

The effect of an extract from mycelium of *Penicillium chrysogenum*  
on plant-pathogen interactions  
and  
Characterisation of elicitors in  
this extract

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## Summary

In agriculture, infection of plants with microorganisms including fungi, bacteria and viruses can cause high losses of yield. Apart from a broad spectrum of indirect and direct techniques to protect plants from damage, the concept of induced resistance provides a promising strategy for the control of diseases. Preliminary studies suggested that an aqueous extract from the dry mycelium of the non-pathogenic ascomycete *Penicillium chrysogenum*, further called 'Pen', can enhance resistance of many plants against several pathogens. The objective of this thesis was to unravel whether Pen can be used as a plant activator in commercial agriculture, to study its mode of action and to narrow down the active principles in Pen.

Pen protected grapevine from downy and powdery mildew (*P. viticola* and *U. necator*), tomato from early blight (*P. infestans*), onion from downy mildew (*P. destructor*) and apple tree from apple scab (*V. inaequalis*) under greenhouse and field conditions without having a direct fungicidal effect. The efficacy of Pen was generally comparable to traditional fungicides such as copper and sulphur and equal to or even better than well-known inducers of resistance such as BABA or BTH. The raw material for extraction of Pen was of constant quality, a prerequisite for a future application in practice. However, Pen often caused phytotoxic side effects such as small necrotic spots or, more rarely, larger necrotic areas. The development of the phytotoxic symptoms was dependent on several parameters, including concentration of Pen, the number of applications, the persistence on the plant tissue, the plant species and variety and environmental conditions. A partially purified fraction of Pen was less toxic than the crude extract.

To study signal transduction pathways involved in Pen-mediated resistance, the model plant *Arabidopsis thaliana* was used, allowing a comparison with the mode of action of other well-known inducers. Pen protected *A. thaliana* from a broad range of pathogens, including an oomycete (*Peronospora parasitica*), two ascomycetes (*Botrytis cinerea*, *Alternaria brassicicola*) and a bacterium (*Pseudomonas syringae* pv. *tomato* DC3000). Pen was still fully protective against *B. cinerea* in *Arabidopsis* transgenes or mutants impaired in the salicylic acid (*NahG*, *npr1*), jasmonic acid (*coi1*), and ethylene (*ein2*) signalling pathway. Pen-mediated resistance against *P. parasitica* was reduced in the transgene *NahG*, but was not affected in the mutants *npr1*, *coi1* or *ein2*, indicating that Pen induced resistance against *P. parasitica* on a salicylic acid-dependent, but NPR1-independent pathway.

Pen triggered early defense-related responses such as an extracellular alkalinisation, an oxidative burst and ethylene production in suspension-cultured cells as well as in intact leaf tissue of numerous mono- and dicotyledon plant species. Cells pretreated with chitin or ergosterol were refractory to a second treatment with the same stimulus but fully responsive to Pen, indicating that Pen contains at least one unidentified elicitor (the 'Pen-elicitor').

To develop new strategies for production of an extract without the undesired phytotoxic side effects, we aimed at purifying and characterizing the Pen-elicitor. Measuring early defense-related responses in suspension-cultured cells is a simple, fast and sensitive bioassay and was thus used as a tool for purification and characterization of the Pen-elicitor. The Pen-elicitor could only be isolated from a high but not from a low penicillin-producing strain of *P. chrysogenum*. The Pen-elicitor was sensitive to protease digestion, to basic hydrolysis, to oxidation by periodate and, to a less extent, to acidic hydrolysis. The Pen-elicitor was not affected by numerous other enzymes and by several chemical treatments. Reversed phase, ion exchange, size exclusion and affinity chromatography revealed that heterogeneity is a characteristic of the Pen-elicitor. Heterogeneity could not be reduced by treating Pen with several specific enzymes or chemicals which do not destroy elicitor-activity, preventing a further analysis.

In conclusion, in this thesis it was shown that Pen has interesting, unique characteristics for an application as a plant protection agent in organic agriculture, provided its phytotoxic side effects can be removed. Our work on *Arabidopsis thaliana* has revealed that Pen has the potential to protect a plant species against a broad range of pathogens, including biotrophic as well as necrotrophic microorganisms belonging to different classes. Furthermore, Pen seems to activate defense mechanisms by way of signal transduction pathways different from known plant activators. We hypothesize that the Pen-elicitor consists of a small, distinct

elicitor-active region, most likely a protein or peptide, which is part of a larger molecule varying in size and/or chemical composition. Although identification of the resistance-inducing substance would considerably facilitate to develop strategies for the preparation and processing of Pen, it is not necessarily a prerequisite for a future usage in practice. As an alternative, improved formulation as well as refined purification steps could make an application of the Pen-extract feasible.

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## Abbreviation List

BABA	$\beta$ -amino butyric acid
BTH	Benzothiodiazole
COI1	Coronatine Insensitive
CPR	Constitutive Expressor of PR proteins
EDS	Enhanced Disease Susceptibility
EF-Tu	Elongation Factor Tu
flg22	Elicitor-active peptide consisting of 22 amino acids from the N-terminal end of flagellin
GMO	Genetically modified organism
HR	Hypersensitive Response
INA	Isonicotinic Acid
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
MAPK	Mitogen Activated Protein Kinase
NIM1	Non-inducible Immunity
NO	Nitric Oxide
NPR1	Non-expressor of PR1
PAD	Phytoalexin Deficient
PAMP	Pathogen-Associated Molecular Pattern
PDF	Plant Defensin
Pen	Aqueous extract of the dry mycelium of the high penicillin-producing strain of <i>Penicillium chrysogenum</i>
Pen <sub>2000</sub>	Pen >2000 Da
Pen <sub>Acetonprec</sub>	Pen precipitated with 80% acetone at $-20^{\circ}\text{C}$
Pen <sub>binding</sub>	Pen binding to ConA
Pen <sub>C8</sub> /Pen <sub>C18</sub>	Pen binding to C8 or C18 reversed phase columns
Pen <sub>charged</sub>	Pen binding to SP-Trisacrylamide (kation exchanger)
Pen <sub>nonbinding</sub>	Pen not binding to ConA
PR	Pathogenesis Related
ROS	Reactive Oxygen Species
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SID	Salicylic Acid Induction Deficient
TCV	Turnip Crinkle Virus
TMV	Tobacco Mosaic Virus





# CHAPTER I

## General Introduction

## Resistance and disease: two possible outcomes of plant-microbe interactions

In agriculture, infection of plants with microorganisms including fungi, bacteria and viruses can cause high losses of yield (Agrios, 1997). The dramatic famine in Ireland in 1845 caused by the late blight disease (*P. infestans*) on potato and the complete breakdown of wine industry in France after the introduction of downy mildew (*P. viticola*) from America to Europe in the 1870ies are only two examples (Dowley et al., 1995; Singh, 2000). To prevent damage due to pathogens, several methods have been developed (Agrios, 1997). On the one hand, indirect techniques are well-known, including the use of high quality propagation material, sanitation (e.g. removal of overwintering sources of inoculum or of infected volunteer plants), avoidance techniques, crop rotation, soil management, plant nutrition and the selection of resistant varieties. On the other hand, diseases are directly controlled by the application of pesticides or, more rarely, antagonists. In addition, the concept of induced resistance provides a promising strategy for the control of diseases (Hammerschmidt, 1995; Agrios, 1997). Breeding for resistant varieties and protection of plants by plant activators, both make use of the plant immune system.

Although agriculture faces serious problems due to microorganisms, plants are resistant against the majority of fungi, bacteria and viruses, which are present in large numbers and high diversity in the environment, i.e. disease is a very rare outcome of plant-microbe interactions. A variety of mechanisms contributes to the defense against potentially pathogenic microorganisms. The first line of the plant defense consists of preformed defense mechanisms such as physical barriers (cuticle, waxes, shape and position of stomata) and constitutively expressed antimicrobials (Agrios, 1997; Wittstock and Gershenson, 2002). In addition, plants can activate several defense mechanisms in response to microorganisms. These include strengthening of the cell wall by enhanced cross-linking and deposition of callose, lignin or silica (Stumm and Gessler, 1986; Schmele and Kauss, 1990; Matern et al., 1995; Sticher et al., 1997), the production of proteinaceous and non-proteinaceous antimicrobials (Sticher et al., 1997; Dixon, 2001), and a hypersensitive response (HR) resulting in localized cell death (Mittler and Lam, 1996).

The efficacy of the concerted action of preformed and inducible defense mechanisms determines whether a particular microorganism can cause disease in a plant species and how severe the disease will be if a microorganism once has managed to invade a plant. If all genotypes of a microorganism are prevented from growing on a particular plant species, the microorganism is called a 'non-host pathogen', the plant a 'non-host plant' and the mechanism 'non-host resistance' (Agrios, 1997). As mentioned before, non-host resistance is the most frequent outcome of plant-microbe interactions. If the inducible defense mechanisms of a plant species are activated too slowly or the concerted action of constitutive and inducible defense mechanisms is insufficient against a particular microorganism, the outcome of the interaction is disease. In this case, the plant is 'susceptible', called a 'host' and the microorganism a 'pathogen'. Nevertheless, inducible defense mechanisms may still restrict the growth or reproduction of a pathogen, resulting in different degrees of disease expression, from very slight symptoms, hardly distinguishable from non-host resistance to complete destruction of a plant. This kind of resistance is often referred to as 'horizontal resistance' or 'basal resistance' (Agrios, 1997). However, if inducible defense mechanisms contributing to basal resistance have already been activated by an appropriate stimulus before a plant comes in contact with a microorganism, plants are less susceptible to a broad range of pathogens. This state of resistance is commonly referred to as 'induced resistance'. The phenomenon of induced resistance has first been described in detail by (Ross, 1961). He showed that the upper, remote, leaves of tobacco plants were more resistant against different viruses when a lower leaf had previously been infected by tobacco mosaic virus (TMV). He called the phenomenon systemic acquired resistance (SAR). The state of induced resistance

either depends on defensive compounds that are produced as a result of the induction treatment, and/or on a faster and intensified activation of defense mechanisms upon challenge inoculation with a pathogen. The latter mechanism is referred to as 'priming', 'sensitization' or 'potentiation' (Ton, 2001). Several resistance inducing agents have been described, including pathogenic and non-pathogenic microorganisms (e.g. plant growth promoting rhizobacteria), extracts from microorganisms, and numerous natural and synthetic compounds.

In addition, within a susceptible plant species, some varieties may become resistant against certain races of a pathogen. In such an interaction, the pathogen is capable of initiating infection, but is immediately arrested at the site of penetration by a hypersensitive response (HR) and a programmed cell death (Schneider, 2002). The resulting 'host resistance' (also referred to as 'race-cultivar-specific' or 'vertical' resistance) is generally controlled by a single dominant resistance (R) gene in the host. The gene product of such an R gene interacts, either directly or indirectly, with the product of a matching dominant avirulence (avr) gene expressed by the pathogen (Agrios, 1997; Holt et al., 2003).

Many studies have shown that significant similarities exist between the different types of resistance. Besides the fact that the same types of defense responses can be activated, there is evidence that signal transduction pathways might be partly convergent (Mysore and Ryu, 2004). Various mutants such as *nho1* and *eds1* compromised in several or all types of resistance have been identified (Parker et al., 1996; Lu et al., 2001; Hammond-Kosack and Parker, 2003). Furthermore, gene expression profiling in *Arabidopsis* revealed that similar sets of genes are activated after inoculation with either non-host pathogens or avirulent pathogens (Tao et al., 2003). Even though significant similarities exist between the different types of resistance, there are also differences (Mysore and Ryu, 2004). In this work, the focus will be on signalling molecules and networks involved in induced resistance.

## Signalling Molecules and Genes Involved in Defense

### Salicylic acid dependent resistance

The involvement of salicylic acid (SA) in the establishment of SAR has been recognized first by (White, 1979) who found that application of synthetic SA is sufficient to make plants more resistant. Only in 1990, it was discovered that also endogenous SA naturally accumulates in pathogen-challenged leaf tissue. Accumulation of SA further correlated with the expression of certain proteins (pathogenesis related (PR) proteins) as well as with enhanced resistance levels in tobacco and cucumber plants (Malamy et al., 1990; Métraux et al., 1990). The use of transgenic NahG plants gave deeper insight into the role of SA in resistance. NahG plants can not accumulate SA, they constitutively express the bacterial NahG gene, encoding a salicylate hydroxylase which converts SA into catechol. NahG tobacco and *Arabidopsis* plants showed enhanced disease susceptibility to several microorganisms, including the bacterium *Pseudomonas syringae* and the oomycete *Peronospora parasitica* (Delaney et al., 1994) (fig. 1). In addition, NahG transgenes did not develop SAR upon induction treatment, indicating that SA is required for induced as well as for non-host resistance.

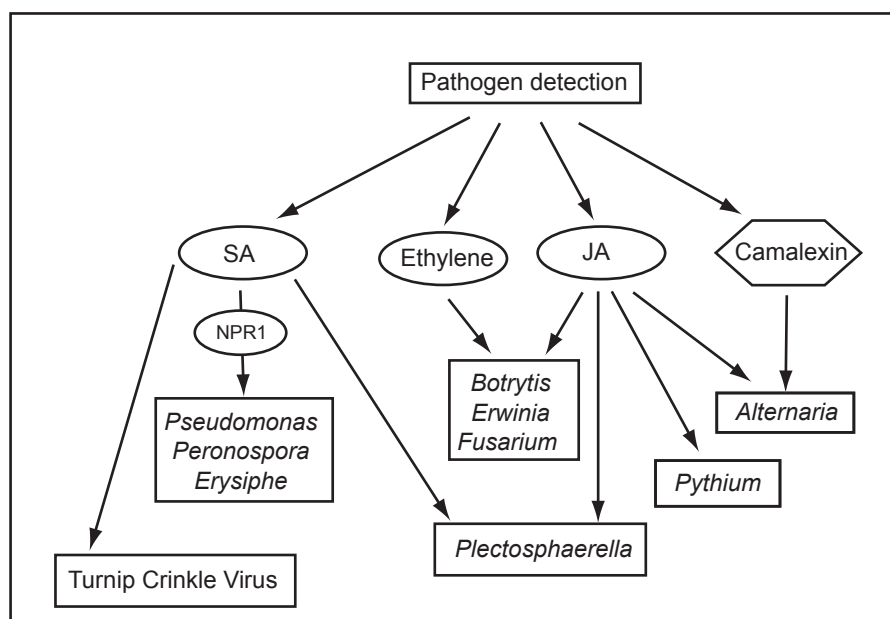
Several genes involved in SA-dependent signalling have been identified. Relatively early, NPR1 (also known as NIM1) has been identified as part of the SA-dependent pathway (Cao et al., 1994; Delaney et al., 1995). NPR1 is a protein containing ankyrin repeats, a structure often involved in protein-protein interactions (Cao et al., 1997; Ryals et al., 1997). The gene product of NPR1 is localized in the nucleus in the presence of SA, suggesting an interaction with transcription factors. NPR1 mutants (*npr1*) do not express PR protein and show enhanced susceptibility to the same set of pathogens as NahG transformants (Delaney et al., 1995). However, the fact that *npr1* plants show normal levels of SA suggests that NPR1 acts downstream of SA. Resistance induced by way of signalling pathways requiring SA and NPR1 is often referred to as systemic

acquired resistance (SAR).

During the last few years, additional genes acting in the SA-dependent signalling pathway have been identified, and double mutant analyses have been used to place them in order in the signalling network (reviewed by (Glazebrook, 2001)) (fig. 2). As an example, the gene products of EDS1, EDS5, PAD4 and SID2 all seem to act upstream of SA (Falk et al., 1999; Jirage et al., 1999; Nawrath and Métraux, 1999; Dewdney et al., 2000). Plants mutated in EDS5 and SID2 show SA levels as low as NahG transformants. In addition, several negative regulators of the SA dependent pathway have been identified, including the gene products of EDR1, MPK4, CPR1 and CPR6 (Clarke et al., 2000; Frye et al., 2000; Petersen et al., 2000; Clarke et al., 2001; Jirage et al., 2001). Mutations in negative regulators result in enhanced constitutive disease resistance, which is either coupled with constitutive expression of PR proteins or with priming.

Although PR proteins have frequently been used as markers for induced resistance, their importance for enhanced disease resistance still remains unclear. For some of them, an antimicrobial effect has been demonstrated in vitro (Sticher et al., 1997). However, other studies have shown that resistance and expression of PR proteins are not necessarily linked. As an example, *Arabidopsis* plants mutated in a gene called DTH9 expressed PR genes normally in response to SA treatment but they failed to develop resistance against *Peronospora parasitica* and *Pseudomonas syringae* pv. *maculicola* (Mayda et al., 2000).

Several natural and synthetic compounds inducing enhanced disease resistance by way of SA/NPR1 dependent signalling pathways have been described. Such compounds are SA, 2,6-dichloroisonicotinic acid (INA), benzothiadiazole (BTH), probenazole and the bacterial protein harpin (Sticher et al., 1997). BTH, the active compound of the 'plant activator' Bion® (Syngenta AG) is a functional analogue of SA and induces resistance in NahG transformants but not in npr1 mutants (Delaney et al., 1995; Lawton et al., 1996).



**Figure 1.** The network of disease signaling in *Arabidopsis*. This model distinguishes several inducible signaling pathways in *Arabidopsis* and their effect against the respective pathogens. Modified from Thomma BPHJ et al. (2001), Current Opinion in Immunology 13: 63-68.

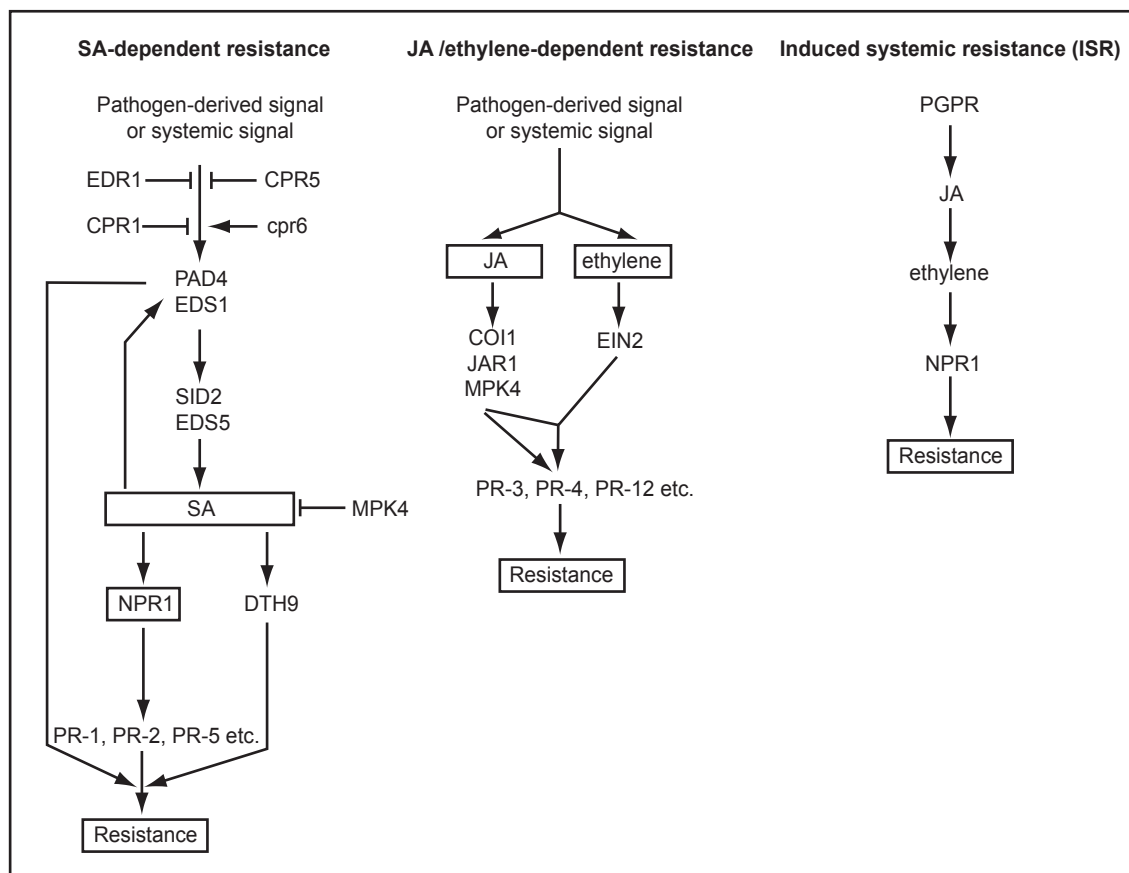
#### Jasmonic acid and ethylene dependent resistance

As reported above, *Arabidopsis* plants deficient in SA response were more susceptible to pathogens such as *Peronospora parasitica*, *Pseudomonas syringae* and *Erysiphe orontii*. However, their resistance level

against *Botrytis cinerea*, *Alternaria brassicicola* and *Erwinia carotovora* was not affected (Thomma et al., 1998; Thomma et al., 1999; Norman-Setterblad et al., 2000). In contrast, mutants affected in production, perception or signalling of the plant hormones jasmonic acid (JA) and/or ethylene were more susceptible to the latter but not to the former set of pathogens. Furthermore, it has been demonstrated that JA and ethylene are involved in resistance of *Arabidopsis* and tobacco against numerous soil microorganisms, including several species of the genus *Pythium*, *Fusarium oxysporum*, *F. solani*, *Thielaviopsis basicola* and *Rhizopus stolonifer* (Staswick et al., 1998; Vijayan et al., 1998; Geerats et al., 2002). Yet, the two hormones are not always required simultaneously. While resistance against *B. cinerea* requires concomitant activation of a JA and an ethylene signalling pathway, resistance against *A. brassicicola* is only dependent on JA (Thomma et al., 1998; Thomma et al., 1999). Pathogen-induced production of JA and ethylene, or exogenous application of these two signalling molecules, induces a particular set of defense-related genes, including PR-3, PR-4 and PR-12 (also called PDF1.2) (van Wees, 1999).

### Induced systemic resistance

It has been demonstrated that some plant growth promoting rhizobacteria (PGPR) can induce resistance against a broad range of pathogens in many plant species (van Loon et al., 1998). This kind of resistance has been referred to as rhizobacteria-mediated induced systemic resistance (ISR) (Pieterse et al., 2000). *Arabidopsis* plants expressing ISR have enhanced defensive capacity against *Fusarium oxysporum*, *Pseudomonas syringae* and *P. parasitica*. It has been demonstrated that a distinct ISR signalling pathway



**Figure 2.** A model describing the positions of *Arabidopsis* genes in signal transduction networks that control the activation of defense responses. Figure modified from Glazebrook J (2001), *Current Opinion in Plant Biology*, 4: 301-308.

exists. This pathway is SA-independent but requires functional JA and ethylene perception as well as functional NPR1. In contrast to resistance against *B. cinerea*, which requires concomitant activation of ethylene- and JA-dependent pathways, JA and ethylene responses are engaged successively in ISR (Pieterse et al., 1998). Expression of ISR is not associated with the accumulation of the marker genes well-known from SA- and JA/ethylene-dependent pathways. SA/NPR1-dependent SAR and JA/ethylene/NPR1-dependent ISR can be activated simultaneously, resulting in an additive protection against *P. syringae* pv. *tomato*. These results suggest that SAR and ISR are distinct pathways without significant crosstalk (van Wees et al., 2000).

### **Other signalling pathways and cross talks among pathways**

There is evidence that besides the 'classical' pathways described above, other signalling cascades may exist. Particularly, several studies suggested that a signalling pathway dependent on SA but not on NPR1 exists (Clarke et al., 2000; Kachroo et al., 2000; Glazebrook, 2001). As an example, (Kachroo et al., 2000) showed that resistance against tobacco mosaic virus is only dependent on SA but does not require NPR1. Furthermore, it has been shown that a signalling pathway independent of JA is involved in basal resistance of *Arabidopsis* to *A. brassicicola* (Thomma et al., 1999; Zhou et al., 1999). Several compounds such as BTH and harpin have been shown to induce resistance by way of SA/NPR1-dependent signalling pathways (Lawton et al., 1996; Dong et al., 1999). In contrast, resistance induced by the non-protein amino acid  $\beta$ -amino butyric acid (BABA) against *P. parasitica* and by the bacterial protein flagellin against *P. syringae* pv. *tomato* does not require ethylene, JA, SA or NPR1 (Zimmerli et al., 2000; Zipfel et al., 2004).

There is growing evidence that JA, SA and ethylene defense signalling pathways do not function independently (Thomma et al., 2001; Kunkel and Brooks, 2002). More likely, they are involved in a complex signaling network in which the different pathways influence each other through positive and negative regulatory interactions. Particularly, there are different studies suggesting that SA and JA signalling are mutually antagonistic. (Thomma et al., 2001) speculate that the existence of multiple defense mechanisms might be the evolutionary answer of plants to challenges from different groups of pathogens. While SA-dependent defense responses such as a hypersensitive response seem particularly suited to restrict the growth of biotrophs such as *P. parasitica*, *P. syringae* and *E. orontii*, these defense responses might even promote growth of necrotrophic microorganisms such as *B. cinerea*, *A. brassicicola* and *Pythium* sp. Indeed, it has been shown recently that growth of the necrotrophic pathogens *Botrytis* and *Sclerotinia* is suppressed in the mutant *dnd1*, which fails to produce a normal HR (Gorin and Levine, 2000). It has also been demonstrated that mutants with reduced levels of SA display enhanced responses to inducers of JA-dependent gene expression (Gupta et al., 2000). Furthermore, (Petersen et al., 2000) have shown that a mutation in MPK4 blocks the JA-inducible expression of PDF1.2 (PR-12) and causes the constitutive activation of SA-dependent signalling. However, (Berrocal-Lobo et al., 2002) have shown that both positive and negative interactions between ethylene and SA signalling pathways can be observed depending on the type of pathogen. Furthermore, both JA and SA contribute to resistance against the fungus *Plectosphaerella cucumerina* in *Arabidopsis*, suggesting that a general antagonism is unlikely (Thomma et al., 2000).

## **Recognition of microorganisms and early events in signal transduction**

### **Early events in signal transduction**

The activation of defense mechanisms involves numerous events, starting with the recognition of an appropriate stimulus. As reported above, many studies have focused on relatively late events in signal transduction, such as the accumulation of secondary signalling molecules, on the activated defense mechanisms, or on the effect on plant-microbe interactions. However, another body of literature has focused on early defense-related events in plant-microbe interaction. The question which molecules are recognized

by plants and how has been of central interest. To study the perception system of plants, suspension cultured plant cells have proven to be useful tools. This system has the advantage that a large number of cells can be stimulated at exactly the same time and early events in signal transduction are easily detectable seconds to minutes after contact with the stimulus (Boller and Felix, 1996). However, cell cultures do not represent the intact biological system, thus results have to be verified in intact plants. For instance, it has been difficult to examine gene-for-gene interactions in cell cultures. Furthermore, there are some difficulties to link results on early defense-related responses measured in cell cultures with results on late defense-responses in intact plants.

One of the earliest observable events in signal transduction, detectable within seconds or minutes, is a change in the ion permeability of the plasma membrane, measurable as an efflux of  $K^+$  and  $Cl^-$  and an influx of  $H^+$  and  $Ca^{++}$  (Sacks et al., 1993; Jabs et al., 1997; Zimmermann et al., 1997). Proton influx results in an alkalinisation of the extracellular medium ('alkalinisation response'), a parameter easily measurable in suspension-cultured cells (Felix et al., 1991; Blume et al., 2000). Elicitor-stimulated increases in cytosolic  $Ca^{++}$  concentration and extracellular alkalinisation can both be inhibited by protein kinase inhibitors such as K-252a and staurosporin, allowing to distinguish elicitor induced responses from unspecific effects such as addition of bases, pK changes or membrane leakage due to membrane active compounds. An inhibition of alkalinisation response by K-252a indicates that rapid changes in protein phosphorylation are involved in receptor-mediated regulation of ion channels (Felix et al., 1991; Blume et al., 2000). Several other studies have also pointed out that protein phosphorylation is required for early signal transduction. It has been shown that mitogen activated protein kinases (MAPK), eukaryotic enzymes involved in various facets of cellular regulation, as well as calcium dependent protein kinases are activated by a large variety of abiotic and biotic stimuli in different plant species relatively early after elicitor perception (Romeis, 2001). Only recently, (Asai et al., 2002) have identified a whole plant MAPkinase signalling cascade activated within the first 30 min after addition of the elicitor flagellin (see below).

The production of reactive oxygen species (ROS) such as  $H_2O_2$  and  $O_2^-$  at the cell surface, (also denoted as 'oxidative burst') was shown to start approximately 2 min after elicitor treatment (Dixon et al., 1994). Like alkalinisation of the extracellular space, an oxidative burst is easily measurable in suspension cultured plant cells. It was demonstrated that an oxidative burst can be induced by a transient increase of cytoplasmic  $Ca^{2+}$  levels (Blume et al., 2000; Lecourieux et al., 2002). Apart from a putative direct antimicrobial effect, ROS seem to be involved in enhanced crosslinking of proline-rich cell wall proteins (Lamb and Dixon, 1997; Grant and Loake, 2000). In addition to ROS, nitric oxide (NO) was found to be generated in tobacco, soybean and *Arabidopsis* upon infection with avirulent bacteria or viruses, as well as upon elicitor treatment (Dangl, 1998). Together, ROS and NO appear to be essential second messengers for the activation of defense-related genes and programmed cell death.

### **Molecules triggering defense responses in plants**

Much work has focused on the molecules which can be recognized by plants and trigger defense responses. On the one hand, proteins encoded by avirulence genes are either directly or indirectly recognized by the gene product of a matching resistance gene (Dangl and Jones, 2001). On the other hand, plants possess sensitive detection systems for numerous microorganism-derived structures (exogenous elicitors) and structures released from plant cell walls during an attempted invasion (endogenous elicitors), so-called general elicitors. Perception of general elicitors is thought to activate defense responses resulting in non-host or induced resistance. The concept of exogenous elicitors is equivalent to the concept of Pathogen-Associated Molecular Patterns (PAMPs) known from animal innate immunity (Nürnberg and Brunner, 2002). Exogenous elicitors or PAMPs are characteristic structures of entire groups or classes of microorganisms, no matter whether these microorganisms are pathogenic or not. These structures allow plant or animal cells to distinguish self from non-self. PAMPs constitute structures that are unique to



microorganisms, have important roles in microbial physiology or structure and are therefore evolutionary highly conserved (Nürnberg and Brunner, 2002). Various exogenous elicitors or PAMPs activating defense responses in plants have been isolated from bacteria, fungi and even algae, and many of these general elicitors have been reported to activate also the animal innate immune system (Nürnberg and Brunner, 2002).

General plant elicitors identified so far belong to chemical classes such as saccharides, lipopolysaccharides, proteins, glycoproteins and sterols. One group of elicitors consists of structures forming the cell wall of microorganisms, including fungal oligosaccharides such as chitin and  $\beta$ -glucans (Shibuya and Minami, 2001), lipopolysaccharides from gram negative bacteria (Dow et al., 2000), as well as sulfated fucan oligosaccharides and  $\beta$ -1,3-glucans from marine algae (Klarzynski et al., 2000; Klarzynski et al., 2003). Some elicitors are associated with the plasmamembrane such as fungal ergosterol (Granado et al., 1995) or bacterial harpin (Wei et al., 1992; Baker et al., 1993; He et al., 1993). Another group of elicitors includes molecules secreted by microorganisms, associated more or less closely with the surface of the microorganisms. This category includes invertase from yeast (Basse et al., 1992), elicitins (a family of low molecular weight proteins) (Ricci et al., 1993; Kieffer et al., 2000) as well as transglutaminase (Hahlbrock et al., 1995; Brunner et al., 2002) typical of *Phytophthora* species, necrosis-inducing proteins from *Phytophthora* and *Fusarium* species (Fellbrich et al., 2002; Keates et al., 2003) and bacterial flagellin (Felix et al., 1999). Enzymes secreted by microorganisms for degradation of plant cell walls such as xylanases, endopolygalacturonases or pectinases can either be perceived by plants directly, i.e. by their protein structure, as shown for xylanase (Enkerli et al., 1999; Poinssot et al., 2003)), or via their enzymatic activity by releasing endogenous elicitors from plant cell walls (reviewed in (Fry et al., 1993)). Furthermore, also proteins localized within intact cells such as bacterial cold shock protein (Felix and Boller, 2003) or elongation factor Tu (EF-Tu) (Kunze et al., 2004) have recently been shown to be perceived by plant cells and to trigger defense responses at very low concentrations.

For many elicitors, the minimal structure required for perception and elicitation of plant defense responses has been identified. The following section gives an overview.

**Oligosaccharides.** Several oligosaccharides from fungal cell walls activating defense responses in plants have been described. (Sharp et al., 1984; Yamaguchi et al., 2000) showed that plants can perceive highly specific structures of glucans derived from the cell walls of the oomycete *Phytophthora sojae* f. sp. *glycinae* or the rice blast fungus *Magnaporthe grisea* respectively. One elicitor is a hepta- $\beta$ -glucoside with a 1,6-linked  $\beta$ -glucopolysaccharide as a backbone and branches at the 3-position of two 6-linked glucosyl residues, the other elicitor is a  $\beta$ -glucopentaose with a 1,3-linked  $\beta$ -oligosaccharide as a backbone branched at the 6-position of one 3-linked residue. Closely related glucosides differing for example only in the position of the glucosyl residues had low or no elicitor activity. However, both elicitors activate defense responses only in particular test systems of specific plant species, namely in soybean cotyledons (heptagluoside) and in rice cell cultures. In contrast, chitin is a potent elicitor in many plant species including *Arabidopsis*, tomato, melon, wheat and barley (Yamaguchi et al., 2000), and the recognized structures, linear  $\beta$ -1,4-linked oligomers of N-acetylglucosamines, are much less complex. However, various systems preferentially recognize chitin fragments of different size. While rice and wheat cell cultures perceive larger oligosaccharides (rice and wheat: hepta- to octamers), tomato cells react equally well to tetra- to decamers (Felix et al., 1993; Yamaguchi et al., 2000). Oligogalacturonides set free from pectic polysaccharides of plant cell walls by fungal pectin lyases and pectinases have been known to act as so called endogenous elicitors (Côté and Hahn, 1994). Oligogalacturonides with a degree of polymerisation from 4 to more than 20 are most active, depending on the test system (Simpson et al., 1998; Shibuya and Minami, 2001). Generally, higher concentrations of oligogalacturonides are required to show elicitor activity compared to other oligosaccharide elicitors (Shibuya and Minami, 2001).



**Glycopeptides.** The minimal structure of yeast invertase inducing ethylene production has been identified as a glycopeptide (Basse et al., 1992). The glycopeptide consists of at least two amino acids, one of them being an asparagine carrying an N-linked glycan side chain with 10-12 mannosyl residues. Glycopeptides with only 8 mannosyl residues had a 100 fold lower activity. Furthermore, they demonstrated that the glycan part alone acts as a suppressor of defense responses induced by the glycopeptide. This result suggests that the glycan part is necessary for binding to a receptor and the peptide part for activation of defense responses.

**Peptides.** For several proteins and glycoproteins, the structure necessary and sufficient to induce defense responses in plants has been identified as a relatively small (13 to 22 amino acids), surface exposed and highly conserved domain. A synthetic peptide comprising 22 amino acids of a highly conserved domain within the N-terminus of bacterial flagellin (called flg22) has been shown to be a potent elicitor of plant defense responses in several plant species (Felix et al., 1999). Tomato cells could also perceive smaller peptides comprising 15 to 21 amino acids. (Meindl et al., 2000) showed that binding of flagellin to a receptor and activation of defense responses can be attributed to distinct N- and C-terminal domains, according to the address-message concept: While the N-terminus of the flagellin peptide is required for binding to a high affinity binding site, the C-terminus is necessary for activation of the receptor. This result explains why peptides consisting of less than 10 amino acids of the N-terminal part of flg22 were inactive as inducers of plant defense responses, and even inhibited the response of tomato cells to flg22 (Felix et al., 1999). (Brunner et al., 2002) identified a sequence consisting of 13 amino acids (Pep-13) as the elicitor-active part of the 42 kDa glycoprotein transglutaminase associated with the cell wall of *Phytophthora* species. They showed that within Pep-13, the same amino acids are indispensable for both the activity of the enzyme and for elicitation of defense-responses. Only recently, the domains recognized within elicitor-active bacterial cold-shock protein (Felix and Boller, 2003) and bacterial elongation factor EF-Tu (Kunze et al., 2004) have been identified as highly conserved domains consisting of 15 or 18 amino acids respectively, the latter denoted as elf18. These two peptides represent a new type of plant elicitor, because they are not derived from external structures but are usually situated in the cytoplasm of intact cells. Until now, intracellular PAMPs have only been known from the animal field. As an example, animal cells have been shown to perceive heat shock proteins (Seo et al., 1995; Hayashi et al., 2001) and bacterial DNA (Hayashi et al., 2001).

**Proteins and enzymes.** In contrast to the peptide-elicitors described above, (Fellbrich et al., 2002) found that the intact protein structure as well as two cystein rich residues were essential for elicitor activity of necrosis inducing protein NPP1, a 24 kDa protein isolated from *Phytophthora parasitica* cell walls. Plant cell wall degrading enzymes secreted by fungi can activate plant defense responses by liberating molecules such as oligogalacturonides and cutin monomers from plant cell walls (Collmer and Keen, 1986; Schweizer et al., 1996). Yet, some authors found that defense responses induced by cell wall degrading enzymes were much stronger and had other kinetics than would be expected by liberated endogenous elicitors only (Rouet-Mayer et al., 1997; Poinssot et al., 2003). (Enkerli et al., 1999) were the first to demonstrate conclusively that plants can sense the proteins themselves. In addition, plant cells might respond to changes in the turgor pressure and subsequent volume increases caused by the enzymatic degradation of their cell wall (Trewavas and Knight, 1994; Felix et al., 2000).

## Induced resistance: from the lab to the field

The idea to use the phenomenon of induced resistance to protect crops against disease is tempting. While avr-R-gene mediated host resistance acts only against a particular race of a pathogen and can easily be overcome by mutations in the pathogen population, induced resistance involves the activation of a more basal set of defense mechanisms and is therefore very stable. However, in contrast to R-avr-gene triggered defense responses, induced resistance normally does not completely prevent disease but rather reduces

it.

Two different strategies have been pursued to make use of induced resistance. One strategy is to upregulate positive regulators of non-host and induced resistance such as NPR1 (SA-dependent signalling) and ETR1 (ethylene-dependent signalling) (Delaney et al., 1995; Berrocal-Lobo et al., 2002) or to downregulate negative regulators such as EDR1 (Frye and Innes, 1998; Frye et al., 2000) by genetic engineering. However, there is growing evidence that constitutive expression of inducible defense mechanisms might be costly and finally result in lower yields (Brown, 2002; Heil and Baldwin, 2002). The other strategy is to activate inducible defense mechanisms by appropriate stimuli only when crops are threatened by pathogens. Different types of stimuli inducing defense have been described, including (i) living pathogenic microorganisms, (ii) living non-pathogenic microorganisms such as plant growth promoting rhizobacteria (iii) more or less purified extracts from microorganisms or preparations from plant cell walls containing exogenous or endogenous elicitors (iv) plant extracts as well as (v) 'natural' and 'synthetic' chemicals referred to as 'inducers'.

The application of pathogenic microorganisms for induction of resistance is hardly feasible for agricultural practice. In contrast, PGPR have been shown to protect plants efficiently under commercial greenhouse as well as field conditions. Various products based on PGPR are available (van Loon et al., 1998). The effect of several synthetic and natural chemicals such as SA, BABA, BTH and probenazole has been documented under field conditions. Some of them have even been commercialized, e.g. BTH as Bion® and probenazole as Oryzemat® (Cohen et al., 1994; Sticher et al., 1997). In contrast, an agricultural application of SA, the 'classical' inducer of SAR, is not feasible because SA is not stable and can be toxic to plants in the doses required to induce resistance (Kessmann et al., 1994). An extract of the giant knotweed *Reynoutria sachaliensis* (sold as Milsana®) activates resistance mechanisms which are particularly effective against powdery mildews (Herger et al., 1988; Herger et al., 1989; Herger and Klingauf, 1990; Konstantinidou-Doltsinis and Schmitt, 1998). Compared to the large body of literature on defense-related responses induced by general elicitors, relatively little is known on their effect on plant-pathogen interactions, particularly on their efficacy on crop plants under field conditions. However, it has been demonstrated that even synthetic peptides inducing early defense-related responses in plant cell cultures such as flg22 and elf18 can induce resistance against *P. syringae* pv. *tomato* in *Arabidopsis* (Zipfel et al., 2004). Other general elicitors have been shown to reduce disease under greenhouse conditions, including laminarin on grapevine against *Botrytis cinerea* and on tobacco against *Erwinia carotovora* (Klarzynski et al., 2000; Aziz et al., 2003), sulfated fucan oligosaccharides on tobacco against tobacco mosaic virus (Klarzynski et al., 2003), and an endopolygalacturonase on grapevine against *Plasmopara viticola* (Poinsot et al., 2003). The bacterial protein harpin induced resistance on *Arabidopsis* against *Peronospora parasitica* and *Pseudomonas syringae* pv. *tomato* (Dong et al., 1999). Harpin has been shown to be effective on several crops against various diseases under field conditions, and is commercially sold (Messenger®, Eden Bioscience) (Wei et al., 1998).

Especially for organic agriculture, for whose products the demand has increased highly in the last decades (Tamm, 2001), it is important to substitute chemicals in plant protection and to apply improved biological methods (Schneider and Ullrich, 1994). The substitution of traditional fungicides such as copper and sulphur, widely used in conventional as well as in organic agriculture, by other, environmental friendly products, has been a major focus of organic agriculture in the last few years (Speiser et al., 2000). The concept of induced resistance is well known in organic agriculture. Induced resistance is supposed to be jointly responsible for a phenomenon called 'plant strengthening', which was observed already by the pioneers of organic agriculture after the application of herb and compost extracts.

However, despite excellent efficacy of many inducers and elicitors (including commercialized products) under controlled conditions, they often fail to perform sufficiently in practice (L. Tamm, personal communication). In addition, substances to be applied in organic agriculture have to fulfill several criteria

(EU Council Regulation (EEC) No. 2092/91, 1991; EU Council Regulation (EEC) No. 1488/97, 1997; IFOAM Basic Standards for Organic Agriculture, 2000; Tamm, 2001). One critical point is the way of production. Only natural products or products identical to natural products may be used. Furthermore, natural products may not be obtained from genetically modified organisms (GMOs). Thus, products such as Bion® (containing the synthetic compound BTH) or Messenger® (containing harpin obtained from genetically modified bacteria) may not be used.

The development of novel plant activators (inducers and elicitors) is therefore of high interest for organic agriculture. To make an application feasible in practice, they have to fulfil several criteria. (i) They have to perform well and reproducibly under field conditions (ii) be consistent with the guidelines of organic agriculture, (iii) be available in sufficient amounts and (iv) in constant quality and (v) to be relatively cheap.

In the 1990ies, attention fell on an aqueous extract from the dry mycelium of the ascomycete *Penicillium chrysogenum*, further called 'Pen'. Preliminary studies suggested that spraying Pen on leaves or adding it to the soil can enhance disease resistance of many plants against several pathogens (E. Mösinger, Sandoz AG Switzerland, personal communication; (Dong and Cohen, 2001, 2002). Pen fulfilled most of the criteria for a plant activator, i.e. (i) *P. chrysogenum* is not a GMO, (ii) the mycelium of *P. chrysogenum* is obtained as a by-product from penicillin production, is thus available in large amounts and is relatively cheap, and (iii) the procedure of penicillin production is highly standardized, thus relatively constant quality can be expected.

## Outline of this thesis

In chapter 2, the effect of Pen on several crop plants against various pathogens in the greenhouse and in the field is reported. Special focus is given to the efficacy of Pen against downy mildews. Furthermore, the question whether the mycelium of *P. chrysogenum* is available in constant quality is investigated. We tested extracts from 30 batches of mycelium in a tomato-*Phytophthora infestans* bioassay. Side effects of Pen are discussed. Significant parts of this work were done by the diploma student Christina Rentsch, by Urs Guyer and Sonia Jiménez-Jiménez.

In chapter 3, the range of action of Pen on the model plant *Arabidopsis thaliana* is investigated, i.e. we tested whether Pen induces resistance against a bacterium (*Pseudomonas syringae* pv. *tomato*), an oomycete (*Peronospora parasitica*) and two ascomycetes (*Botrytis cinerea*, *Alternaria brassicicola*). We addressed the question whether one or several of the well-known secondary signalling molecules SA, JA and ethylene or the key-substance NPR1 are required for Pen-mediated resistance against *P. parasitica* and *B. cinerea*. We also assess the potential of Pen to induce early defense-related responses such as extracellular alkalinisation, ethylene production and an oxidative burst. We used suspension cultured cells and leaf tissue of several plant species as test systems.

The work done in chapter 4 aims at the characterization and identification of the elicitors in Pen responsible for induced resistance. We used extracellular alkalinisation and ethylene production as fast and convenient bioassays to monitor the purification process.

In chapter 5, we discuss our results on the aspect of a future application of Pen in practice.

Chapters 2 to 4 have been written as publications, which will be submitted. Therefore, some parts of the general introduction have also been used for the introductions of the individual chapters.



## CHAPTER II

### **An aqueous extract of the dry mycelium of *Penicillium chrysogenum* induces resistance in several crops under greenhouse and field conditions**

#### **Abstract**

We have examined the effect of Pen, an aqueous extract of the dry mycelium of *Penicillium chrysogenum*, on plant-pathogen interactions. Pen was effective against a broad range of pathogens on several crop plants under greenhouse and field conditions. Pen protected grapevine from downy and powdery mildew (*P. viticola* and *U. necator*), tomato from early blight (*P. infestans*), onion from downy mildew (*P. destructor*) and apple tree from apple scab (*V. inaequalis*) to a similar extent as standard fungicides such as copper and sulphur or well-known inducers such as Bion or BABA. Pen had no direct fungicidal effect and is thus supposed to protect plants by activating their defense mechanisms. The raw material for extraction of Pen was available in constant quality, a prerequisite for an application in practice. However, Pen often caused phytotoxic side effects. The symptoms mostly consisted in small necrotic spots or, more rarely, in larger necrotic areas. The development of the symptoms was dependent on several parameters, including concentration of Pen, the number of applications, the persistence on the plant tissue, the plant species and variety as well as environmental conditions. A partially purified fraction of Pen was much less toxic than the crude Pen extract, but protected grapevines to a similar extent against *P. viticola*. Our data show that Pen has interesting and unique properties as an inducer of plant disease resistance, but more research is needed to further reduce its phytotoxic side effects.

## Introduction

In agriculture, infection of plants with microorganisms including fungi, bacteria and viruses can cause high losses of yield (Agrios, 1997). The complete breakdown of the wine industry in western Europe, particularly in France, after the introduction of downy mildew (*Plasmopara viticola*) from America to Europe in the 1870ies is only one example (Singh, 2000). To prevent damage due to pathogens, several methods have been developed (Agrios, 1997). On the one hand, indirect techniques are well-known, including the use of high quality propagation material, sanitation (e.g. removal of overwintering sources of inoculum or of infected volunteer plants), avoidance techniques, crop rotation, soil management, plant nutrition and the selection of resistant varieties. On the other hand, diseases are directly controlled by the application of pesticides or, more rarely, antagonists. In addition, the concept of induced resistance provides a promising strategy for the control of diseases (Hammerschmidt, 1995; Agrios, 1997).

It has long been known that plants can develop enhanced resistance to a broad spectrum of pathogens upon contact with necrotising pathogens (Ross, 1961). Later, it has been found that resistance can also be induced by various non-pathogenic root-colonizing pseudomonads (van Loon et al., 1998) and by treating plants with various natural or synthetic compounds. Induced resistance can be expressed at the site of treatment only, called local acquired resistance (LAR). If non-treated, remote parts of the plant are protected as well, the phenomenon is referred to as systemic acquired resistance or induced systemic resistance (SAR, ISR) (Kuc, 1983; Pieterse et al., 2000). Mechanisms involved in induced resistance can include (i) strengthening of the cell wall by enhanced crosslinking of cell wall components or deposition of molecules such as lignin, callose or silica (Stumm and Gessler, 1986; Schmele and Kauss, 1990; Matern et al., 1995; Sticher et al., 1997), (ii) production of antimicrobial proteins (pathogenesis-related (PR) proteins) or low molecular substances, so-called phytoalexins (van Loon, 1999; Dixon, 2001), and (iii) a hypersensitive response characterized by rapid cell death and local necrosis (Mittler and Lam, 1996).

The potential to control diseases by inducing resistance through life, non-pathogenic rhizobacteria, by crude extracts from microorganisms (and plants) or by natural or synthetic chemical compounds has long been recognized (Kuc, 2001). Extracts or chemical compounds inducing resistance are often referred to as 'plant activators', 'inducers' or, if derived from microorganisms, 'elicitors'. Inducers do not have a direct impact on pathogens, which clearly distinguishes them from fungicides (Kuc, 1983; Kessmann et al., 1994). Examples for inducers are salicylic acid (SA) (Ward et al., 1991), isonicotinic acid (INA) (Ward et al., 1991), jasmonic acid, benzothiadiazoles (BTH) (the active compound of Bion®) (Friedrich et al., 1996; Gorlach et al., 1996), probenazole (the active compound of Oryzmate®) (Sekizawa and Mase, 1980),  $\beta$ -aminobutyric acid (BABA) (Cohen et al., 1994), the bacteria-derived elicitor harpin (the active compound of Messenger®) (Dong et al., 1999) as well as various crude extracts from microorganism or plants (e.g. Milsana®, an extract from the giant knotweed *Reynoutria sachaliensis*) (Daayf et al., 1997; Konstantinidou-Doltsinis and Schmitt, 1998).

Especially in organic agriculture, for whose products the demand has increased highly in the last decades (Tamm, 2001), it is important to substitute chemicals used in plant protection, e.g. copper and sulphur, and to apply improved biological methods (Schneider and Ullrich, 1994). The concept of induced resistance is well known in organic agriculture. Induced resistance is supposed to be jointly responsible for a phenomenon called 'plant strengthening', which was observed already by the pioneers of organic agriculture after the application of herb and compost extracts. To integrate induced resistance into commercial agriculture, inducing substances have to be available in sufficient quantities and in constant quality. In addition, synthetic chemical compounds not occurring in nature may not be used in organic agriculture (Codex Alimentarius Commission, 1999; OMRI Generic Materials List with the National Organic Program Final Rule Listings, 2001; Speiser et al., 2004).

In the 1990ies, attention fell on an aqueous extract of the mycelium of the ascomycete *Penicillium*

*chrysogenum*, further called Pen. Preliminary studies suggested that spraying this extract on leaves or adding it to the soil can enhance disease resistance of many plants against several pathogens (E. Mössinger, Sandoz AG Switzerland, personal communication; (Dong and Cohen, 2001, 2002). The mycelium of *P. chrysogenum* is obtained as a by-product from penicillin production. It is relatively cheap and available in sufficient amounts, both prerequisites for a potential use in practice.

In this study, we examined the effect of Pen on several plant-pathogen interactions under greenhouse and field conditions. We demonstrate that Pen reduces disease severity of several pathogens on different agriculturally important plant species, including *Plasmopara viticola* on grapevine and *Phytophthora infestans* on tomatoes. We show that mycelium for extraction of Pen is available in constant quality. We conclude that Pen provides an interesting alternative to fungicides such as copper. However, more work is needed to reduce the phytotoxic side effects.

## Material and Methods

### Inducers and fungicides

#### Preparation of the Pen extract

Pen extract was prepared from the dry mycelium of *Penicillium chrysogenum* obtained from Sandoz GmbH (Kundl, Austria). The mycelium of a high penicillin-producing strain of *P. chrysogenum* was produced on industrial scale. To extract penicillin, n-butylacetate was added to the mycelium-medium mixture (1:2) and pH adjusted to 1 to 3 with H<sub>2</sub>SO<sub>4</sub>. The butanol-phase was removed by decantation and the aqueous phase including the mycelium was stored in tanks for 12 to 36 h before removing the remaining butylacetate by distillation (50 to 60°C for 5 min). Then, the mycelium was dried for 3 h at 140°C. The dry mycelium of *P. chrysogenum* does not contain penicillin contaminations because penicillin is not heat stable. Nevertheless, individual batches are checked for absence of penicillin by routine quality assurance systems (Sandoz GmbH, personal communication). To prepare the extract 'Pen', 150 g of the dry mycelium was added to 1 liter demineralized water. The suspension was either shaken at 75 rpm for 16 h at room temperature or autoclaved for 3 h at 120°C. The water soluble part was separated from the mycelium by filtration over a layer filter (K-200, Seitz) or over a cellulose filter (no. 595, Schleicher&Schuell). The crude, aqueous Pen extract was subsequently stored at 5°C in the dark. A fraction >2000 Da (=Pen<sub>2000</sub>) was prepared by dialysing the crude Pen extract in dialysis tubes with a cut-off of 2000 Da (Spectra/Por® 6, Socochem AG) for 48 h at 5°C.

To prepare the standard Pen extract used for most experiments, mycelium of two production batches (97/15 and 99/12) was used. To test variability of batches over time, a total of 30 batches dating from 1993 to 1999 were used, which were extracted as described above.

The crude aqueous Pen extract contained on average 45 g/l dry matter, dialysis reduced the content of Pen<sub>2000</sub> to 12 g/l. All concentrations of Pen are indicated in g dry matter per liter water. If not otherwise mentioned, Pen was applied at concentrations of 45 g/l and Pen<sub>2000</sub> at 12 g/l.

#### Other inducers and fungicides

As reference inducers, either benzothiadiazole (BTH) (Bion®, Syngenta AG) or β-amino butyric acid (BABA, Fluka Chemie GmbH) were used at concentrations of 0.05 g/l (Bion), 0.1 g/l (BABA field) or 1 g/l (BABA greenhouse). In field experiments, the standard fungicides Myco-San (10 g/l), Myco-Sin (7.7 g/l) (both Schaeffle GmbH), sulphur (5 g/l) and copper (0.5 g/l) were used.

#### Testing for fungicidal effects *in vitro*

The effect of the crude Pen extract on growth of *P. infestans* and *C. lagenarium* was examined *in vitro* on



agar plates containing an appropriate growth medium (rye agar or potato carrot agar respectively). Three holes were cut out in equal distances from the center and filled with the test substance. A mycelial plug was placed in the center. Test substances were Pen (45, 30 and 15 g/l), water and the standard fungicides metalaxyl against *P. infestans* (0.01 g/l and 0.1 g/l) (Ridomil®, Syngenta AG) or dithianon against *C. lagenarium* (0.5 g/l) (Delan®, Siegfried Agro AG). Mycelial growth was assessed after 14 d. Furthermore, the crude Pen extract (4.5 g/l) was tested for direct inhibitory effects on a broad range of pathogens (*Alternaria brassicicola*, *Botrytis cinerea*, *Fusarium culmorum*, *Pyricularia oryzae*, *Pythium ultimum*, *Rhizoctonia solani* and *Septoria nodorum*) using industry standard methods (Syngenta AG, Stein, Switzerland). A test substance was considered fungicidal if mycelial growth was limited or prevented as compared to the water control.

The inhibitory effect of Pen on the germination of sporangia of *P. infestans* was tested on agar plates (rye agar) containing water, the fungicide chlorothalonil (0.01 g/l) (Bravo®, Syngenta) or the crude Pen extract (1.6 or 3.3 g/l). Germination rates were assessed after 28 and 50 h.

### Pathogens

Sporangia of the obligate biotrophs of *Plasmopara viticola* and *Pseudoperonospora cubensis* were obtained by washing infected grapevine or cucumber leaves respectively with distilled water. Several isolates of *Phytophthora infestans* were used. For initial experiments, two *P. infestans* isolates from potato plants were used (Syngenta AG, isolates 4-8 and 5-8). For later experiments, two isolates of *P. infestans* were obtained from infected tomato plants (isolates 98002 and 98003). *P. infestans* was grown on rye agar at 18-22°C in the dark. Sporangia were collected from 2 week old cultures by gently scratching with a glass rod. *Colletotrichum lagenarium* was grown on potato carrot agar at 18-22°C in the dark. To obtain conidia for experiments, the fungus was cultivated once on rice polish agar. Conidia were harvested from 6 to 7 day old cultures by gently scratching with a glass slide. All pathogens except the two *P. infestans* isolates from tomato were kindly provided by Syngenta AG (Stein, Switzerland). In field experiments, infection occurred naturally.

### Plant material

**Grapevine.** Seedlings of grapevine (*Vitis vinifera*) cv. 'Chasselas' were used for greenhouse assays. Small seedlings (kindly provided by Syngenta AG, Stein, Switzerland) were transplanted to pots (Ø 8 cm) containing soil (Einheitserde Typ 0, Patzer GmbH & Co) and 3 g/l Tardit 3M (Hauert & Co). Grapevine, tomato and cucumber plants were grown in the greenhouse at a temperature of 18 to 28°C under natural light. In winter time, light intensity was increased by lamps (Radium lamps 250 W/D, 12-15 kLux) and extended to a day period of 16 h light. Plants were used for experiments when they had 5 to 8 fully expanded leaves. Field experiments were carried out on grapevines cv. 'RieslingxSylvaner' and 'Chasselas' (both on rootstock 5BB) in Frick, Switzerland. Soil fertility management and weed control were carried out according to standards of organic agriculture.

**Tomato.** Tomato plants (*Lycopersicon esculentum*) cv. 'Supermarmande' were grown in peat-rich and pre-fertilized soil ('Torf-Spezialsubstrat', Blumenerdenwerk Stender or 'Einheitserde Typ P' (Patzer GmbH & Co). Seedlings were transferred at the 2-leaf stage to pots (Ø 12 cm) containing the same peat-rich and pre-fertilized soil or soil ('Einheitserde Typ 0') mixed with perlit (2:1). Plants were fertilized once a week with a mineral fertilizer. Plants were used for experiments when they had 6 to 8 fully expanded leaves.

**Cucumber.** Cucumber plants (*Cucumis sativus*) cv. 'Aramon F1' were grown in peat-rich and pre-fertilized soil in the greenhouse ('Torf-Spezialsubstrat', Blumenerdenwerk Stender). At the 1-leaf stage they were transferred to pots (Ø 12cm). Plants were fertilized once a week with a mineral fertilizer.

**Apple trees.** Field experiments were carried out on apple trees (*Malus domestica*) cv. 'Rubinette' in Frick, Switzerland. Soil fertility management and weed control were carried out according to standards of organic



agriculture.

*Potato.* Potato plants (*Solanum tuberosum*) cv. 'Agrida' were grown in an experimental field in Frick, Switzerland.

*Onion.* Onion plants (*Allium cepa*) cv. 'Centurion' (set onions) were grown in the field according to commercial practice in Holzikon, Switzerland.

### Experimental design

All experiments in the greenhouse and in the field were conducted in a completely randomized block design with 6 (all greenhouse experiments), 9 (apple tree and grapevine in the field), 4 (potato) or 3 (onion) replicates, according to EPPO guidelines (Guideline for the efficacy evaluation of plant protection products, 1999).

### Treatment of plants

For greenhouse experiments, plants were sprayed by means of a hand-sprayer till near run-off. Treatments were performed 7 d before inoculation. Treated grapevine seedlings were kept in the humidity chamber (100% RH, 20-21°C) for 24h, and then transferred back to the greenhouse. In field experiments, plants were weekly sprayed by means of a 'Balkenspritze', a knap-sack sprayer or a high-pressure hand-sprayer till near run-off.

### Inoculation, incubation and disease assessment

Tomato and grapevine plants were drop inoculated with *P. infestans* or *P. viticola*. Drops of 5-7 µl (40'000 sp/ml) or 10 µl (100'000 sp/ml) were applied on tomato or grapevine leaves respectively. After inoculation, plants were incubated in the humidity chamber (100% relative humidity (RH), 14 h light, 5 kLux) for 48 h at 18 to 20°C (tomato) or 24 h at 20°C (grapevine). Tomatoes were subsequently kept in the humidity chamber but relative humidity was lowered to 80 to 95%. Grapevine plants were transferred to growth chambers (60% RH, 14 h light, 20°C during day, 18°C during night) and brought back to the humidity chamber the evening before scoring in order to initiate sporangia production. Disease of tomato plants was assessed 5 to 7 days after inoculation, disease of grapevine plants after 7 d.

Cucumber plants were sprayed with conidia or sporangia suspensions of *C. lagenarium* or *P. cubensis* (200'000 sp/ml) by means of a hand-sprayer till near run-off. Plants were kept for 24 h in the humidity chamber in the dark (100% RH, 18°C), and then transferred back to the greenhouse. Disease was assessed 7 d after inoculation.

In the field, infection occurred naturally. At least 50 leaves of each grapevine plant and of each apple tree were checked for symptoms. For onions, in each replicate 100 leaves were checked for symptoms.

To assess disease, the parameters of incidence (affected leaves/(total leaves inoculated or number of leaves counted)), severity (percentage of damaged leaf area) and/or lesion diameter were used. Lesion diameters of the largest lesion (onion), the 5 largest lesions (grapevine in the field) or of all visible lesions (tomato) per plant were measured. The necrotic leaf area caused by the treatments was assessed in greenhouse experiments 3 to 14 d after treatment and in field experiments simultaneously with disease assessment.

### Calculations and statistics

Efficacy was calculated according to (Abbott, 1925) as follows: efficacy (%) =  $100(1-a/b)$ , with a = disease severity (or disease incidence or lesion diameter) of treatment and b = disease severity (or disease incidence or lesion diameter) of control. Relative efficacy (%) of a sample was calculated as  $100((\text{efficacy sample}/\text{efficacy standard Pen extract})-1)$ .

Data were analysed by ANOVA followed by a Tukey test at  $\alpha = 0.05$  for multiple comparisons (Zar, 1996). All analyses were done using SPSS (version 10.0, SPSS Inc.).

## Results

### Pen does not contain a major fungicidal activity

The crude Pen extract did not inhibit the growth of *P. infestans* and *C. lagenarium* *in vitro*. In addition, Pen was tested for an inhibitory effect on a broad range of other pathogens, including *Alternaria brassicicola*, *Botrytis cinerea*, *Fusarium culmorum*, *Pyricularia oryzae*, *Pythium ultimum*, *Rhizoctonia solani* and *Septoria nodorum*. Pen only slightly inhibited the growth of one species (*P. oryzae*), but had no or even a growth promoting effect on all other species. Furthermore, tomato plants from which Pen had thoroughly been washed off before inoculation with *P. infestans* were still significantly protected (fig. 3). However, Pen reduced the germination rate of sporangia from *P. infestans* *in vitro* in one experiment.

### Pen protects grapevine against *P. viticola* and *U. necator*

Grapevine plants treated with the crude Pen extract were significantly less infected by *P. viticola* than water-treated control plants (fig. 1). In three field seasons, Pen reduced disease severity on average by 67% and disease incidence by 40%, and was thus comparable to the contact fungicide copper and at least equal to the inducers Bion or BABA (fig. 1 and tab. 1). In 2003, Pen reduced disease severity even by 90% and in 1997, a year with much higher disease pressure, still by 51%. In 1997, the diameters of the five largest lesions per plant were measured in addition. Pen significantly reduced the lesion diameter by 37% (data not shown). In contrast, neither Bion nor copper decreased the mean size of the largest lesions. In 2003, the crude Pen extract as well as Pen<sub>2000</sub>, a size fraction of the crude Pen extract (>2000 Da), were tested. Efficacy of Pen<sub>2000</sub> was comparable to the efficacy of the crude Pen extract. Results from the field could be confirmed under greenhouse conditions on grapevine seedlings, where both Pen and Pen<sub>2000</sub> reduced disease severity in six independent experiments on average by 88% compared to control plants.

In 1998, disease pressure by *U. necator* was very high in the field. Under these conditions, Pen reduced disease severity from 73% in control plants to 3% and was thus as effective as the standard fungicides sulphur or Mycosan (table 1).

### Pen protects apple trees against *V. inaequalis*

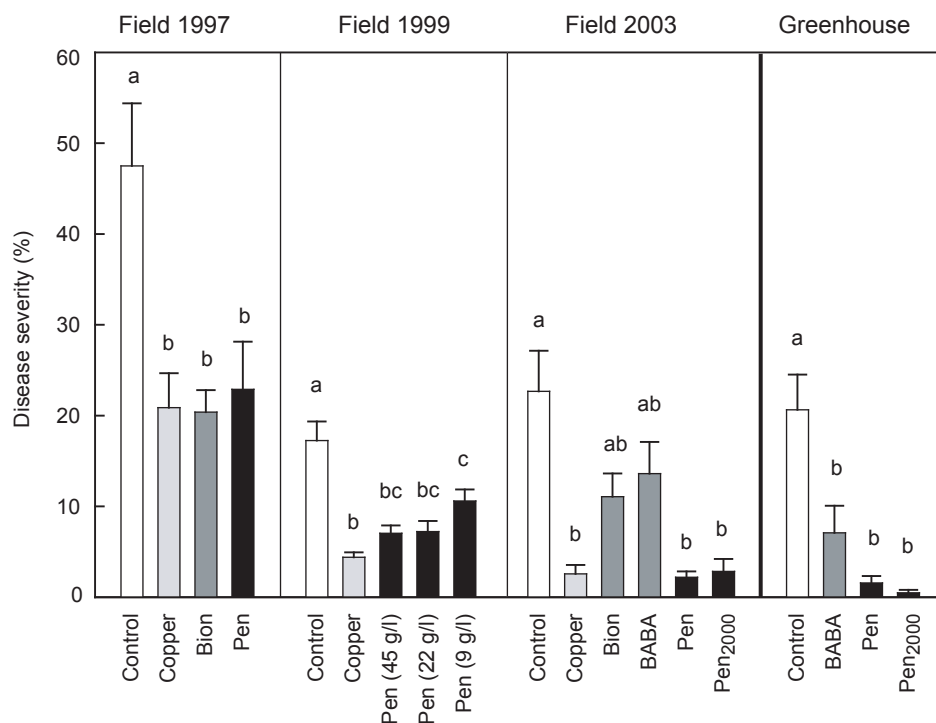
Under field conditions, apple trees treated with the crude Pen extract were significantly less infected by *V. inaequalis*, the causal agent of apple scab, than water-treated control plants (fig. 2 and tab. 1). In two different years, Pen reduced disease severity by 89% and 93% respectively as well as disease incidence by 32 and 42%, although disease pressure was quite high. The efficacy of Pen was comparable to the standard fungicide copper. Pen<sub>2000</sub> reduced disease severity significantly less than the crude Pen extract (fig. 2). However, efficacy of Pen<sub>2000</sub> was still comparable to the efficacy of standard fungicides like sulphur or mycosin/mycosan.

### Pen protects tomato against *P. infestans*

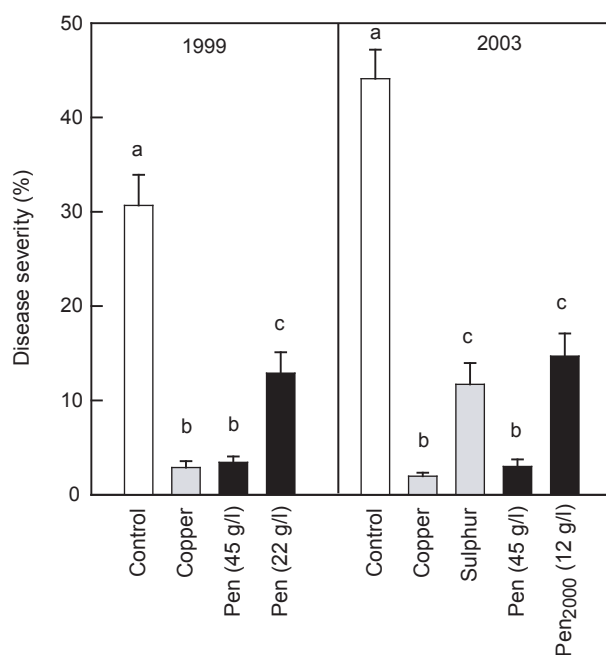
Tomato plants treated with the crude Pen extract were significantly less infected by *P. infestans* than water-treated control plants under greenhouse conditions (fig. 3 and tab. 1). In 33 independent experiments, Pen reduced disease severity on average by 71% and disease incidence by 41%. Bion reduced disease severity on average by 41% and disease incidence by 18% (9 independent experiments) (tab. 1). When Pen was thoroughly rinsed off the leaves prior to inoculation with *P. infestans*, disease severity was still significantly reduced and efficacy was comparable to non-rinsed off Pen (fig. 3).

### Pen protects cucumber against *C. lagenarium* and *P. cubensis*

Pen reduced disease severity of *C. lagenarium* and *P. cubensis* on cucumber in the greenhouse in 6 or 2 independent experiments on average by 24% or 27% respectively. The efficacy of Pen varied largely between experiments (0 to 65%) (tab. 1), but disease reduction was statistically not significant in any of

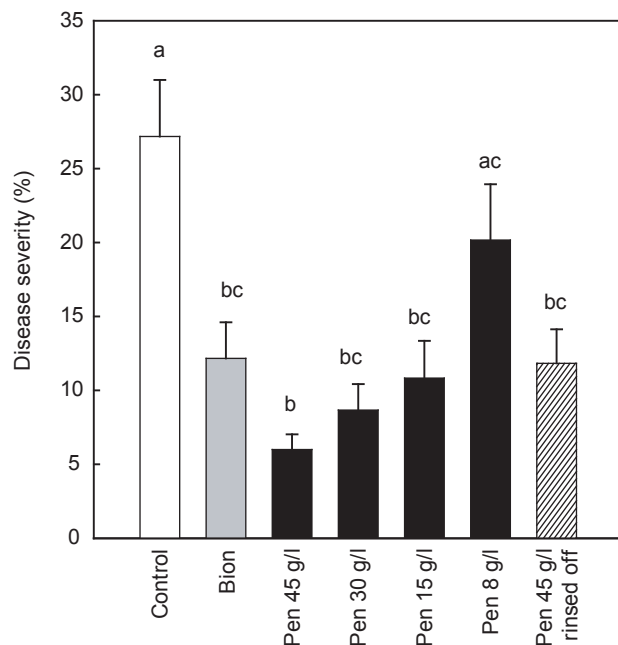


**Figure 1.** Effect of Pen on grapevine against *P. viticola* in the field and the greenhouse. Grapevine plants cv. 'Riesling x Sylvaner' (field) or 'Chasselas' (greenhouse) were treated with water (white), copper (0.5 g/l) (light gray), the inducers Bion (0.05 g/l) or BABA (field 0.1 g/l, greenhouse 1 g/l) (dark gray), the crude Pen extract (45 g/l if not mentioned otherwise) or Pen<sub>2000</sub> (12 g/l) (black). The figure shows results from three field seasons and representative results from one out of six experiments in the greenhouse. Bars show percentage diseased leaf area (mean and standard error). Different letters indicate statistically significant differences (pairwise comparisons, Tukey test,  $p < 0.05$ ).



**Figure 2.** Effect of Pen on apple trees against *V. inaequalis* in the field in 1999 and 2003. Apple trees cv. 'Rubinette' were weekly treated with water (white), copper (0.5 g/l), sulphur (5 g/l) (gray), different concentrations of the crude Pen extract or Pen<sub>2000</sub> (12 g/l) (black). Bars show percentage diseased leaf area (mean and standard error). Different letters indicate statistically significant differences (pairwise comparisons, Tukey test,  $p < 0.05$ ).

the experiments. In contrast, Bion significantly reduced disease severity of both pathogens on average by about 50%. In all treatments, disease incidence was nearly 100%, i.e. almost all leaves were infected but to different degrees.



**Figure 3.** Effect of Pen on tomato against *P. infestans* in the greenhouse. Tomato plants cv. 'Supermarmande' were treated with water (white), Bion (0.05 g/l) (gray) or different concentrations of the crude Pen extract (black) one week before inoculation. In one treatment, Pen was thoroughly rinsed off before inoculation (hatched). The figure shows representative results from one out of 33 experiments. Bars show percentage diseased leaf area (mean and standard error). Different letters indicate statistically significant differences (pairwise comparisons, Tukey test,  $p < 0.05$ ).

#### Pen does not protect potato against *P. infestans*

Pen treatment did not reduce disease severity or incidence of *P. infestans*, the causal agent of early and late blight, on potato. In contrast, copper significantly reduced disease severity from 86% in control to 22%.

#### Pen protects onions against *P. destructor*

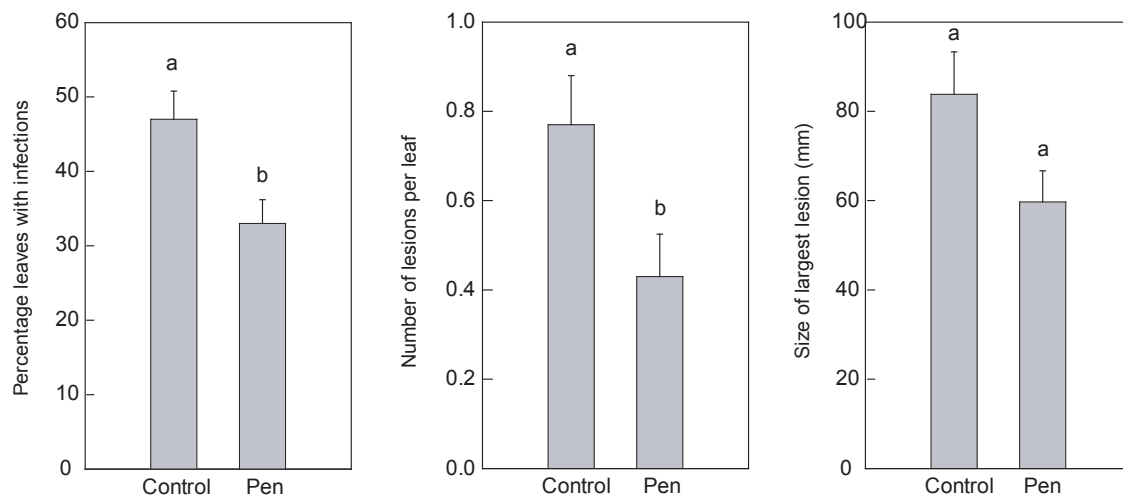
Under field conditions, onion plants were significantly less infected by *P. destructor* than non-treated plants (fig. 4). The percentage of infected leaves and the number of lesions per leaf were significantly reduced by 30% and 44%. Furthermore, Pen reduced the mean size of the largest lesion by 29% from 8.4 to 6 cm ( $p=0.07$ ).

#### The raw material for extraction of the Pen extract is available in constant quality

A total of 30 batches of mycelium of *P. chrysogenum* were extracted with water and tested on tomato plants for disease reducing activity. The efficacy of these extracts was compared to the efficacy of the reference Pen extract (from batches 97/15 and 99/12). The relative efficacy of the batches varied from -18% to +28%, with 80% of the batches within a range of  $\pm 10\%$  (fig. 5). The observed variation in the efficacy of different batches is negligible for an application in practice.

#### Pen can cause negative side effects

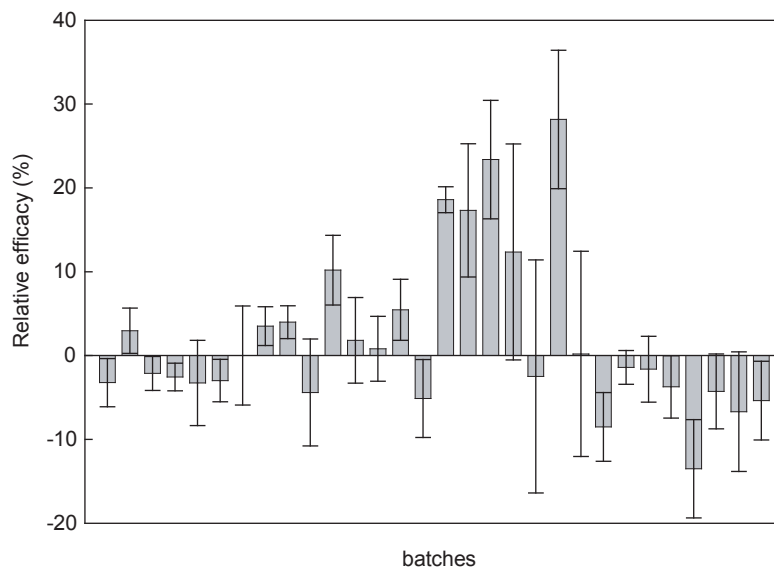
Foliar treatment of plants with the crude Pen extract not only induced resistance but also caused several other symptoms. (i) Treating tomato plants with Pen lead to accelerated senescence of leaves in several experiments. (ii) Tomato plants bent their leaves actively down upon Pen-treatment. This phenomenon was present throughout all experiments but was only observed in tomato. (iii) Pen treatment may cause phytotoxic effects. A speckling developed on leaves and stems of tomato, grapevine and to a lesser extent of cucumber plants after spaying with the crude Pen extract (fig. 6). The symptoms mostly consisted in small necrotic spots not associated with lesions caused by a pathogen. In the field, some grapevine leaves



**Figure 4.** Effect of Pen on onion against *P. destructor* in the field. Plants were non-treated or weekly treated with the crude Pen extract (45 g/l). Percentage diseased leaves, the number of lesions per leaf and the size of the largest lesion per leaf were determined for 100 leaves per replicate. Bars show means and standard errors. Different letters indicate statistically significant differences (t-test,  $p < 0.05$ ).

**Table 1.** Efficacy of Pen against different pathogens in various crop plants compared to the efficacy of standard fungicides or inducers. The table shows the number of experiments performed (N), the mean efficacy of all experiments as well as the minimum and maximum efficacy.

Plant	Pathogen	Location	Treatment	Efficacy (%)			N
				Mean	Min	Max	
Grapevine	<i>P. viticola</i>	Field	Pen	67	52	90	3
			Bion	55	52	57	2
	Greenhouse	Pen	88	67	100	6	
		BABA	68	34	94	4	
Apple tree	<i>V. inaequalis</i>	Field	Pen	91	89	93	2
			Copper	93	90	95	2
Tomato	<i>P. infestans</i>	Greenhouse	Pen	71	17	100	33
			Bion	41	14	60	7
Cucumber	<i>C. lagenarium</i>	Greenhouse	Pen	24	0	64	6
			Bion	58	0	92	6
	<i>P. cubensis</i>	Greenhouse	Pen	27	11	59	2
			Bion	46	28	59	2
Onion	<i>P. destructor</i>	Field	Pen	44	-	-	1
Potato	<i>P. infestans</i>	Field	Pen	0	-	-	1
			Copper	94	-	-	1



**Figure 5.** Variability of efficacy of different production batches. A total of 30 batches of mycelium of *P. chrysogenum* dating from 1993 to 1999 were extracted with water. The efficacy of these extracts was tested using the tomato - *P. infestans* bioassay and compared to the efficacy of the standard Pen extract (from batches 97/12 or 99/15). Relative efficacy was calculated as  $100((\text{efficacy batch}/\text{efficacy standard}) - 1)$ . Efficacy rates of the standard Pen extract varied between 68 and 89% throughout the experiments. The graph shows means  $\pm$  standard errors.

developed larger necrotic areas. On apple trees, no necrotic spots were visible by eye after Pen treatment. However, in 2003, leaves were of a lighter green and stems were slightly shorter after repeated application of Pen (data not shown). In contrast to the crude Pen extract, Pen<sub>2000</sub> was much less toxic to grapevine plants than the crude Pen extract under greenhouse and field conditions (data not shown).

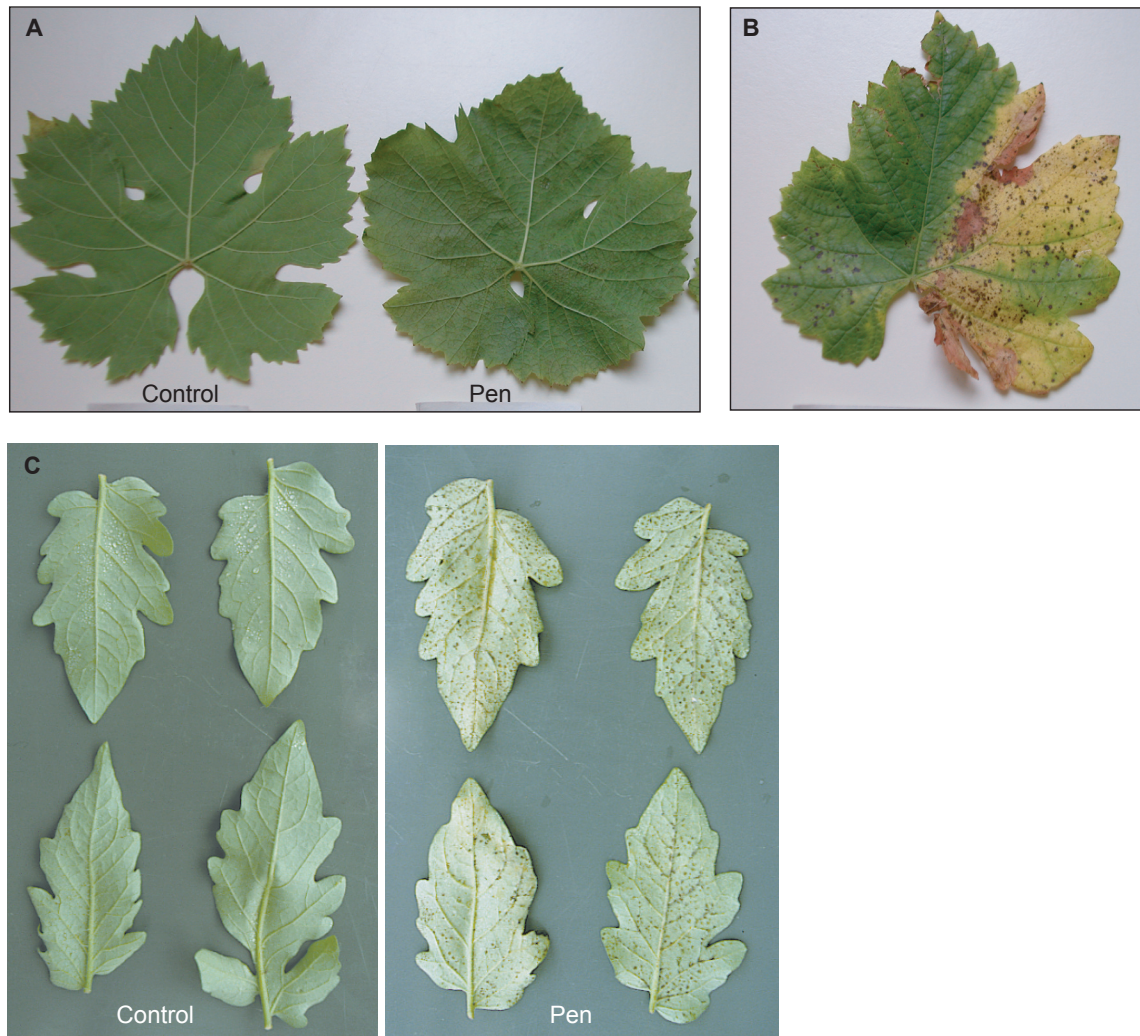
The occurrence of the symptoms was dependent on different parameters, including concentration of Pen, the number of applications, the persistence on the plant tissue, the plant species and variety and environmental conditions. As an example, apple trees did not develop any toxic symptoms in 1999, but some symptoms were visible in 2003 (see above). Furthermore, Pen was much more toxic to the grapevine variety 'Chasselas' than to 'Riesling x Sylvaner' in field experiments.

The phytotoxic effect of extracts from 30 batches of mycelium varied largely from -37% to +239% compared to toxicity of the standard Pen extract (data not shown). Most of the extracts were more toxic than the reference extract. There was no correlation between the toxicity and the efficacy of the extracts.

## Discussion

In this study we report that Pen, an aqueous extract from the dry mycelium of the ascomycete *P. chrysogenum*, protects many plant species against several pathogens under greenhouse and field conditions. There is strong evidence that the protective effect of Pen is not due to a direct toxic effect on pathogens for the following reasons: (i) We showed that Pen has no direct inhibitory effect on the growth of *P. infestans*, *C. lagenarium* and many other pathogens *in vitro*. (ii) When Pen was thoroughly washed from leaves before inoculation, tomato plants were still strongly protected against *P. infestans*. (iii) Pen protects tomato but not potato plants from disease by the oomycete *P. infestans*. This finding strongly suggests that the activity of Pen is plant mediated. (iv) A wide range of bacteria and fungi immediately colonize the Pen extract if it is not kept under sterile conditions. (v) Pen protects plants against a wide range of pathogens





**Figure 6.** Phytotoxic effect of Pen on grapevine (**A** and **B**) and tomato (**C**). **A** and **B**, Grapevine leaves (cv. 'Riesling x Sylvaner') from outdoor experiments after 6 treatments. **A**, Typical leaves of untreated control and Pen-treated (15 g/l) leaves. **B**, Pen-treated leaf with strong phytotoxic symptoms. **C**, Effect of Pen (45 g/l) on tomato (cv. 'Supermarmande') after one treatment.

including oomycetes, ascomycetes and basidiomycetes and even bacteria (chapter 3). In contrast, most fungicides have a much narrower range of activity. Yet, in one experiment, we found an inhibitory effect of Pen on the germination rate of sporangia of *P. infestans*. Furthermore, efficacy of Pen against *P. infestans* was slightly reduced when leaves were rinsed-off before inoculation. In conclusion, our data suggest that Pen-mediated resistance is mainly based on the activation of host resistance mechanisms. However, it can not be completely excluded that Pen can have minor direct inhibitory effect on certain developmental stages of a particular pathogen.

Pen induced resistance against several diseases not only under controlled greenhouse conditions but also under field conditions. Pen-mediated resistance was effective under field conditions on grapevine against powdery (*U. necator*) and downy mildew (*P. viticola*), on apple tree against apple scab (*V. inaequalis*) and on onion against downy mildew (*P. destructor*) as well as against early blight (*P. infestans*) on tomato under greenhouse conditions. Furthermore, Pen was even effective under very high disease pressure, as described

for *P. viticola* in 1997, *U. necator* in 1998 and for *P. destructor* in 2000. Efficacy of Pen on grapevine and apple tree in the field was comparable to the effect of standard fungicides such as copper and sulphur. Furthermore, if compared to other well-known inducers such as BABA or Bion, efficacy of Pen was equal or superior in most plant-pathogen systems. The only exception was cucumber, where Bion performed much better against the two tested pathogens *C. lagenarium* and *P. cubensis*. Particularly, the effect of Pen against downy mildew (*P. viticola*) on grapevine is of outstanding interest, since *P. viticola* is one of the most noxious microorganisms on grapevines worldwide (Emmet et al., 1992). The most effective and widely used product for the control of downy mildews in organic viticulture is copper. Yet, the use of copper is quite problematic because it is known to accumulate in the soil (Ráz et al., 1987). The replacement of copper by other, more environmental friendly products, has been a major focus of organic agriculture in the last few years (Speiser et al., 2000). However, no real alternative products in conformity with the guidelines of organic agriculture have been found yet. Pesticides to be applied in organic agriculture have to fulfil several criteria (EU Council Regulation (EEC) No. 2092/91, 1991; EU Council Regulation (EEC) No. 1488/97, 1997; IFOAM Basic Standards for Organic Agriculture, 2000; Tamm, 2001). One criterion is the way of production. Only natural products or products identical to natural products may be used. Furthermore, natural products may not be obtained from genetically modified organisms. The Pen extract complies with the guidelines, in contrast to other inducers such as Bion (containing the synthetical active compound BTH) or Messenger® (containing the bacterial protein harpin obtained from genetically modified bacteria). In addition, the raw material for production of the Pen-extract is relatively cheap and we have shown that it is available in constant quality, prerequisites for an application in practice.

Evaluating a commercial use, unfortunately, the phytotoxic side effects make an application of the crude Pen extract unfeasible. Particularly grapevines suffered from severe symptoms caused by the crude Pen extract, especially with repeated application as required in practice. In contrast, the crude Pen extract was much less phytotoxic to apple trees. In two years (1998 and 1999), apple trees did not develop any symptoms at all. Thus, an application in this culture could be more favourable. In addition, our data indicate that there are several possibilities to reduce phytotoxicity, e.g. a fraction prepared from the crude Pen extract by dialysis (Pen<sub>2000</sub>) was much less toxic than the crude extract itself. This fraction protected grapevines similarly to the crude extract against *P. viticola*, but its efficacy was reduced on apple trees against *V. inaequalis*. However, Pen<sub>2000</sub> was still as efficient as the frequently used fungicides such as sulphur or MycoSan. Furthermore, there is evidence that airing the crude Pen-extract and/or treating it with bases such as sodium hydroxyde have the potential to reduce phytotoxicity. Inducers are known to be active only if they interact with receptors on the plant membrane or if they are taken up by plant cells. Therefore, the efficacy of Pen<sub>2000</sub>, a fraction containing only substances with a molecular weight larger than 2000 Dalton, might be limited as a result of poor uptake. However, uptake might be improved by formulation. Improved formulation might also allow applying lower, less toxic doses of the crude Pen extract.

In conclusion, we showed that Pen, the aqueous extract from the mycelium of *P. chrysogenum*, induces resistance against a broad range of pathogens in several crops under greenhouse and field conditions. Particularly its effect against downy mildews on grapevine and onion is promising. However, the phytotoxic side effects make an application of the crude Pen extract unfeasible in practice. Yet, our data suggest that phytotoxicity can be reduced by appropriate techniques, which have still to be improved. Furthermore, narrowing down the active principle of Pen might allow developing new strategies for a more specific extraction and/or processing.



## CHAPTER III

### **An extract of *Penicillium chrysogenum* induces resistance in *Arabidopsis thaliana* independently of known signalling pathways and elicits early defense responses**

#### **Abstract**

Pen, an aqueous extract of the mycelium of *Penicillium chrysogenum*, induces resistance in various crop plants against several pathogens. In order to examine signal transduction pathways, the model plant *Arabidopsis thaliana* was used. Pen protected *A. thaliana* from a broad range of pathogens, including an oomycete (*Peronospora parasitica*), two ascomycetes (*Botrytis cinerea*, *Alternaria brassicicola*) and a bacterium (*Pseudomonas syringae* pv. *tomato* DC3000) without having a direct antimicrobial effect. Pen was still fully protective against *B. cinerea* in *Arabidopsis* transgenes or mutants impaired in the salicylic acid (NahG, npr1), jasmonic acid (coi1), and ethylene (ein2) signalling pathway. In contrast, Pen-mediated resistance against *P. parasitica* was reduced in the transgene NahG, but not affected in the mutants npr1, coi1 or ein2. Furthermore, Pen induced early defense-related responses such as an extracellular alkalinisation, ethylene production and an oxidative burst in numerous mono- and dicotyledon plant species. The response to Pen could completely be inhibited by the protein kinase inhibitor K-252a. Cells pretreated with chitin or ergosterol, were refractory to a second treatment with the same stimulus, but fully responsive to Pen. Our data suggest that the Pen extract contains at least one unidentified elicitor inducing resistance via signal transduction pathways different from classical SA/NPR1- or JA/ethylene- dependent pathways.

## Introduction

Plants have evolved sophisticated defense mechanisms against potentially pathogenic fungi, bacteria and viruses, including preformed barriers and constitutively expressed antimicrobials as well as inducible defense mechanisms. Resistance can be induced upon contact with pathogenic or non-pathogenic microorganisms, extracts of microorganisms, or synthetic chemicals, providing protection against a broad spectrum of pathogens. It has been shown that plants can recognize general structures associated with microorganisms, so called elicitors or PAMPs (Pathogen Associated Molecular Patterns) (Nürnberg and Brunner, 2002), including flagellin (Felix et al., 1999) and harpin (Strobel et al., 1996) from bacteria, chitin (Felix et al., 1993; Brunner et al., 2002), ergosterol (Granado et al., 1995) and several cell-wall glucans (Sharp et al., 1984; Yamaguchi et al., 2000) from fungi and laminarins from algae (Aziz et al., 2003). After binding to a specific receptor of the plant, elicitors trigger a signalling cascade, finally resulting in biochemical and mechanical defense mechanisms such as production of phytoalexins (Mansfield, 2000), translation of specific proteins with putative antimicrobial activities (Linthorst, 1991; van Loon, 1999) and mechanical strengthening of the cell walls (Matern et al., 1995; Benhamou et al., 1996; Mauch-Mani and Slusarenko, 1996). Early events in signal transduction include protein phosphorylation (Felix et al., 1991), changes in the ion permeability of the plasma membrane, measurable as an efflux of  $K^+$  and  $Cl^-$  and an influx of  $H^+$  and  $Ca^{++}$ , resulting in a transient alkalinisation of the extracellular space (Sacks et al., 1993; Jabs et al., 1997; Zimmermann et al., 1997), the production of ethylene and an oxidative burst (Felix et al., 1991; Dixon et al., 1994). More downstream in the cascade, one or several of the secondary endogenous signal molecules salicylic acid (SA), jasmonic acid (JA) and ethylene or the protein NPR1 (also called NIM1), are often required for signal transduction (Glazebrook, 2001). It has been shown that depending on the stimulus, specific signal transduction pathways involving one or several of these key regulators are activated, leading to resistance against specific sets of pathogens. In addition, these pathways can influence each other through a network of complex regulatory interactions (Kunkel and Brooks, 2002). Generally it is thought that resistance against necrotrophic pathogens such as *Botrytis cinerea* or *Alternaria brassicicola* is depending on functional ethylene and JA perception, while resistance against biotrophs such as *Peronospora parasitica* and the bacterium *Pseudomonas syringae* is depending on SA and NPR1 (Thomma et al., 2001).

In this study we describe that Pen, an extract from the mycelium of the ascomycete *Penicillium chrysogenum* not only triggers early events in signal transduction in various plant species, but also induces resistance in *A. thaliana* against the downy mildew *Peronospora parasitica*, the necrotrophic ascomycetes *Botrytis cinerea* and *Alternaria brassicicola* and the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato*. We provide evidence that Pen-mediated resistance is independent of ethylene, jasmonic acid and NPR1 and depends partially on salicylic acid.

## Methods

### Elicitors and inhibitors

**Pen.** 150 g of the dry, penicillin-free mycelium of *Penicillium chrysogenum* (see chapter 2) were added to 1 liter demineralized water and autoclaved for 3 hours at 120°C. Before use, the suspension was stirred and then centrifuged or filtrated (paper filter no. 595 Æ 15mm, Schleicher & Schuell). The crude aqueous extract of *Penicillium chrysogenum*, in the following called "Pen", contained 45 g/l dry matter and had an osmolarity of 270 mosmol. For *Pseudomonas* bioassays and experiments with cell cultures and leaf slices, the crude Pen-extract was dialysed against distilled water during 24 hours at 4°C in a dialysis tube with a 2000 Dalton cut-off (Spectrapor). The fraction >2000 Dalton (further called Pen<sub>2000</sub>) with an osmolarity of 20 mosmol

containing 12 g/l dry matter was kept for experiments. All extracts were kept at  $-20^{\circ}\text{C}$ . All concentrations refer to the amount of dry matter in the aqueous extract. Suspension cultured cells do not respond to penicillin or penicillin by-products formed during heating.

**Other elicitors and inhibitors.** Chitin prepared by reacylation of chitosan from crab shells (Fluka) as described by (Felix et al., 1993) was kindly provided by G. Felix. Ergosterol was purchased from Sigma and dissolved in DMSO. The synthetic peptide flg22 containing a highly conserved sequence of 22 amino acids from the N terminus of bacterial flagellin (Felix et al., 1999) was used as a reference in alkalisation and ethylene bioassays. The protein kinase inhibitor K-252a (Fluka) was diluted in DMSO and applied at a final concentration of  $1\ \mu\text{M}$ .

### Test for fungicidal activities in Pen

*Botrytis cinerea* and *Alternaria brassicicola* were grown on agar plates containing their growth medium plus the standard fungicides difenoconazol (0.01 g/l, Score<sup>®</sup>, Syngenta AG) or fludioxonil (0.01 g/l, Celest<sup>®</sup>, Syngenta AG) against *A. brassicicola* or *B. cinerea* respectively, or different concentrations of the crude Pen extract (45, 15, 4.5, 0.45, 0 g/l). Plates were grown for 5 (*B. cinerea*) or 10 days (*A. brassicicola*) at  $24^{\circ}\text{C}$ . To test whether Pen has an inhibitory effect on germination, three  $10\ \mu\text{l}$  drops of spore suspensions containing  $10^5$  sp/ml were applied to water agar plates containing the same concentrations of Pen or a fungicide as described before. Germination was checked under the microscope after 24 hours.

Direct inhibitory effects on the obligate biotroph *Peronospora parasitica* were assessed by rinsing-off experiments. *A. thaliana* wildtype plants (WS) were sprayed with Pen (15 g/l) and washed thoroughly with water 1, 24, 48 and 72 hours after this treatment or not washed at all. Plants were inoculated 73 hours after the Pen-treatment with  $20'000$  sp/ml of *P. parasitica* ecotype EMWA. Three replicates were used per treatment and timepoint.

### Cultivation of plants

Seeds of *Arabidopsis thaliana* wildtype plants ecotype Columbia (Col-0) and Wassilewskija (WS), transgenic NahG plants (Col-0 and WS background), the mutants *nim1/npr1* (Col-0 and WS background), *pad3-1*, *pad2*, *ein2-1* (Col-0 background) and *bai24*, *bai38* and *bai65* (WS background) were sown into soil (H4 substrate, GVZ-Bolltec AG plus vermiculite). Surface sterilized seeds of the jasmonic acid insensitive mutant *coi1* (Col-0 background) were sown on MS (Sigma) agar plates containing  $50\ \mu\text{M}$  methyl-jasmonate (Serva). Homozygous *coi1* seedlings show normal root growth and were transferred to soil one week after sowing. For *Pseudomonas* bioassays, plants were grown individually in small pots (4 x 4 x 6 cm), for all other assays, larger pots (12 x 20 x 6 cm), each containing ten plants, were used. Plants were cultivated in a growth chamber with a 10 h day ( $90-120\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at  $24^{\circ}\text{C}$ ) and 14 h night ( $18^{\circ}\text{C}$ ) cycle at 60% relative humidity. Seeds of BABA insensitive plants (*bai24*, 38 and 65)) were kindly obtained from Brigitte Mauch-Mani (University of Neuchâtel, Switzerland).

### Elicitor treatment

If not mentioned otherwise, three to four weeks old plants were sprayed two days before inoculation with a chromatography spray at 1 bar pressure with crude Pen-extract, demineralized water (control), chitin (5 mg/ml) or ergosterol (0.01mM) till the leaves were covered by a fine film of drops (about 1.8 ml per pot). Treating Pen 1 to 3 days before inoculation gave similar results. If not mentioned otherwise, Pen was used at concentrations of 15 g/l. For *Pseudomonas* bioassays, leaves were pressure infiltrated with a syringe without a needle with Pen<sub>2000</sub> (1.2 g/l) or with 10 mM  $\text{MgCl}_2$  two days before inoculation.

### *Peronospora parasitica* bioassay

The two strains EMWA and NOCO of the biotrophic oomycete *Peronospora parasitica* were maintained on

wildtype plants of ecotypes WS or Col-0 respectively. Sporangia of *P. parasitica* were obtained from heavily sporulating fresh or frozen leaves by gently shaking leaves in tap water. The suspensions were diluted to 100'000 (NOCO on Col-0 wildtype, ein2-1, pad2), 10'000 (NOCO on Col-0 NahG), 7500 (EMWA on WS wildtype, npr1, bai) or 1000 (EMWA on WS NahG) sp/ml. Plants were sprayed with a chromatography spray at a pressure of 1bar till they were wet (2.5-3.5 ml per pot). Inoculated plants were kept at 18°C and 100% relative humidity under short day conditions (10 h light, 60-90  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for one day. Then, relative humidity was reduced to 80% for five days and was increased again to 100% to induce sporulation the night before scoring and harvesting. Disease severity (percentage leaf area covered by sporangiophores) was assessed for each plant separately by checking upper and lower side of the leaves. When disease severity of control plants was below 10%, experiments were excluded from data analysis.

#### ***Botrytis cinerea* bioassay**

*Botrytis cinerea* (strain 424) was cultivated on Pea Agar containing 16% (w/v) peas and 0.5% saccharose at 22°C. Conidia were scratched gently with a brush from 8 days old cultures, suspended in a 1,5% w/v malt medium (SMB) and diluted to 75'000 to 100'000 sp/ml. Plants were sprayed with a chromatography spray at a pressure of 1 bar till they were wet (about 3.5ml per pot). Inoculated plants were kept at 18°C and 100% relative humidity under short day conditions (10 h light, 60-90  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Destroyed leaf area was assessed for each plant five days after inoculation.

#### ***Alternaria brassicicola* bioassay**

*Alternaria brassicicola* (strain 306) was cultivated on 4% Potato Dextrose Agar (Difco) at 22°C in permanent light. Conidia were scratched gently with a rope from 10 days old cultures and diluted in water to concentrations of 100'000 spores per milliliter. The first, second and third pair of true leaves was inoculated by applying two 5- $\mu\text{l}$  drops of the spore suspension. Inoculated plants were kept at 24°C and 100% relative humidity under short day conditions (10 h light, 60-90  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Disease severity was determined by measuring the diameters of the lesions three days after inoculation.

#### ***Pseudomonas syringae* bioassay**

The rifampicin-resistant *Pseudomonas syringae* pv. *tomato* strain DC3000 was grown on King's medium B agar plates (King et al. 1954) and transferred to liquid King's medium B the evening before the experiment. Bacteria were collected by centrifugation and resuspended in 10 mM  $\text{MgCl}_2$  to a final density of  $10^5$  colony-forming units (cfu) per ml. Bacteria were pressure infiltrated into leaves with a syringe without a needle till leaves were water soaked. One, 24 and 48 hours after inoculation, bacteria were reextracted from leaves by cutting two discs from one leaf of each replicate with a cork borer ( $r = 3$  mm). Leaves were ground in 10 mM  $\text{MgCl}_2$  and appropriate dilutions of the extracts were plated on Nyga plates containing rifampicin.

#### **Plant cell cultures and alkalisation assay**

Cell suspension cultures of *Arabidopsis thaliana* (May and Leaver, 1993), tomato (*Lycopersicon esculentum*, line Msk8) (Koorneef et al., 1987; Felix et al., 1991), *Lycopersicon peruvianum* (Felix and Boller, 1995), tobacco (*Nicotiana tabacum*, line 275N, derived from cv. 'Havanna 425') (Felix and Meins, 1987) and rice (*Oryza sativa*, line OC) (Baba et al., 1986) were cultured as described elsewhere. Cell suspensions were used six to ten days after subculture. To measure alkalisation of the growth medium, 3 ml aliquots of the cell suspensions were placed in open 20 ml vials on a rotary shaker at 120 cycles  $\text{min}^{-1}$ . The pH in the medium was continuously measured using a small combined-glass electrode (Metrohm, Herisau, Switzerland) and registered on a pen recorder.  $\Delta\text{pH}_{\text{max}}$  was derived from the recordings. The protein kinase inhibitor K-252a (Fluka) was dissolved in dimethylsulfoxide (5%), and applied as described in (Felix et al., 1991).

### Ethylene biosynthesis and oxidative burst in leaf slices

Fully expanded leaves of *Arabidopsis thaliana* ecotypes Col-0, WS and La-er, tomato (*Lycopersicon esculentum* cv MoneyMaker), tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), grapevine (*Vitis vinifera* cv Chasselas), apple tree (*Malus domestica* cv. 'Rubinette'), rapeseed (*Brassica napus*), brussel sprout (*B. oleracea* ssp. *oleracea* var. *gemmifera*), white cabbage (*B. oleracea* ssp. *oleracea* var. *capitata* f. *alba*), thai cabbage (*B. rapa*), chinese cabbage (*B. rapa* ssp. *chinensis*), rose (*Rosa* sp.), Yucca (*Yucca* sp.) and spider plant (*Chlorophytum comosum*) were cut into 2 mm slices and floated on water over night. For assaying ethylene production, leaf slices (approximately 10 mg fresh weight per replicate) were transferred to 6 ml glass tubes containing 0.5 ml of an aqueous solution and appropriate concentrations of the dialysed Pen extract. Vials were closed with rubber septa and ethylene accumulation in the free air space was measured by gas chromatography after 2 h of incubation at room temperature.

For measuring oxidative burst, active oxygen species released by leaf tissues were measured by a luminol-dependent assay. Slices were transferred to assay tubes containing 0.2 ml H<sub>2</sub>O supplied with 20 µM luminol and 2 µg horseradish peroxidase (Fluka). Luminescence was measured in a LKB 1250 luminometer (LKB Wallac, Turku, Finland).

### Calculations and statistics

In *B. cinerea*, *A. brassicicola* and *P. parasitica* bioassays, four to six replicates were used, one replicate being the mean of the ten plants from one pot. For *Pseudomonas* bioassays, five replicates, each consisting of one single plant, were used. Percentage disease reduction by Pen-treatment (efficacy of Penicillium extract) was calculated for each pot as follows:  $(1 - (\text{destroyed leaf area})_{\text{treated}} / \text{mean}(\text{destroyed leaf area})_{\text{untreated}}) * 100$ .

Data were analysed by ANOVA followed by a Tukey test at  $\alpha = 0.05$  for multiple comparisons (Zar, 1996). All analyses were done using SPSS (10.0, SPSS Inc.).

## Results

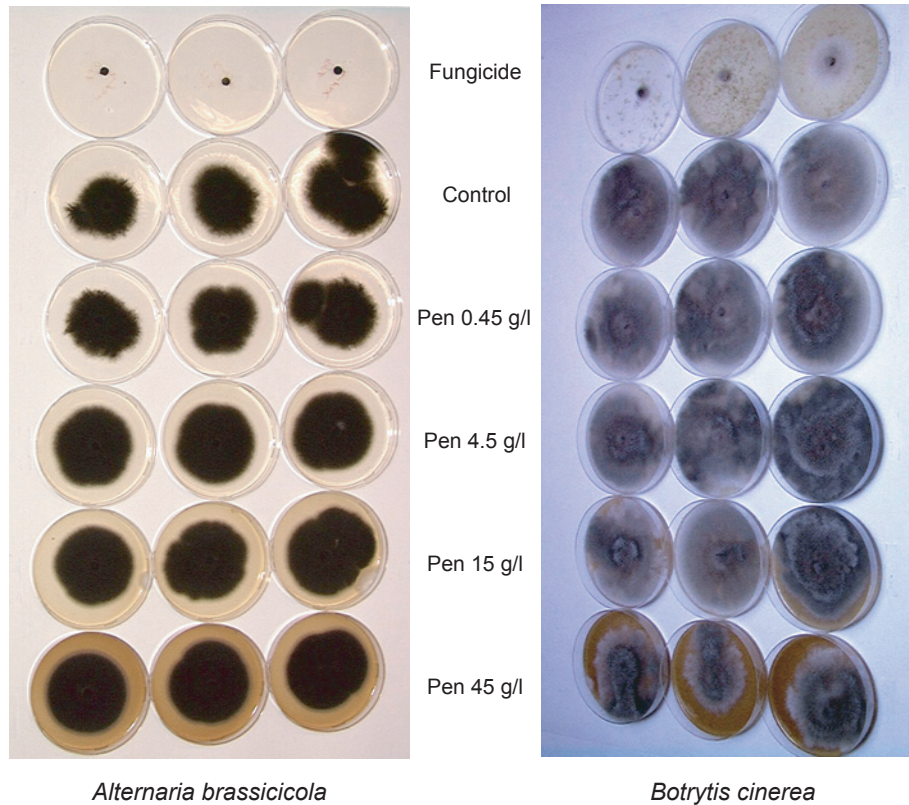
### Pen has no direct inhibitory effect on pathogens

As in chapter 2, Pen was tested for a direct inhibitory effect on the pathogens under consideration. Pen did not inhibit but even promoted growth of *A. brassicicola* (fig. 1). On Pen-containing agar plates, *A. brassicicola* grew faster and more densely, and spores produced four times longer and 40% more germ tubes per spore than spores on control plates. Germination rate of *A. brassicicola* on Pen-containing agar plates was generally slightly increased (+8%), except on plates containing the highest Pen concentration (30 g/l), where germination rate was slightly reduced from 85% to 74% (-14%).

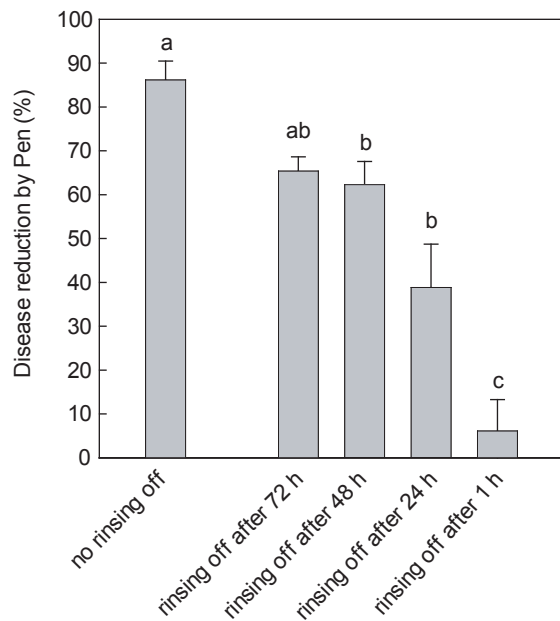
The growth of *Botrytis cinerea* was not affected by Pen except on plates containing the highest Pen concentration (45 g/l). Here, the growth diameter was slightly reduced, but the fungus grew more densely and was sporulating more than on control plates (fig. 1). Germination rate of *B. cinerea* spores was nearly 100% on Pen-containing as well as on control plates, but germ tubes of spores germinating on Pen-containing agar plates were much longer than on control plates.

Pen was tested for a fungistatic effect on *P. parasitica in planta*. Efficacy of Pen was negligible when plants were washed one hour after application (fig. 2). In contrast, Pen significantly reduced disease severity by about 65% when plants were washed 48 or 72 hours after Pen application. Yet, efficacy of Pen was highest on not washed plants (86%).





**Figure 1.** Effect of Pen on growth of *A. brassicicola* and *B. cinerea* in vitro. Different concentrations of the crude Pen extract or the standard fungicides difluconazole (*A. brassicicola*) or fludioxamile (*B. cinerea*) at concentrations of 0.01 g/l were added to the growth media of the fungi. Pictures were taken after 10 d (*A. brassicicola*) or 5 d (*B. cinerea*).



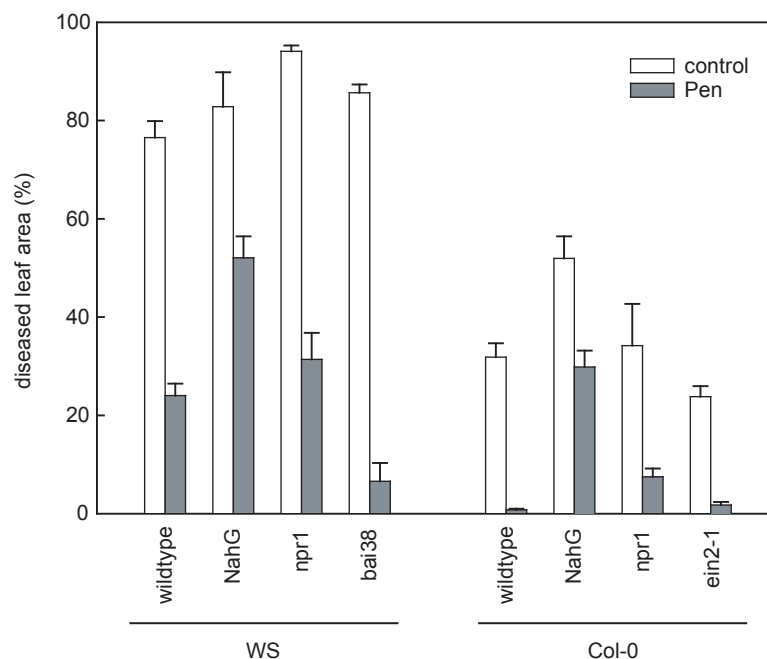
**Figure 2.** Effect of rinsing off leaves on the efficacy of Pen against *P. parasitica*. *A. thaliana* plants (WS) were treated with the crude Pen extract or water 72 h before inoculation. Leaves of Pen- and water-treated plants were either rinsed off thoroughly 1, 24, 48 and 72 h after application or not rinsed off. Plants were inoculated with *P. parasitica* strain EMWA (20'000 sp/ml). Disease severity of control plants was 85±10%, and was not affected by rinsing off. The graph shows means and standard errors. Different letters indicate significant differences (Tukey test,  $p < 0.05$ ).

### Pen protects *A. thaliana* against three different pathogens

*A. thaliana* plants treated with the crude Pen extract were considerably less infected with *P. parasitica*, *B. cinerea*, *A. brassicicola* and *P. syringae* pv. *tomato* DC3000 than water-treated control plants. Pen reduced the leaf area infected by the oomycete *P. parasitica* in Col-0 and WS wildtype plants in five independent experiments on average by 93±3% and 74±7% respectively (mean±SE) (fig. 3 and 5A). Maximal mean protection in both ecotypes was 98%, minimal protection was 82% in ecotype Col-0 and 46% in WS. Average disease severity in control was much higher in WS (76±7%) than in Col-0 (22±4%) plants (fig. 3). However, within one ecotype, there was no significant correlation between disease severity of control plants and the efficacy of Pen (data not shown). When higher Pen concentrations were used (30–45 g/l), protection in WS plants was as high as in Col-0 plants (92±3%, 4 independent experiments) (data not shown).

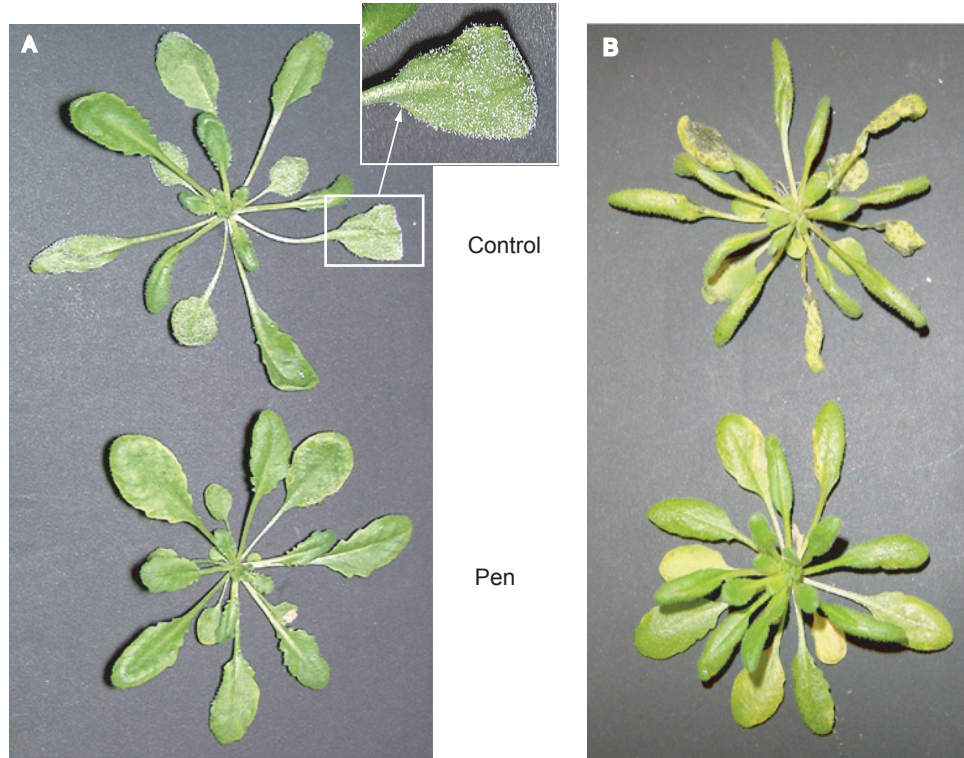
Pen reduced the leaf area destroyed by *Botrytis cinerea* on wildtype *A. thaliana* Col-0 plants in two independent experiments by about 50% (fig. 4 and 5B). In a third experiment, control plants were completely destroyed by the fungus and Pen had no protective effect. In all experiments, the lesions looked quite different in Pen-treated compared to control plants. In control plants, lesions were large, wet and slightly to strongly sporulating. Yet, in Pen-treated plants, lesions were usually restricted, dry and hardly sporulating. However, old leaves (cotyledons, first and second pair of true leaves) of Pen-treated plants often turned yellow to a larger extent after inoculation than leaves of control plants.

Because wildtype plants do not get infected by *A. brassicicola*, experiments were done on the phytoalexin deficient mutant *pad3-1*, which is more susceptible to this fungus (Thomma et al., 1999). Mean lesion diameter was significantly reduced by Pen from 4.8 to 2.8 mm (-46%,  $p < 0.05$ ) and lesions on Pen-treated plants usually did not show the typical ringlike necrosis of control leaves (data not shown).



**Figure 3.** Leaf area infected by *P. parasitica* on Pen-treated compared to water-treated *A. thaliana* plants. *A. thaliana* wildtype plants and mutants (background WS or Col-0) were sprayed with the crude Pen extract (15 g/l) or water 2 d before inoculation with *P. parasitica* (strains EMWA on WS and NOCO on Col-0). Standard sporangia concentrations were used for the different genotypes. The graph shows means and standard errors. Experiments were repeated at least once with similar results. Differences between control and Pen-treated plants were significant in all genotypes (t-test,  $p < 0.05$ ).

Growth of the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 in *A. thaliana* plants (WS) was strongly reduced in leaves treated with a partially purified fraction of Pen (Pen<sub>2000</sub>) (fig. 6). 48 hours after inoculation, there were about forty times more bacteria in leaves of control plants than in Pen-treated leaves. A second experiment gave similar results on ecotype WS as well as on Col-0 (data not shown).

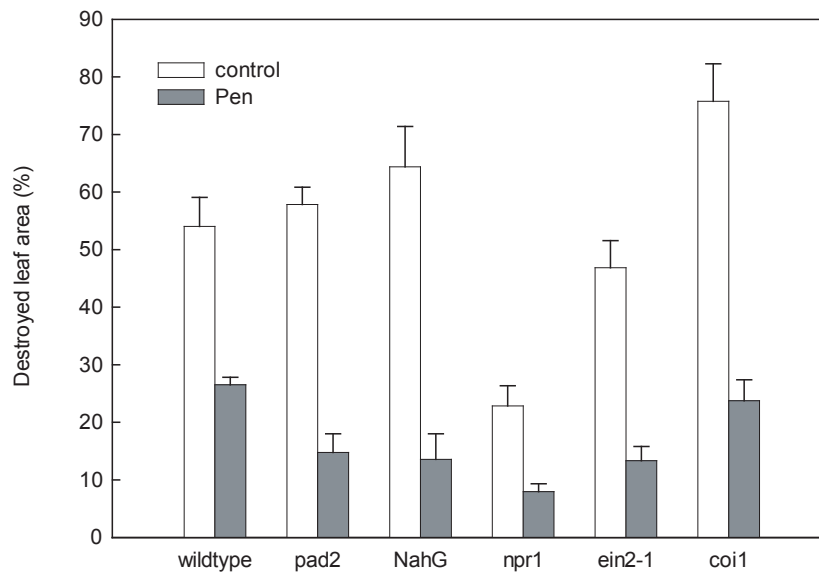


**Figure 5.** Effect of Pen on disease with *P. parasitica* (A) and *B. cinerea* (B). *A. thaliana* ecotype Col-0 plants were treated with the crude Pen extract (15 g/l) or water 2 d before inoculation. A, 3 weeks old wildtype plants were inoculated with 100'000 sp/ml of *P. parasitica* strain Noco. The small picture shows a detail of an infected leaf with a white lawn of sporangiophores. B, 6 weeks old wildtype plants were inoculated with 100'000 sp/ml of *B. cinerea*.

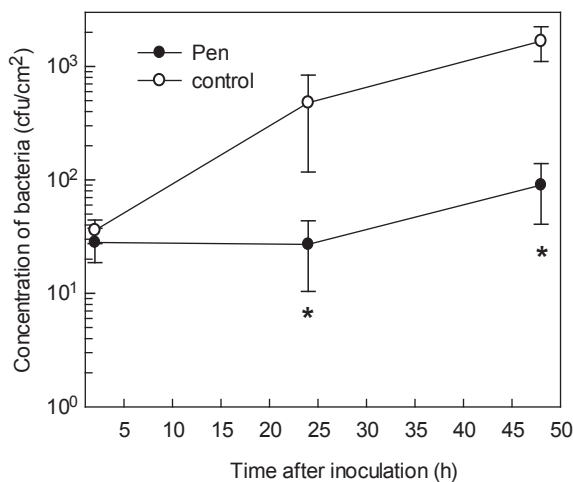
#### Induction of resistance by Pen is independent of NPR1, ethylene and jasmonic acid and partially dependent on salicylic acid

We tested whether the induction of resistance by Pen is dependent on some of the currently known key regulators of plant defense responses against pathogens such as salicylic acid (SA), NPR1, ethylene or jasmonic acid (JA), using the transgene NahG and the mutants *npr1*, *ein2-1* and *coi1*. In addition, we tested the efficacy of Pen on the phytoalexin deficient mutant *pad2*, which is more susceptible to *B. cinerea* than wildtype plants (Glazebrook et al., 1997), and on three  $\beta$ -aminobutyric acid (BABA)-insensitive mutants, one of them identified to be an abscisic acid-insensitive mutant (*bai 38*). Pen significantly reduced infection of *P. parasitica* and *B. cinerea* on all of the tested genotypes (figures 3 and 4). However, efficacy of Pen against *P. parasitica* was significantly lower on the salicylic acid deficient transgene NahG than on wildtype plants or any of the mutants. In contrast, efficacy of Pen against *B. cinerea* on NahG transgenes (Col-0) was comparable to efficacy on wildtype plants (fig. 4). Five independent experiments on NahG plants in Col-0 as well as in WS background gave similar results. While the efficacy of Pen on WS wildtype and *npr1* could be improved by higher Pen-concentrations, the efficacy of Pen on NahG could not be improved (data not shown).





**Figure 4.** Leaf area destroyed by *B. cinerea* on Pen- compared to water-treated *A. thaliana* plants. npr1 mutants are in WS background, wildtype plants and all other mutants are in Col-0 background. Plants were sprayed with Pen (15 g/l) or water 2 d before inoculation with 75'000 to 100'000 sp/ml. The graph shows means and standard errors. Experiments were repeated at least once with similar results. Differences between control and Pen-treated plants were significant in all genotypes (t-test,  $p < 0.05$ ).



**Figure 6.** Effect of Pen on growth of the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (Pst). Growth of Pst in Pen<sub>2000</sub>- compared to water-treated leaves of *A. thaliana* ecotype WS. Leaves were pressure infiltrated with a syringe without a needle with either Pen<sub>2000</sub> (1.2 g/l) or 3 mM MgCl<sub>2</sub> 20 hours before inoculation with 10<sup>5</sup> cfu/ml. Bacteria were extracted 1, 24 and 48 h after inoculation. Graph shows means  $\pm$  standard errors (n=5). The experiment was repeated once with similar results.

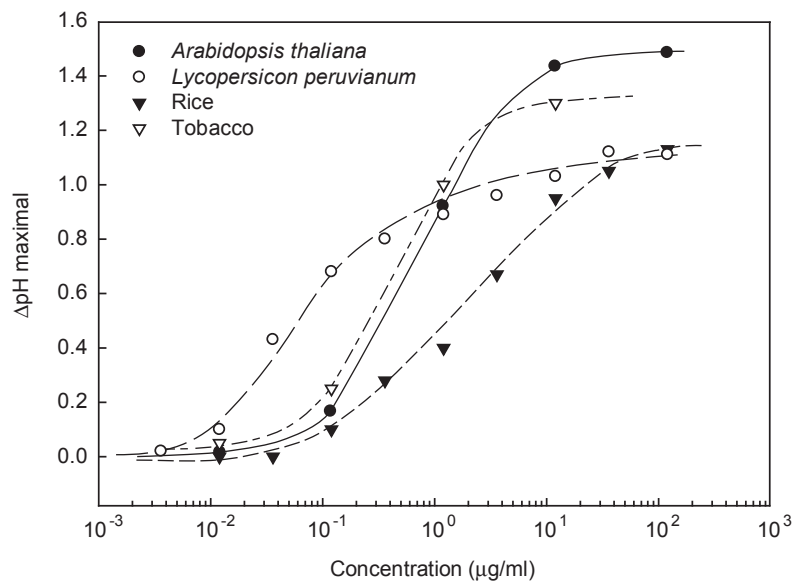
### Pen induces early signalling events in *Arabidopsis thaliana* and a wide range of mono- and dicotyledon plant species

Different elicitors of fungal and bacterial origin have been reported to induce early signalling events like a rapid medium alkalinisation, the production of ethylene and an oxidative burst in plant cell-suspensions. Pen (crude extract and Pen<sub>2000</sub>) reproducibly induced a rapid and strong alkalinisation response in cell cultures of *Arabidopsis thaliana*, tomato (*Lycopersicon esculentum*), wild form of tomato (*L. peruvianum*), tobacco (*Nicotiana tabacum*) and rice (*Oryza sativa*) (fig. 7 and 8). Alkalinisation responses started after a lag-phase of one (rice, *L. peruvianum*) to three (*A. thaliana*) minutes at saturating doses (fig. 8). At low doses, longer lag-phases up to 10 min were often observed. Doses between 0.1  $\mu$ g/ml (*L. peruvianum*) and 2  $\mu$ g/ml (rice)

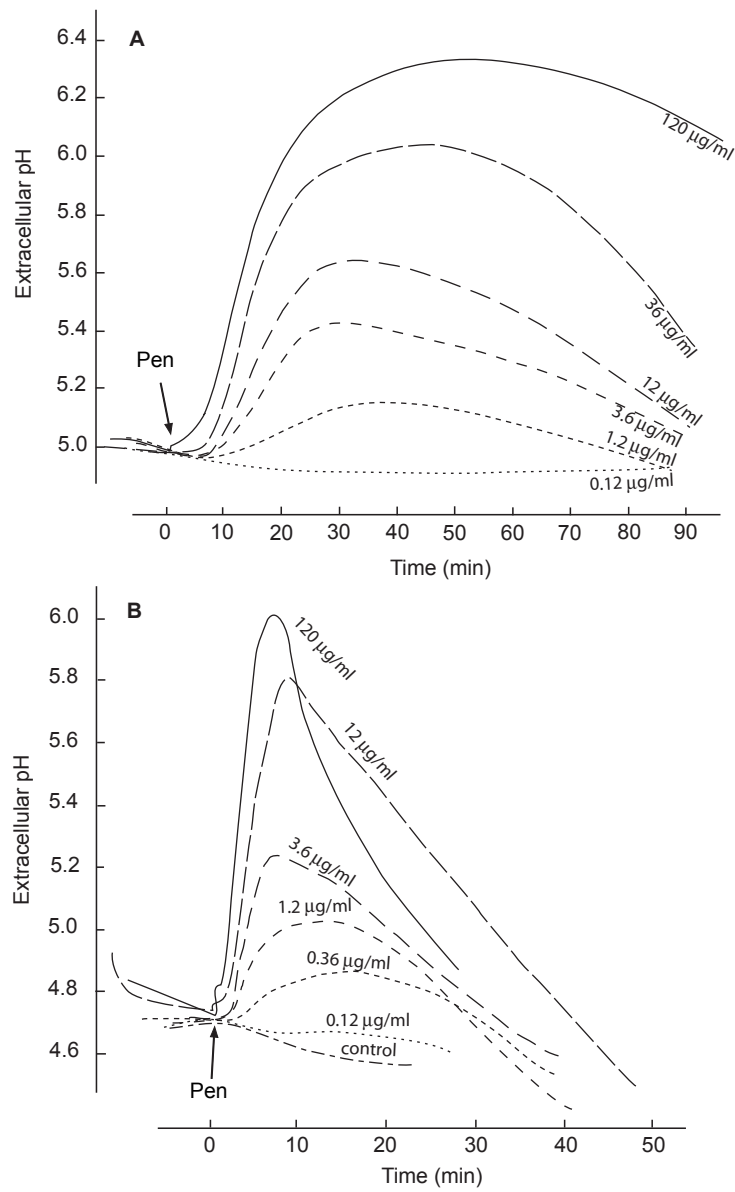
were sufficient for half maximal alkalinisation responses (EC50) (fig. 7). Maximal alkalinisation responses induced by Pen were between 0.8 (tomato) and 1.5 (*A. thaliana*) pH units. Alkalinisation responses could be completely inhibited by the protein kinase inhibitor K-252a (data not shown).

The Pen-mediated alkalinisation responses were compared to responses induced by the well-known fungal elicitors chitin and ergosterol. Similar to Pen, chitin induced alkalinisation responses in cell cultures of all species. However, maximal alkalinisation responses to Pen were always much more pronounced (fig. 9). Furthermore, *A. thaliana* cells pretreated with chitin were still responsive to Pen but were refractory to a second chitin stimulus (fig. 9). In contrast to Pen, ergosterol, an elicitor derived from fungal membranes, had no activity in *A. thaliana* cell cultures (data not shown).

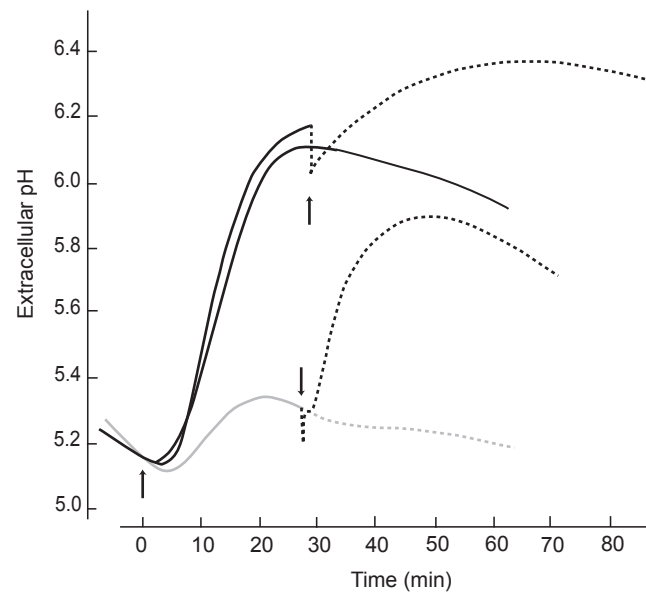
Pen extract induced an oxidative burst in *A. thaliana* and chinese cabbage (data not shown) and strongly induced the production of the stress hormone ethylene in leaf slices of a wide range of plant species, including tomato, tobacco, rice and *A. thaliana* (three different ecotypes) (fig. 10). In addition, five more members of the Brassicaceae family (all of the genus *Brassica*), grapevine, apple tree, rose, yucca and spider plant all responded to Pen with ethylene production (data not shown). Maximal response to Pen was strongest in *A. thaliana* (Col-0, La-er), different *Brassica* species and tomato (25-30 fold induction), followed by grapevine (17 fold induction), tobacco and apple tree (9 fold), and was lowest but still significant in rice.



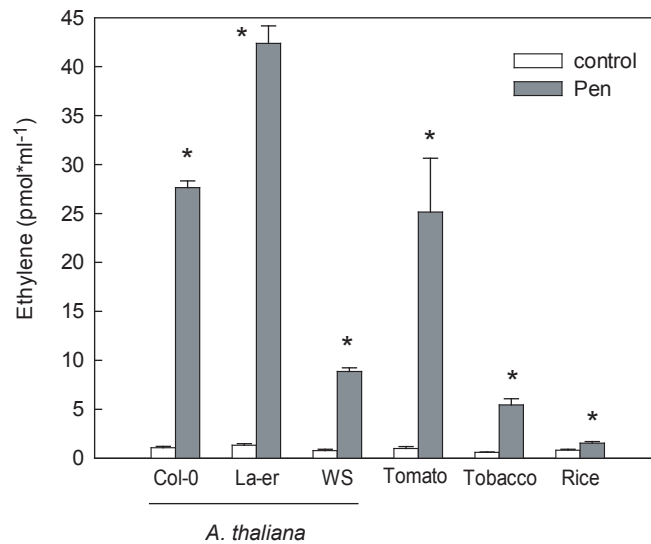
**Figure 7.** Alkalinisation-inducing activity of Pen in suspension-cultured cells of *A. thaliana*, *L. peruvianum*, rice and tobacco. Dose-response curves induced by Pen<sub>2000</sub> (Pen >2000 Da). Points represent maximal alkalinisation responses derived from permanent recording of alkalinisation responses.



**Figure 8.** Alkalinization of the culture medium of *A. thaliana* (A) and rice (B) cells in response to treatment with different concentrations of Pen<sub>2000</sub> (Pen >2000 Da). Curves show typical lag-phases and time courses.



**Figure 9.** Extracellular alkalinisation of *A. thaliana* cell cultures in response to treatment with one or two consecutive stimuli of Pen<sub>2000</sub> (black) or chitin (gray). Straight and dotted lines represent first and second stimuli respectively. Concentrations were 120  $\mu\text{g/ml}$  (Pen), 10  $\mu\text{g/ml}$  (chitin 1<sup>st</sup> stimulus) and 30  $\mu\text{g/ml}$  (chitin 2<sup>nd</sup> stimulus). Arrows indicate when elicitors were added.



**Figure 10.** Ethylene-inducing activity of Pen<sub>2000</sub> in different plant species. Pen<sub>2000</sub> was added to leaf slices of tomato, tobacco, rice and three different ecotypes of *A. thaliana*. Different concentrations of Pen were used (0.06 (WS), 0.24 (Col-0, La-er, tomato) and 0.36 (tobacco, rice) mg/ml). Ethylene production was measured after 2 h in a gas chromatograph. Asterisks indicate significant differences between control and Pen-treatment (t-test,  $p < 0.05$ ).

## Discussion

In this study we report that Pen, the aqueous extract of the dry mycelium of *P. chrysogenum*, protects *A. thaliana* plants against a wide range of pathogens and induces early defense-related responses. When the leaves of *A. thaliana* were sprayed or infiltrated with Pen before inoculation, plants were more resistant against two different strains of the oomycete *Peronospora parasitica*, the ascomycetes *Botrytis cinerea* and *Alternaria brassicicola* and the virulent bacterium *Pseudomonas syringae* pv. *tomato* DC3000. Our data from the greenhouse and the field confirm that Pen can protect plants against various pathogens including oomycetes (*Phytophthora infestans*, *Plasmopara viticola*) and ascomycetes (*Venturia inaequalis*, *Uncinula necator*) (chapter 2).

There is strong evidence that Pen has no major direct inhibitory effect on *P. parasitica*, *B. cinerea* and *A. brassicicola*. Our *in vitro* experiments with *B. cinerea* and *A. brassicicola* showed that Pen can even promote growth and germination of the two necrotrophs, probably due to its high sugar content. Furthermore, efficacy of Pen against *B. cinerea* was improved if leaves were washed before inoculation. These findings suggest that the indirect protective effect of Pen against necrotrophs such as *B. cinerea* might even be larger than observed because it is counteracted by direct growth promotion. To test for a fungistatic effect of Pen against the obligate biotroph *P. parasitica*, we used an *in vivo* assay. We showed that the time of persistence of Pen on the plant tissue is of crucial importance for its efficacy. If Pen was washed from leaves after 1 h, no inhibitory effect on the growth of *P. parasitica* was observed, indicating that washing was effective and one hour of exposition to Pen is not enough to induce resistance. If Pen persisted on the leaves for 24 to 72 h, disease severity was significantly and strongly reduced. However, efficacy of Pen was even higher when leaves were not washed at all. Thus, the protective effect of Pen against *P. parasitica* seems to be mainly plant-mediated, although a minor fungistatic effect can not be excluded. The hypothesis that Pen acts via the activation of plant defense mechanisms was further supported by the facts that Pen induced early defense-related responses and that the efficacy of Pen against *P. parasitica* was reduced on SA-deficient NahG compared to wildtype plants.

Our data suggest that Pen does not induce resistance on one of the well-known signalling pathways which require either both SA and NPR1 (denoted as 'Systemic Acquired Resistance' (SAR)) (Sticher et al., 1997), ethylene and/or JA (Thomma et al., 1998; Thomma et al., 1999) or JA, ethylene and NPR1 (denoted as 'Induced Systemic Resistance' (ISR)) (Pieterse et al., 1998). Pen was fully protective against *B. cinerea* in *Arabidopsis* transgenes or mutants impaired in the salicylic acid (NahG, npr1), jasmonic acid (coi1), and ethylene (ein2) signalling pathway. The same was observed for flg22-induced resistance against *P. syringae* pv. *tomato* (Zipfel et al., 2004) and for BABA-mediated resistance against *P. parasitica* (Zimmerli et al., 2000). In contrast, Pen-mediated resistance against *P. parasitica* was impaired in the transgene NahG, suggesting an important role of SA, but was not affected on the mutants coi1, ein2 and npr1. This finding supports the hypothesis that besides a SA/NPR1-dependent pathway which is activated by many stimuli, including the chemical inducers benzothiadiazole (BTH) and probenazole and the bacteria-derived elicitor harpin (Lawton et al., 1996; Dong et al., 1999; Yoshioka et al., 2001), a pathway only dependent on SA but independent of NPR1 exists (Glazebrook, 2001; Shah, 2003).

Our finding that Pen induces resistance against biotrophs (*P. parasitica*) as well as necrotrophs (*B. cinerea*, *A. brassicicola*) is in contrast to the hypothesis that distinct, mutually antagonistic signalling pathways lead to resistance against the two groups of pathogens (Thomma et al., 2001; Kunkel and Brooks, 2002). However, also BABA induced resistance against *P. parasitica* and *P. syringae* as well as against *B. cinerea* (Zimmerli et al., 2000; Zimmerli et al., 2001).

The preceding interpretations of our results are all based on the assumption that Pen contains only one elicitor inducing resistance. As an alternative model, Pen might contain two or more elicitors inducing resistance by way of distinct signalling pathways. This hypothesis could explain why the efficacy of Pen

against *P. parasitica* on NahG transformants is only reduced but not completely suppressed. A simultaneous activation of both signalling pathways would result in enhanced resistance levels, as reported for a simultaneous activation of SAR and ISR, (van Wees et al., 2000). Similarly, the finding that Pen was equally efficient against *B. cinerea* on all tested mutants could have been the result of different elicitors activating distinct signalling pathways. However, in this case, each of the elicitors alone had to be sufficient to induce the observed level of resistance. To test the 'two-elicitor-hypothesis', experiments should be repeated with purified Pen extracts. In addition, the analysis of double mutants could provide more insights.

Pen induced typical early defense-related responses, including an alkalinisation of the extracellular space, an oxidative burst and ethylene production. Pen-mediated alkalinisation responses were completely inhibited by the protein kinase inhibitor K-252a. Because protein phosphorylation is necessary for elicitor-mediated activation of early defense responses, application of K-252a allows to distinguish responses elicited by signal substances from unspecific effects such as pK changes, addition of bases and leakage of cell membranes due to addition of detergents (Felix et al., 1991; Boller and Felix, 1996). Pen stimulated alkalinisation responses and ethylene production in suspension cultured cells and leaves of numerous plant species belonging to different families, including even monocots such as rice. This finding is consistent with the observation that Pen induces resistance in a broad range of plant species including *Arabidopsis*, tomato, grapevine, apple tree and cucumber (chapter 2). In contrast, several elicitors such as a hexa- $\beta$ -glucoside (Sharp et al., 1984) and a  $\beta$ -glucopentaose (Yamaguchi et al., 2000) isolated from fungal cell walls and bacterial EF-Tu (Kunze et al., 2004) seem to induce defense responses only in a limited set of species. Similarly to Pen, chitin has been shown to induce early defense responses in many species including tomato, melon, soybean, parsley, wheat and rice (Shibuya and Minami, 2001). It can be argued that the elicitor activity of Pen might be identical to chitin or ergosterol, two well-known elicitors typical of higher fungi. However, there is substantial evidence that Pen contains at least one more elicitor, which is responsible for Pen-mediated resistance against pathogens. (i) Pen induced much stronger alkalinisation and ethylene responses than chitin. (ii) Pen was active in *Arabidopsis* cell cultures, which do not respond to ergosterol. (iii) Cells which were refractory to chitin were still fully responsive to Pen. (iv) Pen was very sensitive to protease digestions, indicating that a protein part is necessary for induction of alkalinisation and ethylene responses (see chapter 4). (v) Most importantly, we found that neither chitin nor ergosterol protected *A. thaliana* plants against *P. parasitica* and *B. cinerea* (unpublished data), indicating that, at least under our conditions, chitin or ergosterol are not sufficient to induce resistance.

In conclusion, Pen triggers early defense-related responses in numerous plant species and provides resistance against several pathogens in *Arabidopsis*. There is strong evidence that Pen induced resistance against *P. parasitica* on a salicylic acid-dependent, but NPR1-independent pathway. Whether Pen contains only one elicitor or several different elicitors inducing resistance via distinct signalling pathways still has to be elucidated.

## CHAPTER IV

### Purification and characterisation of the elicitor activities of Pen

#### Abstract

Pen, an aqueous extract of the dry mycelium of a high penicillin-producing strain of *P. chrysogenum*, induces early defense-related responses and resistance against several diseases in a broad range of plant species. The objective of this study was to purify and characterize the unidentified elicitor contained in Pen, denoted as Pen-elicitor. The Pen-elicitor could only be isolated from a high but not from a low penicillin-producing strain of *P. chrysogenum*. The Pen-elicitor was sensitive to protease digestion, to basic hydrolysis, to oxidation by periodate and, to a less extent, to acidic hydrolysis. Elicitor-activity was not affected by numerous other enzymes, including glucanases, chitinases, mannosidases, laminarinases and glycosydases as well as by chemical deglycosylation. Reversed phase, ion exchange, size exclusion and affinity chromatography revealed that heterogeneity is characteristic for the Pen-elicitor. Heterogeneity could not be reduced by treating Pen with specific enzymes or chemicals not affecting the elicitor-activity, which prevented a further analysis. We hypothesize that the Pen-elicitor consists of a small, distinct elicitor-active region, most likely a protein or peptide, which is part of a larger molecule varying in size and/or chemical composition.

## Introduction

When attacked by pathogens, plants activate several defense mechanisms, including the production of phytoalexins and antimicrobial proteins as well as the strengthening of the cell walls by enhanced cross-linking or deposition of molecules such as lignin, callose or silica (Sticher et al., 1997; Fritig et al., 1998; Heath, 2000; Dixon, 2001). Much attention has been paid to the perception of pathogens by plants and the signal transduction leading to the activation of defense mechanisms. Some of the earliest reactions of plants to pathogens include changes in the permeability of ion channels, measurable as an intracellular accumulation of  $\text{Ca}^{2+}$  and an extracellular alkalisation, the formation of reactive oxygen species and the synthesis of the stress hormone ethylene (Dixon et al., 1994; Kombrink and Somssich, 1995; Nürnberger and Scheel, 2001).

Several microorganism-derived compounds, so called exogenous elicitors, inducing early defense-related responses or the expression of antimicrobials, have been identified. Elicitors can be of fungal, bacterial or viral origin and include proteins, glycoproteins, saccharides, lipids and glycolipids. They can be membrane-, cell wall- or cell surface-associated structures, molecules actively secreted into the medium or located in the cytoplasm of intact cells. Examples of bacterial elicitors are flagellin (Felix et al., 1999), cold shock protein (Felix and Boller, 2003) and lipopolysaccharides from the cell wall of gram-negative bacteria (Dow et al., 2000). Some examples of elicitors isolated from higher fungi and oomycetes are chitin (Felix et al., 1993) and oligoglucans (Sharp et al., 1984; Yamaguchi et al., 2000) derived from cell walls, ergosterol (Granado et al., 1995), and various proteins and glycoproteins (Basse and Boller, 1992; Ricci et al., 1993; Nürnberger et al., 1994; Enkerli et al., 1999; Fellbrich et al., 2002). In addition, microbial enzymes secreted for degradation of plant cell walls can activate defense responses indirectly by releasing elicitor-active fragments from plant cell walls, so-called endogenous elicitors (Fry et al., 1993). Furthermore, plant cells might respond to changes in the turgor pressure and subsequent volume increases caused by the enzymatic degradation of their cell wall (Trewavas and Knight, 1994; Felix et al., 2000).

Usually, only a small part within a large molecule is required for recognition and elicitation of defense responses. Examples are a glucopentaose and a heptaglucoside from fungal cell wall glucans (Sharp et al., 1984; Yamaguchi et al., 2000), a sequence of 22 amino acids from bacterial flagellin (Felix et al., 1999) and a glycopeptide consisting of 12 mannosyl residues and at least 2 amino acids from yeast transglutaminase (Basse et al., 1992). To purify new elicitors and to elucidate minimal structures, simple, sensitive and fast bioassays are required. Thus, monitoring early defense-related responses in suspension-cultured cells or in leaf slices are valuable and widely used methods. However, cell cultures and leaf slices do not represent the intact biological system and it needs to be confirmed whether their response corresponds to the reaction of the intact plant (Boller and Felix, 1996). Therefore, it has to be verified whether highly purified elicitors can really affect plant-pathogen interactions. However, only few studies have focused on this aspect.

In chapters 2 and 3 we have shown that Pen, an aqueous extract from the mycelium of the non-pathogenic fungus *Penicillium chrysogenum*, induces resistance in various plant species against several pathogens, such as in grapevine against *Plasmopara viticola* and *Uncinula necator*, in apple tree against *Venturia inaequalis*, in cucumber against *Coletotrichum lagenarium*, in tomato against *Phytophthora infestans* and in *Arabidopsis thaliana* against *Peronospora parasitica*, *Botrytis cinerea*, *Alternaria brassicicola* and *Pseudomonas syringae* pv. *tomato*. Furthermore, we have shown that Pen induces early defense-related responses in suspension-cultured cells and leaf slices of many species. In this study, we attempted to purify and identify the elicitor/-s contained in Pen. We used medium alkalisation and ethylene production as fast and convenient assays to follow the purification process. Here, we show that a protein structure is important for the activity of the Pen-elicitor. However, identification of the elicitor failed so far because of its heterogeneity in size, charge and polarity.



## Methods

### Elicitors

#### Extracts from dry mycelium of *P. chrysogenum*

An aqueous extract was prepared from the dry mycelium of a *P. chrysogenum* strain used by industry for penicillin production (see chapter 3 for details) by autoclaving 150 g of the dry mycelium in 1 l water for 3 h. The resulting crude aqueous extract (= Pen) was dialysed in tubes with a 2000 Dalton cut-off (Spectra/Por® 6, Socochim SA) against water. If not mentioned otherwise, the Pen fraction larger 2000 Dalton (Pen<sub>2000</sub>) containing 12 mg/ml dry matter was used for further chemical and enzymatic treatments and purification steps. For initial experiments, Pen was shaken three times with 100% 2-butanol (1:1) to remove apolar substances like ergosterol, then precipitated with 80% acetone for three hours (−20°C). The resulting pellet was dissolved in the initial amount of water (=Pen<sub>Acetoneprec</sub>). To obtain a Pen extract with a limited range of molecular weight, Pen was first dialysed in tubes with cut-off 1 kDa and afterwards in tubes with cut-off 2 kDa. The fraction 1 to 2 kDa was lyophilised and resuspended in the initial amount of water.

#### Extracts from fresh mycelium of *P. chrysogenum*

Fresh mycelium of two strains of *P. chrysogenum* was obtained from Sandoz GmbH (Kundl, Austria). One strain produces high amounts of penicillin and is used for penicillin production on industrial scale (the dry mycelium used for Pen extraction originates from this strain) (=the high penicillin-producing strain), the other strain produces only low amounts of penicillin (=the low penicillin-producing strain). Both strains were grown by Sandoz GmbH in mini-fermentors using the same growth medium as for industrial production at identical conditions. The culture filtrates were obtained by centrifugation and sterile filtration.

#### Extracts from the high penicillin-producing strain

4 ml mycelium (ground in liquid nitrogen or intact) was mixed with 16 ml liquid (water ± 3ml culture filtrate of the high-penicillin producing strain). Triton (final concentration 1%), n-butylacetate (20 ml) or water was added and pH was lowered to 3 with H<sub>2</sub>SO<sub>4</sub> in half of the samples. Tubes were shaken at room temperature and samples were taken after 0, 1, 3, 6, and 24 h, boiled for 10 min (95°C) and frozen. Butylacetate was evaporated before testing the fractions in alkanisation assays.

#### Extracts from the low penicillin-producing strain

**Extraction by enzymes.** Mycelium of the low penicillin-producing strain was digested with enzymes listed in table 1 under “other enzymes” and with pronase as well as protease V8. Samples were digested over night at room temperature with a final enzyme concentration of 10 mg/ml in 10 mM TrisHCl (pH 6.8) and boiled before testing

**Extraction by heat.** Mycelium was heated with or without glucose or penicillin (benzylpenicillin natrium, Sandoz GmbH) (i) on a heating plate (magnetic stirrer) at 200°C for 1 min to 3 h (ii) in water (pH 2 and 6) at 95°C for 1 to 24 h (iii) in an oven at 140°C for 1 to 12 h. Water was added to the resulting pellets and water soluble fractions were tested in alkanisation assays.

**Extraction of cell wall glucans.** Ground mycelium was treated according to (Yamaguchi, Yamada et al. 2000)) to extract cell wall glucans.

**Extraction with butylacetate.** Mycelium was treated according to the protocol of Sandoz GmbH for extraction of penicillin as described in chapter 2. Samples were taken after all steps and tested in alkanisation and ethylene bioassays

**Extraction with SDS and Mercapthoethanol.** Ground mycelium was treated with 2% SDS and 0.5% Mercapthoethanol for 1 to 24 h at room temperature or at 95°C. Such a treatment had no effect on the elicitor activity of the Pen extract.

*Extraction with ethylenediamine.* Ground mycelium was extracted with 100% ethylenediamine for 10 d at room temperature. The supernatant was dried, the pellet was dissolved in water and pH was adjusted to 5. The soluble part was tested in alkalisation assays.

*Extraction with enzymes of the high penicillin-producing strain.* Ground mycelium of the low penicillin-producing strain (4 ml) was reacted with culture filtrate of the high penicillin-producing strain, a protein extract from mycelium of the high penicillin-producing strain, or with a combination of the two (16 ml) at pH 3 or 6 (room temperature, 0 to 24 h). Samples were boiled before they were tested in alkalisation bioassays. Proteins were extracted from ground mycelium of the high penicillin-producing strain by shaking in water containing  $\text{CaCl}_2$ ,  $\text{MgCl}_2$  (1 mM) and NaCl (10 mM) (pH 7.9). The procedure was repeated at pH 5 and the two extracts were combined for the extraction experiment.

#### Other elicitors and inhibitors

Chitin used in *A. thaliana* cell cultures kindly provided by G. Felix was prepared by reacylation of chitosan from crab shells (Fluka) as described by (Felix, Regenass et al. 1993). In *L. peruvianum* cell cultures, chitin hexamers (Seikagaku Corp, Tokyo, Japan) were used. Ergosterol (Sigma) was dissolved and diluted in DMSO. The synthetic peptide flg22 containing a highly conserved sequence of 22 amino acids from the N terminus of bacterial flagellin (Felix, Duran et al. 1999) and elongation factor Tu (EF-Tu) purified from *E. coli* (Kunze et al., 2004) were used as references in alkalisation and ethylene bioassays and as controls for protease digestions. The protein kinase inhibitor K-252a (Fluka) was diluted in DMSO and applied in final concentration of 1  $\mu\text{M}$ .

#### Plant cell cultures, alkalisation and ethylene bioassays

Cell suspension cultures of *Arabidopsis thaliana*, tomato (cell line Msk8), *Lycopersicon peruvianum*, and rice (cell line OC), were used six to ten days after subculture (see chapter 3 for details on maintenance). Alkalisation of the growth medium was either continuously measured and recorded as described before (chapter 3) in 3 ml aliquots of the cell culture, or in 0.5 ml aliquots, placed into 24-well-plates, after a certain time period. This time period was derived from permanent recordings for each single batch of cells as the average time needed for maximal response.

For ethylene bioassays, *A. thaliana* plants ecotypes Col-0, WS and La-er were grown in growth chambers. Leaf slices of fully expanded leaves were floated on water over night. Ethylene biosynthesis was measured as described before (chapter 3).

#### Purification by chromatography

*Reversed phase chromatography.* Pen<sub>2000</sub> containing 0.1% trifluoroacetic acid (TFA) was run on C8 or C18 pre-columns (Chromabond) with 0.1% TFA and eluted with 50% acetonitrile. Binding fractions (=Pen<sub>C8</sub> or Pen<sub>C18</sub>) dissolved in 0.1% TFA were then run on C8 or C18 HPLC columns (Pharmacia Biotech C8 SC 2,1/10 and C18 SD 2,1/10) using a SMART system (Pharmacia Biotech) and eluted with different linear acetonitrile gradients. Absorption was detected at three different wavelengths ( $\lambda=214, 280, 395$  nm). Fractions were tested in alkalisation assays.

*Ion exchange chromatography.* Pen<sub>Acetoneprec</sub> containing 10 mM Na-acetate (pH 4.8) was run on a cation exchange column (SP-Trisacryl M, Biosapra) and eluted with 0.5 M NaCl (Pen<sub>charged</sub>). A small part of the Pen extract which could only be eluted with 1 M NaOH did not induce alkalisation responses. Pen<sub>charged</sub> was precipitated twice with 80% acetone and resuspended in 20 mM TrisHCl (pH 8) or in 20 mM Na-acetate (pH 4.8) for anion- or cation exchange on MonoQ (PC 1,6/5, Pharmacia biotech) or MonoS (PC 1.6/5, Pharmacia Biotech) HPLC columns respectively using the SMART system. Binding fractions were eluted with a linear gradient of 0 to 0.5 M NaCl, and tested in alkalisation assays.

*Size exclusion chromatography.* Pen<sub>Acetoneprec</sub> was dissolved in 70% formic acid and run with 70% formic

acid on a HPLC superdex peptide column (PC 3,2/30, Pharmacia Biotech) using the SMART system.

**Affinity chromatography.** Pen<sub>2000</sub> or Pen<sub>charged</sub> containing 100 mM Na-acetate or 20 mM MES (both pH 5.2), CaCl<sub>2</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub> (all 1mM) and NaCl (0.15 M) were run on columns containing Concanavalin A (ConA) sepharose (Sigma). The binding fraction (Pen<sub>binding</sub>) was eluted with 0.2 M  $\alpha$ -methyl-D-mannopyranoside. The non-binding fraction of the first run containing elicitor activity was run a second time on ConA to avoid contamination with ConA binding substance due to an overload of the column. The fraction binding to ConA and the flow-through of the second run (Pen<sub>nonbinding</sub>) were acetone precipitated (80% acetone, 3 h, -20°C), dried and resuspended in the same volume of water as the starting material.

### Enzymatic treatments

Pen<sub>Acetoneprec</sub> was digested with several enzyme preparations listed in table 1 under "other enzymes" and with pronase and protease V8. Samples were digested over night at room temperature with a final enzyme concentration of 10 mg/ml (pH~4.8). Pen<sub>2000</sub> and flg22 (5  $\mu$ M) dissolved in 50 mM MES (pH 5.2) were digested with the crude protease preparations pronase, protease K, chymotrypsin, carboxypeptidase, ficin, trypsin and protease V8 (5 mg/ml) (table 1) at 37°C over night. Pen<sub>C8</sub> and fractions of Pen<sub>C8</sub> purified one to three times on C8 or C18 HPLC columns were digested with the highly purified enzymes Trypsin, GluC, O-glycosidase, PNGase F and EndoH (tab. 1) according to the recommendations of the suppliers. Trypsin digestion was performed in 20 mM TrisHCl (pH8), digestion with GluC in 20 mM NaAcetate at pH 4 (optimal conditions for cleaving at glutamate) or 20mM MES at pH 6 (optimal conditions for cleaving at aspartate) respectively. flg22 (Trypsin) and EF-Tu (GluC) were digested as controls under identical conditions. The culture filtrate of the new *P. chrysogenum* strain (see below) was digested with the supernatant of two different *A. thaliana* cell cultures (1:1) and with 5 mg/ml pronase or protease K at room temperature over night (pH~6).

All samples except the culture filtrate were boiled for 10 min at 95°C to stop the reaction.

### Chemical treatments

**Basic and acidic hydrolysis.** Pen<sub>2000</sub> was reacted with 0.1 and 1 M NaOH or HCl respectively at room temperature or 95°C. Samples were taken after 0, 10, 30 and 60 min and neutralized immediately to pH 5. Dilution of Pen by neutralization was taken into account for the calculation of the concentrations used in alkalisation assays.

**Oxidation by periodate (NaIO<sub>4</sub>).** Pen<sub>2000</sub> was reacted with 50 mM sodium periodate (NaIO<sub>4</sub>) at 37°C for 24 h in the dark. The reaction was stopped by precipitating the mixture with 80% acetone for at least three hours at -20°C and the resulting pellet was dissolved in the initial amount of water.

**Reduction by sodium-borohydride (NaBH<sub>4</sub>).** Pen<sub>C8</sub> was reacted with 60 mM NaBH<sub>4</sub> in 30 mM K<sub>2</sub>HPO<sub>4</sub> at 95°C for 90 min. The reaction was stopped by lowering the pH to 4.3 with acetic acid and placing the mixture on ice. The sample was desalted on a PD-10 column (Amersham Pharmacia).

**Chemical deglycosylation with TFMS.** Chemical deglycosylation with trimethylfluorosulfonic acid (TFMS) was performed according to the protocol of GlycoFree Deglycosylation Kit. 5 mg of lyophilized Pen (fraction 1-2 kDa, binding to C8) was reacted with 150  $\mu$ l of a mixture containing 16% toluene and 84% TFMS (Sigma) in a glass vessels which had been cooled down in a dry-ice-ethanol-bath. After shaking 3 times gently, the sample was kept for 4 h at -20°C. The reaction was stopped by adding 450 $\mu$ l of a mixture containing 60% pyridine, 20% methanol and 20% H<sub>2</sub>O and 1.2 ml of a 0.5% solution of ammonium bicarbonate. The sample was desalted on a PD-10 column (Amersham Pharmacia), dried and dissolved in water. Part of the fraction containing elicitor activity was analysed on a C18 HPLC column as described before.

**Acetylation.** 8 mg of Pen<sub>C8</sub> or Pen<sub>C18</sub> were reacted with 2 ml of a mixture containing 25% acetic anhydride and 75% methanol (v/v) for 10 h at room temperature. After adding ammonium bicarbonate to a final concentration of 50 mM, the sample was reacted for one more hour. Reaction was stopped by acetone

precipitation (80%, 1 h at  $-20^{\circ}\text{C}$ ). Supernatant and pellet were dried and both fractions were dissolved in the initial amount of water.

**Table 1.** Enzymes and their documented activities used for different digestions of Pen extract and for extraction of Pen elicitor activity from mycelium of *P. chrysogenum*.

Enzyme	Activities
<b>Proteases</b>	
Carboxypeptidase from bovine pancreas (Boehringer)	Cuts C-terminal amino acids
Chymotrypsin from bovine pancreas (Boehringer)	Cuts at phe, tyr and trp
Ficin from <i>Ficus carica</i> (Boehringer)	Protease mixture, cuts preferentially at basic amino acids and leu, gly
Glu-C, sequencing grade (Roche)	Cuts at glu 1000 times more often than at asp (at pH 4 and 8)
Pronase from <i>Streptomyces griseus</i> (Fluka and others)	Protease mixture, cuts almost any peptide bond
Protease from <i>Staphylococcus aureus</i> strain V8 type XVII-B (Sigma) = Protease V8	Contains endoproteinase GluC
Protease K from <i>Tritirachium album</i> (Boehringer)	Protease mixture, cuts preferentially next to N-substituted hydrophobic aliphatic and aromatic amino acids
Trypsin, crude preparation (Fluka) and sequencing grade (Promega)	Cuts at lys and arg
<b>Glycosidases</b>	
O-Glycosidase (Roche)	Cleaves the disaccharide Gal $\beta$ (1-3) GalNAc from ser or thr in O-glycosylated peptides or proteins
EndoH (New England BioLabs)	Cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins
PNGase F = N-Glycosidase F (New England BioLabs)	Cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins
<b>Other enzymes</b>	
Chitinase (Sigma)	
Cytohelicase from <i>Helix pomatia</i> (IBF)	Laminarinase activity
$\beta$ -Glucanase (BDH)	
$\alpha$ -Glucosidase (Boehringer, Sigma)	
$\beta$ -Glucosidase (Boehringer, Sigma)	
Lyticase from <i>Derskovia xanthineolytica</i> (Sigma)	$\beta$ -1,3-glucanase
Lysing enzyme from <i>Asparagillus niger</i> (Sigma)	Pectinases, cellulases
Lysing enzyme from <i>Cytophagia</i> sp. (Sigma)	Glucanases, proteases
Lysing enzyme from <i>Rhizoctonia solani</i> (Sigma)	Glucanases and proteases
Lysing enzyme from <i>Trichoderma harzianum</i> (Sigma)	Cellulases, proteases and chitinases
Lysozyme from egg (Fluka)	Muramidase
$\alpha$ -Mannosidase from almonds (Sigma)	
Supernatant from suspension cultured <i>A. thaliana</i> and <i>L. peruvianum</i> cells	Chitinases and proteases
Zymolyase from <i>Arthrobacter luteus</i> (Seikagaku)	$\beta$ -1,3-glucan laminarinpentaohydrolase (main activity), $\beta$ -1,3-glucanase, protease, mannanase

### ***In vitro* assays for detection of pectolyase activity**

PectinC from Sigma (10 mg/ml) and fresh suspension cultured *A. thaliana* cells washed several times with mannitol (150 mosmol) were digested with the culture filtrate of the high penicillin-producing strain (10  $\mu\text{l/ml}$ ), pectin lyase (11  $\mu\text{g/ml}$ ) or Pectolyase Y23 (20  $\mu\text{g/ml}$ ) (Seishin Pharmaceuticals, Tokyo) in 0.1 M

Na-acetate (pH 5.1) containing 10 mM CaCl<sub>2</sub>. Substrate plus water and enzymes alone served as controls. Samples were taken after 0, 1 or 2 h and 20 h. Pectolyase activity was determined with two different assays. Absorption spectra from 200 to 350 nm were scanned for each sample to detect changes in the absorption between 230 to 240 nm. At these wavelengths, olefinic bonds of unsaturated uronic esters resulting from pectolyase activity are known to absorb. In an assay according to (Nedjma et al., 2001), 400 µl H<sub>2</sub>O and 50 µl 1 M NaOH were added to 100 µl of a sample and heated for 5 min at 80°C. Then, 600 µl 1 M HCl and 500 µl 0.04 M thiobarbituric acid (TBA) were added and heated again for 5 min at 80°C. After cooling down, absorption at  $\lambda=550$  nm was measured.

## Results

### **The Pen-elicitor can only be isolated from the mycelium of a high penicillin-producing strain of *P. chrysogenum***

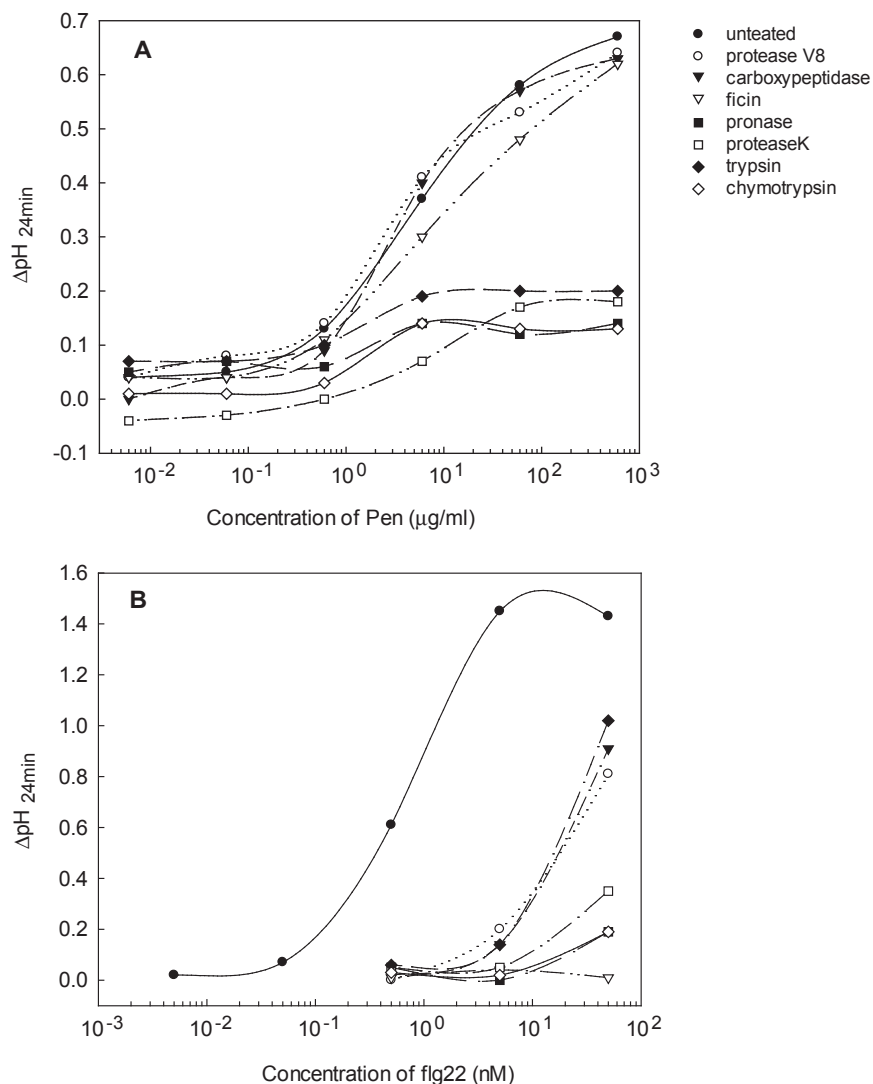
We have shown before that Pen, an aqueous extract from the dry mycelium of a high penicillin-producing strain of *P. chrysogenum*, contains an unidentified elicitor (the 'Pen-elicitor') inducing early defense-related responses. Here, we extracted fresh mycelium of a low and a high penicillin-producing strain of *P. chrysogenum* to test whether (i) the Pen-elicitor is specific for the high penicillin-producing strain and (ii) the drying process of the mycelium has an influence on the Pen-elicitor. Both *P. chrysogenum* strains were grown in mini-fermentors under conditions identical to industrial production. The Pen-elicitor could only be extracted from the mycelium of the high but not the low penicillin-producing strain of *P. chrysogenum*, although the mycelium of the low penicillin-producing strain was extracted under a wide range of conditions, including the application of several solvents, heat treatments under different pH values and digestions with numerous enzymes (see material and methods and tab. 1). An alkalisation-inducing activity was only detectable in the water soluble part of an extract with ethylenediamine in one experimental setup (data not shown). To avoid alkalisation responses due to chitin, an elicitor liberated particularly at low pH values from fungal cell walls, all extracts were tested in cell cultures which had been desensitized for chitin by a pretreatment with this elicitor as described by (Felix et al., 1998).

Intact and ground mycelium of the high penicillin-producing strain of *P. chrysogenum* was extracted at pH 3 or 6 with and without a solvent or a tenside for different time periods. All extracts from intact but not from ground mycelium induced alkalisation responses in *A. thaliana* cell cultures pretreated with chitin. Best alkalisation-inducing activity was found in samples extracted for a short time (10 min to 1 h) with a tenside at pH 6 (data not shown). Cells desensitized for Pen reacted to extracts from the fresh mycelium of the high penicillin-producing strain only with a faint additional alkalisation of the medium (data not shown). To distinguish elicitor-induced alkalisation responses from unspecific effects such as membrane leakage due to membrane active compounds (e.g. tensides) or addition of bases, the protein kinase inhibitor K-252a was added to cell cultures short before the extracts were added. Alkalisation responses were completely inhibited by K-252a.

### **The Pen-elicitor is sensitive to protease digestion, to basic hydrolysis and to oxidation by periodate**

Pen, the aqueous extract of the dry mycelium of the high penicillin-producing strain of *P. chrysogenum*, was digested with various enzyme preparations including chitinase, cytohelicase, glucanase, glucosidase, two glycosydases, mannosidase, zymolyase, lyticase, several other lysing enzymes, the supernatant of different cell cultures and various preparations containing protease activities (for details see tab. 1). Only pronase, protease K, chymotrypsin and trypsin strongly reduced the alkalisation-inducing activity of Pen

(fig. 1A). All other enzymes, including the proteases carboxypeptidase, ficin and protease V8 had no effect on the Pen-elicitor. All of the seven tested crude proteases largely reduced the alkalisation-inducing activity of the peptide flg22 (fig. 1B), which was digested to check the activity of the proteases. Like crude trypsin, highly purified trypsin strongly reduced the alkalisation-inducing activity of Pen, indicating that impurities are not necessary for the inactivation of the Pen-elicitor (data not shown).

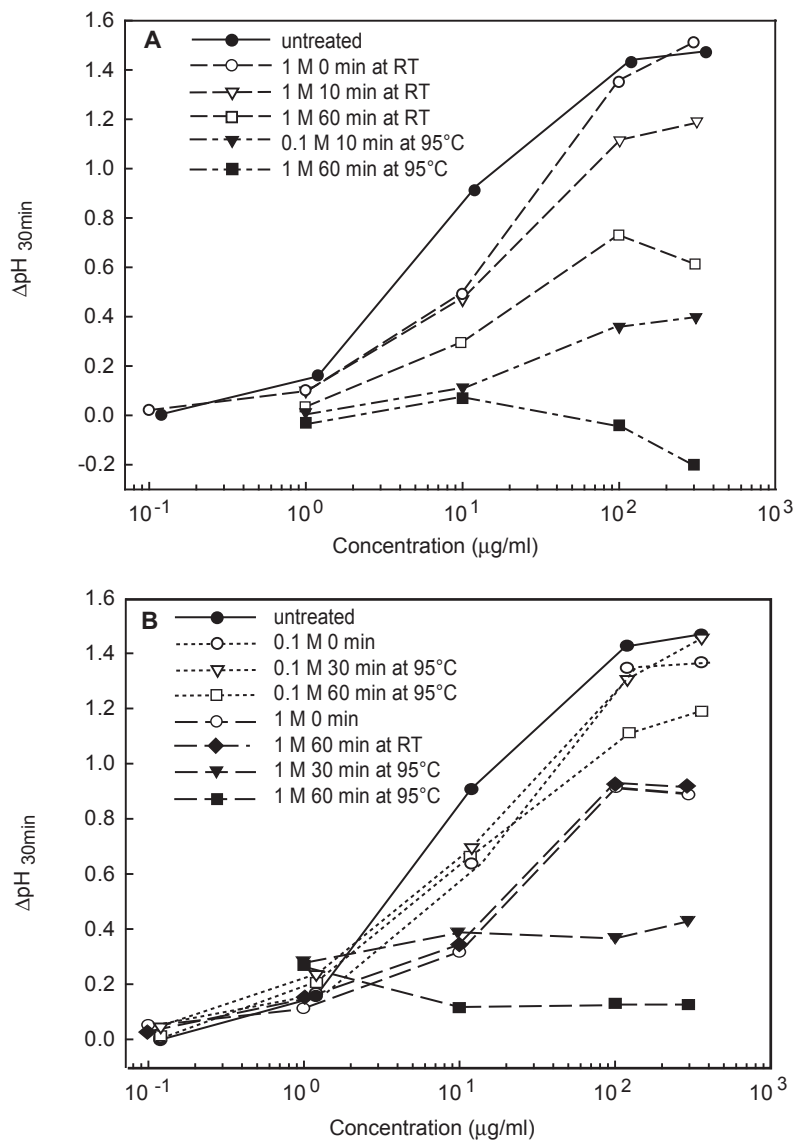


**Figure 1.** Effect of treatment with different proteases on alkalisation-inducing activity of Pen<sub>2000</sub> (A) and flg22 (B). Samples were digested with 5 mg/ml enzyme at 37°C over night and reaction was stopped by boiling (95°C, 10 min). Different doses of Pen<sub>2000</sub> (A) or flg22 (B) untreated or treated with protease V8, carboxypeptidase, ficin, pronase, protease K, trypsin or chymotrypsin were added to *A. thaliana* cell cultures 47 min after a pretreatment with chitin (10 mg/ml). pH change was measured after 24 min. Cells were refractory to a second chitin stimulus (20 mg/ml). Proteases alone did not induce alkalisation, and samples taken immediately after adding the proteases had same elicitor activities as the untreated control (data not shown).

The Pen-elicitor was very sensitive to basic hydrolysis, but quite robust to acidic hydrolysis, i.e. boiling Pen for 10 min with 0.1 M NaOH was sufficient to destroy the alkalisation-inducing activity almost completely



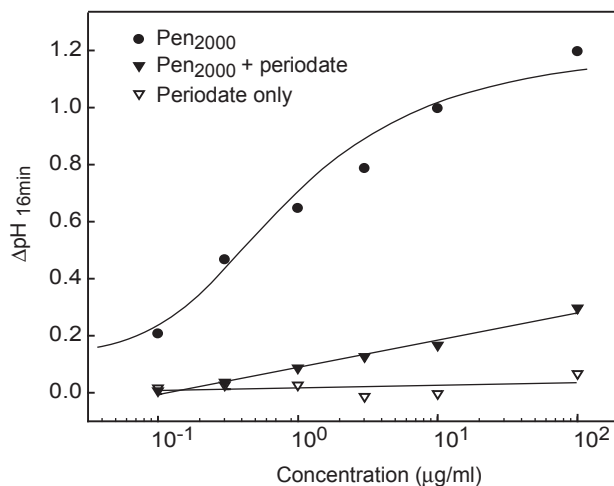
(fig. 2A), whereas boiling Pen for one hour with 0.1 M HCl had almost no effect (fig. 2B). Boiling Pen for 30 min with 1 M HCl was necessary to destroy the alkalisation-inducing activity completely. The maximal response of cells to Pen treated with 1 M NaOH at room temperature decreased with time and was less than half of the initial value after 1 h (fig. 2A). However, the concentration inducing a half maximal response (EC50) was hardly changed.



**Figure 2.** Effect of treatment with NaOH (A) and HCl (B) on alkalisation-inducing activity of Pen<sub>2000</sub>. Different doses of treated or untreated Pen were added to *A. thaliana* cell cultures and pH was measured after 30 min. Pen<sub>2000</sub> was reacted with 0.1 M or 1 M HCl or NaOH respectively at 95°C or at room temperature. Samples were taken after 0, 10, 30 and 60 min and neutralized immediately to pH 5.

Reacting Pen with the strong oxidizing agent sodium periodate (NaIO<sub>4</sub>) completely destroyed alkalisation-inducing activity in three independent experiments (fig. 3). Surprisingly, when the reaction was done in large (250 ml) instead of small (< 1 ml) amounts of Pen, periodate only slightly reduced the alkalisation-inducing activity of Pen. Treatment of Pen with the strong reducing agent sodium borohydride (NaBH<sub>4</sub>) did

not affect the alkalinisation-inducing activity (data not shown). Furthermore, Pen was insensitive to chemical deglycosylation by TFMS (data not shown).



**Figure 3.** Effect of treatment with NaIO<sub>4</sub> on alkalinisation-inducing activity of Pen<sub>2000</sub>. Dose-response curves for untreated Pen (closed circles), Pen treated with 50 mM NaIO<sub>4</sub> for 24 h at 37°C in the dark (closed triangles), and periodate only (open triangles) in *L. peruvianum* cell cultures. PH was measured after 16 min. Amount of periodate added corresponds to the amount of periodate added with the digested Pen extract. All fractions were precipitated with acetone and resuspended in water.

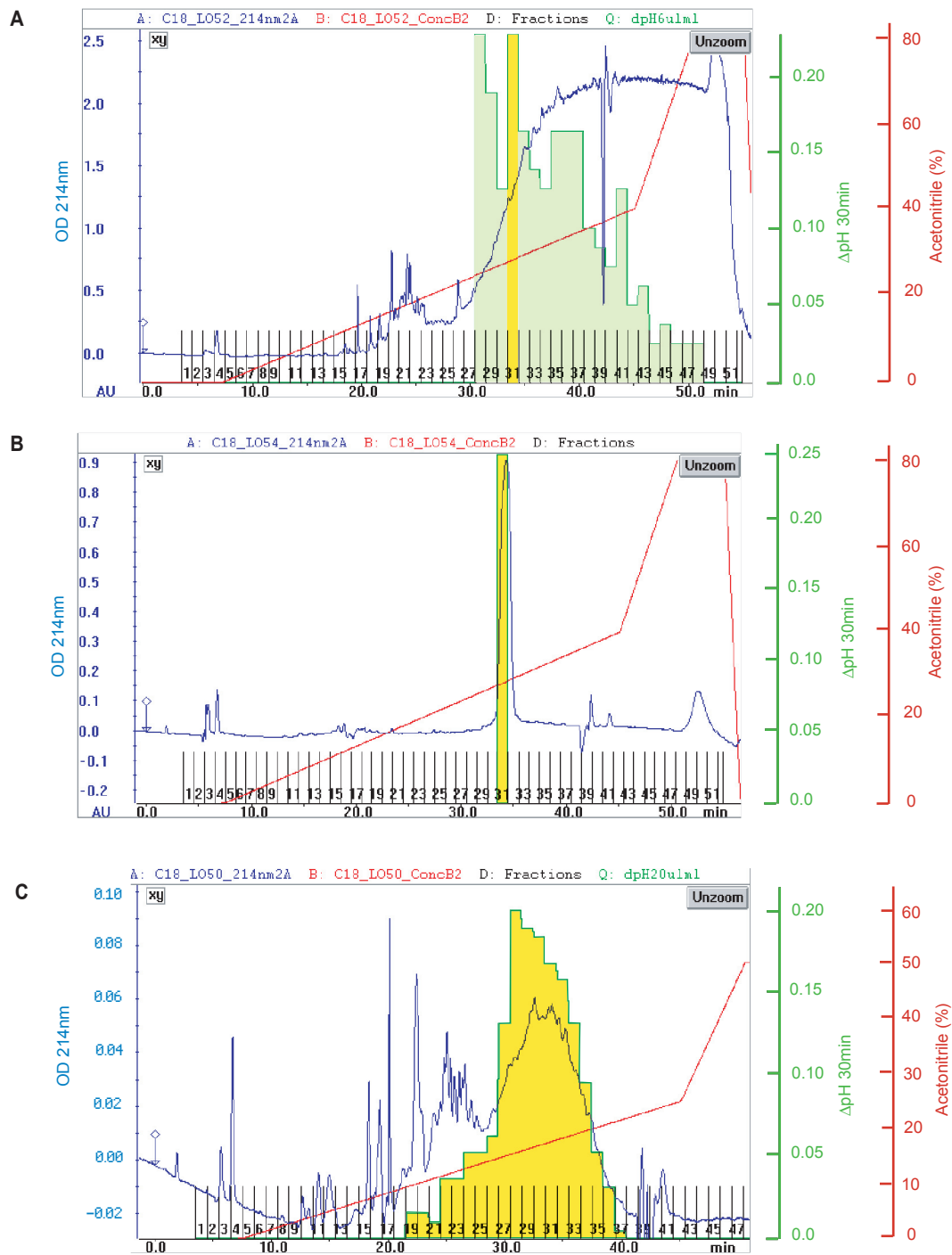
### The Pen-elicitor is heterogeneous in polarity, size and charge

A considerable part of the Pen-elicitor was binding to C8 and C18 alkyl chains in reversed phase chromatography. However, the resulting chromatogram did not show distinct peaks, and the alkalinisation-inducing activity was spread over a wide range of fractions (fig. 4A). A Pen extract with a restricted range of molecular size (1-2 kDa) behaved as heterogeneously as the normally used Pen extract (data not shown). Running a selected fraction again under identical conditions lead to a sharp peak at the same position and alkalinisation-inducing activity was only found in the corresponding fraction (fig. 4B). However, running a selected fraction on the same column but with a shallower elution gradient resulted in a chromatogram with a strongly broadened peak and alkalinisation-inducing activity was concomitantly spread over a wide range of fractions (fig. 4C). Running Pen on ion exchange and size exclusion columns resulted in a chromatogram similar to the one of reversed phase chromatography, and alkalinisation-inducing activity was spread over many fractions as described before (data not shown).

It was suspected that the heterogeneity of the Pen-elicitor results from variable structures bound to a distinct, small elicitor-active region. To remove such structures, fractions with a defined reaction (fig. 4B) were either digested with enzymes which do not inactivate the Pen-elicitor ('non-inactivating enzymes'), e.g. endoproteinase GluC or glycosidases, or exposed to specific chemical treatments, e.g. chemical deglycosylation or acetylation. However, none of the treatments affected the reaction of the fraction in reversed phase chromatography (data not shown).

Part of the elicitor activity of Pen was binding reversibly to the lectin Concanavalin A (Pen<sub>binding</sub>), whereas another part did not (Pen<sub>nonbinding</sub>). In alkalinisation assays, Pen<sub>binding</sub> was more active than Pen<sub>nonbinding</sub> (fig. 5A). In contrast, in ethylene bioassays, leaves showed a stronger response to Pen<sub>nonbinding</sub> than to Pen<sub>binding</sub>, the latter hardly inducing any ethylene production (fig. 5B). In both bioassays, the maximal response to any of the two fractions alone and even to a combination of the two, was much lower compared to the original Pen extract (fig. 5B). No elicitor activity was detectable after boiling ConA-sepharose for 10 min to 1 h in water or SDS (data not shown).

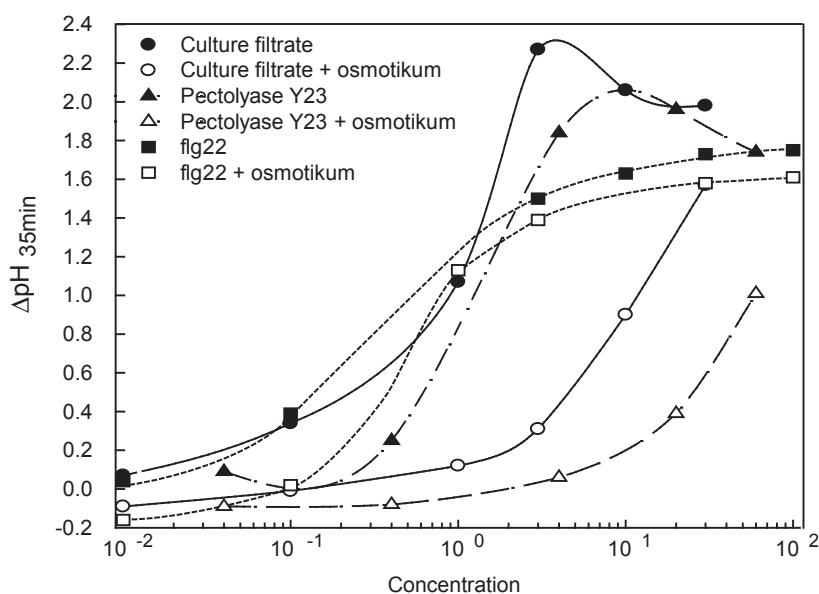




**Figure 4.** Purification of the Pen elicitor by reversed phase chromatography. The graph shows absorption at 214 nm (blue lines), acetonitrile concentration (red lines) and alkalisation-inducing activity of the fractions (green and yellow area). Alkalisation-inducing activity of an aliquot of each fraction (6 (A), 10 (B) and 20  $\mu\text{l/ml}$  (C)) was tested in *A. thaliana* cell cultures, pH was measured after 30 min. **A**, PenC18 run on a C18 HPLC column with 0.1% TFA and eluted with a linear gradient of acetonitrile (0 to 40%). **B**, Fraction 31 run again with the same gradient on the same column after acetonitrile had been evaporated. **C**, A selected fraction from the 1st run (fraction 28) run on the same column but eluted with a more shallow acetonitrile gradient (0 to 24%).

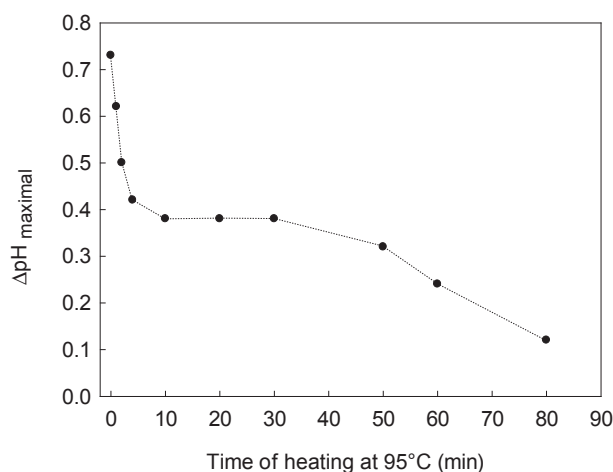
### The culture filtrate of *P. chrysogenum* contains an elicitor- and a pectolyase- activity

Not only the Pen extract but also the culture filtrate of the high penicillin-producing *P. chrysogenum* strain contained an elicitor activity inducing very strong alkalinisation responses in *A. thaliana*, tomato, *L. peruvianum* and rice cell cultures as well as ethylene production in *A. thaliana* leaf slices. As a control, no alkalinisation-inducing activity was found in the growth medium alone. As in the aqueous extracts from the mycelium, no alkalinisation-inducing activity was found in the culture filtrate of the low penicillin-producing *P. chrysogenum* strain (data not shown). The culture filtrate of the high penicillin-producing strain behaved different to Pen: The alkalinisation response upon treatment with the culture filtrate started after a very short lag phase (less than one minute) and was then very pronounced, i.e. up to 2.5 pH units compared to maximal 1.5 pH units induced by Pen (fig. 6). In contrast to Pen, the elicitor in the culture filtrate was insensitive to digestion with pronase or trypsin (data not shown), but was only partly heat stable (fig. 7). Furthermore, *A. thaliana* cell cultures pretreated with Pen responded with further alkalinisation adding the culture filtrate as a second stimulus, but they were refractory to a second stimulus with Pen (data not shown).

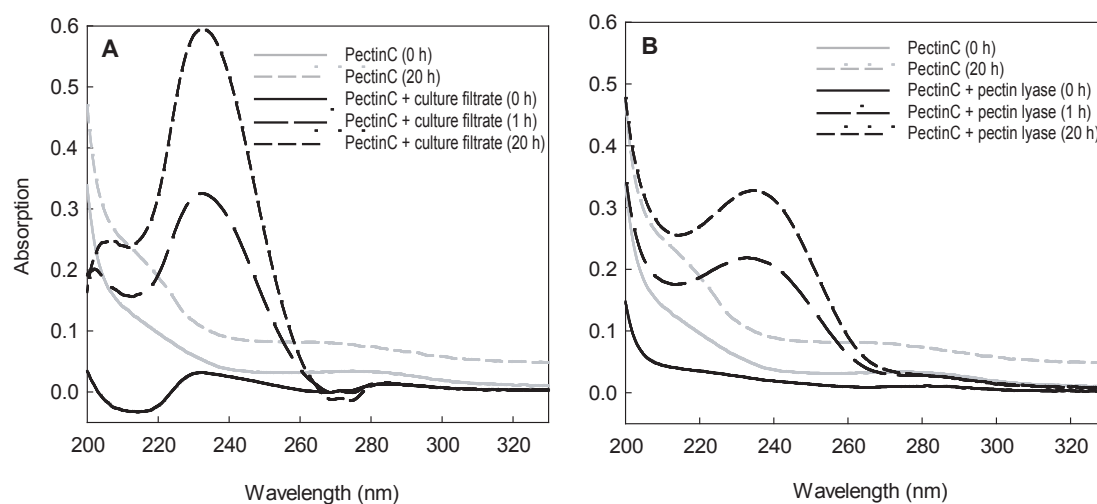


**Figure 6.** Effect of increased extracellular osmotic potential on alkalinisation-inducing activity of the culture filtrate of the high penicillin-producing strain of *P. chrysogenum*, pectolyase Y23 and flg22. Dose-response curves of the culture filtrate (circles), pectolyase Y23 (triangles) and flg22 (squares) in *A. thaliana* cell cultures with and without increasing the osmotic potential of the extracellular medium. Osmotic potential was increased from 150 to 450 mosmol with mannitol 1 min before elicitors were added, pH was measured after 35 min. Concentrations are in  $\mu\text{l/ml}$  (culture filtrate),  $\mu\text{g/ml}$  (pectolyase Y23) or nM (flg22).

Very high alkalinisation responses, as induced by the culture filtrate, are also typical of pectolyases (G. Felix, unpublished data). As demonstrated for pectolyases (G. Felix, unpublished data), the alkalinisation response to the culture filtrate could be suppressed by increasing the osmolarity of the medium of the cell cultures, whereas the response to flg22 was not affected (fig. 6). In two different *in vitro* assays, a pectolyase activity was detected in the culture filtrate (fig. 8 and 9).



**Figure 7.** Effect of heat treatment on alkalisation-inducing activity of the culture filtrate of the high penicillin-producing strain of *P. chrysogenum*. Aliquots (1  $\mu$ l/ml) of the culture filtrate boiled for different times at 95°C were added to *A. thaliana* cell cultures. Maximal alkalisation responses were derived from permanent recordings of the alkalisation responses.

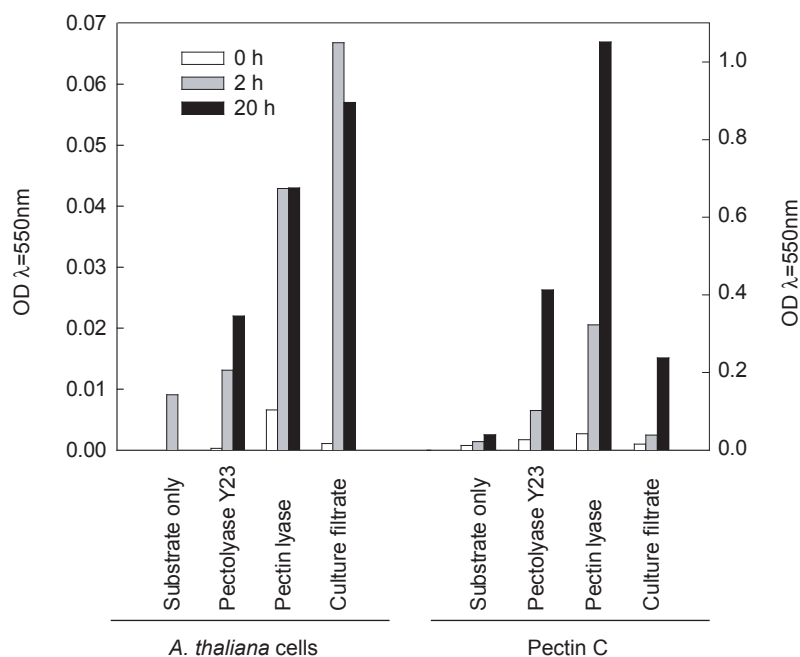


**Figure 8.** Detection of pectolyase activity in the culture filtrate of the high penicillin producing strain of *P. chrysogenum*. Curves show absorption spectra ( $\lambda=200-330$  nm) of pectinC (20  $\mu$ g/ml) digested with the culture filtrate (20  $\mu$ l/ml) (A) or pectin lyase (10  $\mu$ g/ml) (B) at 37°C. Samples were taken after 0 h (straight lines), 1 h (long dashed lines) and 20 h (short dashed lines), boiled and scanned in a photospectrometer. Absorption of pectinC alone is shown in gray, absorption of the digested pectinC in black, absorption of the enzymes alone was subtracted.

## Discussion

We have demonstrated before that Pen, the aqueous extract from the dry mycelium of a high penicillin-producing strain of *P. chrysogenum*, elicits early defense-related responses in cell cultures and leaf slices of a wide range of plant species and induces resistance against various diseases in several plants (chapters 2 and 3). Furthermore we have shown that Pen contains at least one elicitor different from the well-known fungal elicitors chitin and ergosterol. This unidentified elicitor has been referred to as the Pen-elicitor. The objective of this study was to characterize and identify the Pen-elicitor.

The fact that the Pen-elicitor is recognized by a broad range of plant species let us suggest that the Pen-elicitor represents a microorganism-derived structure which is generally recognized by plants. Such structures are referred to as general elicitors, which are conceptually identical to PAMPs (pathogen-associated



**Figure 9.** Detection of pectolyase activity in the culture filtrate of the high penicillin-producing strain of *P. chrysogenum* in two different substrates. PectinC (10 mg/ml) and fresh *A. thaliana* cells were digested with culture filtrate (10  $\mu$ l/ml), pectolyase Y23 (20 mg/ml) or pectin lyase (10 mg/ml) for 0, 2 and 20 h at 37°C. Samples were reacted with thiobarbituric acid and absorption at  $\lambda=550$  nm was measured in a photometer. Absorptions of the enzymes alone were subtracted.

molecular patterns) (Nürnberg and Brunner, 2002). PAMPs or general elicitors consist of structures unique for microorganisms which are highly conserved because they have important roles in microbial physiology or structure. Thus, they generally occur in whole classes of microorganisms. Examples of general elicitors are  $\beta$ -glucans, chitin fragments and ergosterol typical of higher fungi, extracellular proteins typical of oomycetes, and lipopolysaccharides and flagellin typical of bacteria (Ricci et al., 1993; Granado et al., 1995; Felix et al., 1999; Dow et al., 2000; Shibuya and Minami, 2001; Hahlbrock et al., 2003). As an alternative hypothesis, the Pen-elicitor might be an enzyme liberating endogenous elicitors from plant cell walls. However, the latter hypothesis is very unlikely, because the fresh mycelium of *P. chrysogenum* was dried for several hours at very high temperature before Pen was extracted by autoclaving.

The Pen-elicitor could only be extracted from a high but not from a low penicillin-producing strain of *P. chrysogenum*, although both strains were grown under identical conditions and a wide range of extraction methods were applied. Moreover, the Pen-elicitor was not found in any of several tested commercially available crude fungal preparations (data not shown). This finding is in strong contrast to the fact that the Pen-elicitor, which is isolated from a non-pathogenic fungus, is generally recognized by plants. Three explanations for this result are discussed in the following. (i) The Pen-elicitor might represent a substance newly formed by a Maillard reaction during heating of the mycelium. However, this hypothesis is highly unlikely because the Pen-elicitor could be extracted from fresh, unprocessed mycelium of the high penicillin-producing strain without heating. Furthermore, none of the heat treatments released Pen elicitor-activity from the mycelium of the low penicillin-producing strain. (ii) The Pen-elicitor might be present in all strains (and even in other fungal species), but is only set free in sufficient amounts under particular conditions, as reported for several cell wall components (Ruiz-Herrera, 1992; Yamaguchi et al., 2000). As an example, specific enzymes expressed only by particular strains of a fungus might be required for the extraction of the

Pen-elicitor. This speculation was based on the finding that only the culture filtrate of the high but not of the low penicillin-producing strain of *P. chrysogenum* contained an additional elicitor activity, which was most likely a pectolyase. We aimed at verifying this hypothesis by digesting mycelium of the low penicillin-producing strain with the culture filtrate and a protein extract from ground mycelium of the high penicillin-producing strain. We could show that enzymes in the culture filtrate of the high penicillin-producing strain are not sufficient for extraction of the Pen-elicitor from the mycelium of the low penicillin-producing strain. However, we were not able to verify the effect of intracellular enzymes, because surprisingly, no elicitor-activity could be extracted from the mycelium of the high penicillin-producing strain if the mycelium was ground before extraction. This result might indicate that the required enzyme has been destroyed during the process of grinding. (iii) The Pen-elicitor might represent a molecule which is expressed by many fungi, but only during some developmental stages. It is known that the production of antibiotics is often initiated under suboptimal growth conditions and is frequently coupled to particular developmental or morphological stages (Keller and Hohn, 1997; Kim et al., 2003). General regulators control the transition from one developmental stage to another, activating many different genes. Fungal strains which have been selected for high production of antibiotics might more readily change into such a developmental stage and thus express the Pen-elicitor.

In order to characterize the Pen-elicitor, we digested Pen with various enzymes. We showed that several crude protease preparations such as pronase, protease K, chymotrypsin and trypsin reduced elicitor activity of Pen to a large extent, suggesting that a protein part is necessary for alkalisation-inducing activity of Pen. Digestion with highly purified trypsin gave similar results, indicating that proteases and not other putatively present enzymes were responsible for the effect of the crude enzyme preparations. Furthermore, the Pen-elicitor was very sensitive to basic but much less sensitive to acidic hydrolysis, a characteristic typical of proteins. Furthermore, we have evidence that the elicitor-active domain contains the basic amino acids lysine and/or arginine but not glutamate, because trypsin (cleaving next to lysine and arginine), but not GluC (cutting next to glutamate and with less sensitivity to aspartate), destroyed the elicitor-activity.

Several experiments indicated that no glyco part is required for elicitor activity of Pen. Particularly, none of the following treatments affected elicitor-activity: (i) digestion with several preparations containing carbohydrate-digesting enzymes such as glucanases, glucosidases, mannosydases, mannanases, laminarinases and chitinases, (ii) enzymatic deglycosylation by N- and O-glycosydases, and (iii) chemical deglycosylation by TFMS. On the other hand, the strong oxidizing agent periodate repeatedly destroyed elicitor-activity of Pen, rather suggesting that a sugar moiety is necessary for elicitor activity. Periodate has been described to specifically destroy carbohydrates by cleaving carbon-carbon bonds with vicinal hydroxyl groups as well as hydroxy-aldehydes and ketones (Basse and Boller, 1992). However, it is feasible that periodate not only oxidizes carbohydrates but also other structures such as proteins. Furthermore, we found that one part of the elicitor-activity of Pen was binding to ConcanavalinA, a lectin which specifically and reversibly binds  $\alpha$ -D-mannose und  $\beta$ -D-glucose. In alkalisation bioassays, the fraction binding to ConA induced higher maximal alkalisation responses than the non-binding fraction, whereas in ethylene bioassays, only the non-binding fraction induced responses, while the binding fraction did not. There are two possible explanations for these results are: (i) The crude Pen extract contains two different elicitors, one of them being a glycopeptide, whereby the glycopeptide might induce alkalisation and the other ethylene response. (ii) The Pen-elicitor could contain a glyco part which is not necessary for elicitor activity. Molecules containing one to several carbohydrate residues are expected to be large and putative receptors on the membrane might be less accessible to them in intact leaf tissue than in cell cultures. This hypothesis could explain why leaf slices, as opposed to cell cultures, did not respond to the ConA binding fraction. In general, the results of the ConA-column are difficult to interpret: In the alkalisation as well as the ethylene bioassay, the maximal response to any of the two fractions and even to a combination of the two was much lower than to the original Pen extract. Thus, part of the Pen-elicitor was either destroyed during affinity chromatography,

e.g. by columns contaminated with proteases, or bound irreversibly to the column. The latter hypothesis is rather unlikely, because boiling ConA with and without SDS did not release any elicitor activity. In conclusion, most of our results indicate that only a protein part but not a sugar part is necessary for the elicitor activity of Pen.

Running Pen on reversed phase columns, elicitor activity was spread over a wide range of fractions. When a selected fraction was run a second time under identical conditions, elicitor activity was eluted at the very acetonitril concentration and the chromatogram showed a sharp peak at this position. This finding clearly proves the results of the first run. However, when such a fraction was run on the same column but with a shallower elution gradient, activity was spread over a broad range of fractions and the peak flattened more than expected. Thus, the Pen-elicitor seems not to be a discrete substance but consists of molecules with heterogeneous characteristics. Separating Pen on ion exchange and size exclusion chromatography gave similar results. We hypothesize that the Pen-elicitor consists of a small, distinct elicitor-active region, most likely a protein or peptide, which is part of a larger molecule varying in size and/or chemical composition. This hypothesis is supported by several studies in the literature which have shown that characteristic but relatively small structures within large molecules are often sufficient to induce early defense-related responses. Examples are (i) a domain of 22 amino acids from the N-terminal end of bacterial flagellin (Felix et al., 1999), (ii) a glycopeptide consisting of 10 mannosyl residues and two amino acids from yeast invertase (Basse et al., 1992), and (iii) a heptaglycoside from glucans of the fungal cell wall (Yamaguchi et al., 2000). The heterogeneity of the Pen-elicitor might either result from the relatively harsh treatment of the mycelium before extraction, including addition of a solvent, acidification and heating at 130°C for several hours and/or from modifications of proteins including glycosylation and phosphorylation (Fryksdale et al., 2002). It is known that most proteins secreted by fungi as well as most fungal cell wall proteins are glycosylated (Ruiz-Herrera, 1992; Peberdy, 1994), and glycosylation can result in heterogeneous reactions of proteins (Fryksdale et al., 2002). A glycoprotein elicitor in a yeast extract reacted as heterogeneous as the Pen-elicitor, preventing an identification of the elicitor-active molecule (Basse and Boller, 1992). This elicitor could only be identified when a relatively pure yeast glycoprotein was used as a starting material for the purification (Basse et al., 1992). We hypothesize that removing parts of the Pen-elicitor complex which are not necessary for elicitor activity by specific, non-inactivating chemicals or enzymes could result in a (more) homogeneous elicitor fraction. Therefore, we digested an apparently homogeneous fraction (fig. 5B) with non-destructive enzymes such as endoproteinase GluC and several glycosydases as well as by chemical deglycosylation. However, none of the treatments changed the reaction of the fraction in reversed phase chromatography. Possible explanations for this result are (i) digestions/treatments did not work under the selected conditions, (ii) changes were not detectable with the selected methods, or (iii) the Pen-elicitor does not have the suspected structure.

In conclusion, we have demonstrated that a protein domain is necessary for the elicitor-activity of Pen. Furthermore, we have shown that heterogeneity is characteristic for the Pen-elicitor. This fact prevented a further analysis, e.g. by mass spectrometry.

## CHAPTER V

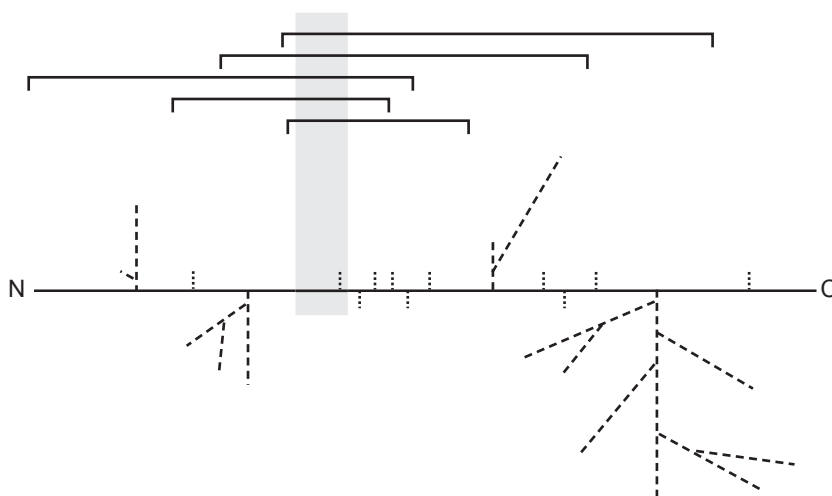
### General Discussion



The substitution of traditionally used chemical fungicides such as copper and sulphur has been a major focus of organic agriculture in the last few years (Speiser et al., 2000). A promising strategy is provided by the concept of induced resistance (Agrios, 1997). We have demonstrated that Pen, an aqueous extract from the dry mycelium of a high penicillin-producing strain of *P. chrysogenum*, reduced disease levels caused by several pathogens on various crop plants in the greenhouse and the field, i.e. Pen protected grapevine, tomato and onion from downy mildews (*P. viticola*, *P. infestans*, *P. destructor*), apple tree from apple scab (*V. inaequalis*) and grapevine from powdery mildew (*U. necator*) (chapter 1). The efficacy of Pen was comparable to the efficacy of copper, a fungicide commonly used to control downy mildews in conventional and organic agriculture. Furthermore, against the listed pathogens, Pen performed generally better than the well-known inducers BABA and BTH. All our results presented in chapters 3 and 4 suggest that Pen-mediated resistance is mainly based on the activation of plant defense mechanisms and that Pen has no major fungicidal effect. The lack of any direct antimicrobial activity has often been made a prerequisite for an extract or a compound classified as a 'plant activator' (Kessmann et al., 1994). However, for an application in practice, a simultaneous antimicrobial and inducing activity of a compound is not a problem but might rather be an advantage. The combined application of an inducer and a protective fungicide might increase the efficacy of any of the protective agents alone. On the one hand, the efficacy of inducers often decreases with disease pressure. The simultaneous application of a protective fungicide might decrease the viable inoculum, thus reducing disease pressure. On the other hand, the effect of contact fungicides is dependent on their even distribution and on their persistence on the plant tissue, i.e. if some parts of a leave have not been sprayed or if the fungicide has been rinsed off by rain, an infection gets possible. Then, the development of the disease can be stopped or slowed down in plants which are in an induced state. Therefore, effective plant protection strategies might include the simultaneous application of contact fungicides and inducers. However, the fact that Pen lacks a direct fungicidal effect made the investigation of its mode of action much easier.

Despite the interesting resistance inducing activities of Pen, its phytotoxic side effects have made an application in practice unfeasible so far. The attempt to identify batches of mycelium containing less phytotoxic activity failed. We hypothesized that knowing the chemical structure and/or the origin of the Pen-elicitor(s) would allow (i) to develop improved, more selective methods for the extraction and/or (ii) to identify processes to enrich the elicitor-activity and to remove the toxic compounds. In addition, for a possible future registration as a plant protection agent, at least one of the active ingredients should be identified. Therefore, one focus of this thesis was the purification and identification of the elicitor(s) contained in Pen inducing resistance in plants. The purification of unknown elicitors from complex extracts requires a simple, sensitive and fast bioassay. Measuring early defense-related responses in suspension-cultured cells has been proven to be a useful tool with the required characteristics (Boller and Felix, 1996). However, it has always to be kept in mind that cell cultures are not competent for the intact biological system, i.e. they might react differently from intact plants. Thus, compounds selected on cell cultures will have to be verified on plant-pathogen systems. In chapter 4, we demonstrated that the alkalisation-inducing activity of Pen could be destroyed by several proteases, including a highly purified protease preparation, indicating that a protein part is necessary for the activity. Whether this treatment also resulted in a loss of the resistance-inducing characteristics still has to be verified. Furthermore, we have shown that the Pen-elicitor is very heterogeneous in size, charge and polarity. This fact prevented the purification of a homogeneous fraction and further analysis. Similarly, Basse and Boller (1992) were not able to purify an elicitor from a crude yeast extract to homogeneity, but only succeeded when they used a prepurified preparation of yeast invertase as a starting material (Basse et al., 1992). We hypothesize that the Pen-elicitor consists of a, small, homogeneous, elicitor-active region which is part of a large molecule, as reported for many other elicitors (Basse et al., 1992; Felix et al., 1993; Felix et al., 1999; Brunner et al., 2002). We speculate that this large molecule might be a glycoprotein because part of the Pen-elicitor was binding to the lectin ConA, which selectively binds mannose and glucose residues.

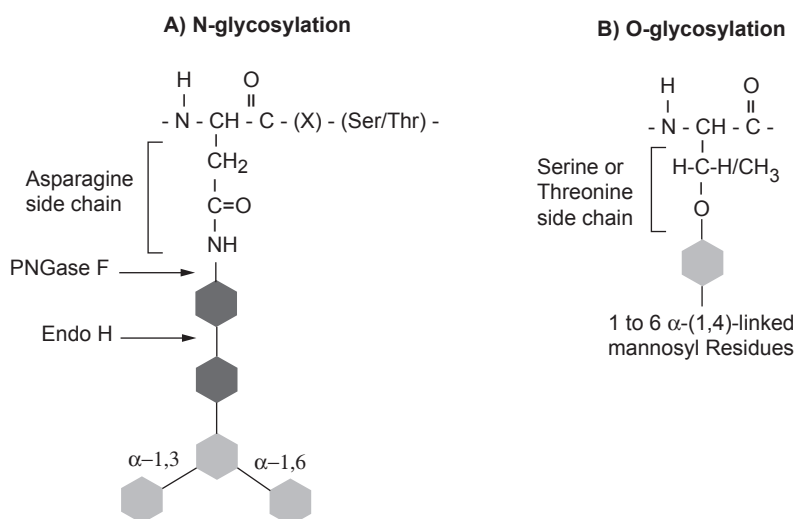
Furthermore, glycoproteins are known for their heterogeneous characteristics (Basse et al., 1992; Basse and Boller, 1992; Ruiz-Herrera, 1992; Fryksdale et al., 2002). We hypothesize that the harsh treatment of the mycelium from which Pen is extracted might unspecifically split a formerly homogeneous molecule into molecules varying in size and chemical composition (fig. 1). Thus, elicitor-active molecules may contain variable numbers of glycosidic side-chains which in turn may vary in their composition, either originally or due to the processing of the mycelium. Using unprocessed mycelium for the extraction of the Pen-elicitor could unravel whether all or part of the heterogeneity of the Pen-elicitor is a result of the processing. Yet, for technical reasons, non-processed mycelium of the high penicillin-producing strain of *P. chrysogenum*, the raw material for the dry mycelium, could only be provided from the producing company at the very end of this work.



**Figure 1.** A model of the Pen-elicitor and putative structures contributing to its heterogeneity. It is hypothesized that the Pen-elicitor consists of a small, homogeneous structure, which is part of a glycoprotein. The straight line represents the protein, the dashed and the dotted lines saccharides which are N- or O-linked respectively to the protein. The gray box marks a region necessary and sufficient for elicitor-activity. The black brackets show possible molecules with elicitor-activity resulting from the harsh chemical treatment of the mycelium of *P. chrysogenum*, resulting in the heterogeneity of the Pen-elicitor.

Saccharide side chains are either added to the side-chain  $\text{NH}_2$  group of an asparagine (N-glycosylation) or to the side-chain OH group of a serine or threonine (O-glycosylation) (fig. 2) (Alberts et al., 1994). Only asparagines in the sequences Asn-X-Ser/Thr (where X is any amino acid except proline) become N-glycosylated. The saccharide side chains of N-glycosylation-sites are highly variable, consisting of few to several hundreds of sugar residues. Many saccharide side-chains are rich in mannose, but they can also contain other sugar residues such as N-acetyl-glucosamine, galactose or glucose. Although N-linked saccharides are highly diverse, they consist of a common core region in all eukaryotic species, i.e. two N-acetyl-glucosamines binding to asparagine and three  $\alpha$ -mannoses (fig. 2A) (Alberts et al., 1994). In contrast, O-linked saccharides are very short, consisting of 1 to about 7 sugar residues, in fungi generally  $\alpha$ -1,4-linked mannoses (fig. 2B) (Peberdy, 1994; Chaffin et al., 1998). In fungi, the saccharides seem to be linked to the OH-group via an  $\alpha$ -mannose. In contrast, in higher eukaryotic species the saccharides are linked via an N-acetyl-glucosamine (Timpel et al., 1998). Because O-glycosylation seems to differ between fungi and higher eukaryotic groups, it can be speculated that O-glycosylated structures could be PAMPs. Although we speculate that the Pen-elicitor is part of a glycoprotein, it remains unclear whether the Pen-elicitor itself is a

peptide or rather a glycopeptide. None of the enzymatic treatments with the endoglycosidases PNGase F, Endo H or O-glycosidase reduced or destroyed the elicitor-activity of Pen. While Endo H is quite specific, cleaving only high mannose structures ( $n=2-150$ ) and some hybrid oligosaccharides, PNGase F is reported to hydrolyze nearly all types of N-glycan chains. Using PNGase F, Basse et al., (1993) could demonstrate that N-glycosylation is required for the elicitor-activity of yeast invertase. Although a positive control to check the enzyme activities was missing in our experiments, we speculate that N-glycosylation is not required for the elicitor-activity of Pen. The removal of O-linked glycans by enzymatic methods is known to be difficult and usually requires a combination of enzymes (Fryksdale et al., 2002). Furthermore, it is not known whether O-glycosydase, the enzyme used in this study, can remove O-linked saccharides when they are linked to the protein via a mannose and not via an N-acetyl-glucosamine. However, O-glycosylation has been reported to be highly sensitive to alkali treatment (Ruiz-Herrera, 1992), a feature characteristic of the Pen-elicitor. Yet, the fact that chemical deglycosylation by TFMS did not affect the Pen-elicitor contradicts the hypothesis that



**Figure 2.** N-glycosylation (**A**) and O-glycosylation (**B**) of proteins. **A.** Core region of N-linked saccharides. Although N-linked saccharides are highly diverse, all consist of the shown core region of 2 N-acetylglucosamines and 3  $\alpha$ -mannoses binding to a side-chain  $\text{NH}_2$  group of an asparagine. Only asparagines in the sequences Asn-X-Ser/Thr (where X is any amino acid except proline) become glycosylated. Arrows indicate the cleavage sites of the endoglycosydases PNGase F and Endo H. Figure modified from Alberts et al. (1994). **B.** O-glycosylation in fungi. O-linked saccharides consist of one to seven mannose residues (unbranched), linked to the side-chain OH group of a serine or threonine. Dark grey polygons are N-acetylglucosamines, light grey polygons are  $\alpha$ -mannoses. The linkage type is indicated at the lines connecting the sugar residues.

an O-glycosylation is necessary for the activity of the Pen-elicitor, although a positive control was missing.

Glycosylation is very characteristic of secreted proteins, but is very rare in proteins of the cytosol (Alberts et al., 1994). In fungi, many of the secreted (glyco)proteins are structurally associated with the cell envelope, i.e. the plasma membrane and the cell wall, while some are also found in the medium (Peberdy, 1994). There is growing evidence that the properties (expression, distribution and chemical characteristics) of secreted proteins is strain-specific and is dependent on multiple factors, e.g. growth conditions and the growth state of the fungus (Chaffin et al., 1998). Therefore, a particular (glyco)protein might not be continuously found and is not always found in the same fraction, i.e. in the medium or in the cell wall. This fact might explain

why the Pen-elicitor, although generally recognized by plants, could only be detected in a high but not in a low penicillin-producing strain of *P. chrysogenum*. As an example, the expression of the Pen-elicitor might be coupled to a particular morphological stage during which fungi produce antibiotics and might thus be preferentially produced in high penicillin-producing strains.

Some cell wall-associated (glyco)proteins bind very loosely to the cell wall and can be extracted very easily, e.g. with buffers or detergents. Other (glyco)proteins can only be extracted with drastic chemical treatments, e.g. with strong bases or acids, or with hydrolytic enzymes such as glucanases and zymolyase, indicating that they are covalently linked to the cell wall saccharides. According to (Chaffin et al., 1998), secreted (glyco)proteins can be categorized into four different groups, i.e. (i) hydrolytic enzymes and proteins with cell wall targets (ii) hydrolytic enzymes and proteins with extracellular targets (iii) morphology-associated (glyco)proteins which are in general covalently bound to cell-wall glucans and (iv) other enzymes or proteins occasionally detected in cell walls (table 1). Because the Pen-elicitor can be extracted from unprocessed mycelium of *P. chrysogenum* by very mild treatment, i.e. shaking in water for 10 min at room temperature, we hypothesize that the Pen-elicitor might belong to the first or the second group. The first group includes enzymes whose function is postulated to be within the fungal cell wall (Chaffin et al., 1998). These enzymes are thought to be involved in the cell wall biosynthesis or the remodelling that accompanies growth and division of cells. One example is the enzyme transglutaminase, crosslinking proteins by an amide-bond between the side chain  $\text{NH}_2$  group of a lysine and the side chain  $\text{CONH}_2$  group of a glutamine. A sequence of 13 amino acids (Pep-13) of the 42-kDa glycoprotein transglutaminase of *Phytophthora sojae* has recently been identified as a new PAMP (Brunner et al., 2002). The second group includes proteins and enzymes whose substrates are not found in the cell wall but in the environment. The action of these enzymes may provide access to nutrients for the organism. These (glyco)proteins are at least transiently associated with the cell wall during their translocation across the cell wall to the external environment. However, in some cases, the distribution of some of these enzymes is variable and, under some growth conditions, may be primarily cell wall-associated. Several (glyco)proteins belonging to this group have been identified as PAMPs, including invertase (Basse et al., 1992), as well as xylanase and polygalacturonase, two enzymes contributing to the degradation of plant cell walls (Enkerli et al., 1999; Poinssot et al., 2003).

In conclusion, we speculate that the Pen-elicitor consists of a small, homogeneous structure, which is part of a large molecule, most likely a glycoprotein. An O-glycosylated region might be necessary for the activity of the Pen-elicitor. We hypothesize that the Pen-elicitor is part of a secreted (glyco)protein, which is either loosely associated with the cell wall or secreted to the medium. Knowing which genes are upregulated in the high compared to the low penicillin-producing strain might help to identify candidates. In addition, if the heterogeneity of the Pen-elicitor is mainly caused by the harsh treatment of the mycelium of *P. chrysogenum*, using an extract from the unprocessed mycelium could make purification and identification more feasible.

## Outlook

In this thesis, it has been demonstrated that Pen, an aqueous extract from the dry mycelium of *P. chrysogenum*, has interesting, unique properties for an application as a crop protection agent in organic agriculture, provided the phytotoxic side effects can be removed. Yet, during the time of the thesis, it was not possible to unravel the structure of the Pen-elicitor. The analysis of an extract from unprocessed mycelium, which is now also available, will show whether the heterogeneity of the Pen-elicitor is a result of the processing. Although identification of the resistance-inducing substance would considerably facilitate to develop strategies for the preparation and processing of Pen, it is not necessarily a prerequisite for a future usage in practice. Improved formulation as well as refined purification steps could make an application of the Pen-extract feasible.



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## Curriculum Vitae

5th of March 1973	Born in Basel, Switzerland
1980-1989	Primary and secondary school in Binningen (CH)
1989-1992	High school (Gymnasium) in Oberwil (CH), Matura Type D
1993-1998	Studies in Biology I, University of Basel, Switzerland
1998-1999	Diploma thesis at the Botanical Institute in Basel under the supervision of PD Dr. Jürg Stöcklin and Prof. Dr. Christian Körner entitled 'Die Wirkung von erhöhtem CO <sub>2</sub> auf die sexuelle Reproduktion von Magerwiesenpflanzen'
June 1999	Diploma in Biology I
2001-2004	PhD thesis in Botany titled 'The effect of an extract from mycelium of <i>Penicillium chrysogenum</i> on plant-pathogen interactions and characterisation of elicitors in this extract' at the Research Institute of Organic Agriculture (FiBL), Frick (CH) under the supervision of Dr. Lucius Tamm (FiBL), Prof. Dr. Thomas Boller and PD Dr. Andres Binder (both University of Basel)

## Publications

**Thürig B, Körner C, Stöcklin J** (2003) Seed production and seed quality in a calcareous grassland in elevated CO<sub>2</sub>. *Global Change Biology* 9: 873-884

**Thürig B, Binder A, Boller T, Guyer U, Jiménez S, Rentsch C, Tamm L** An aqueous extract of the dry mycelium of *Penicillium chrysogenum* induces resistance in several crops under controlled and field conditions. Submitted