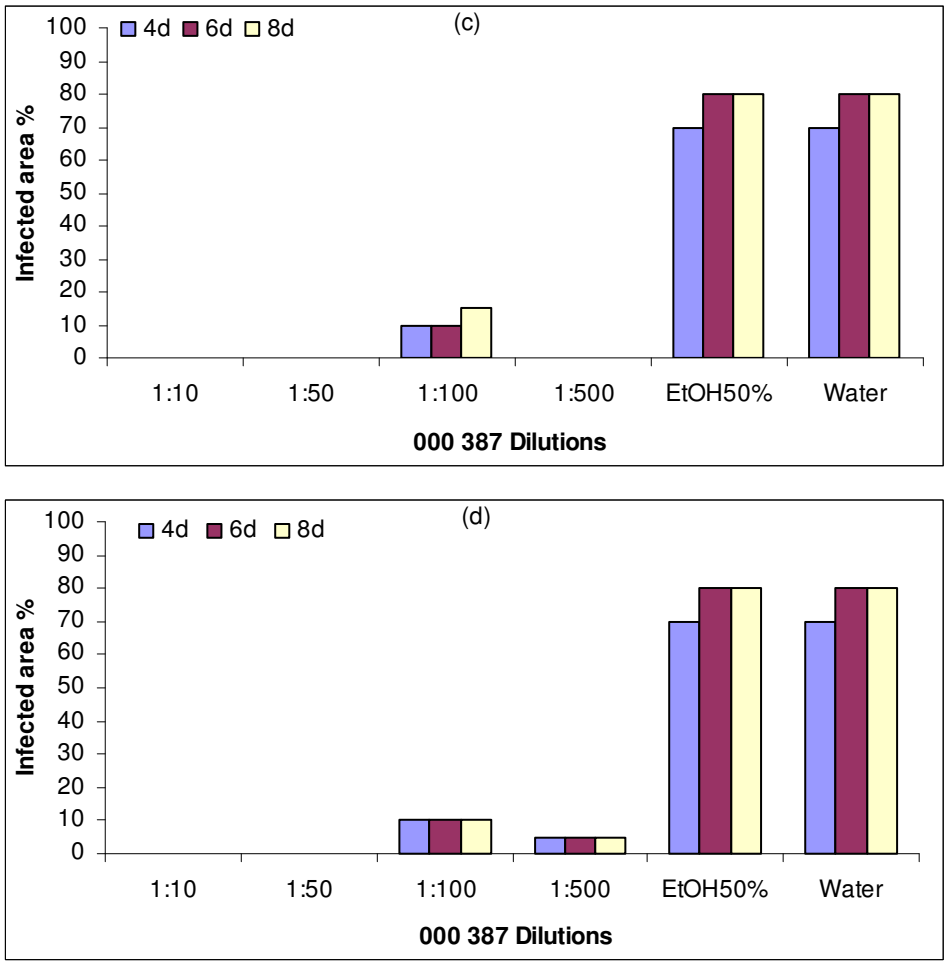


Figure 28: Effect of different dilutions of 000 387 CF on *P. infestans* in: 1st infection-local leaves (a), 1st infection-systemic leaves (b), 2nd infection-systemic leaves (c) and on the infection of the systemic leaves of control experiment (d) of intact tomato plants. CF = culture filtrate.

Parallel with testing extract 000 387 CF in tomato plants, fractionation steps were done to identify its active ingredient. The final identification step proved that the extract contains Cycloheximide as active ingredient (Obrig *et al.,* 1971). Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus*. Cycloheximide exerts its effect by interfering with peptidyl transferase activity of the 60S ribosome, thus blocking translational elongation (Tang *et al.*, 1999).

Further experiments were executed to find other promising extracts on the intact tomato plant system.

Based on the promising results of the extract 014 008-2 from detached leaves test system, this extract was also a candidate to be tested in an intact tomato plant system to check its ability for an inducing effect in plants. The extract was fractioned into; Fraction 1, Fraction 2 and Fraction 3 (mycelium extract). The extract was diluted (1:5) and then tested in the plant system. Table 5 shows the visual evaluation of the

necrosis on the leaves which varied among the different fractions, where, fraction 1-treated plants showed the lowest infection rate.

Table 5: Visual estimation of the effects of 014 008-2 extract fractions (induced resistance effect) on the necrosis development on intact tomato plants infected with *P. infestans*. BThr. = BreakThru® S 240 0.06%; results are the averages of three replications.

Extract fraction	Necroses (% / plant)
1	10
2	50
3	80
BThr 0.06%	80
Water	80

The microscopic analyses of the leaves of treated plants revealed that mycelium and sporangiophores treated with fraction 3 grew normally as in the controls (Table 6 and Figure 30b). From leaves of plants treated with fraction 1, misshaped sporangiophores without sporangia were formed. Treatment with the fraction 2 resulted in a weak sporulation compared to the control, where, the sporangiophores were abnormal in shape and they did not produce sporangia (Figure 30a).

Table 6: Microscopic analysis of 014 008-2 extract fractions (induced resistance effect) on the fungal sporulation and sporangia development on intact tomato plants infected with *P. infestans*. BThr. = BreakThru® S 240 (0.06%); - = sporulation was not appeared; + = sporulation appeared.

Extract fraction	Growth of mycelium	Sporulation development	Sporangia formation
1	+	-	-
2	+	+/-	- (sporangiophores without sporangia)
3	+	+	+
BThr 0.06%	+	+	+
Water	+	+	+

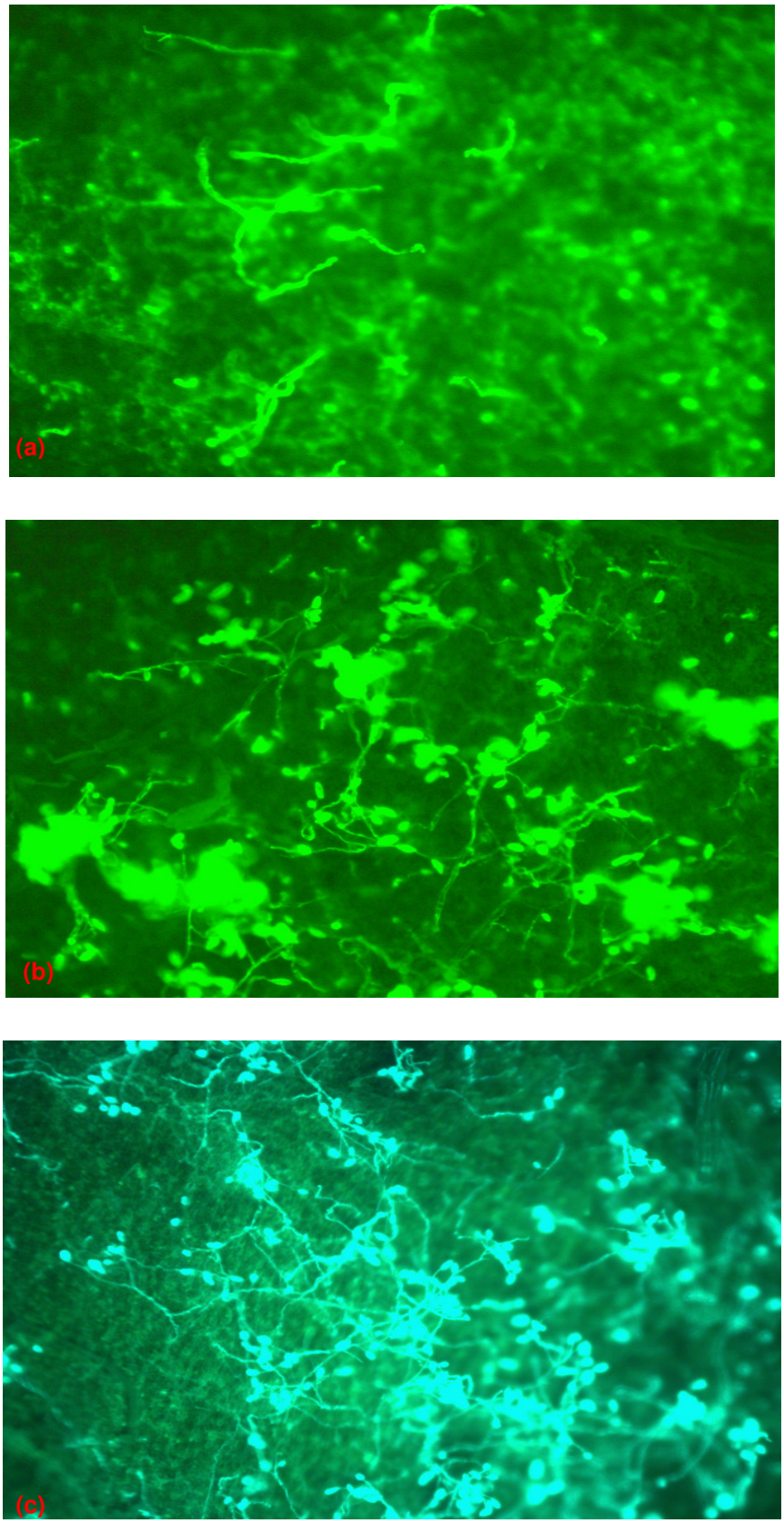


Figure 29: Fluorescence micrographs showing tomato plants treated with 014 008-2 fractions and inoculated with *P. infestans*. Sporangiophores without sporangia in plants treated with fraction 1 (a), Sporangiophores carry normal sporangia in control plants (b), Normal sporangial growth in plants treated with the ineffective substance (014326-2) (c).

4 DISCUSSION

We were successful in establishing and developing rapid suitable techniques to screen a high number of Actinomycetes extracts for their action against *Phytophthora infestans*, the causal agent of tomato and potato late blight. Within the framework of a project financed by the DBU (Deutsche Bundesstiftung Umwelt), alternative phytosanitary compounds from Actinomycetes extracts have been screened. Based on the Pflanzenschutzgesetz §2 Nr. 10, plant strengthen (protection) compounds (Pflanzenstärkungsmittel), should not act directly against the pathogen, but via an induction of resistance mechanisms. The important phytpathogen *P. infestans* was chosen as a model.

4.1 *In vitro P. infestans* test system:

With the aim to test thousands of Actinomycetes extracts in an *in vitro* screening test for their action on *P. infestans*, a high throughput screening test had to be developed.
In previous reports, many *in vitro* investigations have been done to find effective extracts from plants or from microorganisms against different kinds of pathogens (Soylo *et al.*, 2005; Hammer *et al.*, 1999; Bianchi *et al.*, 1997; Chaudhuri and Sen, 1982). Some investigations concentrated on finding substances certainly effective against *Phytophthora spp.* (Soylo *et al.*, 2006; Soylo *et al.*, 2005; Bowers and Locke, 2004; Jende *et al.*, 1999).

Plate diffusion test: Measuring the fungal mycelium growth, using MGA (mycelium growth area) and WGA (weight of growth area) methods, was found to be not practicable for a high throughput screening mainly due to the time duration (nearly 14 days for each test), the place required and a consistent test setup. MGA and WGA methods were at least helpful for collecting enough information on the activity of the extracts tested *in vitro* growth of both *P. infestans* types used (the wild type and the transformant 208m2). MGA and WGA methods were also not efficient in measuring the fine young mycelium inside the medium. *P. infestans* is a facultative parasite which is known to grow in different kinds of media (Judelson and Roberts, 2002; Peters *et al.*, 1998; Xu, 1982). Testing different eligible media, mycelium growth was optimal in rye-glucose and V-8 media, so both media have been considered for further optimizations.

24-well multi plate test: This test aimed to benefit from the fluorescence properties of the transformant 208m2, a GFP-expressing strain using a fluorescence reader. Starting inoculum was a suspension with a constant amount of sporangia, which was determined from a regression line formula for 1 ml suspension (correlation between the sporangia fluorescence and the sporangial amount). The inoculum's concentration was measured in fluorescence reader always before starting a test.

The mycelium did not grow well in the liquid medium and aggregated at the bottom of the wells quenching the fluorescent reader beam, so that no quantitative relation between growth and fluorescence could be established. It was also recognised that V-8 medium was turbid despite centrifugation and had a high self-fluorescence compared to rye-glucose medium which was highly transparent and only slight self-fluorescing.

The mycelium growth was detected best using 485/528 nm Ex/Em – filters combination which was the nearest to the expressed GFP (S65T) fluorescence (488/510 nm Ex/Em) (Azeddine *et al*., 2003; Si-Ammour *et al*., 2003). The high consistent growth rate was achieved at 23 °C on solid rye-glucose medium. Fluorescence microscopy showed that sporangia formation was optimal when the mycelium was incubated at 15 to 18 °C, but formation of sporangia was inhibited at 23 °C. Due to the high fluorescence of the sporangia that could affect the measurement of the mycelium growth, a temperature of 23 °C was chosen for the test. Applying the extracts to the medium 24 h before inoculation, allowed complete diffusion for the extracts into the medium.

96-well multi plate test: The 24-well multiplate system was used only to establish the screening test and to confirm the results of the substances tested in the plate diffusion test, but, for high throughput handling, it was essential to use 96-well plates. Another important reason using the 96 well plates was the pipetting of small extract volumes delivered from the industrial partner into the same form of test plates with a multichannel pipette.

The extracts that reduced growth of *P. infestans* (Pi+) were also tested on their effect on *in vitro* growth of *Erwinia amylovora*. One Pi effective extract (000 387 CF) showed no efficiency on the bacterial growth, and this was probably due to a selective effect on

Oomycetes. In the screening of the extracts on growth inhibition of *P. infestans*, the focus was also laid on those that did not show any inhibition on mycelium growth (Pi $^{-}$), to test them in the parsley cell culture for the induction of furanocoumarin phytoalexins. These extracts were considered as candidates of potential resistance inducers that could be classified as plant strengthen agents.

The extracts tested against *P. infestans* (Pi), were classified depending on their effectiveness to inhibit mycelium growth. Analysis of the results of this screening part led to a classification of the extracts into those that showed a clear inhibition of mycelium growth. In the framework of the screening for a plant strengthen agent they had to be excluded from the Pc+ list. The same was true for extracts which action was intermediate and thus unclear. Extracts that had no influence on the growth of *P. infestans in vitro* were chosen for further investigations in the parsley cell culture system to detect their potential of induction of phytoalexins. Settings of the positives borders for the exclusion list were based on the estimation that in the screening of Actinomycetes extracts that act directly against an organism should be in the 'promille range' (Dr. Markus Kempf, Sourcon Padena GmbH, personal communication). At the end of screening tests, only 0.62% (52 extracts) from a total of 8335 tested extracts was active against *P. infestans* (Pi+).

4.2 Phytotoxicity test of solvents and extracts

Phytotoxicity is an injury or damage to a plant due to chemical treatment. This injury includes height reduction, stand reduction, vigor reductions, color changes, root pruning, stunting, changes in growth patterns, changes in physiology, burning, necrosis, chlorosis, sterility, elongation and etiolating, growth inhibition, plant growth regulation, flecking, weak plants, thickening, catching in the boot, streaking and shedding of the leaves, enhancement of maturity, death, and death of the pollen (Chase and Simone, 2001; Beckett and Davis, 1988).

The aims of these tests were: firstly, to test the phytotoxicity on tomato leaf tissues of ethanol (EtOH) and dimethyl sulfoxide (DMSO) as extract solvents. DMSO was found to be toxic even at low concentrations (6%), therefore, it has not been used further. EtOH was toxic only at high concentrations (more than 60%). Secondly, to test the

phytotoxicity of the extracts and to find the concentrations nonphytotoxic to the plant tissues.

The promising extracts tested in the detached leaves had a lower positive effect than they had in the *in vitro* tests, and it was thought that this might be due to two reasons, either 60% EtOH as solvent was not efficient enough to promote penetration of the extract compounds into the plant tissues or evaporation of the solvent was too rapid. Break-Thru® S 240 was chosen as an alternative for the application of the promising extracts, because it was found to be not phytotoxic at low concentrations (below 0.05%). BreakThru is an organosilicone surfactant with good properties as a dispersing and wetting agent, enhancing the entrance of the dissolved extracts (Stachecki *et al.*, 2004; Schlachter *et al.*, 1999). So, the extracts delivered in 60% EtOH by the cooperation partner, were dried up and re-dissolved in BreakThru.

4.3 Detached leaves test system

Positive classified extracts from *in vitro* growth inhibition tests (Pi+) and from parsley cell culture tests (Pc+) (Chapter III) were examined in detached leaves. Pi+ extracts that showed a direct growth inhibition *in vitro*, were retested in detached leaves for their efficiency in plant tissues. Extracts inducing furanocoumarin phytoalexin formation in parsley cell cultures (Siegrist *et al.*, 1998), were re-tested in the same way.

Plant tissues are capable to defence themselves against pathogens by both preformed and induced mechanisms. One of the most effective induced responses is the hypersensitive reaction (HR) (Goodman and Novacky, 1994; Keen, 1990). HR results in small brown necrotic lesions at or near the site of infection produced by the host plant during incompatible interaction. The difference between a susceptible and a resistance plant often is based on how fast and powerful a plant is able to activate its own defence system. In other words, the resistant plant is able to fend off the attack in time. In contrast, a susceptible plant must be induced to respond faster to become resistant (Grover and Growthaman, 2003; Dangl and Jones, 2001).

This secondary screening test concentrated on three monitoring factors. Firstly, infected leaf area representing the spread of mycelium in the leaf. Secondly, area of

sporulation representing the completion of the asexual propagation cycle, and thus the epidemic potency. Thirdly, formation of necroses as an induction of HR.

The extracts were applied to the detached leaves 24 h before inoculation (spray application) to allow them to enter the plant tissues and to allow the potential establishment of inducible defence responses. Only 2 extracts (010 290-1 and 014 008-2) out of 52 Pi+ and 1 extract (010 678-3) out of 42 Pc+, induced limited dark brown necrosis, resulting in an inhibition of the mycelium spread in the tissues, leading to a conclusion that the extracts induced HR.

Extract 010 290 (Pi+) was able to reduce infection and inhibit sporangia formation. This extract had an influence on the size of the infected area at low concentrations (25 and 10%) which was reduced to 10 and 35%, respectively, compared to control treatment. A significant influence on sporulation level has also been recognized at the applied concentrations, which was reduced to 0 and 5% of the leaf area, respectively, compared to the control. Small necrotic lesions were also observed on the leaves. Microscopic analysis showed that most sporangia from the inoculum did not germinate on the leaf surface, and germ tubes produced abnormal morphological alterations. These results indicated direct actions of the extracts on the one hand, and perhaps induced resistance in the plant cells on the other hand.

Extract 014 008-2 (Pi+), was always effective in EtOH and in BreakThru® S 240 solvents. Visual estimations showed that it had a high influence on the sporulation level (0, 5%) as well as on the infected area (25, 25%) at low concentrations tested (25, 10%, respectively) compared to control treatments.

The same trend was also found in one of the Pc+ extract (010 678-1). This extract has been fractioned into three major fractions (F1, F2 and M (mycelium extract)). F1 and F2 fractions showed no influence on the fungal development stages. But, the M fraction induced dark brown lesions after inoculation on leaves treated with the standard screening concentration of 25%. The infection area and the sporulation were reduced to 15% compared to control plants. Actually it is not clear, if the action of Pc+ extract fraction 010 678-1 M is based only on defence inducing effects or also on an additional

direct effect against the pathogen. Further purifications of the active compounds are essential to answer this question.

Further fractionations of these extracts are currently under way by the cooperating partner to identify the active compound.

4.4 Plant test system

The HR is characterised by rapid cell death at the infection site and in the local surrounding region leading to small necrosis. This HR was leading to a systemic induced resistance. Therefore, the extracts 000 387 CF and 014 008-2 induced necrosis on detached leaves were tested on intact plants. The other extracts that were available only in small volumes (1.5 ml) during the time of the screening, could not be produced in time in larger volumes for the treatment of whole plants.

Extract 000 387 CF was phytotoxic at concentrations higher than 1%. Nevertheless, interesting results were obtained in the systemic leaves of plants treated with concentrations lower than 1%, where, the symptoms (infected area) varied (between 0 to 10%) depending on the extract concentrations applied to the plants. These results suggested that the extract could be promising either by inducing resistance or by exerting a direct effect against the pathogen due to systemic transport in the plant. The later fractionating and identification performed by our cooperation partner, however, proved that the active ingredient of 000 387 CF was Cycloheximide.

Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus*. Cycloheximide exerts its effect by interfering with peptidyl transferase activity of the 60S ribosomes, thus blocking translational elongation. It is widely used in biomedical research to inhibit protein synthesis in eukaryotic cells studied *in vitro*. It is inexpensive and acts rapidly. Due to significant toxic side effects, including DNA damage, teratogenesis, and other reproductive effects (including birth defects and toxicity to sperm), cycloheximide is generally used only in *in vitro* research applications, and is not suitable for human use as a therapeutic antibiotic compound. Although it has been used as a fungicide in agricultural applications, this application is now decreasing as the health risks have become better

understood (Obrig *et al.*, 1971; Tang *et al.*, 1999). Due to the mentioned effects, the extract has been deleted from the positive list.

The fractions of a promising extract from detached leaves tests (014 008-2) were applied to the plants (2 true leaves stage) to investigate their resistance-inducing effects in the systemic leaves, and to study the fungal structures microscopically after the treatment. Microscopic investigations using the fluorescence microscope showed that the extract fractions did not prevent mycelium growth in plant tissues. Fraction 1 was a promising fraction, because it prevented the sporulation of the fungus; there were no sporangia formed in all plants treated with this fraction, and the infected area on leaves of plants treated with this fraction was only 10% compared to controls. Fraction 2 was found to be less effective than Fraction 1, and the sporulation area was 50% compared to the control. The sporangiophores were abnormally shaped without sporangia formation.

In the literature, morphological abnormalities of mycelia were described in consequence of the treatment with extracts from plants or microorganisms; lysis of cell wall structures indicated that the cell wall lost stability substantially (Jende *et al.*, 1999). Extracts were found to be effective mainly on the infection structures of *P. infestans* in tomato leaves. The swelling of fungal structures and the lysis of hyphal cell walls indicated that extracts influenced the fungal cell wall. Possible explanations for the alterations in hyphal morphology were cytoplasmic coagulation, hyphal shrivelling, hyphae without cytoplasm and blistering (Soylu *et al.*, 2006; Soylu *et al.*, 2005).

A clear investigation of the observed effect would be necessary on the microscopic level (electron microscopy). These results were obtained at the end of the screening project, so due to time constraints, it was not possible to carry out further experiments within the frame of this thesis.

Chapter II

Screening and identification of phytosanitary compounds derived from Actinomycetes extracts against growth of *Erwinia amylovora*

2 MATERIALS AND METHODS

2.1 NB medium

- 20 g/l Sucrose
- 8 g/l NB (Sigma Nutrient Broth) powder
- 18 g/l Agar
- pH 7.0

Medium components were mixed with 1 L of distilled water and then the pH of the mixture was adjusted to 7.0. After that, the medium was autoclaved at 115 °C for 20 min. The autoclaved medium was distributed into flasks each has 25 ml liquid medium, and the medium was kept at 4 °C until use. Solid medium was used only for long time preservation of *Erwinia amylovora*, and it was prepared by adding agar to the liquid medium before it was autoclaved.

2.2 Subculturing of the bacterium

Erwinia amylovora isolate Hoh-1 was kindly obtained by Dr. Jürgen Siegrist. *E. amylovora* was sub-cultured on NB medium by adding 20 µl of a 2.0 OD_{600} bacterium culture to 25 ml flasks of NB liquid medium. The bacterium culture was incubated for 16 h at 28 °C under shaking (160 rpm). The fresh bacterium suspension was adjusted to 0.4 – 0.5 OD_{600} to be used in the test.

2.3 Preparation of the extracts

The following part was done by Sourcon-Padena GmbH (Tübingen, Germany): (not mentioned details are confident information)

Isolation of Actinomycetes: Strains were isolated from soil. Soil samples were collected from all over Europe conform depending on the Rio-convention. The isolated bacteria were stored on HA-Agar medium (contents/l: 4 g of yeast extract, 4 g of malt extract, 4 g of glucose, at pH 7.3).

Cultivation of Actinomycetes: Strains were cultivated in three different liquid media at 27 °C on incubation shakers (Fa. Noctua, Mössingen) for 5 to 14 d depending on the produced biomass. Then, the resulted culture broth was run through an extraction-roboter (constructed by Sourcon-Padena GmbH).

Fractionation of positive extracts: This step was analyzed using HPLC and HPLC-MS techniques, where, the active extracts from the different screening tests were fractionated in their sub-fractions to be screened again in the tests described before.

The following part was done as PhD work in AlPlanta - RLP AgroScience GmbH: The extracts were obtained in 96-well plates as dry extracts (200 µl per well). The crude extracts were dissolved in 100 µl/well of dimethyl sulfoxide (DMSO) (40X end concentration, 115 µl end volume). The plates were shaken at 175 rpm for 1 h at 23 °C until they were well dissolved. The dissolved extracts were either tested directly or kept at 4 °C until used in the tests.

2.4 Measurement of bacterial concentration

The bacterial concentration was optimized by measuring the optical density (OD) of the bacterial suspension using the photometer at OD_{600} nm. 1 ml of the bacterial dilution was measured in a cuvette and adjusted to 0.4 – 0.6 OD_{600} to be used in the test.

2.5 Establishing a screening test system of extracts using 96-well multi plate

The system was established to screen a high number of extracts for the effectiveness to inhibit cell multiplication of *E. amylovora* in 96-well multi plates. The wells of the plates were pipetted with 100 µl of NB liquid medium. As described in Table 1, 5 µl of dissolved extracts were pipetted into the medium. As a positive control, streptomycin (5 µl of 0.01% end concentration), 5 µl of DMSO (the solvent of the extracts) as a negative control were included. After that, 10 µl of bacterium suspension (0.4 – 0.6 OD_{600}) were added to 3 out of 5 wells. For each sample (extract) treatment, 3 repetitions of extract+bacteria and 2 repetitions of extract without bacteria treatment were performed. The test plates were shaken with 175 rpm at 28 °C on a rotary shaker (Certoma, Tsram). The bacterial growth in plates was monitored by measuring the OD_{600} at 0, 6 and 24 h after inoculation using a ELISA Reader. The effect of the extract on the bacterial growth was calculated by subtracting the difference of the average of OD_{600} values of extracts without bacteria from the average of OD_{600} values of extracts+bacteria between 0 - 6 h as well as between 0 - 24 h. Finally, the data representing the bacterial growth after 6 h and after 24 h were transferred to appropriate diagrams. The extracts which reduced or prevented the bacterial growth

were fractioned and retested again in the test system to identify the effective compound.

Table 1: Pipetting scheme of a 96-well plate for standard screening of potentiality bacteriostatic Actinomycetes extracts. S = Sample, E+B = Extract with bacteria, E-B = Extract without bacteria, Str. = Streptomycin 0.01%, DMSO = Dimethyl sulfoxide, B = Bacterium, - = Empty

Str.	-	+B	+B	+B	-B	-B	-	-B	-B	-B	-B	-B	Medium
DMSO	-	+B	+B	+B	-B	-B	-	E+B	E+B	E+B	E-B	E-B	S7
S1	-	E+B	E+B	E+B	E-B	E-B	-	E+B	E+B	E+B	E-B	E-B	S8
S2	-	E+B	E+B	E+B	E-B	E-B	-	E+B	E+B	E+B	E-B	E-B	S9
S3	-	E+B	E+B	E+B	E-B	E-B	-	E+B	E+B	E+B	E-B	E-B	S10
S4	-	E+B	E+B	E+B	E-B	E-B	-	E+B	E+B	E+B	E-B	E-B	S11
S5	-	E+B	E+B	E+B	E-B	E-B	-	E+B	E+B	E+B	E-B	E-B	S12
S6	-	E+B	E+B	E+B	E-B	E-B	-	E+B	E+B	E+B	E-B	E-B	S13

2.6 Assessment of potential effective substances of the Actinomycetes against *E. amylovora* in *in vitro* and in *in vivo* apple plantlets (induction of resistance)

The extracts that had a direct effect against *E. amylovora in vitro* and which had at the same time an inducing effect in parsley cells to produce furanocoumarins, were selected to be tested on apple plantlets.

2.6.1 Preparation of *in vitro* apple explants

MLO6 medium (contents / L):

- 4.464 g MS medium van der Salm modification
- 30 g Sucrose
- 3.5 g Micro agar (per 500 ml)
- 1 ml Thiamine (1 mg/ml stock solution) + Vitamins stock MS (1 mg/l end concentration)

Explants of apple (Gala Royal), obtained by Dr. Wolfgang Jarausch (RLP AgroScience GmbH, Neustadt/WS, Germany) in plastic boxes containing MLO6 medium (MS-medium, van der Salm modification) (Duchefa, NL), were kept at 16 h day light at 22 °C and 8 h dark at 18 °C.

2.6.2 Establishing a test system for effective extracts from parsley cells (Pc)

This test system was established to test the promising extracts from the Pc test system against the bacterial growth in *in vitro* cultured apple plantlets (23 °C, 16h/8h light/dark cycle). For each promising extract, 15 explants (five explants were grown in each plastic box) were used. The experiments started by spraying the explants with the extracts in different concentrations (Table 2). Explants were sprayed with 1 ml of each concentration using a sterilised glass bottle sprayer, and then explants were left for 2 - 3 d until the substances were dried (Figure 1). Positive control substances were sprayed on the explants to compare the efficiency of the promising extracts with the positive controls (Bion® (BTH), Prohexadion-Calcium, Streptomycin). As a negative control, explants were only inoculated by the bacterial suspension by cutting the leaf tips of water sprayed plantlets. Two - 3 d after treatment, the tips of leaves were cut using a small scissor dipped into a bacterial suspension. Three d after inoculation, the symptoms started to appear on the leaves, monitored each 3 d for 6 weeks and evaluated by calculating the percentage of the number of the necrotic (brown) apple shoots to the total inoculated shoots per 3 boxes (15 explants).

Table 2: Promising substances and their concentrations tested on *in vitro* apple explants against *E. amylovora* growth, CF = culture filtrate.

Substance	Concentration
Prohexadion-Calcium	0.25 %
Prohexadion-Calcium	0.025 %
Prohexadion-Calcium	0.0025 %
Bion (BTH)	0.2 %
Bion (BTH)	0.02 %
CF 000 391	10 %
CF 000 391	1 %
Streptomycin	0.01%
Tubercidin (012 228)	0.01 %
Streptothricin	0,01 %

At the end of the experiment, the promising extracts from *in vitro* explants tests were tested on intact plants in the greenhouse.

Figure 1: Treating *in vitro* explants with different concentrations of substances (a), infecting the treated explants with *E. amylovora* suspension (b).

2.7 Testing of promising extracts from Actinomycetes against *E. amylovora* on intact apple plants in the greenhouse

2.7.1 Preparation of plants

Roots induction solution (contents / L) (Jarausch *et al.,* 1996)**:**

- 30 g/l Sucrose
- 5 mg/l IBA

Explants produced in MLO6 medium, were transferred into roots induction solution for 4 d in darkness.

Rooting Medium (RM) (contents / L):

- 2.2 g ½MS macro and micro elements without vitamins

- 1 ml Vitamin stock solution (MS)
- 10 g Sucrose
- 7 g/l Micro agar

After 4 d (rooting induction period), explants were transferred into rooting medium for 14 d. After that, *in vitro* grown apple plants were transferred into small pots (one explant per pot) containing a mixture of (1:5) Perlite: Peatmoss. The pots were kept in a chamber in the greenhouse and covered with transparent covers to allow air and light entrance and to avoid fast water evaporation. After 14 d, the covers were removed and the plants were irrigated regularly each 2 d and left for 4 months until they had the appropriate size for the experiments.

Shoots of the plants were sprayed with the promising extracts or fractions from *in vitro* tests. Different concentrations (20x, 10x, 5x, and 1x) were dissolved in DMSO or in Greemax solvent to facilitate the entrance into the leaf tissues. The concentrations were sprayed with 1.5 ml/plant on the shoots of the plants (10 plants for each concentration), and then the plants were left for 4 h until the spray solution was dried. Greemax and DMSO were also sprayed on the plants as control treatments to determine their effect on the plants. Bion®, which is known as a resistance inducer in plants, was sprayed (0.002%) as a positive control. The negative control plants, were inoculated with the bacterial suspension after spraying with water. Two - 3 d after treatment, the tips of the upper 4 leaves for each plant were cut using a scissor dipped into a cell suspension of *E. amylovora* (2.0 of OD_{600}) to allow an infection. After inoculation, the plants were kept under controlled greenhouse conditions (27 °C and 80% humidity) and the development of symptoms was monitored weekly for 6 weeks using a scale of disease evaluation (Table 3). At the end of the experiment, data were analysed and the most effective substances against the bacterial cells were selected to be fractionated and identified.

Table 3: Scale of disease evaluation used in greenhouse apple plantlets infected with *E. amylovora*.

0	No browning symptoms
1	Browning of leaf tissues near the cutting site and the leaf vein
2	Browning of 2 to 3 leaves near the cutting site or shoot apex
3	Browning with new shoot growth
3.5	Browning of the half part of the shoot without new shoot growth
4	Browning of the whole shoot (explant death)

3 RESULTS

3.1 Screening extracts against *Erwinia amylovora* using 96-well plates

Some of the extracts tested against *P. infestans* were also tested for a direct action on the growth of *E. amylovora*, in order to check whether the extracts had a general toxic effect or a selective action against the bacterial pathogen could be observed (Table 4 and Figure 2). The results showed that effective and ineffective extracts against *P. infestans* had weak effects against the bacterial growth of *E. amylovora*.

Table 4: Some extracts effective and ineffective against *P. infestans* mycelial growth were tested against *E. amylovora* in 96-well plates. CF = culture filtrate; MyEx = mycelium extraction.

Extract number	Effect on *P. infestans*	Effect on *E. amylovora*
010413 CF	Moderate	Weak
000140 CF	Moderate	Weak
000140 MyEx	Moderate	Weak
000387 CF	Strong	Weak
000387 MyEx	Moderate	Weak
000386 CF	Weak	Weak
000065 CF	Weak	Weak
000389 CF	Moderate	Weak
000389 MyEx	Weak	Weak

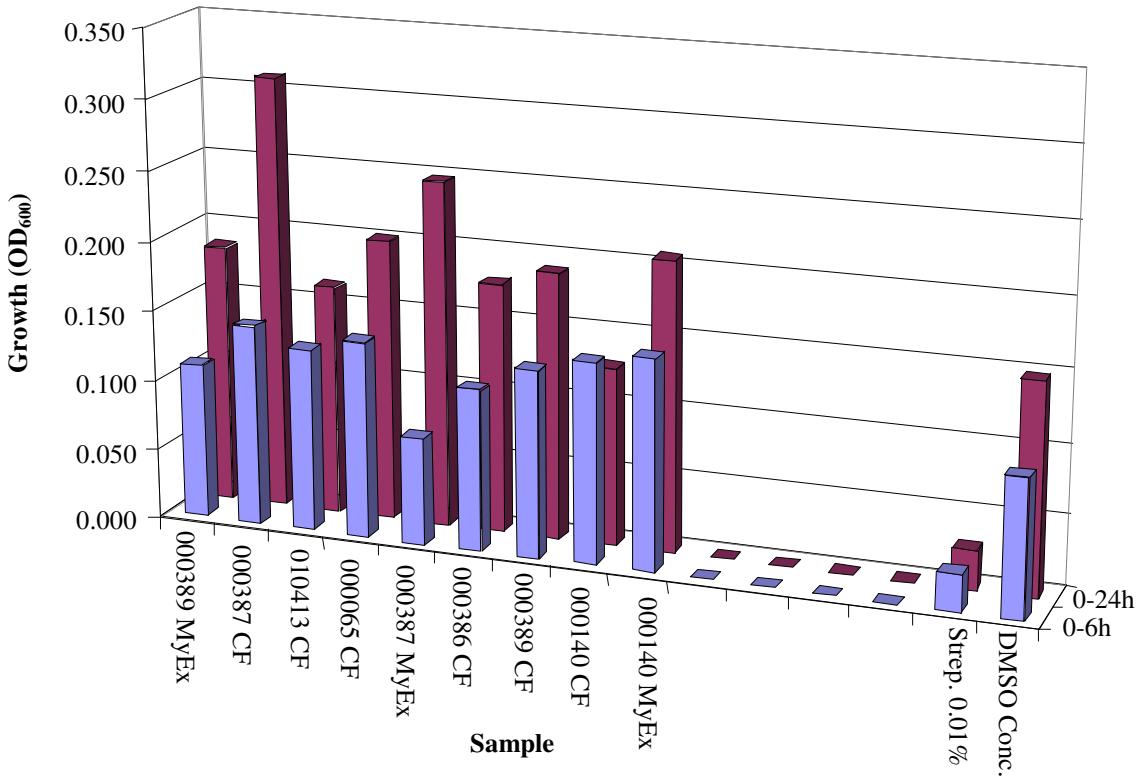


Figure 2: Some extracts effective against *P. infestans* mycelia growth were tested also against the bacterial growth of *E. amylovora* after 6 and 24 h in 96-well plates. CF = culture filtrate; MyEx = mycelium extract; OD = optical density of the bacteria; h = hours. Experiments were repeated at least twice (2 replications for each treatment).

Around 5236 extracts were screened for their direct effect against the bacterial growth to *E. amylovora* using a 96-well plate test. Growth was calculated as percentage of bacterial growth of the negative control (DMSO), i. e, the percentage of bacterial growth was 100%, that means the growth of the bacteria in extract treatment was the same as in the DMSO (as solvent of the extract) amended medium. On the other side, if the bacterial growth was less than 100%, the extract had an effect on the bacterial growth, and if it was more than 100%, the extract would have a stimulating effect on bacterial growth.

The action of the negative control (DMSO) and the positive control (streptomycin) on the bacterial growth were calculated as shown in Table 5.

Table 5: Shows the action of the negative (DMSO) and the positive (streptomycin) controls on the bacterial growth 6 and 24 h after inoculation. The data are the average of results obtained from the control treatments. OD = optical density; SD = standard deviation.

Substance	Bacterial growth after 6 h		Bacterial growth after 24 h	
	OD	SD	OD	SD
DMSO	0.121	0.017	0.208	0.025
Streptomycin	0.023	0.024	0.001	0.018

Only 60 extracts (from the 5236 tested) were found to be effective against the growth of *E. amylovora* and they varied in their effectiveness. Depending on the results of the OD of the relative growth of the bacterial cells, the extracts could be sorted into three groups:

(i) If the effect of the extract resulted in OD < 0.6, the extract was classified as effective as streptomycin and considered as a highly active extract.

(ii) If the effect of the extract resulted in OD = 0.6, it was regarded as a moderately active extract.

(iii) If the effect of the extract resulted in OD > 0.6, it was regarded as a weakly active extract.

Extracts that had comparable effect like the streptomycin control (classified as strong) were selected and tested again for further fractionation and to check the stability during the storage at 4 °C, in some cases, after months (Figure 3). It was found that the tested extracts varied in their effectiveness, in some cases being not effective anymore as before, due to the instability of the active ingredients.

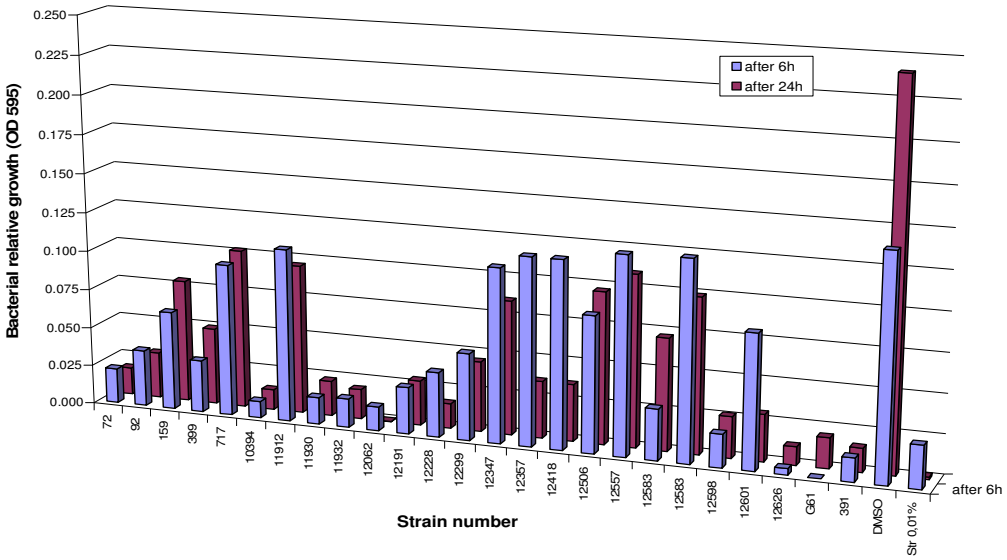


Figure 3: Some Actinomycetes strains effective against *E. amylovora* bacterial growth tested again against the bacterial growth of *E. amylovora* in 96-well plates. OD = optical density of the bacteria, DMSO = dimethyl sulfoxide, Str. = Streptomycin.

The most effective extracts were selected for fractionation respectively purification and identification of the active ingredients to be tested in the same tests system against the

bacterial growth. After further tests and further fractioning steps, some active substances (antibiotics) were identified (Table 8).

As an example, strain 000 391 was selected to explain how further fractionation, optimisation, and purification steps were performed. The strain 000 391 was cultivated in 3 types of media and the extracts were tested in the 96-well plate test system for growth inhibition of *E. amylovora* (Figure 4). It was found that the strain is most effective when cultured in medium 3. (Due to corporate secret of the project corporation partner, the medium ingredients are not allowed to be published).

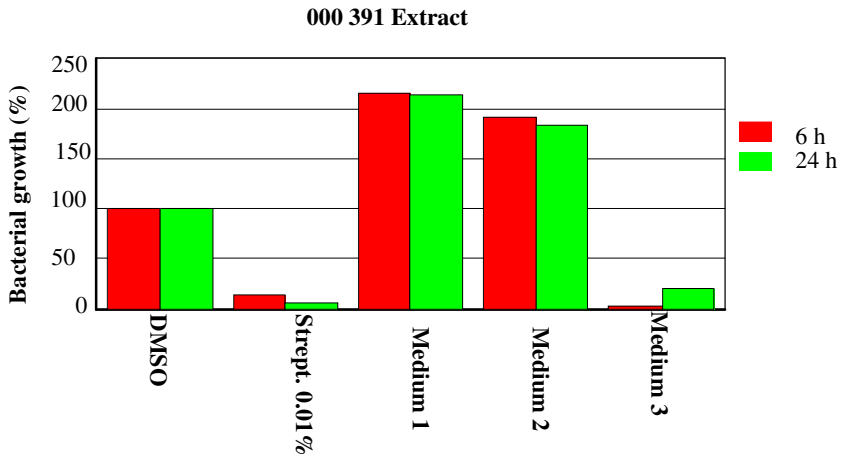


Figure 4: Growth inhibiting effect of the crude extracts of strain 000 391 on growth of *E. amylovora* depending on the medium.

In order to confirm the results obtained in Figure 4, the most effective extract (Medium 3) was separated into three fractions (Figure 5) and was then tested again. It was found that fraction 2 and 3 showed no effect, whereas fraction 1 carried the active ingredient.

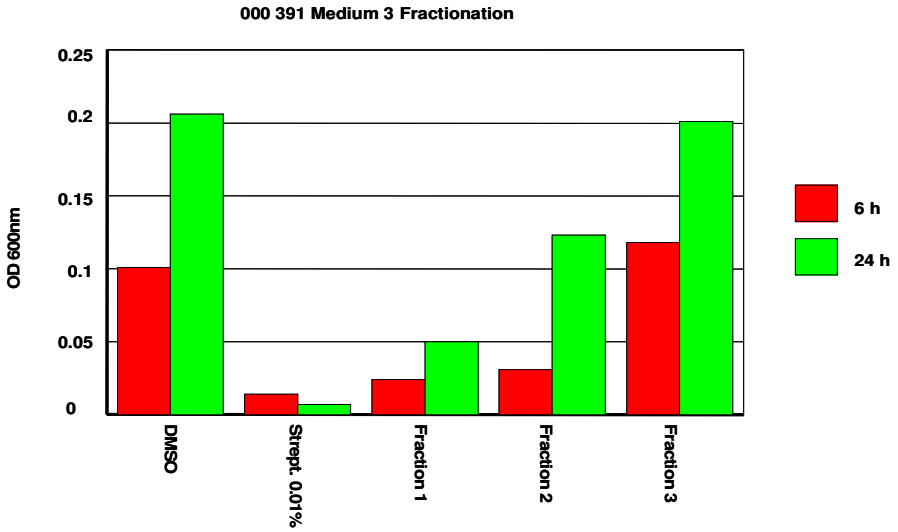


Figure 5: Effect of three extract fractions of strain 000 391 cultivated in medium 3 on growth inhibition of *E. amylovora*.

Depending on the results from Figure 5, several purification steps were carried out to purify the active ingredient from fraction 1 (Purification steps were performed by the corporation partner Sourcon Padena GmbH):

1st purification step (solid phase extraction):

For further optimisation and purification steps, the broth of a 10 L Bioreactor was run on Amberlite XAD16. The resulted components were analysed using HPLC-MS (Figure 6). A high number of substances were detected in fraction 1, so, there was a necessity to perform other purification techniques.

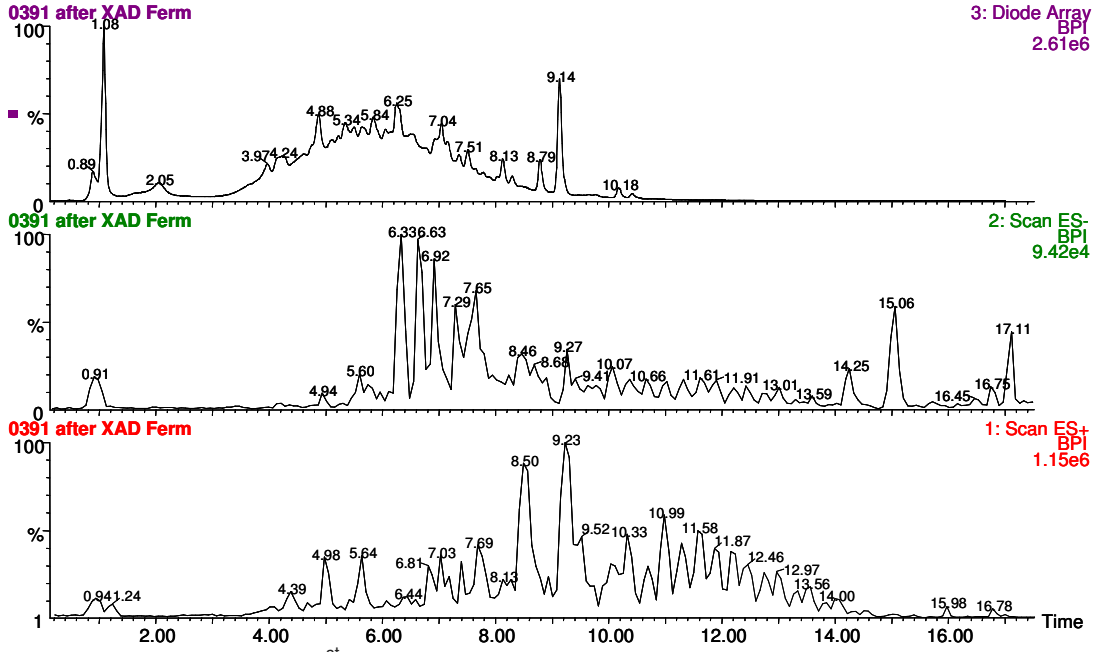


Figure 6: Chromatogram for the 1st purification step of fraction 1 of 000 391. Three detection possibilities were done using; 1: Mass spectrometer in a positive mode (ESI+), 2: Mass spectrometer in a negative mode (ESI-), 3: UV-vis detector (DAD). Each peak represents at least one substance.

2nd purification step (liquid-liquid-phase extraction):

In this step, butanol was used in liquid-liquid extraction phase at pH 4 and the resulting peaks (substances) were compared with those of the previous step (Figure 8). It was found that the extraction step with butanol (right part of Figure 7) efficiently reduced the number of detectable purified substances (Figure 7).

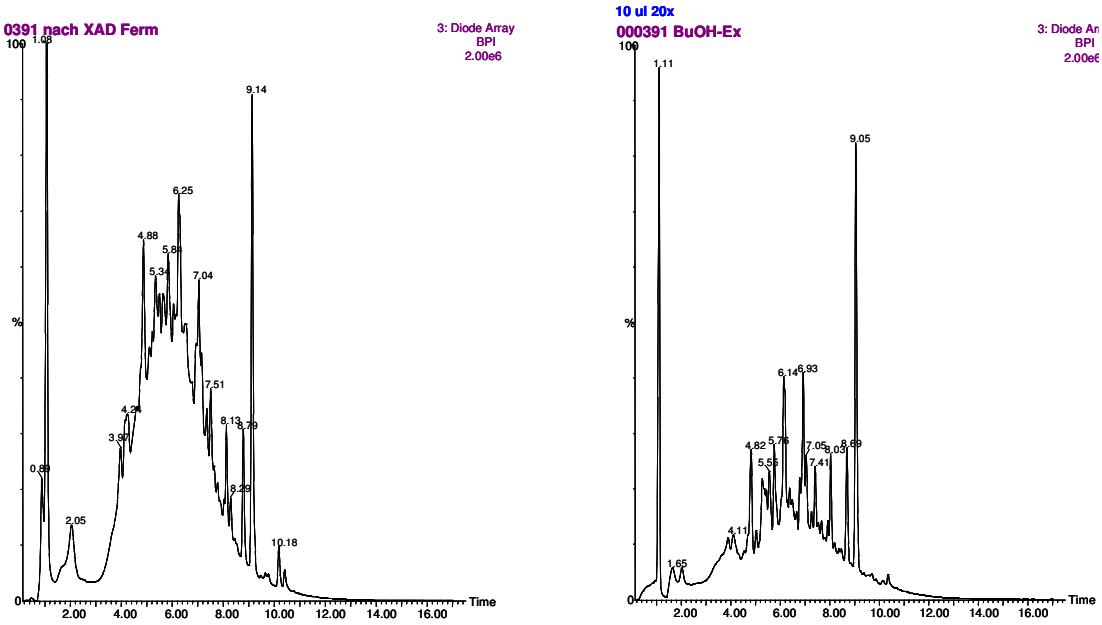


Figure 7: A comparison of the HPLC signals using UV-vis detector before (left) and after (right) the extraction of the extracts with butanol.

3rd purification step (gel chromatography):

The purification in this step was done by using gel chromatography running on Sephadex LH2O, because of the differences in the mass of the substances and in order to separate the substances in their single components. In Figure 9, the chromatogram (UV-vis signal) shows a clear main peak compared to the peaks in Figure 7. The new recognised peaks (substances) were tested again in the 96-well plate test system for growth inhibition (Figure 8). From 6 fractions tested, only one was effective. In addition, a clear signal represented this fraction in the HPLC-Chromatogram (UV-vis) (Figure 9).

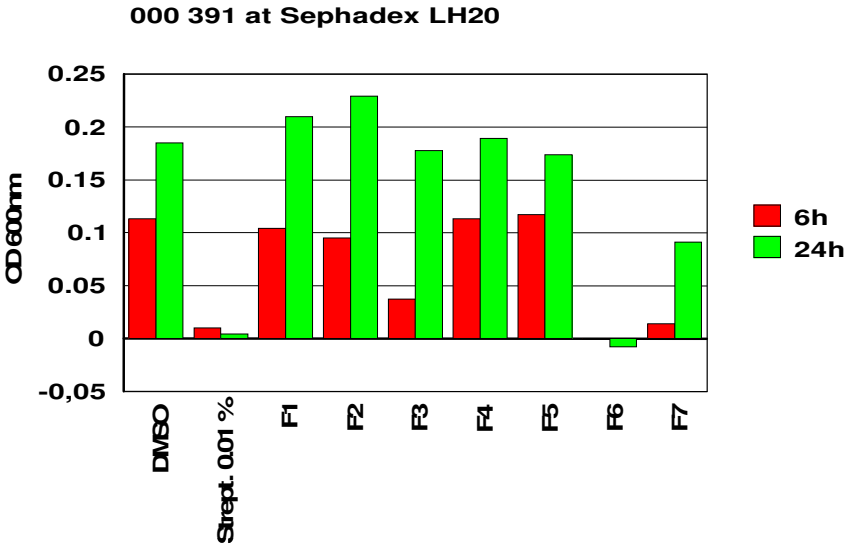


Figure 8: Effect of 6 fractions of strain 000 391 tested on growth inhibition of *E. amylovora* in the 96-well plate test system.

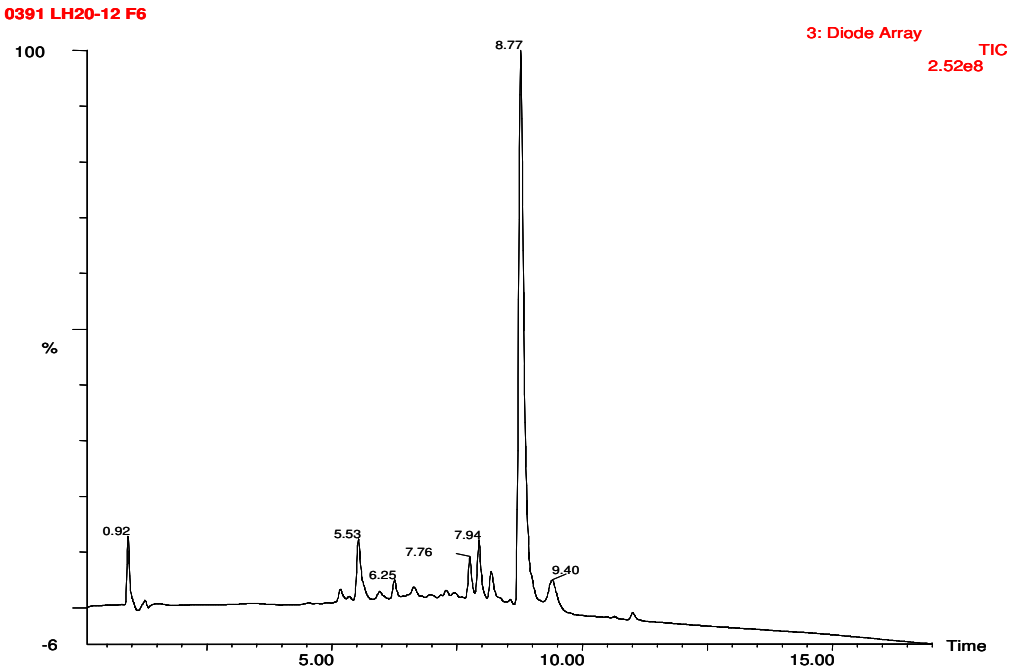


Figure 9: HPLC-Chromatogram (UV-vis Signal) of the positive identified fraction 6 (F6).

4th purification step (preparation of HPLC and identification of the chemical structure of the active ingredient):

After the identification of the positive fraction (fraction 6) of strain 000 391, the fraction was further purified until the main substance peak appeared clearly (Figure 10). With the help of NMR, the active (effective) substance was identified as Chuanghsinmycin (structure elicitation steps using NMR were thankfully performed by Ellen Graf, group of Prof. Dr. Roderich Süssmuth, Rudolf-Wiechert-Professor of Biological Chemistry, Technical University of Berlin, Institute of Chemistry, Organic Chemistry).

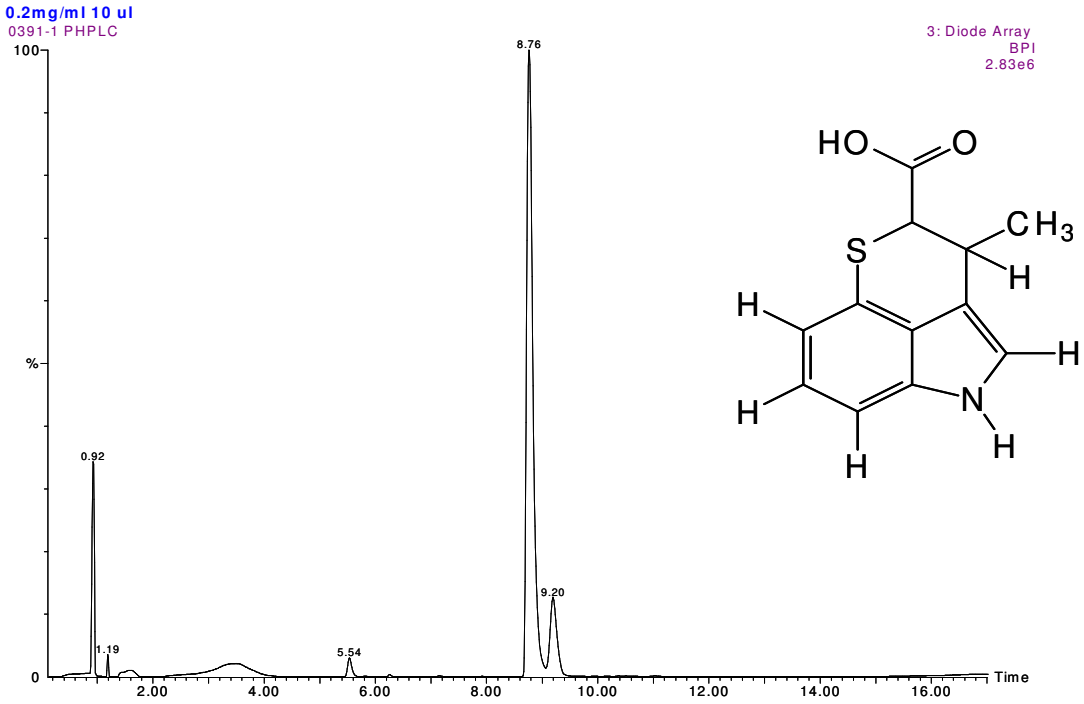


Figure 10: HPLC-Chromatogram (UV-vis Signal) of the active identified fraction 6 (F6). NMR technique was used to identify the chemical structure of the active substance. The chemical structure represents Chuanghsinmycin.

3.2 *In vitro* apple plantlets as test system for testing extracts against the fire blight disease

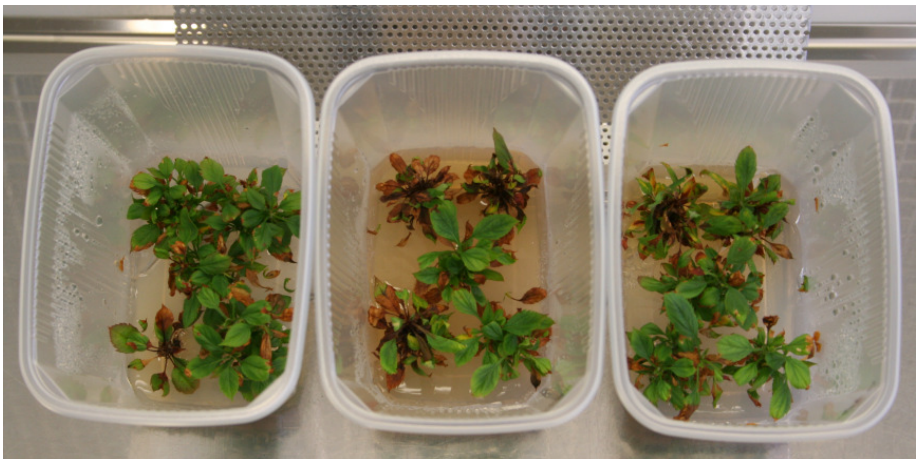
The promising extracts from the parsley cells screening test were tested against *E. amylovora* in *in vitro* apple plants. Different concentrations from each extract were sprayed on plant shoots. After monitoring the symptoms for about 3 weeks in 3 days intervals, the results showed that the substances varied - depending on the applied concentration – in their effectiveness in reducing the disease symptoms resulting from the bacterial infection. Different positive controls (Prohexadion-Calcium®, Bion® and streptomycin) were also included in this test system; they also showed different efficiencies in reducing the necrotic symptoms on plant shoots. Table 5 shows the percentage of the diseased (brown) shoots to the total number of infected shoots (of 15 plantlets). Prohexadion-Calcium showed in all concentrations a slight effect against *E. amylovora*, however, in all plantlets treated with the 0.25% concentration a phytotoxicity has been recognized. Bion® showed also a slight effect in reducing the infection. The effect of Bion® in 0.2% concentration was higher than when applied at the 0.02% concentration which was not suitable for controlling the disease. The culture filtrate 000 391 exhibited at the lower concentration (1%) a higher effect against the disease than at the higher concentration (10%). Streptomycin proved to be strongly

and successfully effective against *E. amylovora*, but it led, however, to chloroses in the leaves. Tubercidin exhibited only an insufficient effect against the bacterial disease. It was possible with streptothricin showed a high effectiveness against the fire blight disease; however, it caused a slight chlorosis in the leaves. The percentage of diseased shoots to total shoots in untreated infected plants was approximately at 90% level (Figure 11).

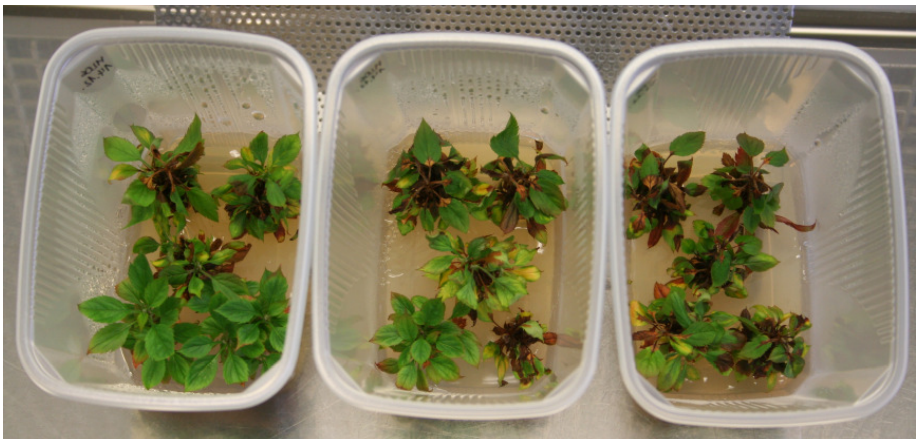
Table 5: The effectiveness of different substances tested against *E. amylovora* on *in vitro* growth of apple plantlets (Royal Gala). CF = culture filtrate.

Substance	**Total number of shoots / 15 plants**	**Total number of diseased shoots / 15 plants**	**(%) of diseased shoots to total shoots**
Prohexadion-Calcium (0.25%)	33	7	21.2
Prohexadion-Calcium (0.025%)	37	17	51.5
Prohexadion-Calcium (0.0025%)	40	17	42.5
Bion (0.2%)	46	11	23.9
Bion (0.02%)	46	21	45.6
000 391 CF (10%)	41	9	21.9
000 391 CF (1%)	39	7	17.9
Streptomycin (0.01%)	34	0	0
Tubercidin (0.01%)	41	12	29.2
Streptothricin (0.01%)	44	1	2.2

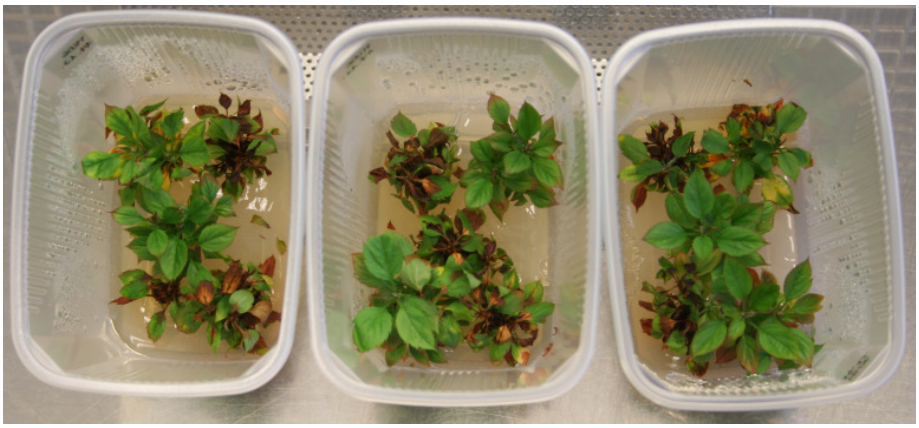
Five healthy – untreated *in vitro* plantlets



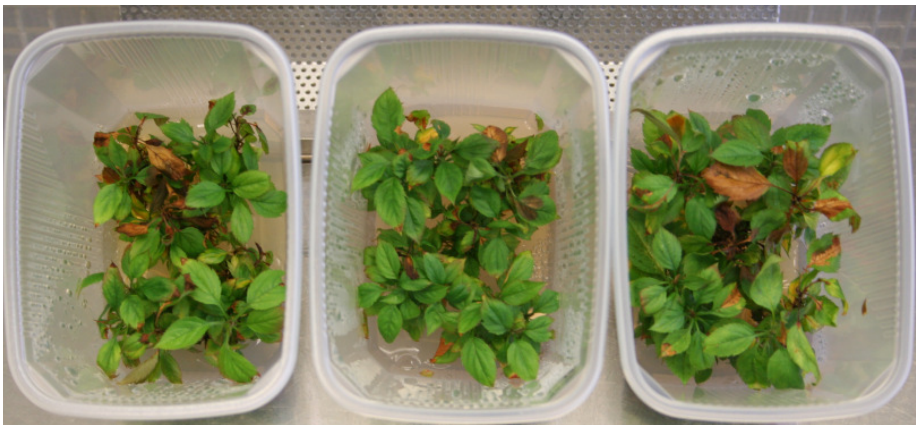
Prohexadion-Calcium (0.25%)



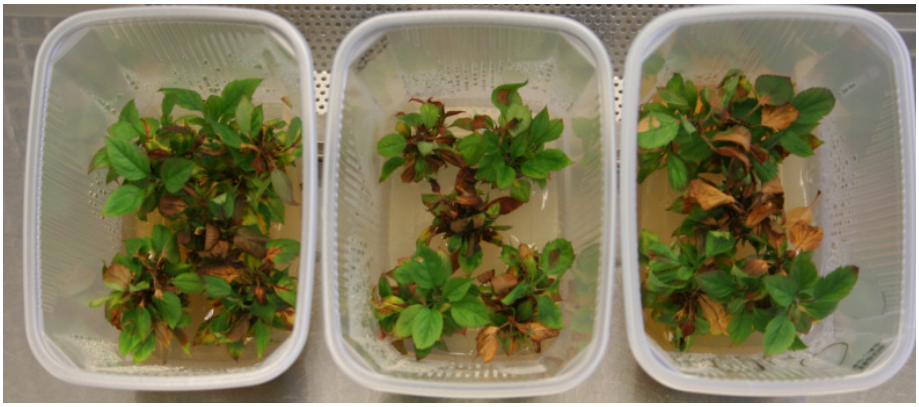
Prohexadion-Calcium (0.025%)



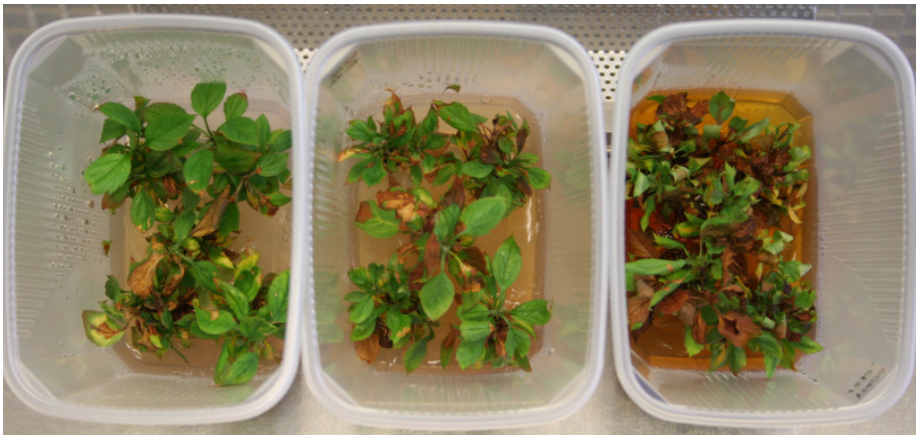
Prohexadion-Calcium (0.0025%)



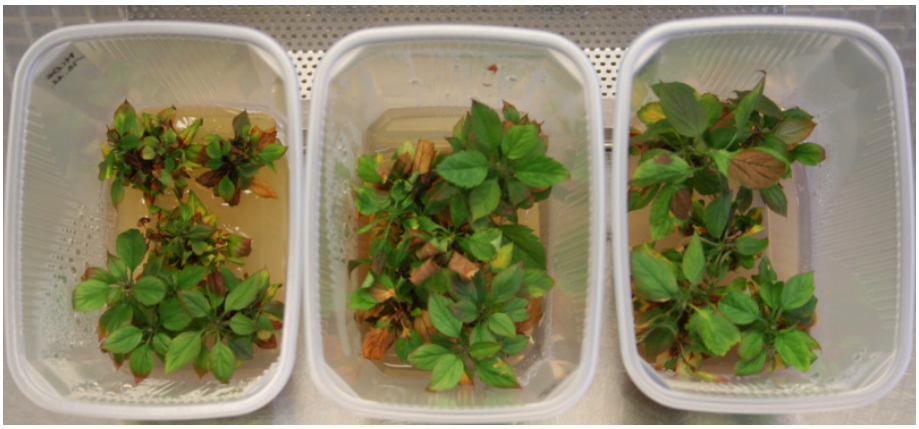
Bion 0.2%



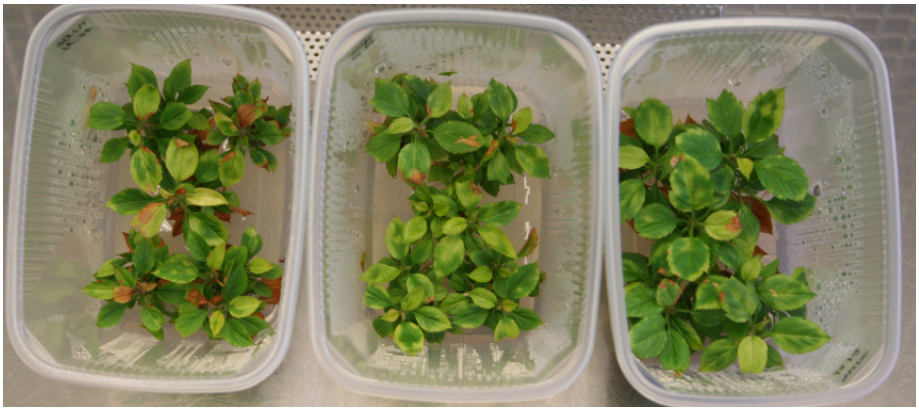
Bion (0.02%)



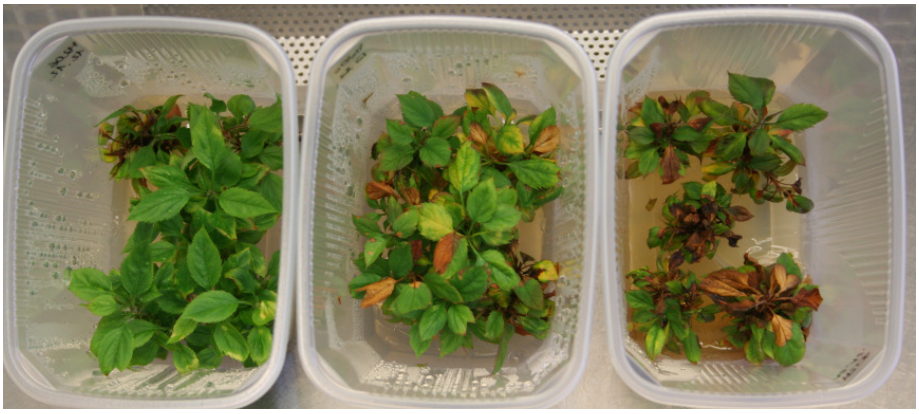
CF 000 391 (10%)



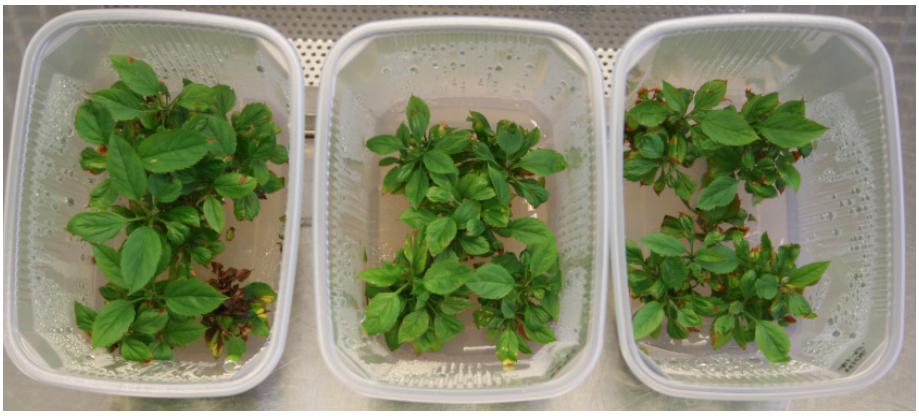
CF 000 391 (1%)



Streptomycin (0.01%)



Tubercidin (0.01%)



Streptothricin (0.01%)

Figure 11: Apple plantlets infected with *E. amylovora* and treated with different substances tested against fire blight disease on *in vitro* cultivated apple plantlets (Royal Gala). 4 weeks after inoculation. CF: culture filtrate.

3.3 Intact apple plants as test system against fire blight

A highly effective promising extract from the *in vitro* test (strain 012 228) was applied on greenhouse plants. In this experiment, the following concentrations were used: 1x, 5x, 10x and 20x. The results showed that concentrations tested varied only slightly in their effectiveness on the symptoms appearance (Table 7). It was found that the highest concentrations applied (10x and 20x) were only slightly effective in reducing the symptoms.

Table 7: Effect of different concentrations of strain 012 228 against *E. amylovora* on greenhouse apple plants (Royal Gala).

Substance	Scale of symptoms
Negative control	3.9
Positive control (Bion®)	1.2
012 228 (1x)	3.8
012 228 (5x)	3.0
012 228 (10x)	2.4
012 228 (20x)	2.9

Due to the culture conditions, *in vitro* apple plantlets have soft leaves with a thin cuticle of the epidermal cells. Substances to be tested, thus, can easily enter the tissue, and effects can be were easily detected compared to plants grown under 'natural' conditions. In order to try a more 'realistic' test system, greenhouse plants were chosen for the application of extracts to be tested.

Extracts of strain 012 228 as well as of other strains showing activity against *E. amylovora* in *in vitro* apple plants or in greenhouse apple plants were fractionated to identify their effective substances, and these fractions were then retested *in vitro* against bacterial growth.

In parallel with the *in vitro* fractions test, the active substance in strain 012 228 has been identified by HPLC. Table 8 shows the different effective strains and the effective substances that could so far be identified.

Table 8: The identification of different effective compounds from Actinomycetes strains tested against *Erwinia amylovora* on apple plants.

Strain number	**Identification**
012 228	5´- O –Sulafamoyl Tubercidin
014 038	5´- O –Sulafamoyl Tubercidin
012 357	5´- O –Sulafamoyl Tubercidin
000 092	Factumycin and derivate
000 072	Factumycin and derivate
012 598	Factumycin and derivate
012 601	Factumycin and derivate
012 557	Factumycin and derivate
012 583	Factumycin and derivate
000 391	Chuanghsinmycin
013 587	Chuanghsinmycin
000 037	Unclear
000 717	Unclear
011 932	Unclear
012 347	Unclear
000 707	Unclear
000 159	Unclear
012 299	Unclear
011 930	Unclear
000 399	Unclear
013 721	Chloramphenicol
012 311	Unclear
011 912	Unclear
012 031	Unclear
011 903	Unclear
014 068	Unclear
010 394	Unclear

As shown in Table 8, strain 012 228 was identified as *Streptomyces tubercidicus*, produced an adenosine-derivative antibiotic which had been identified as tubercidin (Figure 12). It acts as an anti-metabolite inhibitor on the phosphoglycerate kinase (Drew *et al*., 2003).

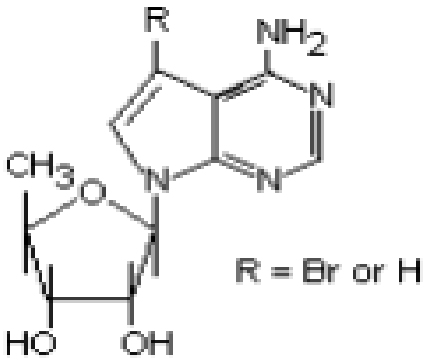


Figure 12: The chemical structure of tubercidin, the active substance in strain 0122 228.

4 DISCUSSION

Applying pesticides frequently resulted in an emergence of resistant strains of the pathogen or in the case of treatments with streptomycin to control *E. amylovora* strains resistant to this antibiotic may be selected (Manulis *et al.*, 1999). Many copper containing compounds, as well as Flumequine, Aliette and Oxolinic acid, have been tested as alternatives to the use of antibiotics. They either revealed insufficient efficacy under field conditions or showed negative side effects (Vanneste, 2000).

In the present study the extracts of Actinomycetes and beside the positive standard control streptomycin have been tested for their antagonistic effects to *E. amylovora* in different test systems such as bacterial *in vitro* growth, *in vitro* explants and in greenhouse apple plants. The principle of this test was to find effective substances that have a direct effect on *E. amylovora* as well as an indirect effect on the fire blight disease.

4.1 *In vitro* growth test system

A suitable high throughput screening test has been established in 96-well multi plates to test about 5236 of Actinomycetes extracts for their effectiveness against *E. amylovora*. Streptomycin was used as a positive control for the test. The OD of bacterial growth was measured at 0, 6 and 24 h after the application of the bacteria using ELISA reader indicating the effect of the extracts on the growth. Only 0.01% (60 out of 5236 extracts) from the total tested extracts revealed antimicrobial activity against *E. amylovora* growth.

An extract was classified as positive, when the OD of the growth after 24 h was equal or lower than the OD of the growth in the streptomycin control. The positive extracts from *in vitro* growth tests or from the Pc test system (Chapter III) were fractionated by several fractionation and purification steps by our cooperation partner (Sourcon-Padena GmbH, Tübingen, Germany) and retested again to find the active substance against *E. amylovora*. Identified active substances were tested on *in vitro* apple explants.

4.2 Testing promising extracts in *in vitro* and in *in vivo* apple explants

The aim of these studies was to test active substances identified in in vitro *growth inhibition tests and in the furanocoumarin induction test with parsley culture cells (Pc).*

Extracts were sprayed 3 days before inoculation with *E. amylovora*, allowing enough time to penetrate leaf tissues. Three substances (Prohexadion-Calcium, Bion and Streptomycin) were included also as effective standard compounds. Prohexadion-calcium (Phd-Ca) is known as a plant growth regulator that suppresses shoot growth in fruit trees. Phd-Ca reduces longitudinal shoot growth by inhibiting the last step in gibberellin biosynthesis. The compound does not have any direct activity against E. amylovora, but it increases host resistance against fire blight. Prohexadion-Calcium is a foliar applied plant growth regulator which reduces vegetative growth by inhibiting the synthesis of gibberellin, a naturally occurring plant hormone. Specifically it decreases the length of shoot internodes, and thus decreases the need for pruning, allows more light to penetrate the tree canopy, increasing fruit coloration, and, due to increased air circulation, decreases the incidence of fire blight. Beside these factors, the compound is thought to be responsible for resistance inducing effects (Rademacher, 2000).
Bion® was used as a resistance-inducer against several pathogens in many monocotyl and dicotyl plants (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996), and also against *E. amylovora* (Zeller V and Zeller W, 1998). Streptomycin is a known effective antibiotic against bacteria that has been limited to be used in Europe in fruit-growing due to the appearance of new resistant strains against the antibiotic (Vanneste, 2000). Three extracts had a direct effect in *in vitro* growth test (CF 000 391, tubercidin and streptothricin), were also tested. One of these substances (tubercidin) induced also compounds in crude extracts of cells of parsley, the furanocoumarins (Pc+).

Prohexadion-Calcium showed only a slight effect against *E. amylovora*, however, slight phytotoxic symptoms appeared on leaves treated with a 0.25% concentration. It was found also that Bion® was more effective at higher concentrations (0.2%) than of lower ones (0.02%) in reducing fire blight symptoms. The culture filtrate 000 391 showed a stronger effect in reducing the disease symptoms at the lower concentration (1%). The active component of 000 391 CF was identified later as chuanghsinmycin. Streptomycin controlled strongly the bacterial growth and no fire blight symptoms

appeared when plants were treated with 0.01% which was the same concentration used in the *in vitro* growth test system, but, this concentration caused chlorosis in the leaves. 0.01% of tubercidin (identified from the crude extract 012 228) exhibited only an insufficient effect against the bacterial disease which reduced the symptoms to 29.2%. Streptothricin was tested as an alternative promising antibiotic to protect apple plants against fire blight disease. The disease symptoms were reduced to 2.2% when the plants treated with streptothricin 0.01%; however, slight chlorosis in the leaves appeared.

Due to the culture conditions, *in vitro* apple plantlets have soft leaves with a thin cuticle of the epidermal layer. Substances to be tested, thus, can more easily enter the tissue, and effects can be better detected as in plants grown under 'natural' conditions. In order to try a more 'realistic' test system, greenhouse plants can be chosen for the application of the extracts.

Only extract 0122 228, which had a direct effect (bacterial *in vitro* growth test) on *E. amylovora*, has been tested in greenhouse plants before it was identified. This extract showed an effect on apple greenhouse plants by reducing the bacterial symptoms on the leaves (depending on the symptoms scale used: browning appeared only on 2 to 3 leaves near the cutting site or shoot apex) when the extract was 10 fold concentrated. Lower and higher concentrations showed no promising effects on the bacterial growth compared to the control.

The active ingredient of this extract was finally identified as tubercidin. This substance is known as an antibiotic produced by *Streptomyces tubercidicus* which acts as an anti-metabolite on the phosphoglycerate kinase (Drew *et al.*, 2003), and this effect may explain the positive mentioned effects on *E. amylovora*.

The remaining positive extracts have to be tested in the *in vitro* apple plant tests and in greenhouse plants. Some of the extracts that had an effect on the bacterial growth in *in vitro* tests and in *in vitro* plants were identified and the identification of others is still investigated.

Chapter III

Parsley cell culture as screening system for detecting potentially resistance inducing extracts of Actinomycetes

2 MATERIALS AND METHODS

2.1 Optimization of a screening test system using parsley cell suspensions cultures

This system was established because of the phenomenon of induced resistance as a principle for plant protection based on the activation of defence mechanisms. A major hallmark of induced disease resistance is the ability of plants (cells) to defend themselves against a broad spectrum of pathogens by activation of defence responses. Cells of parsley (*Petroselinum crispum)* are known as a model in triggering a cascade of events when induced by different micro-organisms, elicitors, or by certain chemical substances leading to gene activation. One result of gene activation is the secretion of furanocoumarin phytoalexins into the medium of the cells which can be measured by a fluorescence reader (Hahlbrock *et al.*, 1995; Katz *et al.*, 1998). Substances produced by Actinomycetes tested *in vitro* for their direct effect against *P. infestans* or *E. amylovora* were also tested in the parsley cell suspension system, in order to check their ability to trigger the induction of defence responses. Optimization steps had to be done to optimize the available parsley cell suspensions cultures for a high throughput screening of Actinomycetes extracts and fractions.

2.2 Gamborg B5 medium

Gamborg B5 medium (Gamborg *et al.*, 1968) was prepared as follows (Contents / L):

- 20 g Sucrose: Prepared always fresh.
- 0.1 g Inositol: Prepared always fresh.
- 20 ml KNO_3: prepared by solving 150 g of KNO_3 in 1 L of distilled water and kept at room temperature until use.
- 10 ml $MgSO_4$: prepared by solving 50 g of $MgSO_4x7H_2O$ in 1 L of distilled water.
- 10 ml $CaCl_2$: prepared by solving 22 g of $CaCl_2xH_2O$ or 15 g of $CaCl_2x2H_20$ in 1 L of distilled water.
- 10 ml NaH_2PO_4: prepared by solving 15 g of $NaH_2PO_4x2H_2O$ or 13.27 g of $NaH_2PO_4xH_2O$ in 1 L of distilled water; prepared always fresh.
- 10 ml $(NH_4)_2SO_4$: prepared by mixing (13.4 g of $(NH_4)_2SO_4$ and 7.45 g of Na_2EDTA Titration complex 3 (diluted in 70 - 80 °C hot water (stirred for long time) in 1 L of distilled water.

- 2.5 ml $FeSO_4$: was prepared by solving 5.57 g of $FeSO_4x7H_2O$ in 1 L of distilled water. The solution was prepared was always fresh and kept at room temperature under dark conditions.
- 10 ml Trace Elements: prepared by mixing the following substances: 1000 mg of $MnSO_4xH_2O$, 300 mg of H_3BO_3, 200 mg of $ZnSO_4x7H_2O$, 25 mg of $Na_2MoO_4x2H_2O$, 75 mg of KJ, 2.5 mg of $CuSO_4x5H_2O$, and 2.5 mg of $CoCl_2x6H_2O$ in 1 L of distilled water. The stock solution was kept at 4 °C.
- 10 ml 2,4–D: by solving 10 mg of 2,4-D in 1 ml EtOH and the volume was filled up with distilled water to 100 ml. The solution was prepared always fresh.
- 1 ml Vitamins: prepared by mixing the following compounds: (10 mg of thiamine, 1 mg of pyridoxine x HCl, and 1 mg of nicotinic acid) in 1 ml of distilled water. The vitamin mixture was divided into 1 ml tubes and frozen at –21 °C.
- 7.5 g Agar (only for callus medium).
- After mixing the different solutions of the medium, the pH should be between 4.2 and 4.6 and after calibration the pH must be 5.5.

The liquid medium was divided into 500 ml Erlenmeyer flasks, each flask contained 150 ml medium. Then, the medium was autoclaved at 115 °C for 16 min and then it was kept at room temperature in the dark until use.

When the solid medium was needed, it was prepared by adding the agar to the liquid medium before autoclaving. Then, solid medium was left until it was cooled to 50 - 60 °C and poured into Petri-dishes, each contained 25 ml medium. Finally, the medium was kept at room temperature under dark conditions until use.

Due to the limited labour time available, and to obtain a reproductive quality of the medium for the quantitative screening assay, a commercially available salt mixture was used (Duchefa, NL).

2.3 Cultivation and propagation of parsley cells

In the standard subculturing procedure, 15 ml of 7 d old parsley cells suspension were sub-cultured in 150 ml of fresh Gamborg B5 liquid medium and shaken at 110 rpm for 7 d in darkness at 23 °C on a rotary shaker.

Cultivation of the cells in the solid medium was for callus production purposes. 1 ml from cell suspension were put onto Gamborg B5 solid medium and incubated for at least 4 weeks in darkness at 23 °C until the calli appeared. 75% of the produced callus

was transferred to a fresh liquid medium and incubated at the same conditions for at least 3 weeks until the new cells propagated continuously in liquid medium to have fresh cells from time to time. The rest of the callus (25%) was also propagated by transferring it to new solid medium as a source in the case of contamination of the suspension (Figure 1).

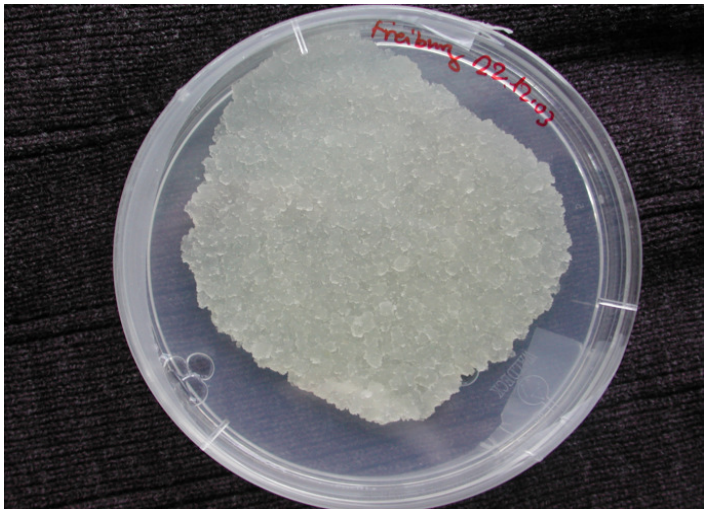


Figure 1: Callus of parsley cells grown on Gamborg B5 solid medium which was used as a permanent nutrient source for the cells.

2.4 Time course of parsley cells growth

This experiment was conducted to establish the subculturing time point and to determine the optimum amount of the cell culture in the log growing phase for the 24-well plate test. 15 ml cell suspension of 7 d old cells was added to autoclaved Gamborg B5 medium (7 flasks of each 150 ml medium). Flasks were incubated at 23 °C for maximum 10 d, shaken at 110 rpm in darkness. Each 24 h one flask was taken and the fresh weight per 10 ml suspension was measured after filtration the medium through a Buchner funnel equipped with a filter paper. The wet filter paper was weighed before and after filtration; the procedure was repeated daily for 10 d. The resulting fresh weights were depicted in a growth curve.

2.5 Description of a parsley cell test system

Extracts screened for their activity to inhibit growth of *E. amylovora in vitro* were also tested in the parsley cell suspension culture for the ability to induce defence responses; these could easily be measured because of the formation of phytoalexins – the blue fluorescing furanocoumarins. With this test set up, a priming active compound

should also be analyzed. As a control of a typical priming substance, BTH (Bion®) was chosen. The inducibility of cell cultures was tested by adding different elicitors as described before.
The additional effect of priming and/or induction of an induced resistance besides the direct bacteriostatic or bactericide action of the extracts screened could enhance the effectivity of the active compound against *E. amylovora*.

Optimum concentration of the primer of cells: Primer is a compound that sensitizes the plant cells to an elicitor in a process called conditioning by activating mechanisms in cells to respond more effectively to a low dose of an elicitor, whereas, untreated cells show only a weak reaction (Siegrist *et al.*, 1998). Bion® or its active ingredient BTH (benzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester), a typical chemical activator, was used as a primer. Bion® concentrations tested were 2.5, 1.25, 0.63, 0.5, 0.25, 0.125, 0.063, 0.03, 0.016, 0.008 and 0.004 mM. After 48 h, the concentration that had no priming effect to produce furanocoumarins was selected and considered as standard for the test.

Optimum concentration of the elicitor of cells: Elicitors are substances often derived from the cell walls of different pathogens which stimulate plant cells defence leading to enhanced resistance (Nürnberger, 1999; Hahn, 1996; Ebel and Cosio, 1994). To optimize an appropriate elicitor for the test, elicitor from *Phytophthora parasitica* cell wall, Pep-13 elicitor, and Messenger® or Harpin (HrpN) elicitor were also tested. Pep-13 elicitor was avoided due to its instability with Bion® primer in the cells (results not shown). The elicitor from *Phytophthora parasitica* cell wall has been optimized for the cells. After the use up of the *Phytophthora parasitica* elicitor, the concentration of harpin was optimized. The maximum concentration not eliciting the cells to produce furanocoumarins was considered as standard for the test.

Extracts, primer, and negative control treatments (extracts solvent) were added in 24 h before adding the autoclaved elicitor to the 24-well plate; each well contained 1 ml cell suspension as explained in Table 1. Plates were covered and sealed and then incubated for 24 h at 23 °C shaken at 150 rpm in darkness. The accumulation of furanocoumarins was measured using the fluorescence reader at 340/400 nm Ex/Em.

Table 1: Testing scheme of a 24-well plate for standard screening of potential Actinomycetes extracts to induce parsley cells to produce furanocoumarins. Pr: Primer = 10 µl/well, Ex: Extract = 10 µl/well, Nc: Negative control (extract solvent) = 10 µl/well, E: Elicitor = 10 µl/well, +: with elicitor, -: without elicitor.

Pr + E	**Pr + E**	**Pr + E**	Pr – E	Pr - E	Pr – E
Nc + E	**Nc + E**	**Nc + E**	Nc – E	Nc - E	Nc – E
Ex1 + E	**Ex1 + E**	**Ex1 + E**	Ex1 – E	Ex1 - E	Ex1 – E
Ex2 + E	**Ex2 + E**	**Ex2 + E**	Ex2 – E	Ex2 - E	Ex2 – E

Two screening tests (test A and test B) were performed per week due to the large number of extracts to be tested. Test A: Cells were propagated always on Thursdays and used for the test on Mondays. Test B: Cells were propagated always on Fridays and used for the test on Tuesdays (Table 2).

Table 2: Testing plan performed for parsley cells per week.

Test	Thursday	Friday	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday
A	Propagation day	-	-	-	Priming day	Eliciting day	measurement day	-
B	-	Propagation Day	-	-	-	Priming day	Eliciting day	measurement day

Messenger (Harpin) elicitor is a protein produced by the fire blight bacterium *E. amylovora*. It was found that Messenger elicits resistance to pathogens and insects and enhances growth of a wide range of plants that are treated with the protein by conventional means (Wei *et al.*, 1992). Different concentrations (50, 25, 10, 5, 2.5, 1, 0.5, 0.1 and 0.05 ng/ml) of harpin (HrpN) were tested in the cells. The fluorescence of produced furanocoumarins was measured 24 h after adding harpin.

A combination was made between the optimized concentrations of the primer and the elicitor should enhance the production of furanocoumarins in the cells.

2.6 Calculation (assessment) of Pc test system results

Due to the high fluctuating data in this test system, the frontiers were calculated de novo after the addition of each new data test plate.

Extracts were considered as positive if the values were higher than the overall average + 2x of the overall standard deviation (SD). The results were divided into 3 parts depending on the number of the positive extracts needed each period.

Part 1: Average = 2836. SD = 2910. Positive fluorescence value = > 8656

Part 2: Average = 3005. SD = 3372. Positive fluorescence value = > 9750

Part 3: Average = 1712. SD = 1410. Positive fluorescence value = > 4532

3 RESULTS

3.1 Time course of the parsley suspension culture used for the standard screening test

This investigation was performed to select the optimum cultivation time of suitable cells weight for screening tests. Cells weight increased gradually over the time course in Gamborg B5 medium and at 23 °C. The standard cell suspension for the test should be on the one side not too high which quenches the beam of the fluorescence reader and on the other side the suspension should have a sufficient density for an efficient screening. In 1 – 3 d old cells, it was found that there were not enough cells propagated in the suspension to establish a screening test. Cells older than 5 d were found to be too dense and quenched the beam of the florescence reader. The suitable cell amount (not too high and not too low) was obtained at the 4th d after subculturing; the fluorescence of the enhanced furanocoumarins was detected in suitable amounts. Additionally, the younger cells at 4th d after subculturing secreted more furanocoumarins in the suspension, than older cells (Figure 2).

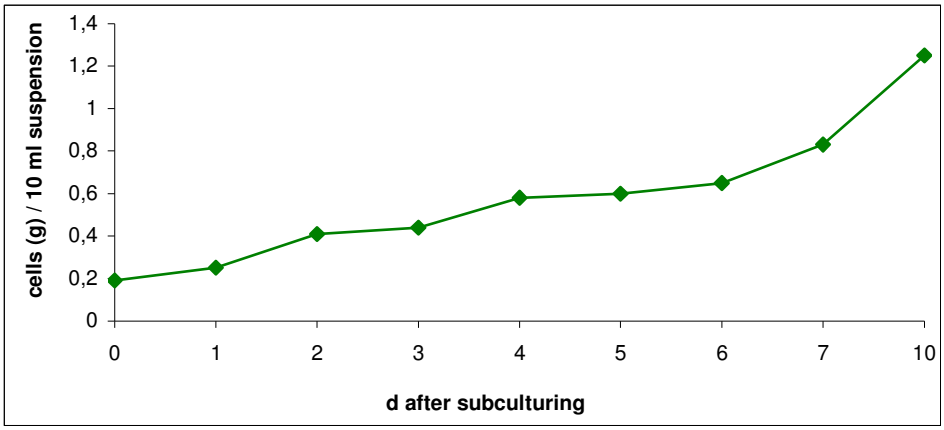


Figure 2: Time course of parsley cells growth in Gamborg B5 medium 23 °C. d = day, g = gram.

3.2 Testing of *E. amylovora* effective extracts in parsley cells test system

Extracts exerting a direct effect on *E. amylovora in vitro*, were also tested in suspension culture of parsley cells for their ability to prime the response of the cells to elicit a defence reaction like furanocoumarins formation (Figure 3). About 0.6% of *in vitro* tested extracts (60 extracts) showing a direct effect against bacterial growth. 12 extracts were as effective as the standard compound streptomycin. These extracts were selected to be tested in the parsley cells for their priming eliciting effect (cells defence response). Extracts solvents and the media were also tested in parsley cells

test system to differentiate their effect from the effect of the extracts. It was found that some extracts had a higher effect on the cells to produce furanocoumarins when they were re-dissolved in EtOH 60%; this might be due to the solubility. Finally, the most effective extracts were selected to be tested against *E. amylovora* in *in vitro* apple plants.

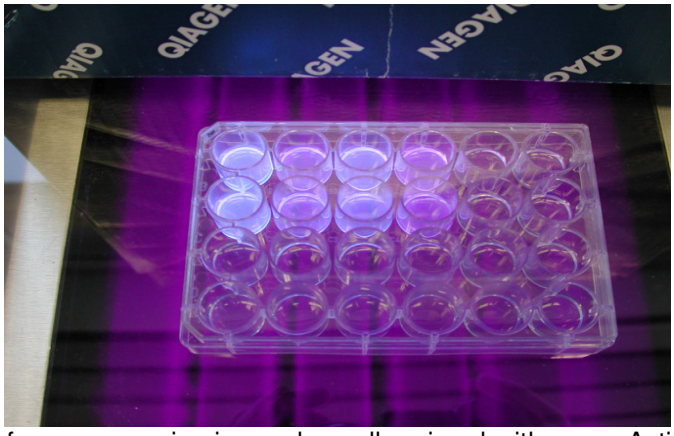


Figure 3: Production of furanocoumarins in parsley cells primed with some Actinomycetes substances in 24-well multi plates.

3.3 Optimizing a screening test system of *P. infestans* extracts in parsley cells

Due to a limited amount of *Phytophthora parasitica* (*Phytophthora sojae*) cell wall preparation to be used as standard elicitor in the test, the commercially available elicitor 'messenger' from EDEN Bioscience was included in the tests. The 'messenger' presents a preparation of *E. amylovora* HrpN-Protein which showed a constant quality over the experimental period.

3.3.1 Optimizing Bion primer and Harpin (HrpN) elicitor

It was necessary to find a non priming concentration of Bion® for the test when it is added alone to the cells. Finding a non eliciting concentration of HrpN elicitor, was also important for the test to exert non eliciting activity in the cells when used alone, but to display an activating effect in the cells to produce furanocoumarins when it applied in combination.

Bion® Optimization:

The activity of priming parsley cells with Bion® has been investigated using different concentrations (2.5, 1.25, 0.63, 0.5, 0.25, 0.125, 0.063, 0.03, 0.016, 0.008 and 0.004 mM).

No priming response (furanocoumarins production) was determined in cells treated with concentrations lower than 0.5 mM as well as in the control treatments (untreated cells and H_2O). The cells started to respond to the primer when they were treated with concentrations higher than 0.5 mM of the primer. The highest Bion® concentration which did not induce the cells to produce furanocoumarins (0.5 mM) was selected as standard for the test (Figure 4).

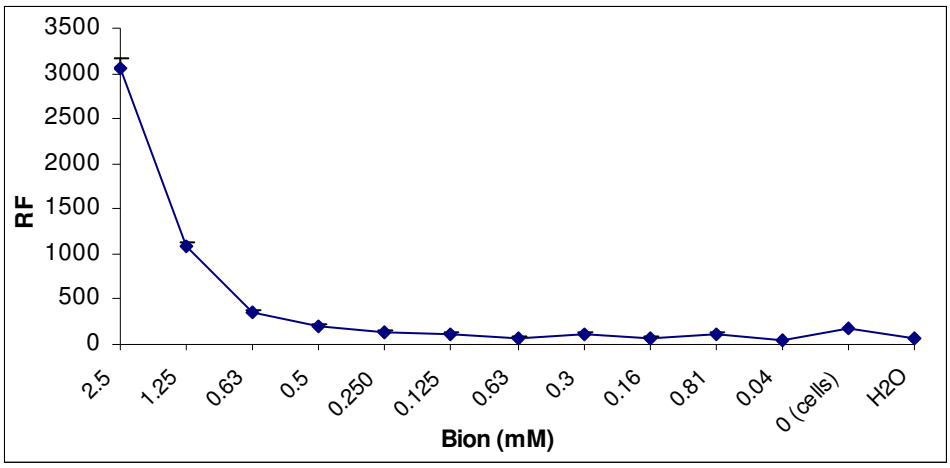


Figure 4: Furanocoumarin induction in parsley cells depending on the Bion® concentration. The relative fluorescence (RF) was measured 24 h after elicitor addition at 340/400 nm Ex/Em. Error bars are standard deviations of 3 replications.

Harpin (HrpN) optimisation:

To test the potential priming activity response, the elicitor (HrpN) should be used at a certain concentration, which did not induce furanocoumarins, if no priming substance was added 24 h before.

Different concentrations (50, 25, 10, 5, 2.5, 1, 0.5, 0.1 and 0.05 ng/ml) of HrpN were tested. No eliciting response (furanocoumarins production) was recognized in cells treated with concentrations lower than 5 ng/ml of the elicitor as well as in the control treatments (H_2O and cells untreated with elicitor). The cells started to respond to the elicitor when they were treated with concentrations higher than 5 ng/ml HrpN. The highest HrpN concentration which did not induce the cells to produce furanocoumarins (5 ng/ml) was selected as standard for the test (Figure 5).

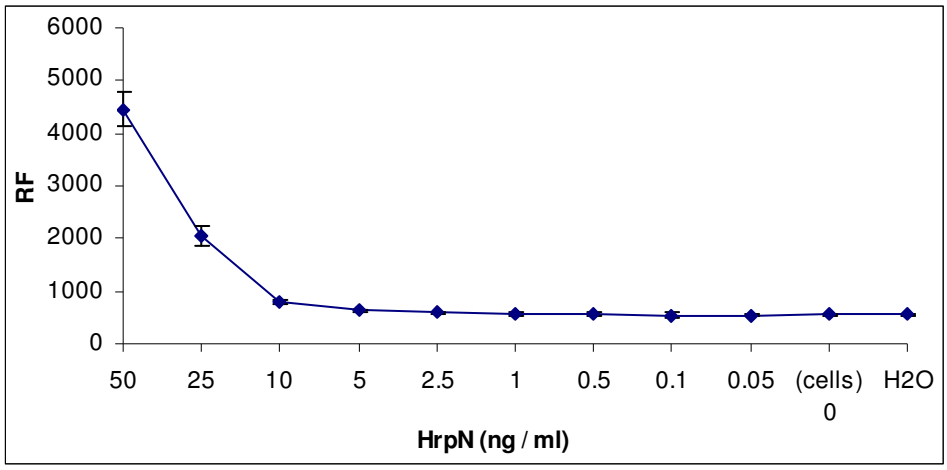


Figure 5: Furanocoumarin induction in parsley cells depending on the HrpN concentration. The relative fluorescence (RF) was measured 24 h after elicitor addition at 340/400 nm Ex/Em. Error bars are standard deviations of 3 replications.

3.4 Testing of *P. infestans* extracts in parsley cells test system

Extracts tested for their direct effect on *P. infestans* mycelial growth (Pi extract) were also tested in parsley cells to check their ability for priming. As shown in Table 3, results were divided into three different groups due to their effects in induction of furanocoumarins production over the time in the cell culture used. Each group had a positive relative fluorescence value. When fluorescence values of the extracts were lower than this value, they were selected as not effective extracts. Around 8780 extracts were tested in parsley cells for their priming effect, but, only approximately 490 extracts (0.06%) could prime the cells to produce furanocoumarins (Pc^+).

Due to the aim of the project, to find substances showing no direct effect on the pathogen, but induced plant defence, the number of extracts was reduced to 42.

Table 3: RF positive values of different extracts tested in parsley test system using 24-well multi plates. RF = relative fluorescence, SD = standard deviation.

Group	Average	SD value	Positive RF value
A	2836	2910	> 8656
B	3005	3372	> 9750
C	1712	1410	> 4200

4 DISCUSSION

4.1 Parsley cells test system

This system was established because of using the phenomena of induced resistance as one of the important principles in plant protection, which is based on the activation of several defence mechanisms. A major hallmark of induced disease resistance is the ability of plants (cells) to defend themselves against a broad spectrum of pathogens by triggering specific defence responses (Siegrist *et al.*, 1998; Hahlbrock *et al.*, 1995). The culture of parsley cells, a fungal elicitor model system, has been used over the last years in studying the interactions of the cultured parsley cells with cell wall elicitors from different pathogens. Many investigations of different defence responses (Nürnberger *et al.*, 1994) have been performed, which include early oxidative burst (Kauss and Jeblick, 1995), rapid alterations in ion transport across the plasma membrane (Conrath *et al.*, 1991), the activation of a mitogen-activated protein kinase activity (Ligterink *et al.*, 1997), the synthesis and secretion of furanocoumarin phytoalexins and various cell wall phenolics (Hahlbrock and Scheel, 1989; Kauss *et al.,* 1993) and the transcriptional activation of various defence-related genes (Somssich *et al.*, 1989; Thulke and Conrath, 1998).

Due to the use of some synthetic chemical compounds to control plant diseases of food crops that could be harmful to the environment and health of users and consumers, it is necessary to find alternative control methods. One of these alternatives is using natural compounds as direct effective compounds or resistance inducers compounds. A high number of chemicals, antibiotics, bio-extracts and microorganisms were examined either *in vitro* or *in vivo* against *P. infestans* (Baysal, 2001; Jeun, 1999; Goodwin *et al.*, 1996; Gisi and Cohen, 1996). Since these extracts or substances can be active against fungal and bacterial pathogens, are biodegradable to non toxic products, and are potentially suitable for use in integrated pest management programs and they could become a new class of safer disease control agents (Soylu *et al.*, 2006; Shmutterer, 1990).

Furanocoumarins are toxic compounds found primarily in species of the *Apiaceae* and *Rutaceae*. They have adverse affects on a wide range of organisms, ranging from bacteria to mammals. Some of the furanocoumarins are photoactive where their toxicity is enhanced in the presence of UV radiation. UV radiation (< 400 nm) induces

the synthesis of furanocoumarins, which are often produced in tissues exposed to the light (Nigg *et al.*, 1993; Berenbaum *et al.*, 1991; Berenbaum and Zangari, 1986; Berenbaum *et al.*, 1984). In the previously published studies with parsley cells (Katz *et al.*, 1998; Siegrist *et al.*, 1998; Thulke and Conrath, 1998), it was concluded that priming and the resulting augmentation of various cellular defence responses is a major mechanism of induced resistance in plants (Conrath *et al.*, 2001).

Using the fluorescence properties of elicitor-induced furanocoumarin phytoalexins in the culture fluids of parsley cells was very helpful in detecting new substances inducing resistance in plants and differentiating this property from direct antimicrobial effects on the pathogen. Parsley cells and elicitor preparations from different pathogens offered a high efficient test system to test a large number of substances within only 3 d, whereas, in intact plants a minimal period of 14 d is necessary for a preliminary evaluation of a test compound.

To select the optimal cultivation time of a suitable cell density, a course of growth of the parsley suspension culture was performed. The suspension density on the one side should not be to high to quench the beam of the fluorescence reader during data recording and on the other side the suspension should have a sufficient density for an efficient detection of the furanocoumarins. The optimal time point was obtained at the 4^{th} d after subculturing, and this was used as a standard for starting the screening test. This test system has been performed in 24-well plates, but in order to carry out a high throughput screening, it was necessary to perform two screenings per week.

Primers are compounds that sensitize the plant cells to an elicitor in a process called conditioning. Bion® (BTH active ingredient) has been used by many investigators for parsley cells to induce the production of furanocoumarins in the cell suspension (Conrath *et al.*, 2001; Nürnberger *et al.*, 1994). It was also used as a control for optimizing our screening test system. The standard priming concentration should be the highest concentration which could not yet induce the cells to produce furanocoumarins. In the case of Bion®, the standard concentration was optimized at 0.5 mM.

Elicitors are substances often derived from the cell walls of different pathogens which stimulate plant cell defence leading to enhanced resistance (Nürnberger, 1999; Hahn, 1996; Ebel and Cosio, 1994). Finding a suitable elicitor was necessary for the screening test. Many elicitors were investigated in the cell suspension tests. Two elicitors were optimized for the screening test; an elicitor extracted from *Phytophthora parasitica* cell wall and harpin (Messenger® or HrpN) a protein produced by the plant pathogenic bacterium *E. amylovora*. The elicitor extracted from *Phytophthora parasitica* cell wall was not used for the whole screening test period because it was used up and was not available commercially. Harpin was an alternative elicitor and it was commercially available from EDEN Bioscience in constant quality over the time of screening. The standard elicitor concentrations used for the screening tests, were 0.4 mg/ml and 5 ng/ml, respectively and are regarded as highest concentration which could not yet induce the cells to produce furanocoumarins.

4.2 Testing Actinomycetes extracts for their priming effect in parsley cell culture

As mentioned above, a higher objective of this screening test was to find compounds priming the cells, which in combination with the elicitor (added 24 h later) could induce in the cells an enhanced production of furanocoumarin phytoalexins.

The extracts tested against the *in vitro* growth of mycelium of *P. infestans* (Pi), were tested again in Pc system, for finding resistance inducing compounds. It was found that only 0.06% (490 extract) out of 8780 extracts could induce the production of furanocoumarins in the cell culture (Pc+). Depending of the aim of the project (see chapter I), to search for plant strengthen (protection) products (Pflanzenstärkungsmittel), extracts displaying a direct effect on the mycelium growth (Pi+), were deleted from the Pc+ list and thus the number of Pc+ reduced to 42 extracts.

In the case of the positive extracts resulting from the *E. amylovora* screening tests, only extracts exerting a direct effect on *E. amylovora in vitro* as the standard compound streptomycin (12 extracts), were tested in the suspension culture of parsley cells for their ability to prime the response of the cells to elicit a defense reaction like furanocoumarin formation; the extracts, however, varied in their inducing effects.

It was found that the different media (containing the extracts), had an inducing effect on the cells to produce furanocoumarins. This phenomenon could not be elaborated due to the confidential information regarding the media. If the effect of the media was excluded, the effect of the extracts in the cell system was low.

Chapter IV

Establishment of transgenic tomato plants to study gene silencing effect as a potential control strategy for *Phytophthora infestans*

2 MATERIALS AND METHODS

2.1 Preparation of media

Media hormones: The following hormones (Table 1) were dissolved in an appropriate amount of solvent (about 0.3 ml 1N NaOH or 95% EtOH) and ddH_2O water was added to 20 ml.

Table 1: The different hormones used for the media. BAP: benzyl aminopurine, NAA: naphthalene acetic acid, IAA: indol acetic acid, GA: gibberellic acid.

Content	Weight (mg)	Solvent	Final Volume (ml)	Final Concentration ($mg.ml^{-1}$)	Liquid storage (ºC)
Kinetin	2	1N NaOH	20	0.1	0-5
BAP	10	1N NaOH	20	0.5	0-5
Zeatin	10	1N NaOH	20	0.5	0-5
NAA	10	95% EtOH	20	0.5	0-5
IAA	10	25% EtOH	20	0.5	0-5
GA	20	25% EtOH	20	1.0	0-5

Media vitamins: The following vitamins were used (Nitsch and Nitsch, 1969) (contents / L):

- 1000X Vitamin Mixture (100 ml) Vitamins + myo-inositol
- 50 mg Thiamine x HCl
- 200 mg Glycine
- 500 mg Nicotinic acid
- 50 mg Pyridoxine x HCl
- 50 mg Folic acid
- 5 mg Biotin
- 10 g Myo-inositol

50 mg folic acid were dissolved in 0.4 ml 1N NaOH and diluted to 10 ml with ddH_2O. The different vitamin solutions were dissolved in 80 ml ddH_2O and the final volume was adjusted to 100 ml and then stored at -20 ºC.

Media antibiotics: The following antibiotics were used in selection of transformation (1000X Antibiotics):

- 100 mg/ml kanamycin (stock solution)
- 150 mg/ml carbincillin (stock solution)

2 g kanamycin and 3 g carbincillin were dissolved separately in 20 ml ddH_2O and then filter sterilized, aliquoted and stored at -20 ºC until use. 1/1000 volume from the stored antibiotics mixture was added to each <55 ºC cooled media.

Media types: The compositions of the different types of media are reported in Table 2.

Table 2: The different media were used in the different callus developmental stages. * = First two weeks use 2 mg of Zeatin in 1 litre of medium, and then change to half the concentration for the third and fourth week, ** = only for the media with 1 mg of Zeatin.

Contents/L	Seeds Germination (A)	Pre-culture and Co-Cultivation (B)	Shoot Induction, Initiation, and Regeneration (C)	Shoot Elongation (D)	Rooting of Transformed Shoots (F)	Unit
MS Salts	4.3	4.3	4.3	4.3	2.15	g
Sucrose	15	30	30	15	15	g
N&N Vitamin Mix (1000X)	1	1	1	1	1	ml
NAA stock		2				ml
BAP stock		2				ml
Zeatin			2/1*			mg
IAA					5	mg
GA			1**			mg
Kanamycin			100	100	50	mg
Timentin			500	500	500	mg
Agar	7	7	7	7	7	g

YEP (Yeast Extract Peptone) medium: The following contents (Table 3) were used in preparing YEP medium:

Table 3: Contents of YEP medium.

YEP Medium	g / l
Yeast extract	10
Peptone	10
NaCl	5

The pH of the media was adjusted to 7.0 before autoclaving (20 min at 121 ºC). Antibiotics were added after cooling the media to 50 - 55 ºC.

2.2 Production of *in vitro* plants

Seeds Sterilization

Tomato seeds of two cultivars (Hellfrucht and Rentita) were put in a sterile box containing 70% ethanol for 1 min under the clean bench. Then, seeds were transferred to a solution containing 5.25% sodium hypochlorite (NaClO) and 0.1% Tween-20 for 30 - 40 min with swirling from time to time. After that, seeds were rinsed 6 - 7 times in sterile distilled water using a sterile beaker, and then transferred to a sterile Petri-dish. Sterilized seeds were emerged in vessels containing medium (A) and the vessels were kept under light in a culture room (25 ºC, 16 h photoperiod, light intensity of 30 - 40 µEm-2s-1). In order to have a uniform germination, the vessels were placed in the dark for one day and then transferred to the culture room. Finally, 8 - 9 d old cotyledons were ready for co-cultivation with *Agrobacterium*.

2.3 Pre-culturing of explants

Eight to nine days old seedlings were removed from culture vessels and placed in sterile Petri dishes using sterile distilled water. The cotyledons were cut with sterile scissors as shown in Figure 1. One to four small holes were made on the cotyledons pieces using scalpel in order to increase infection opportunity of *Agrobacterium* and to enhance transformation efficiency. The cotyledon pieces were then placed in Petri dishes containing medium B that were sealed with parafilm and precultured for 24 hours at 25 ºC under low light conditions (10 µEm-2s-1).

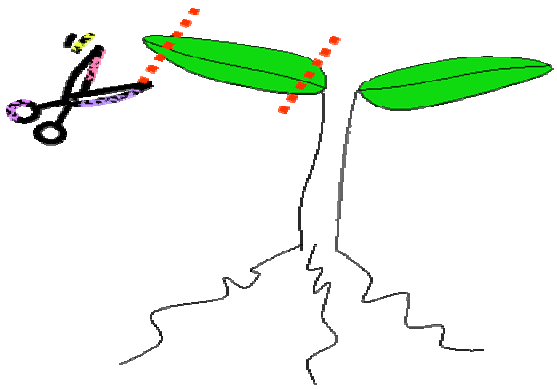


Figure 1: Procedure of cutting the cotyledons to be cultured for callus production.

2.4 Constructs used in *Agrobacterium* transformation

Agrobacterium strain GV3101 carrying pPCV702SM GpG construct. GpG construct contains part of *gfp* gene (+ oriented GFP) in addition to its inverted sequence (- oriented GFP) separated by a spacer sequence. *Agrobacterium* strain ATHV carries pBINmGFP5 construct.

2.5 Preparation of *Agrobacteria*

Both types of *Agrobacteria* were streaked on selective fresh plates 2 - 3 d before they were used. A single colony from the plates was peaked (or a loop from glycerol stock was taken) and then the cells were grown in tubes which contain 6 ml of YEP medium with appropriate antibiotics. Then the cells were incubated at 28 ºC overnight under shaking (200 rpm). The overnight cultures were diluted by liquid MSO 2% to appropriate concentration (OD = 0.5 for *Agrobacterium* ABI).

Liquid solution of MSO 2% contains /L:

- 4.3 g MS Salts
- 100 mg Myo-Inositol
- 0.4 ml Thiamine x HCl (1mg/ml)
- 20 g Sucrose

2.6 Co-cultivation

The diluted *Agrobacterium* cultures (8 ml) were poured into the cotyledons placed in medium B for 30 min with an occasional swirling. The *Agrobacterium* suspension was aspirated from the plates and co-cultivated for 48 hours.

2.7 Regeneration

2.7.1 Initiation of calli and regeneration of shoots

After 48 h from co-cultivation, cotyledon explants were transferred to Petri dishes with medium C and were put in adaxial-side (upside) up position. A Cotyledons cultures were regularly transferred each two weeks to fresh medium C.
The initial calli (with shoot primordial) formed 2 weeks after culture, were excised into small pieces and then transferred to fresh medium D to be sub-cultured.

2.7.2 Differentiation of transgenic shoots

After two or three weeks from culturing on regeneration and selection medium D, three types of regenerating tissues were defined and transferred to different types of media:
1. Compact and green colour calli (with or without green primordial, small adventitious buds) were transferred to medium C contain 1 mg/l Zeatin.
2. Well shaped shoots (1 cm in length) were transferred to medium D.
3. Black/brown colour callus was a sign of kanamycin killing. Loosen (or watery) white calli did not have regeneration capacity. Both calli were discarded.

2.7.3 Rooting of transgenic shoots

When shoot stems elongated to 2 - 4 cm, they were excised from calli and transferred to the rooting medium (E) in transparent plastic container boxes to form a root system and to elongate. When the plantlets were about 10 cm long, they were cut at the internodes and transferred again to medium E. After rooting of the explants, they were transferred to soil, and grown in the greenhouse for later experiments and crossing.

2.8 Transplanting of shoots to greenhouse

The transformed plantlets were removed from culture vessels and the agar was washed gently off from the roots using tap water. Small amount of Rootone® or other rooting powder was added to the washed roots. The pots were filled with moistened soil and planted with transplants. The pots were put on trays, and then the trays were covered with a transparent plastic cap to keep high humidity inside. The trays were put under light in growth room. After that, plastic caps were removed and the plants were irrigated with fertilizers 5 - 7 d after transplanting. The transgenic plants were transplanted to larger pots 2 - 3 weeks after they were transferred to the greenhouse.

2.9 Detection of inserted genes

2.9.1 Detection of GFP fluorescent protein

Plants transformed with *gfp* gene marker were checked for the fluorescence expression by exposing them to UV-A light (Black Ray hand lamp). The plants appeared with fluorescent green colour compared to wild type plants which appeared with red colour (the fluorescent colour of the chlorophyll).

2.9.2 Detection of *gpg* gene

Plants transformed with the *gpg* construct were checked for presence of the inserted T-DNA using PCR technique as the following:

DNA extraction

Samples were collected from three transformed plants to be used in the detection test. The wild type tomato plants were used as a negative control. The total genomic DNA of the treatments was extracted according to Sambrook and Russel (2002). Leaf materials have been cut from the plants (around 20 mg) and put in tubes in LN (liquid nitrogen). The plant material was grinded using plastic pestle after adding 400 µl of extraction buffer (200 mM Tris/HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The tubes were vigorously mixed using vortex for 5 sec. 1 x volume (400 µl) of phenol : chloroform (1:1) was added for DNA isolation. The tubes were mixed and centrifuged for 5 min at 13000 rpm. The supernatants were taken (around 400 µl) and another 400 µl of chloroform were added to the supernatant in order to get rid of phenol residues. The samples were mixed and centrifuged for 5 min at 10000 rpm. The upper phase was transferred to a new reaction tube and 40 µl of 3 M of sodium acetate and 1 ml of EtOH 99.8% were added for DNA precipitation. The samples were mixed and centrifuged for 5 min at 13000 rpm. The supernatant was discarded and DNA pellets were washed in 1 ml of 70% EtOH and then centrifuged for 5 min at 13000 rpm. EtOH 70% was discarded and the samples were dried using a vacuum centrifuge (speed vac.) for 5 min without heating. DNA pellets were dissolved in 100 µl TE buffer and the samples were kept at 5 °C until use for PCR analysis.

PCR analysis

The DNA concentration was measured using a photometer and then adjusted to 100 ng/µl to be used in the PCR analysing. The constructed plasmid (pPCV702SM GpG (537); 11465 bps) was used as positive control in the PCR reaction.

PCR reaction components (total volume of 25 µl per sample):

- Distilled water 13.25 µl
- Buffer (5 x) 5 µl
- dNTP's (10 mM) 1 µl
- DMSO (1%) 0.25 µl
- 5´→ 3´ (35S for. primer) (10 P mol) 1µl
- 3´→ 5´ (Ps+VNb rev. primer) (10 P mol) 1 µl
- Taq DNA polymerase 0.5 µl
- DNA template 2 µl

PCR thermal cycle conditions:

- Incubation	5 min	94 °C
- Denaturation	30 sec	94 °C
- Annealing	45 sec	60 °C
- Elongation	30 sec	72 °C
- Number of cycles	30	
- Final elongation	12 min	72 °C

Preparation of agarose gel

A 1% agarose gel was prepared and dissolved using microwave in 1 x TAE buffer (pH 8.5). The gel was stained with 1 - 2 µl of Ethidium bromide (0.5 µg/ml) before adding the samples. Samples (25 µl) of PCR products were separated on the agarose gel at 100 V for 90 min. The bands were visualized under a UV light transillminator.

RNA extraction

RNA extraction was executed to check the efficiency of transgenic *gpg*-gene expression and detection of SiRNAs (Hamilton *et al.*, 2002).

0.2 g of young tomato leaves were grinded using a pre-cooled sterile mortar. 666 µl (added 3 times) of TRIZOL (total of 2 ml end volume) were added while grinding. The

new material was transferred to 2 microfuge tubes (1.5 ml each) and kept on ice until use. After that, the tubes were incubated for 5 min at room temperature (RT). Chloroform was added (at least 1/5 of the volume). Tubes were shaken on a vortex for 30 sec, left for 2 min until precipitation (this step was repeated 3 times). Tubes were centrifuged for 15 min at 13000 rpm at 26 °C. The supernatant was transferred to new tubes. Tubes were filled with isopropanol 100% (at least 1vol) for precipitation, shaken gently for 5 times and incubated at RT for 30 min. Samples were centrifuged for 30 min at 13000 rpm at 4°C and the supernatant was discarded. The pellet was washed with 350 – 400 µl of EtOH 80% and then centrifuged for 5 min at 13000 rpm at 4°C. The pellet was dried for 15 min at RT, and then re-dissolved in 25 – 30 µl of formamide 50%. The dissolved RNA was transferred to new tubes and vortexed.

RNA optical density (OD) was measured at 260/280 nm Ex/Em (RNA concentration should be at least 1.0 – 2.4 µg/µl). 1µg of RNA samples was run on agarose gel (1%, 1X TBE).

Northern blot analysis (Patrice Dunoyer) was done according to standard protocols (Sambrook and Russel, 2002).

2.10 Silencing of GFP fluorescence

These experiments were conducted in order to check if (how) the *gfp*-gene can be silenced in the plants. Silencing was performed by infiltrating tomato leaves using *Agrobacterium* carrying the desired constructs (GpG and GFP).

2.10.1 Preparation of *Agrobacteria*

The selected recombinant *Agrobacterium* strain containing the desired vector was grown in 40 ml of LB medium containing the necessary antibiotics (20 µl of 100 mg.ml^{-1} kanamycin for *Agrobacterium* with (pBINmGFP5) and 100 µl of 300 mg/ml of streptomycin, 100 µl of 100 mg/ml of spectomycin, 100 µl of 15 mg/ml of rifampicin for *Agrobacterium* with (pPCV702SM GpG) at 28 °C for 24 h with shaking at 200 rpm.

The cultures were transferred to sterile centrifuge Falcon tubes and centrifuged at 5000 x g for 10 min at 15 °C. The pellet was taken and re-suspended in 2 - 5 ml in induction medium and the OD_{600} adjusted to around 1.0 OD by adding induction medium. The *Agrobacterium* suspensions were kept at 22 °C (RT) for 2 - 3 h or overnight until use.

Induction (MMA) medium (for 10 ml suspension): 100 µl of 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) at pH 5.6, 10 µl of 20 µM acetosyringone (AS),

100 µl of 2 mM $MgSO_4$, and then the volume was completed up to 10 ml with sterilized water.

2.10.2 Infiltration of tomato leaves using vacuum method

Agrobacteria suspension was poured in empty sterile Petri dishes and the selected leaves were put on the suspension. A heavy metal grid was placed on the top of the leaves to ensure that they were completely submerged in the suspension. Petri dishes were placed in the exiccator and a continuous vacuum in the range of 1 to 0.1 mbar was removed for 4 min. The vacuum was broken rapidly and the infiltrated leaves were removed from *Agrobacterium* suspension and briefly rinsed with water to remove the bacteria from the leaf surface. The leaves were placed on trays on wet Kleenex paper. The trays were sealed and placed at 23 °C under 16 h light conditions for 3 weeks. The leaves were monitored for the silencing weekly. The infiltration plan was done as shown in Table 4.

Table 4: Infiltration crossing plan between transformed tomato leaves and transformed *Agrobacteria.* GFP = green fluorescence protein.

Plant type	Type of infiltrated *Agrobacterium*	
	***gfp*-Agrobacterium**	***gpg*-Agrobacterium**
***gfp*-plant**	X	X
***gpg*-plant**	X	X
Wild type-plant	X	X

2.10.3 Infiltration of tomato leaves with a syringe

Leaves were infiltrated at six intercostals areas, by pressing 1 ml syringe filled with the desired bacterial suspension on the lower surface of the leaf and injecting a small volume into the leaf tissue. Leaves were mock-infiltrated with water as a negative control. Different leaf ages on the experimental plant were tested for infiltration, transformation and silencing effectivity.

2.10.4 Infection of transformed leaves with Transformant 208m2

Detached leaves of *gpg* and *gfp* transformed tomato and of wild type plants were infected with the transformant 208m2 of *P. infestans* (100 000 sporangia ml^{-1}). In this experiment, tomato leaves of wild type plants were used as control for the experiment.

Detached leaves of *gpg* and *gfp* transformed plants were infected to check whether the fluorescence of 208m2 can be silenced.

3 RESULTS

3.1 Initiation of calli, regeneration, and differentiation of transgenic shoots

Two parts (cotyledons and hypocotyls) of 9 days old plants have succeeded to form calli after 4 to 6 weeks after transformation. It was found that cv. 'Hellfrucht' was more efficient and faster in producing calli from cotyledons and hypocotyls than cv. 'Rentita', so, 'Hellfrucht' was selected to be used in further transformation steps (Figure 2).

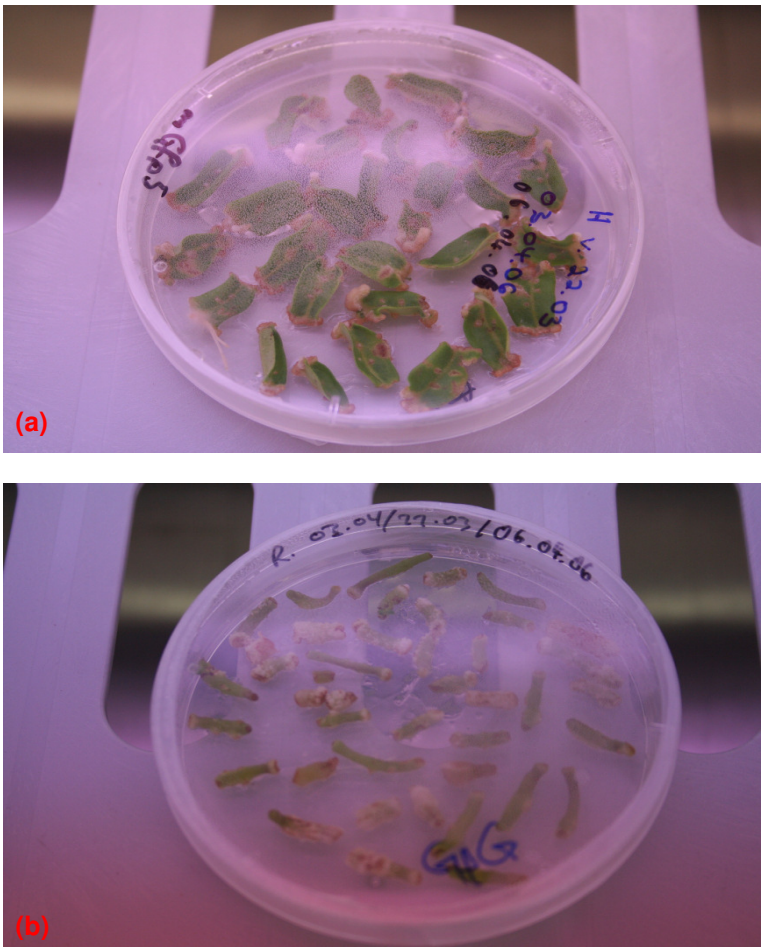


Figure 2: Calli formation from cotyledons (a) and from hypocotyls (b).

Embryogenic calli showed a green fluorescent colour (in the case of *gfp*-transformation) under the fluorescence microscope, as well as the new regenerated shoots (Figure 3).

Proliferations regenerated from embryogenic calli, which appeared also with fluorescence green colour under the UV-A light, were transferred to medium E for rooting induction (Figure 4).

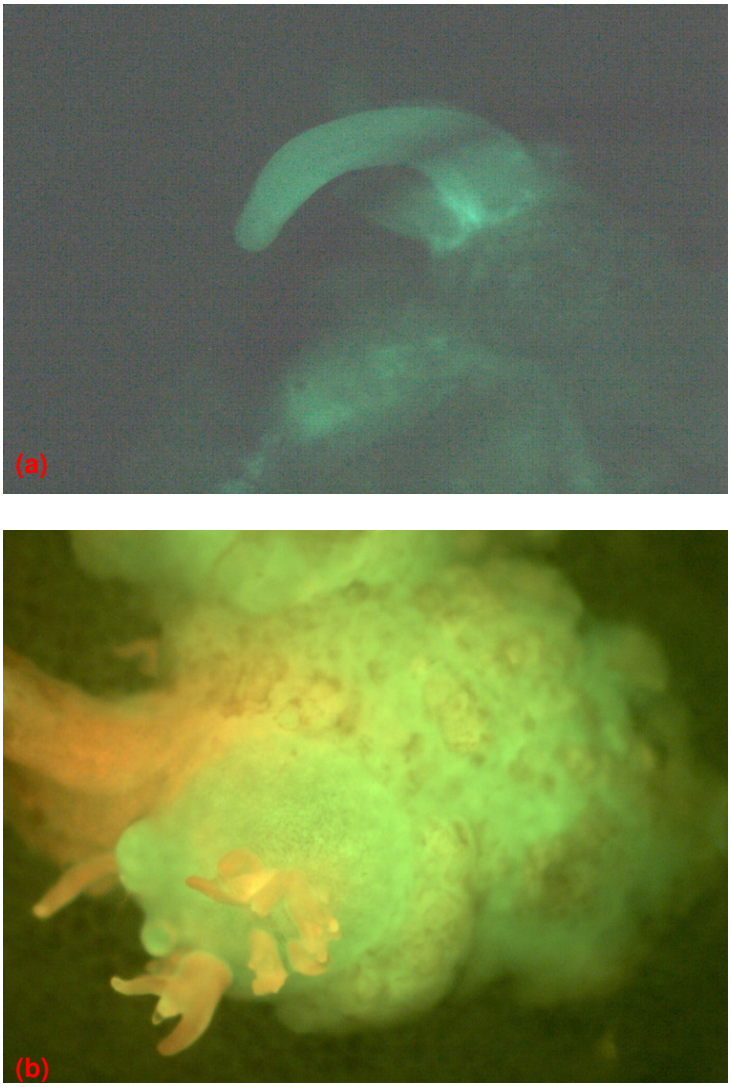


Figure 3: Fluorescent calli and the new regenerated shoots. The transgenic shoots appear with green colour under the fluorescent microscope (a) where they started to produce chlorophyll (b).

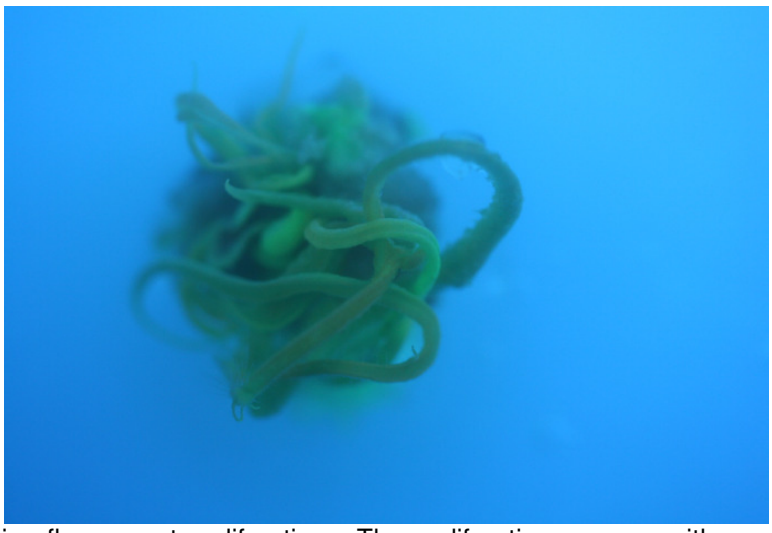


Figure 4: New growing fluorescent proliferations. The proliferations appear with green colour under the UV-A light.

3.2 Detection of GFP protein in plants transformed with *gfp*-gene

After roots and leaves formation (Figure 5), green transgenic tomato explants were recognised when they were exposed to UV-A light. Plants transformed with *gpg*-gene, did not show any green fluorescence as well as the wild type plants but red fluorescence chlorophyll under the UV-A light.

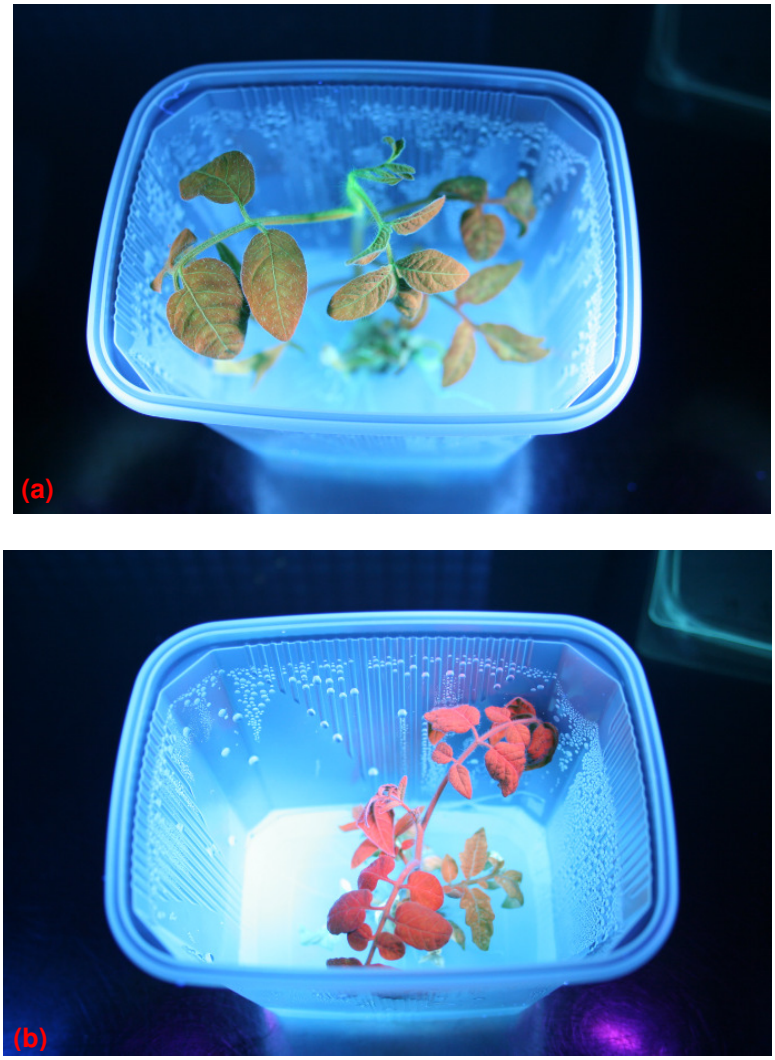


Figure 5: *In vitro* transgenic tomato plants under UV-A light. The *gfp* expressing tomato plant (a) and the *gpg* transgenic plant (b).

3.3 Transplanting of *in vitro* explants to greenhouse

After 6 months from transplanting the explants to greenhouse conditions, the plants were left to grow until the formation of flowers and fruits. Some selected pictures were taken for the both transformed plants (*gfp* and *gpg* transformed plants) (Figure 6).

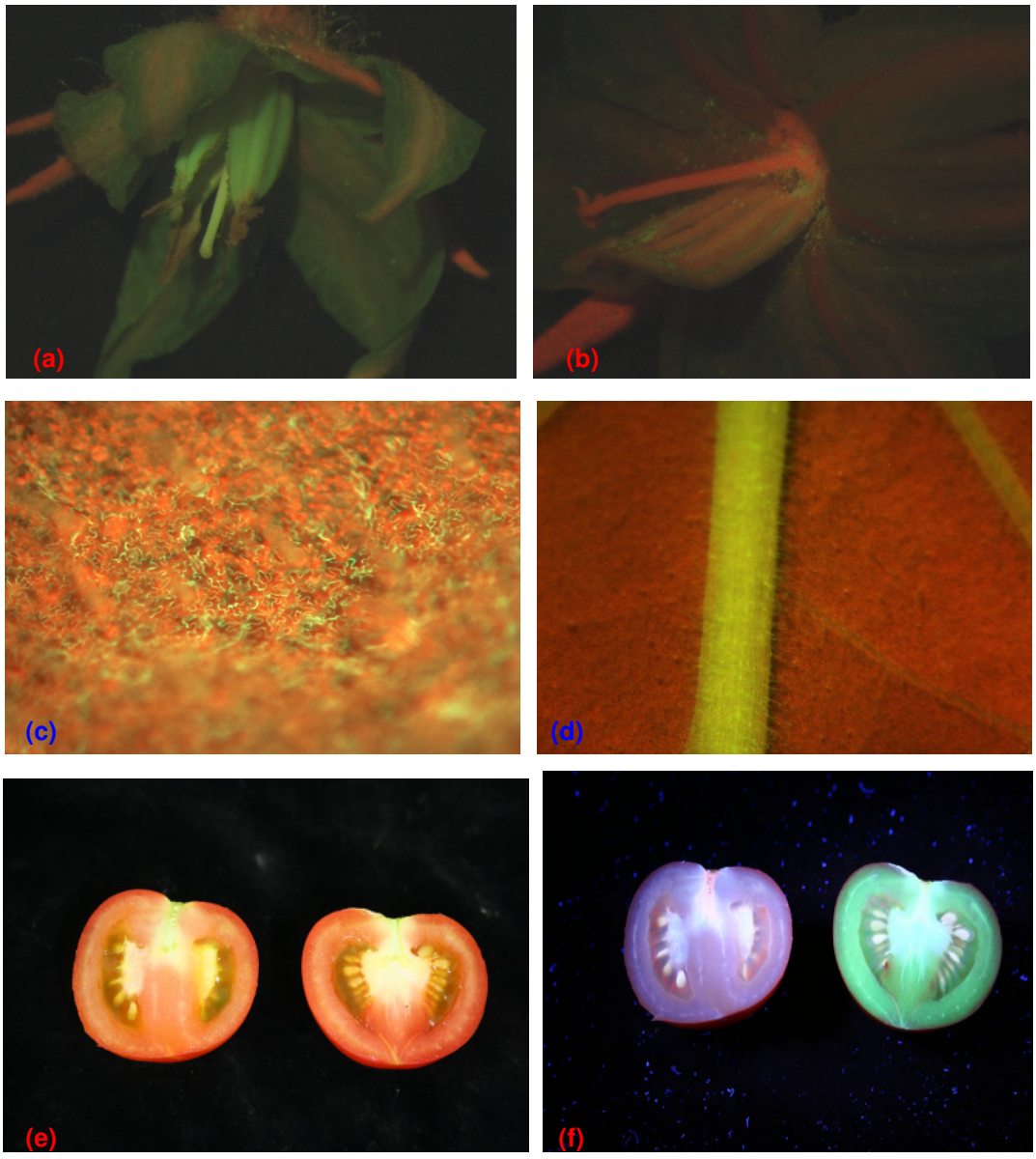


Figure 6: Flowers of transgenic tomato plants under fluorescence microscope (FITC light filter), Flower of *gfp* transgenic tomato plant appears with the fluorescent green colour (a), flower from *gpg* transgenic plants appears with red colour (b), low leaf epidermal layer of *gfp* expressing tomato (c) and major green fluorescing veins of *gfp* expressing tomato (d). Tomato fruits photographed under normal light (e); left: fruit of *gpg*-transformed plant, right: fruit of *gfp* expressing tomato plant. Tomato fruits photographed under UV-A light (f); left: *gpg*-transformed fruit, right: *gfp*-transformed fruit (d).

3.4 Detection of *gpg*-transformed plants by PCR

The PCR products amplified using genomic DNA of three samples from the transformed plants and one sample from a non-transformed plant (negative control). The results of gel staining showed that the samples of transformed plants had the same band size of plasmid control (257-bp), whereas, no band was detected in the wild type plant (Figure 7).

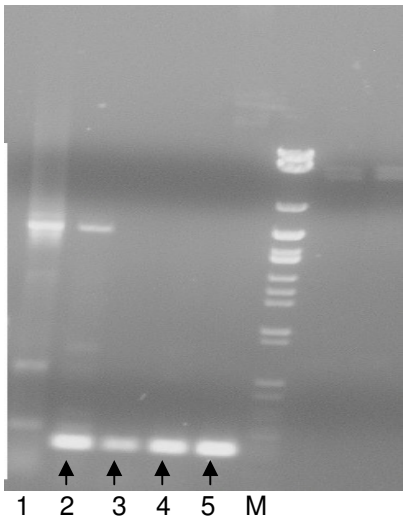


Figure 7: Agarose gel electrophoresis of the PCR products amplified from genomic DNA of tomato transformed and non transformed plants with pPCV702SM GpG (537). Lane 1: sample of wild type plant (negative control), lane 2 to lane 4: samples of *gpg*-transformed plants, lane 5: plasmid (positive control) treatment, M: marker.

3.5 Detection of siRNAs produced in *gpg*-transformed plants

To check if siRNAs were produced in plants transformed with the *gpg* gene construct, Northern blot analysis was performed to check the efficiency of *gpg* gene expression in plants. Results of RNA analysis of 3 samples showed that the *gpg* gene was expressed in plant cells (Figure 8).

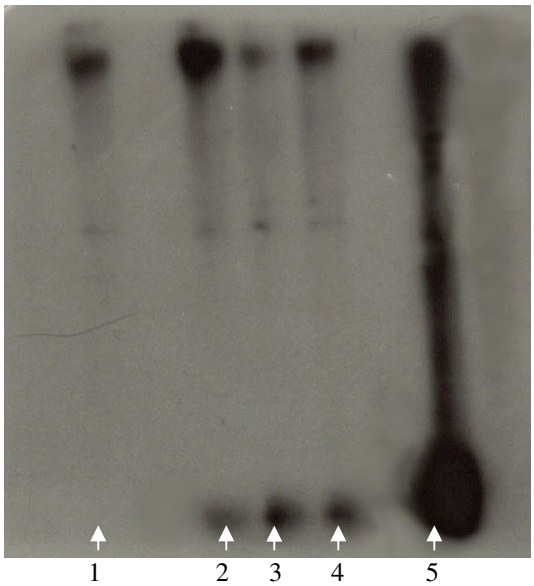


Figure 8: Agarose gel electrophoresis of the Northern blot RNA of tomato transformed and non transformed plants with pPCV702SM GpG (537). Lane 1: sample of none transformed plant (WT plant), lane 2 to lane 4: samples of *gpg*-transformed plants, lane 5: siRNAs of a *gfp*-expressing *Nicotiana benthamiana* C16-plant (positive control).

3.6 Infiltration experiments

The results of the infiltration showed that the mechanical infiltration (syringe infiltration) was more reproducible in tomato leaves than vacuum infiltration. Successful infiltration of *Agrobacteria* harbouring the *gfp*-gene constructs (pBINmGFP5 and pPCV702SM GpG) into wild type plants could be shown by the green fluorescence of GFP in the leaf tissue (Figure 9c). The fluorescence was observable approximately 3 d after infiltration. Infiltration of *gfp*-expressing plants with *Agrobacterium* carrying *gpg* construct, also led to a transiently enhanced fluorescence at the infiltration site which then turned some days later into a reduced green fluorescence (Figure 9b). This phenomenon is due to part-transcriptional gene silencing (PTGS) (Hamilton and Baulcombe, 1999) that spreads through the leaf, best to be seen at the major vein compared to normal fluorescent veins of *gfp*-expressing plants (Figure 9a).

It was also found, that the fluorescent green colour of *gfp-Agrobacterium* was not observed in *gpg*-expressing tomato leaves (Figure 9d), as well as it appears in leaves infiltrated with water, and this probably was due to the produced siRNAs in the plant cells which prevents at the end the production of GFP.

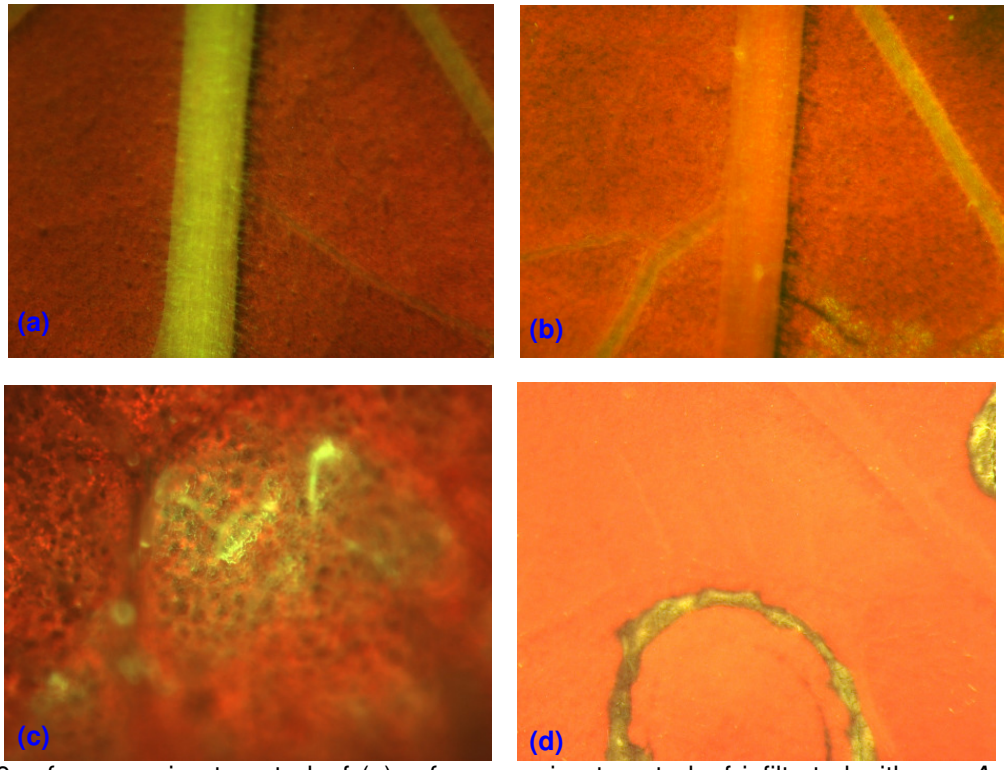


Figure 9: *gfp*-expressing tomato leaf (a), *gfp*-expressing tomato leaf infiltrated with *gpg-Agrobacterium* (b), wild type leaf infiltrated with *gfp-Agrobacterium* (c), *gpg*-expressing tomato leaf infiltrated with *gfp-Agrobacterium* (d) under filter fluorescence microscope (FITC light filter).

3.7 Infection of transformed leaves with Transformant 208m2

Detached leaves of *gpg* and *gfp* transformed and of wild type tomato plants were infected with the transformant 208m2 of *P. infestans* ($1x10^5$ sporangia ml^{-1}), to analyse, if siRNAs produced in the case of *gpg*-expressing tomato plants (Figure 8). This could lead to a silencing of *gfp* gene in the mycelium. In fluorescence microscopic analysis, the mycelium, however, showed that the fungal GFP expression unchanged over the observation time of 7 d and the same was observed in *gfp*-expressing tomato and wild type controls (Figure 10).

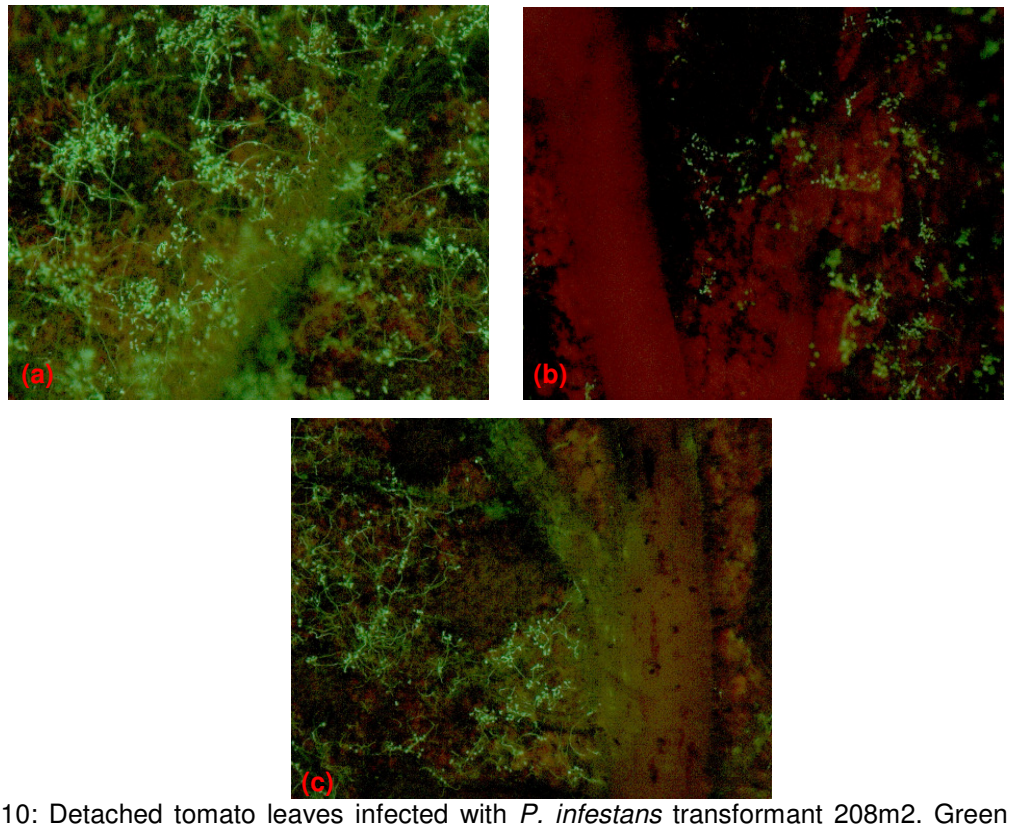


Figure 10: Detached tomato leaves infected with *P. infestans* transformant 208m2. Green fluorescent sporangiophores with sporangia grown in tomato *gfp*-expressed leaves (a), in *gpg*-expressed leaves (b) and in wild type leaves (c).

4. DISCUSSION

This study has been started approximately one year before the deadline of the PhD work with the transformation of tomato tissues, to get plant material for the experiments described below. The idea of this study was to silence the GFP-fluorescence of transgenic *P. infestans* (208m2) through the plant as a model for silencing fungal genes responsible for plant infection.

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants (Tinland, 1996; Smith and Townsend, 1907).

Tomato (*Lycopersicon esculentum*) is one of the most important crops as a genetic model for improving other dicotyledonous crop plants (Ling *et al.*, 1998; Frary and Earle, 1996; Van Roekel *et al.*, 1993; McCormick *et al.*, 1986). The first report of tomato transformation was by McCormick *et al.* (1986). In spite of the success of tomato transformation, most of the procedures relied on cumbersome either feeder layers (petunia, tomato, or tobacco), time consuming media formulations, or successive subcultures that produced significant variability between genotypes. Transformation efficiencies have ranged always; 6% (Vidya *et al.*, 2000), 7 to 37% (Ling *et al.,* 1988), 11% (Frary and Earle, 1996), 9% (Van Rocekel *et al.,* 1993), 14% (Hamza and Chupeau, 1993), and 25% (Hu and Phillips, 2001).

We have succeeded in producing two types of transgenic tomato plants; the first, carrying the *gfp*-gene and the second, carrying inverted *gfp*-gene fragments (gpg). From the cultivars transformed (‘Hellfrucht’ and ‘Rentita’) only ‘Hellfrucht’ could be successfully transformed with the protocol used. It was found that ‘Hellfrucht’ cultivar was faster and produced more calli than it was in ‘Rentita’ cultivar. From approximately 200 cotyledon pairs and 100 hypocotyls, 5 individual ‘lines’ of the ‘T0’ generation both of GFP- and GpG-transformants could be regenerated. The GFP-expressing plants could be easily identified by the green fluorescence under UV-A light. In the case of the non fluorescing GpG-transformant, the integration of gene into the genome could be proven by PCR analysis of genomic DNA.

Molecular biology experienced as a significant shift in thinking in recent years with growing evidence that microRNAs (miRNAs and siRNAs) play a major role in the control of eukaryotic gene expressing during development. Small interfering RNAs (siRNAs) are 21 to 25 nucleotide long non-coding RNA molecules that regulate transmission of protein-coding mRNAs, either by affecting degradation of target mRNAs or the attenuation or termination of translation of without mRNA degradation (Eckardt, 2004). siRNAs are processed from long double-stranded RNA (dsRNA) precursors arising from mRNAs, transposons, viruses, or heterchromatic DNA (Bartel and Bartel, 2003).

The production of siRNAs in our GpG-transformed plants has been detected by Northern blot. The results proved that siRNAs are produced, but in an amount lower than it was in a GFP-expressing *Nicotiana benthamiana* plant that was systemically silenced by *Agrobacterium* transformation with the GpG-construct (the positive control). The bases was established for the following experiments that should lead to the hypothesis testing, if a transgenic host plant (GpG-tomato) producing siRNAs of a target gene (gfp) of a compatible biotrophic phytopathogene (*P. infestans*) can silence the expression of this gene.

In the biotrophic host plant–pathogen interface, the oomycete or fungal pathogen penetrates the plant cell wall but not the host plasma membrane. Infection typically begins when an asexual sporangium lands on the plant and releases zoospores, which encyst and produce germ tubes. Sporangia can also germinate directly. The germ tubes differentiate appresoria which participate in the penetration of the underlying plant cells. When pre-existing openings are present, as is common on potato tubers, however, appresorial penetration is not required. Once entry into the host occurs, intercellular hyphae and haustoria develop (Figure 11) which form biotrophic feeding relationships with the plant. *P. infestans* is regarded as a hemibiotroph, since only living tissue is colonized (Agrios, 1997; Fry and Goodwin, 1995).

Biochemical and genetic evidence supports the existence of multiple small RNA pathways in plants that may have a wide variety of functions in plant growth and development (Dunoyer *et al.*, 2004; Xie *et al.*, 2004). In plants, siRNAs have been studied for years in relation to posttranscriptional gene silencing (PTGS), also known

as RNA interference (RNAi), which is believed to represent a natural defense mechanism against viral infection and the activity of transposable elements (Vance and Vaucheret, 2001; Waterhouse *et al*., 2001).

Some investigations have been done to silence certain genes in *P. infestans*, but all previous trials have been conducted on the *in vitro* level. *P. infestans* gene PiBzp1 which encodes a transcription factor that regulates pathways required for host infection, has been silenced and no further appresoria has been formed, due to a defect in the recognition of the host substrate (Judelson and Roberts, 2002; Latijnhouwers and Govers, 2003; Whisson *et al*., 2005). Such investigations in plant tissues are still missed till to date.

In gene silencing experiments, vacuum and mechanical (syringe) infiltrations were tried. It was found that the efficiency of vacuum infiltration for detached tomato leaves was comparatively low as could be seen from only few areas were expressing GFP. This may be due to the structure of tomato leaf tissues itself or due to the structure and number of the opening sites (like stomata) on the lower side of the leaf. In contrast, infiltration of tomato leaves using a syringe was highly effective. Fluorescence microscopic investigations showed that intercostals fields could be infiltrated and mesophyll cells from the entire area were found to be transformed in the wild type and the GFP-expressing leaves. It was also found that no green fluorescence appeared when the GpG-tomato leaves were infiltrated, and this may be due to the production of siRNAs in the plant cells preventing the expression of GFP.

The green fluorescence of GFP-tomato leaves disappeared gradually starting from the major leaf vein expanding to the other veins when it was infiltrated with the GpG-*Agrobacterium*, indicating a silencing of the *gfp*-gene in the cells. Systemic silencing over the whole plant however could not be observed. This may be due to the comparatively low expression of the *gfp*-gene in the ‘T0’ plants compared to the homozygous GFP-expressing *N. benthamiana* plants (C16) usually used and a model for GFP-silencing.

After the infection of the leaves of GpG-plants with the Transformant 208m2 (GFP-expressing strain), the expressed GFP has not been silenced. This could be explained

due to different reasons; firstly, the siRNAs produced in the leaf cells could not pass the membrane system of the haustoria (Figure 11). Secondly, the siRNA amounts in the GpG-tomato tissue was comparatively low as could be seen on the northern blot result. This gpg-siRNAs amount could be to low in the case of passing the membrane system to induce the silencing mechanism in 208m2. Thirdly, gene silencing potentially works in *P. infestans in vitro* (Blanco and Judelson, 2005; Whisson *et al.*, 2005; Latijnhouwers and Govers, 2003).

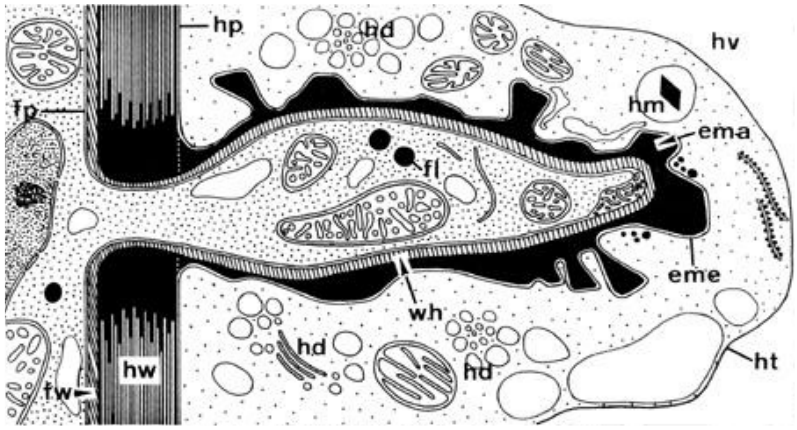


Figure (11): haustorial apparatus of *P. infestans* in potato. Ema = extrahaustorial matrix; eme = extrahaustorial membrane; fl = lipid bodies; fp = pathogen plasmamembrane; fw = pathogen cell wall; hd = host golgi; hm = host microbody; hp = host plasmamembrane; ht = host tonoplast; hv = host vacuole; hw = wall apposition; wh = haustorium wall. From Hohl and Stossel, Can J Bot 54, p. 903 (1976).

The present study aimed to silence the GFP fluorescence of the transformant 208m2 through the plant host with the long-term objective to transform the host plant with inverted repeats of essential pathogen genes, which siRNAs could induce silencing of these genes in the attaching *Phytophthora*. The experiments to date are made with 'T0' plants harbouring presumably only one integrated transgene. Therefore crossings as self-fertilization are actually performed in order to get homozygous and gpg/gfp heterozygous plants with enhanced siRNAs contents.

VI SUMMARY

Within the framework of two projects, financed by DBU (Deutsche Bundesstiftung Umwelt) and ProInno ("Förderung der Erhöhung der Innovationskompetenz mittelständischer Unternehmen"), respectively, in co-operation with an industrial partner, alternative phytosanitary compounds from natural sources have been screened. High throughput screening systems were developed and used for testing of large numbers of extracts of Actinomycetes in 96-well multiplates against *Phytophthora infestans* and *Erwinia amylovora*, the causal agents of tomato late blight and apple fire blight, respectively. These important phytopathogens were chosen as models.

According to the Pflanzenschutzgesetz (PflSchG) §2 Nr. 10a, plant strengthening compounds (Pflanzenstärkungsmittel) should not act directly against the pathogen, but via an induction of plant resistance mechanisms. Therefore, one of the projects (DBU) aimed to exclude direct action on *P. infestans*. Based on the GFP-fluorescence of the *P. infestans* transformant 208m2, a fluorescence optical measurement of mycelium growth respectively growth inhibition was developed, to test the influence of the extracts and extract fractions. Only 52 out of 8335 extracts significantly inhibited the mycelium growth (Pi+) in this test, and thus had to be excluded according to §2 Nr.10a PflSchG for a potential commercial application. Searching for resistance inducing activity, all extracts were re-tested in parsley cell culture as a model for putative resistance induction characterised by formation of furanocoumarin phytoalexins. Only 42 out of the whole set of extracts tested, induced furanocoumarins (Pc+) significantly.

In order to test if the induction correlates with a successful defence of host tissue against *P. infestans*, detached tomato leaves were treated with these Pc+ extracts. Only one extract resulted in formation of small sharp necrosis symptoms after pathogen inoculation, leading to strongly reduced infection and inhibited sporulation. The identification of the active ingredient is currently performed.

When comparing the 52 direct acting substances (Pi+) with the potentially resistance inducing compounds (Pc+), three were also found actively inhibiting *P. infestans* in the host tissue.

These three extracts also induced limited dark brown necrosis, suggesting an induction of hypersensitive reaction (HR). Infection area and sporulation level were reduced to levels below 25% of total leaf area. Microscopic investigations showed non-germinated or abnormally shaped germinated sporangia. Promising extracts were fractionated by the cooperation partner. Fraction 1 of one Pi+-extract (014 008-2) reduced the

sporulation level and the size of the infection area to 5 and 25%, respectively, compared to the control. Application of fraction 2, although less effective than fraction 1, produced sporangiophores that were morphologically abnormal carrying no sporangia, indicating a possible highly specific action on a certain developmental step of the pathogen.

In case of the extracts tested *in vitro* against *E. amylovora*, only 60 out of 5236 extracts inhibited bacterial growth. Only extracts showing a similar effect as the streptomycin positive control (12 extracts), were also tested in the cell culture of parsley for a potential resistance induction. The effect of such promising extract (000 391 CF) was compared in *in vitro* apple plantlets with two purified substances identified in the *in vitro* growth inhibition screening (tubercidin and streptothricin) and two commercially available resistance inducers (Prohexadion-Calcium, Bion®) in addition to streptomycin as control. Streptothricin was found nearly as effective as the streptomycin control (2 and 0% diseased shoots, respectively). 000 391 CF with chuanghsinmycin as active ingredient in unknown concentration, however, was less effective together with tubercidin; both compounds were originally classified effective in the *in vitro* screening (approximately 20 and 30%, respectively). The resistance inducers showed a maximal effectiveness of 20-25% approximately; however, in the highest concentration applied, they caused phytotoxic effects.

As an alternative strategy of plant defence, an experiment was performed to silence the GFP-fluorescence of transformant 208m2 as a model for silencing fungal genes responsible for plant infection via the transgenic host. Two types of 'T0' transgenic tomato plants were produced from 'Hellfrucht' cultivar, one carrying the *gfp*-gene and the other carrying inverted repeat fragment of the *gfp*-gene (gpg). GFP-expressing plants could be identified by their green fluorescence under UV-A light. *gpg*-transformants were verified by PCR analysis of their genomic DNA and the formation of siRNAs by Northern blotting. In order to test the silencing effects in the plants *Agrobacteria* harbouring the *gfp*-gene or *gpg*-construct in a binary vector were infiltrated into tomato leaves. Leaves of agro-infiltrated (*gfp*) wild type plants were found to fluoresce, but no green fluorescence appeared when *gpg*-tomato leaves were infiltrated, due to the production of siRNAs resulting in gene silencing. The green fluorescence of *gfp*-tomato leaves disappeared gradually starting from the major vein expanding to small veins after infiltration of *gpg-Agrobacteria* due to the same phenomenon. However, no systemic silencing over the whole plant was observed; this

may be due to the comparatively low expression of the *gfp*-gene in the‘T0’ plants used. However, the GFP in the *P. infestans* transformant 208m2 was not found to be silenced when leaves of *gpg*-plants were infected; this might be due to unsuccessful passing of siRNAs through haustorium’s membrane system, or due to the comparatively low amount of siRNAs in the *gpg*-tomato tissues.

VII ZUSAMMENFASSUNG

Im Rahmen zweier Projekte, finanziert durch die DBU (Deutsche Bundesstiftung Umwelt) und ProInno ("Förderung der Erhöhung der Innovationskompetenz mittelständischer Unternehmen") und in Kooperation mit einem industriellen Partner sollten alternative Substanzen natürlicher Herkunft für den Einsatz im Pflanzenschutz in einem Screening untersucht werden. Dazu wurden "high throughput" Biotestsysteme entwickelt und eingesetzt, um eine große Anzahl von Extrakten aus Actinomyceten in 96-well Microtiterplatten gegen *Phytophthora infestans* und *Erwinia amylovora* den Erregern der Kartoffelfäule bzw. Braunfäule und des Feuerbrands, zu testen. Wegen ihrer problematischen Bekämpfung waren diese wichtigen Phytopathogene als Modelle ausgewählt worden.

Entsprechend § 2 Nr. 10a des Pflanzenschutzgesetzes (PflSchG) dürfen Pflanzenstärkungsmittel keine direkte Wirkung gegen das Pathogen zeigen, sondern müssen durch die Aktivierung pflanzeneigener Abwehrmechanismen wirken. Aufgrund dessen zielte eine der Projektvorgaben (DBU) daraufhin, die direkte Wirkung der Extrakte auf das Wachstum von *P. infestans* auszuschließen. Basierend auf der GFP-Fluoreszenz der transgenen *P. infestans* Linie 208m2 wurde ein Verfahren zur fluoreszenzoptischen Erfassung des Mycelwachstums bzw. der Wachstumshemmung entwickelt, um den Einfluss von Extrakten und Extraktfraktionen darauf zu untersuchen. Von 8335 untersuchten Extrakten hemmten nur 52 das Mycelwachstum signifikant (Pi+) in diesem Test und mussten deshalb entsprechend § 2 Nr. 10a PflSchG für eine potentielle kommerzielle Verwendung als Pflanzenstärkungsmittel ausgeschlossen werden. Um eine potentiell resistenzinduzierende Eigenschaft zu finden, wurden alle Extrakte in einer Petersilie-Zellkultur getestet, einem Modell für eine mögliche Resistenzinduktion charakterisiert durch die Bildung von Furanocumarin-Phytoalexinen. Dabei wurden nur 42 Extrakte gefunden, welche die Furanocumarinbiosynthese signifikant induzierten (Pc+).

Um zu überprüfen, ob diese Furanocumarininduktion mit einer erfolgreichen Abwehr gegen *P. infestans* im Wirtsgewebe korreliert, wurden isolierte Tomatenblätter mit diesen Pc+-Extrakten behandelt. Bei nur einem Extrakt fand sich die Bildung kleiner scharf konturierter Nekrosen nach Pathogeninokulation, was zu einer stark reduzierten Infektion und gehemmter Sporulation führte. Die Identifikation der aktiven Komponente wird aktuell durchgeführt.

Bei Vergleich der 52 direkt wirkenden Extrakte (Pi+) mit den potentiell resistenzinduzierenden (Pc+), wurden drei Extrakte gefunden, die auch im Wirtsgewebe

das Pathogenwachstum hemmten. Diese drei Extrakte induzierten ebenfalls kleine braun gefärbte Nekrosen, was die Induktion einer hypersensitiven Reaktion (HR) vermuten lässt. Die Fläche der Infektion und die Stärke der Sporulation waren gegenüber der Kontrolle auf ca. 25 % der Gesamtblattfläche reduziert. Mikroskopische Untersuchungen dieser Ansätze zeigten nicht gekeimte Sporangien und abnorm geformte Keimhyphen. Die Erfolg versprechenden Extrakte wurden durch den Kooperationspartner einer weiteren Fraktionierung unterworfen. Die Fraktion 1 von einem der Pi+-Extrakte (014 008-2) reduzierte die Sporulationsstärke und die Größe der infizierten Blattfläche auf 5% bzw. 25 % verglichen mit der Kontrolle. Fraktion 2 desselben Extraktes bewirkte trotz geringerer Effizienz der Infektionshemmung, daß morphologisch abnorme Sporangiophore gebildet wurden, die keine Sporangien trugen, was möglicherweise auf eine hochspezifische Wirkung auf einen Entwicklungsschritt des Pathogens schließen lässt.

Von den Extrakten, die *in vitro* gegen *E. amylovora* getestet wurden, hemmten nur 60 von 5236 das Bakterienwachstum. Nur Extrakte, die einen der Positivkontrolle vergleichbaren Effekt aufwiesen wurden ebenfalls in der Petersiliezellkultur auf eine potentielle Resistenzinduktion getestet.

Der Effekt eines solchen als positiv bewerteten Extrakts (000 391 CF) wurde auf *in vitro*-Apfelpflanzen mit zwei aufgereinigten Substanzen, die ebenfalls positiv im Screening auf Wachstumshemmung waren (Tubercidin und Streptothricin), verglichen. Ebenfalls getestet wurden zwei käufliche Resistenzinduktoren (Bion und Prohexadion-Calcium) und die Positivkontrolle Streptomycin. Streptothricin war fast so effektiv wie die Streptomycin-Kontrolle (2 % bzw. 0% erkrankte Sprosse). Der Extrakt (000 391 CF) enthält Chuangsinmycin als aktive Substanz in unbekannter Konzentration, war jedoch weniger effektiv zusammen mit Tubercidin (ca. 20% bzw. 30% erkrankte Sprosse); beide Substanzen waren ursprünglich im *in vitro*-Screening als positiv bewertet. Die Resistenzinduktoren zeigten eine maximale Effizienz von 20-25%, waren jedoch in den höchsten Konzentrationen phytotoxisch.

Um "Gene Silencing" als eine alternative Strategie des Pflanzenschutzes zu testen, wurde versucht, die GFP-Fluoreszenz der transgenen *P. infestans* Linie 208m2 zu unterdrücken, als ein Modell für das Silencing pilzlicher, für die Infektion notwendiger Gene, durch den transgenen Wirt. Zwei Typen transgener Tomaten ("T0") wurden hergestellt aus dem Kultivar 'Hellfrucht', einer transformiert mit einem *gfp*-Gen, ein anderer mit einem inverted repeat Fragment von *gfp* (gpg). Die GFP-exprimierenden Pflanzen konnten anhand ihrer grünen Fluoreszenz unter UV-A Strahlung identifiziert werden. Die *gpg*-Transformanten

konnten mittels PCR-Analyse ihrer genomischen DNA identifiziert und die Bildung der entsprechenden siRNAs mittels Northern Blot verifiziert werden.

Um zuerst das Gene Silencing in den Pflanzen zu testen, wurden Agrobakterien, die mit binären Vektoren die entweder das gfp-Gen oder das gpg-Konstrukt beinhalteten, in einige ihrer Blätter infiltriert. Blätter von agro-infiltrierten (*gfp*) Wildtyp Pflanzen fluoreszierten danach an den Infiltrationsstellen. Keine Fluoreszenz konnte jedoch in Blättern infiltrierter *gpg*-Pflanzen beobachtet werden, was auf das Vorhandensein von siRNAs und somit gene silencing beruht. Die Grünfluoreszenz von *gfp*-Tomatenblättern verringerte sich allmählich von der zentralen Blattader ausgehend nach der Infiltration mit *gpg*-Agrobakterien aufgrund desselben Phänomens. Es konnte jedoch kein systemisches silencing d.h. über die infiltrierten Blätter hinaus beobachtet werden, was auf die relativ geringe Expression des *gfp*-Gens in den vorhandenen 'T0'-Pflanzen zurückzuführen sein könnte.

Die GFP-Fluoreszenz in 208m2 konnte nicht erkennbar `gesilenced` werden, wenn Blätter von *gpg*-Pflanzen infiziert wurden. Dies könnte entweder auf der nicht möglichen Passage von siRNAs durch das Membransystem der Haustorien beruhen, oder in einer nicht ausreichenden Menge von siRNAs im gpg-Blattgewebe begründet sein.

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Education

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1998 – 2001:	M.Sc. degree 'Plant Protection' - Dept. of Horticulture and Plant Protection, University of Jordan, Amman, Jordan.
1993 - 1997:	B.Sc. degree 'Plant Protection' - Dept. of Plant Protection, Baghdad University, Baghdad, Iraq.
1991 – 1993:	Secondary School – Al-Husain Secondary School – Irbid - Jordan
1981 – 1990:	Primary School – Yubla Primary School – Irbid – Jordan

Professional experiences

2002 – 2004:	Research Assistance, Zentrum Grüne entechnik, Dienstleistungszentrum Ländlicher Raum (DLR) Rheinpfalz, Neustadt/Weinstrasse, Germany.
July – Sep., 2002:	Research Assisstant**,** Institute of Phytopathology, University of Hohenheim, Stuttgart, Germany.
2001 – 2002:	In Bee Keeping Field / Biodiversity Project (UNDP), Faculty of Agriculture, The University of Jordan, Amman, Jordan.
May – Sep., 2001:	Graduate Research Assistance, Dept. of Plant Production, Jordan University of Science and Technology, Irbid, Jordan
1998 – 2000:	Graduate Research and Teaching Assistance, Faculty of Agriculture, Dept. of Plant Protection (Teaching of Plant Pathology Labs), The University of Jordan, Amman, Jordan.
1997 – 1998:	Research Assistant, Biotechnology Center, Faculty of Agriculture, Jordan University of Science and Technology, Irbid, Jordan.

Research interests

- Plant transformation technique
- Molecular biology technology
- Induced resistance in plants (Plant – Pathogen interactions)
- *In vitro* tissue culture technique
- Establishing and developing *in vitro* and *in vivo* screening test systems for Actinomycetes extracts against different plant pathogens (*Phytophthora infestans* fungal-like and *Erwinia amylovora* bacterium).

Seminars & workshops

- Fifty fifth plant protection meeting: The University of Goettingen – Germany, 25-28 Sep., 2006.
- Fifty fourth German Plant Protection Conference. The University of Hamburg – Germany, 20-23 Sep., 2004. (Poster: Development of environment and consumer-friendly plant protection preparations on the basis of natural substances
- Seventh Arab Congress of Plant Protection. University of Jordan - Jordan, 22-26 Oct., 2000. (Seminar: Chemical induced resistance of tomato plants against *Verticillium dahliae*).
- Plant Protection Congress. Hamburg - Germany, 2004. (Poster: Effect of biological plant preparations at the base of microbial extracts).

Publications

- Rida A. Shibli; Mohammed A. Shatnawi; Ihsan Q. Swaidat. (2003). Growth osmotic adjustment, and nutrient acquisition of bitter almond under induced sodium chloride salinity in vitro. Communications in Soil Science and Plant Analysis, 34 (13&14), 1969-1979.
- Shibli, R.A., Sawwan, J., Swaidat, I.Q., and Tahat, M.M. (2001). Increased Phosphorous Mitigates the Adverse Effects of Salinity in Tissue Culture. Communications in Soil Science and Plant Analysis, 32 (3&4), 429-440.
- Sawwan, J., Shibli, R.A., Swaidat, I.Q., and Tahat, M.M. (2000). Phosphorus regulates osmotic potential and growth of African Violet under In Vitro-Induced Water Deficit. J. Plant Nutrition, 23 (6), 759-771.
- Swaidat, I., Buchholz, G., and Krczal, G. Developing highthrouput screenig test for Actinomycetes extracts gainst *Phytophthora infestans* and *Erwinia amylovora* (under preperation).
- Swaidat, I., Buchholz, G., and Krczal, G. Establishment of transgenic tomato plants to study gene silencing effect as a potential control strategy for *Phytophthora infestans* (under preparation).