European Journal of Plant Pathology (2006) 114:185–197
DOI 10.1007/s10658-005-4512-6

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

Barbara Thuerig [1,*], Andres Binder[2], Thomas Boller[3], Urs Guyer[1,4], Sonia Jiménez[1], Christina Rentsch[1] and Lucius Tamm[1]
[1]*Research Institute of Organic Agriculture, Ackerstrasse, 5070, Frick, Switzerland;* [2]*University of Basel, Botanical Institute, Schönbeinstrasse 6, 4056, Basel, Switzerland;* [3]*University of Basel, Botanical Institute, Hebelstrasse 1, 4056, Basel, Switzerland;* [4]*Current address: Institute of Plant Science, ETH centre, 8092, Zurich, Switzerland; *Author for correspondence (Phone: +41-62-8657272; Fax: +41-62-8657273; E-mail: barbara thuerig@fibl.org)*

Accepted 17 October 2005

Key words: apple tree, grapevine, induced resistance, *Phytophthora infestans*, *Plasmopara viticola*, tomato

Abstract

We have examined the effect of Pen, an aqueous extract of the dry mycelium of *Penicillium chrysogenum*, on plant–pathogen interactions. Pen controlled a broad range of pathogens on several crop plants under greenhouse and field conditions. Pen protected grapevine from downy and powdery mildew (caused by *Plasmopara viticola* and *Uncinula necator*), tomato from early blight (caused by *Phytophthora infestans*), onion from downy mildew (*Peronospora destructor*) and apple trees from apple scab (caused by *Venturia inaequalis*) to a similar extent as fungicides such as copper and sulphur or well-known inducers such as benzothiadiazole or β-aminobutyric acid. Pen had no major 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 commercial application. Under certain conditions, Pen caused phytotoxic side effects. The symptoms mostly consisted of small necrotic spots or, more rarely, of 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 and environmental conditions. In grapevine, a partially purified fraction of Pen was much less toxic than the crude Pen extract, but protected the plants to a similar extent against *P. viticola*. Our data show that Pen has interesting and unique properties as a plant protection agent, but more research is needed to further reduce its phytotoxic side effects.

Abbreviations: Pen – an aqueous extract of the dry mycelium of *Penicillium chrysogenum*; Pen_{2000} – a size fraction of Pen (>2000 Da) gained by dialysis.

Introduction

In agriculture, infection of plants with microorganisms including fungi, bacteria and viruses can cause high yield losses. The complete breakdown of the wine industry in western Europe, particularly in France, after the invasion of downy mildew (*Plasmopara viticola*) from America to Europe in the 1870s 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 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; Kuc, 2001).

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 treating plants with various natural or synthetic compounds such as salicylic acid (SA) (White, 1979), isonicotinic acid (INA) (Ward et al., 1991), jasmonic acid (Cohen et al., 1993), benzothiodiazole (BTH) (Friedrich et al., 1996; Gorlach et al., 1996), probenazole (Sekizawa and Mase, 1980), β-aminobutyric acid (BABA) (Cohen et al., 1994) or the bacterial protein harpin (Dong et al., 1999), by various crude extracts from microorganisms or plants (Daayf et al., 1997), as well as by certain non-pathogenic root-colonizing pseudomonads (van Loon et al., 1998). Extracts or chemical compounds inducing resistance are often referred to as 'plant activators', 'inducers' or, if derived from microorganisms, 'elicitors'. Classical inducers do not have a direct impact on pathogens, which clearly distinguishes them from fungicides (Kuc, 1983).

Especially in organic agriculture, the product demand for which has strongly increased 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 responsible for a phenomenon called 'plant-strengthening', already observed by the pioneers of organic agriculture after the application of herb and compost extracts. Yet, inducers to be used in commercial agriculture have to be available in sufficient quantities and in constant quality and have to be effective under field conditions. In addition, compounds to be used in organic agriculture have to be natural and must not derive from genetically modified organisms (Codex Alimentarius Commission, 1999; OMRI, 2001; Speiser et al., 2004). Although several inducers are commercially available, none of them fulfils all of these criteria.

In the 1990s, an aqueous extract of the mycelium of the ascomycete *Penicillium chrysogenum*, called Pen, was developed (E. Mösinger, Sandoz AG, Switzerland, personal communication). 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. The mycelium of *P. chrysogenum* is obtained as a by-product from penicillin production and is thus relatively cheap and available in sufficient amounts. The main objectives of this study are (i) to examine the effect of Pen on several plant–pathogen interactions under greenhouse and field conditions with a special focus on the pathosystems grapevine – *Plasmopara viticola* and tomato – *Phytophthora infestans*, (ii) to assess the quality of the raw material for the production of the aqueous extract and (iii) to evaluate any potential side effects of Pen.

Materials and methods

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 an industrial scale. To extract penicillin, *n*-butylacetate was added to the mycelium–medium mixture (1:2) and pH adjusted to 1–3 with H_2SO_4. The butanol phase was removed by decantation and the aqueous phase including the mycelium was stored in tanks for 12–36 h before removing the remaining butylacetate by distillation (50–60 °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 contamination because penicillin is not heat-stable. Nevertheless, individual batches are checked for absence of penicillin by routine quality assurance systems (Sandoz GmbH, Austria, personal communication). To prepare the Pen extract, 150 g of the dry mycelium was added to 1 l 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_{2000}) was prepared by dialysing the crude Pen extract in dialysis tubes with a cut-off of 2000 Da (Spectra/Por® 6, Socochim SA) 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^{-1} dry matter; dialysis reduced the content of Pen_{2000} to 12 g l^{-1}. All concentrations of Pen are indicated in g dry matter per litre water. If not otherwise mentioned, Pen was applied at concentrations of 45 g l^{-1} and Pen_{2000} at 12 g l^{-1}.

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^{-1} (Bion), 0.1 g l^{-1} (BABA field) or 1 g l^{-1} (BABA greenhouse). In field experiments, the standard fungicides Myco-San (10 g l^{-1}) (Schaette GmbH), sulphur (5 g l^{-1}) and copper (0.5 g l^{-1}) were used.

Testing for fungicidal effects in vitro

The effect of the crude Pen extract on growth of *Phytophthora infestans* and *Colletotrichum 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 centre and filled with the test substance. Test substances were Pen (45, 30 and 15 g l^{-1}), water and the standard fungicides metalaxyl against *P. infestans* (0.01 g l^{-1} and 0.1 g l^{-1}) (Ridomil®, Syngenta AG) or dithianon against *C. lagenarium* (0.5 g l^{-1}) (Delan®, Siegfried Agro AG). A mycelial plug was placed in the centre. Mycelial growth was assessed after 14 days. Furthermore, the crude Pen extract (4.5 g l^{-1}) 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 *Stagonospora 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^{-1}) (Bravo®, Syngenta AG) or the crude Pen extract (1.6 or 3.3 g l^{-1}). Germination rates were assessed after 28 and 50 h.

Pathogens

Sporangia of the obligate biotrophs *Plasmopara viticola* and *Pseudoperonospora cubensis* were obtained by washing infected grapevine or cucumber leaves, respectively with distilled water. Several isolates of *P. infestans* were used. For initial experiments, two *P. infestans* isolates from potato plants were used (Syngenta AG). For later experiments, two isolates of *P. infestans* were obtained from infected tomato plants. *Phytophthora infestans* was grown on rye agar at 18–22 °C in the dark. Sporangia were collected from 2 week-old cultures by gently agitating 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 scraping 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 individual pots containing peat-rich and pre-fertilized soil. Grapevine plants were grown in the greenhouse at a temperature of 18–28 °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 5–8 fully expanded leaves. Field experiments were carried out on grapevines cv. 'Riesling×Sylvaner' 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. In addition, plants were fertilized once a week with a mineral fertilizer. Tomatoes were grown as described for grapevine seedlings and used for experiments when they had 6–8 fully expanded leaves.

Cucumber
Cucumber plants (*Cucumis sativus*) cv. 'Aramon F1' were grown in peat-rich and pre-fertilized soil in the greenhouse. Plants were fertilized once a week with a mineral fertilizer and grown as described for grapevine seedlings.

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. 'Agria' were grown in an experimental field in Frick, Switzerland.

Onion
Onion plants (*Allium cepa*) cv. 'Centrurion' (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 trees and grapevine in the field), 4 (potato) or 3 (onion) replicates, according to EPPO (1999) guidelines.

Treatments on plants

For greenhouse experiments, plants were sprayed with a hand-sprayer until near run-off. Treatments were performed 7 days before inoculation. Treated grapevine seedlings were kept in the humidity chamber (100% RH, 20–21 °C) for 24 h, and then transferred back to the greenhouse. In field experiments, plants were sprayed weekly using a power sprayer with boom, a knapsack sprayer or a high-pressure hand-sprayer until 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 spores ml^{-1}) or 10 μl (100,000 spores ml^{-1}) were applied on tomato or grapevine leaves, respectively. After inoculation, plants were incubated in the humidity chamber (100% RH, 14 h light, 5 kLux) for 48 h at 18–20 °C (tomato) or 24 h at 20 °C (grapevine). Tomatoes were subsequently kept in the humidity chamber but RH was lowered to 80–95%. 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 5–7 days after inoculation, disease of grapevine plants after 7 days. Cucumber plants were sprayed with conidia or sporangia suspensions of *C. lagenarium* or *P. cubensis* (200,000 spores ml^{-1}) using a hand-sprayer until 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 days after inoculation.

In the field, infection occurred naturally. At least 50 leaves of each grapevine plant and 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^{-1} or affected leaves*number of leaves counted^{-1}), severity (percentage of damaged leaf area) and/or lesion diameter were used. Lesion diameters of the largest lesion (onion), the five 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 3–14 days after treatment and in field experiments simultaneously with disease assessment.

Calculations and statistics

Efficacy was calculated according to Abbott (1925) as follows: efficacy (%) = $100(1 - ab^{-1})$, 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 batch was calculated as $100(ab^{-1} - 1)$ with a = efficacy of the batch and b = efficacy of the standard Pen extract. Data were analysed by ANOVA followed by a Tukey test at $\alpha = 0.05$ for multiple comparisons (Zar, 1996).

Results

Fungicidal activity of Pen in vitro

The crude Pen extract did not inhibit the growth of *Phytophthora infestans* and *Colletotrichum 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 *Stagonospora nodorum*. Pen only slightly inhibited the growth of one species (*P. oryzae*), but had no 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 (Figure 1). However, Pen reduced the germination rate of sporangia from *P. infestans in vitro* in one experiment.

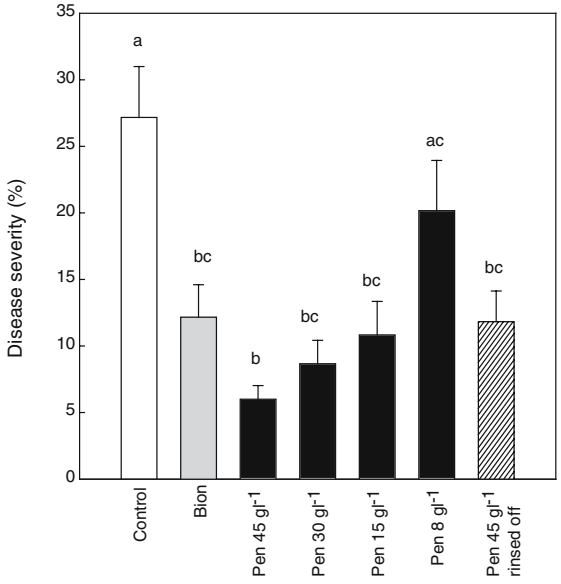

Figure 1. Effect of Pen on tomato against *Phytophthora infestans* in the greenhouse. Tomato plants cv. 'Supermarmande' were treated with water (white), Bion (0.05 g l^{-1}) (grey) or different concentrations of the crude Pen extract (black) one week before inoculation. In one treatment, Pen was thoroughly 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, $P < 0.05$).

Grapevine

Grapevine plants treated with the crude Pen extract were significantly less infected by *P. viticola* than water-treated control plants (Figure 2, Table 1). In three field seasons, Pen reduced disease severity on average by 67%, and was thus comparable to the contact fungicide copper and at least equal to the inducers Bion or BABA. In 2003, Pen reduced disease severity by 90% and in 1997, a year with much higher disease pressure, by 52% (Figure 2). In 1997, the diameters of the five largest lesions per plant were also measured. 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_{2000}, a size fraction of the crude Pen extract (> 2000 Da), were tested. Efficacy of Pen_{2000} was comparable to the efficacy of the crude Pen extract (Figure 2, Table 1). Results from the field could be confirmed under greenhouse conditions on grapevine seedlings, where both Pen and Pen_{2000} reduced disease severity in six independent experiments on average by 88% or 68%, respectively, compared to control plants (Table 1). In 1998, disease pressure by *U. necator* was very high in the field. Under these conditions, Pen reduced disease severity from 73% (control) to 3% (Pen) and was thus as effective as the standard sulphur or Myco-san fungicides (Table. 1).

Apple trees

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. In two different

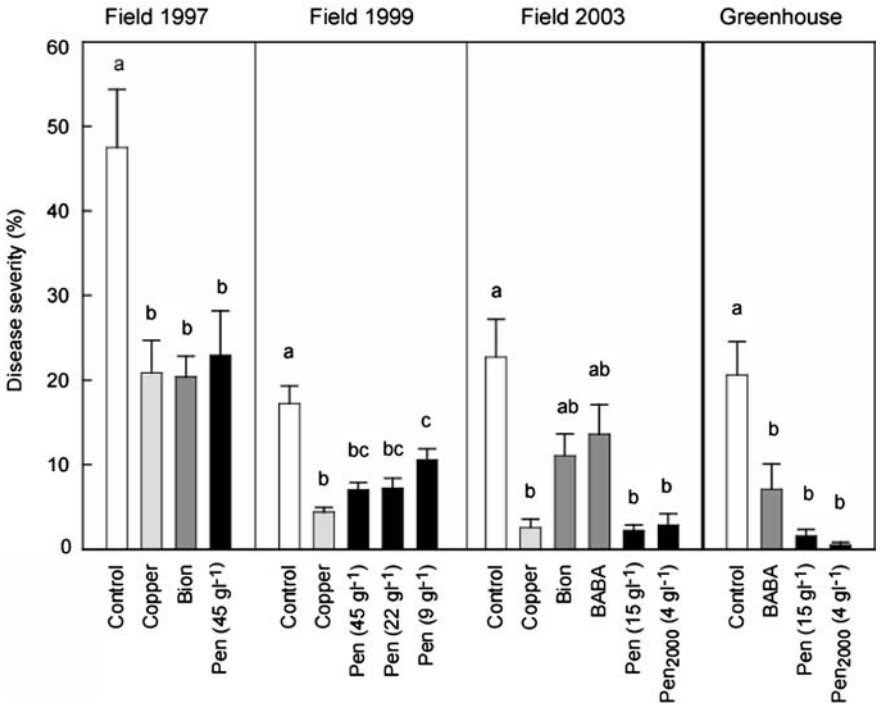

Figure 2. Effect of Pen on grapevine against *Plasmopara viticola* in the field and the greenhouse. Grapevine plants cv. 'Riesling×Sylvaner' (field) or 'Chasselas' (greenhouse) were treated with water (white), copper (0.5 g l^{-1}) (light grey), the inducers Bion (0.05 g l^{-1}) or BABA (field 0.1 g l^{-1}, greenhouse 1 g l^{-1}) (dark grey), the crude Pen extract or Pen_{2000} (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, $P < 0.05$).

years, Pen reduced disease severity by 89% and 93% (Figure 3 and Table 1), respectively, although disease pressure was quite high. The efficacy of Pen was comparable to the standard copper fungicide. Pen_{2000} reduced disease severity significantly less than the crude Pen extract (Figure 3, Table 1). However, efficacy of Pen_{2000} was still comparable to the efficacy of standard fungicides like Myco-san or sulphur.

Tomatoes

Tomato plants treated with the crude Pen extract were significantly less infected by *P. infestans* than water-treated control plants under greenhouse conditions (Figure 1 and Table 1). In 33 independent experiments, Pen reduced disease severity on average by 71% (Figure 1, Table 1). Bion reduced disease severity on average by 41% (seven independent experiments). 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 treatments (Figure 1).

Cucumbers

Pen reduced disease severity of *C. lagenarium* and *P. cubensis* on cucumber in the greenhouse in six or two independent experiments on average by 24% or 27%, respectively. The efficacy of Pen varied largely between experiments (0–65%) (Table 1), but disease reduction was statistically not significant in any of the experiments. In contrast, Bion significantly reduced disease severity of both pathogens on average by around 50%.

Potatoes

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

Table 1. Efficacy of Pen against different pathogens in various crop plants compared to the efficacy of standard fungicides or inducers

Plant	Pathogen	Location	Treatment	Parameter	Disease of control			Efficacy (%)			*n*
					Mean	Min	Max	Mean	Min	Max	
Grapevine	*P. viticola*	Field	Pen	Diseased leaf area (%)	29	17	48	67	52	90	3
			Pen_{2000}		23	–	–	88	–	–	1
			BABA		23	–	–	40	–	–	1
			Bion		36	23	48	55	52	57	2
			Copper		29	17	48	73	56	89	3
		Greenhouse	Pen	Diseased leaf area (%)	18	10	27	88	67	100	6
			Pen_{2000}		21	10	50	68	12	97	6
			BABA		29	17	50	52	0	83	4
	U. necator	Field	Pen	Diseased leaf area (%)	73	–	–	93	–	–	1
			Sulphur					97	–	–	1
			Myco–san					97	–	–	1
Apple tree	*V. inaequalis*	Field	Pen	Diseased leaf area (%)	38	31	44	91	89	93	2
			Pen_{2000}		44			67	–	–	1
			Copper		38	31	44	93	90	95	2
			Sulphur		44	–	–	73	–	–	1
			Myco-san					77	–	–	1
Tomato	*P. infestans*	Greenhouse	Pen	Lesion diameter (mm)	19	10	38	71	17	100	33
			Bion		20	10	30	41	14	60	7
Cucumber	*C. lagenarium*	Greenhouse	Pen	Diseased leaf area (%)	33	12	59	24	0	64	6
			Bion					58	0	92	6
	P. cubensis	Greenhouse	Pen	Diseased leaf area (%)	55	17	92	29	11	59	2
			Bion					46	28	59	2
Onion	*P. destructor*	Field	Pen	Number of lesions per leaf	0.78	–	–	44	–	–	1
Potato	*P. infestans*	Field	Pen	Diseased leaf area (%)	33	–	–	0	–	–	1
			Copper					94	–	–	1

The table shows the examined parameters, the number of experiments performed (*n*), the mean disease of the untreated control over all experiments, the minimum and maximum disease of the control, the mean efficacy of the plant protection product in all experiments as well as its minimum and maximum efficacy.

significantly reduced disease severity by 94% from 33% (control) to 4% (copper) (Table. 1).

Onions

Under field conditions, onion plants treated with Pen were significantly less infected by *P. destructor* than non-treated plants (Figure 4). The percentage of infected leaves was significantly reduced by 30% from 47% (control) to 33% (Pen), and the number of lesions per leaf by 44% from 0.77 (control) to 0.43 (Pen) ($P < 0.05$). Furthermore, Pen reduced the mean size of the largest lesion by 29% from 8.4 (control) to 6 cm (Pen) ($P = 0.07$).

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 ±10% (Figure 5). The observed variation in the efficacy of different batches is negligible for an application in practice.

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 led to accelerated senescence of leaves in several experiments; (ii) tomato leaves showed bending after Pen treatment, and this phenomenon was present throughout all experiments; (iii) a speckling developed on leaves and stems of tomato, grapevine and to a lesser extent cucumber

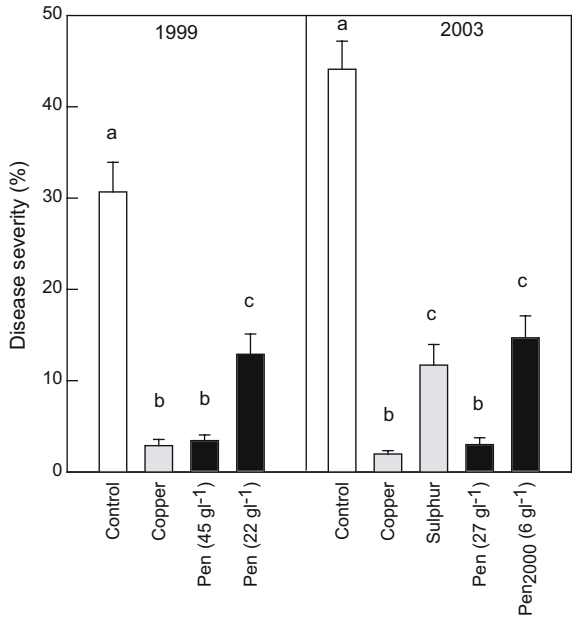

Figure 3. Effect of Pen on apple trees against *Venturia inaequalis* in the field in 1999 and 2003. Apple trees cv. 'Rubinette' were treated weekly with water (white), copper (0.5 g l^{-1}), sulphur (5 g l^{-1}) (grey), different concentrations of the crude Pen extract or Pen_{2000} (6 g l^{-1}) (black). Bars show percentage diseased leaf area (mean and standard error). Different letters indicate statistically significant differences (pairwise comparisons, Tukey test, $P < 0.05$).

plants after spaying with the crude Pen extract (Figure 6). The symptoms consisted mostly of small necrotic spots not associated with lesions caused by a pathogen. In the field, some grapevine leaves developed larger necrotic areas. On apple trees, no visible necrotic spots were present 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_{2000} was much less phytotoxic to grapevine plants than the crude Pen extract under greenhouse and field conditions (Figure 7). 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 phytotoxic symptoms in 1999, but some symptoms were visible in 2003 (see above). Furthermore, Pen was more phytotoxic to the grapevine variety 'Chasselas' than to 'Riesling×Sylvaner' in field experiments. The phytotoxic effect of extracts from 30 batches of mycelium varied largely from 37% to +239% compared to the phytotoxicity of the standard Pen extract (data not shown). Most of the extracts were more phytotoxic than the reference extract.

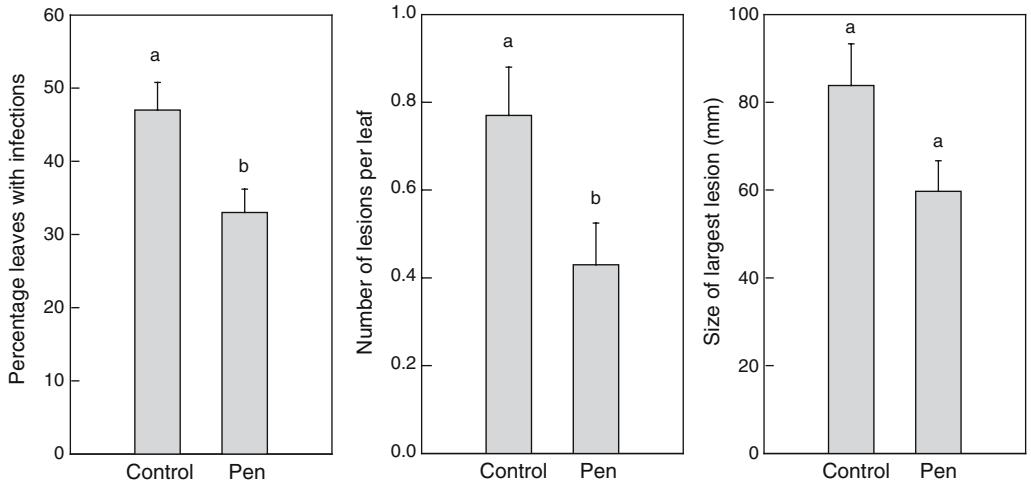

Figure 4. Effect of Pen on onion against *Peronospora destructor* in the field. Plants were non-treated or treated weekly with the crude Pen extract (45 g l^{-1}). Percentage diseased leaves, the number of lesions per leaf and the size of the largest lesion per leaf were determined for 100 leaves per replicate. Bars show means and standard errors. Different letters indicate statistically significant differences (*t*-test, $P < 0.05$).

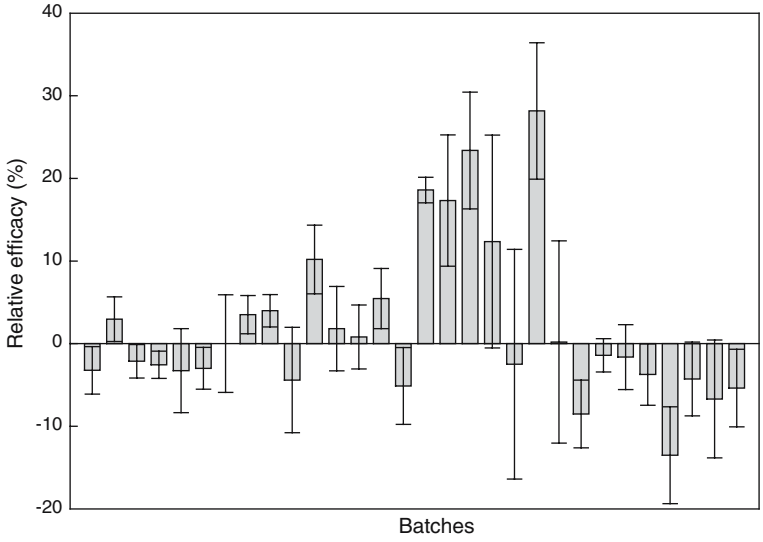

Figure 5. Variability of efficacy of different production batches of *Penicillium chrysogenum*. A total of 30 batches of mycelium of *P. chrysogenum* dating from 1993 to 1999 were extracted with water. The efficacy of these extracts was tested using the tomato – *Phytophthora infestans* bioassay and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

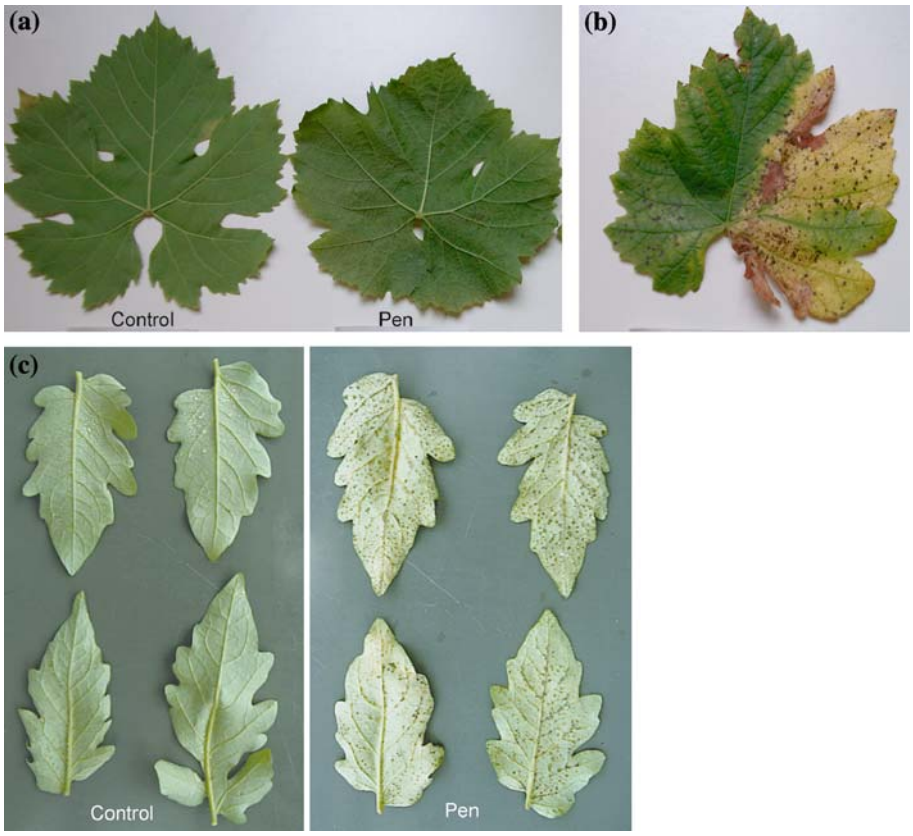

Figure 6. Phytotoxic effect of Pen on grapevine (a and b) and tomato (c). (a) and (b), Grapevine leaves (cv. 'Riesling×Sylvaner') from outdoor experiments after 6 treatments. (a), Typical leaves of untreated control and Pen-treated (15 g l^{-1}) leaves. (b) Pen-treated leaf with strong phytotoxic symptoms. (c) Effect of Pen (45 g l^{-1}) on tomato (cv. 'Supermarmande') after one treatment.

There was no correlation between the phytotoxicity and the efficacy of the extracts.

Discussion

In this study we report that Pen, an aqueous extract of 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

Figure 7. Phytotoxic effect of the crude Pen extract compared to the partially purified fraction Pen_{2000} on grapevine in outdoor experiments. Plants of the variety 'Riesling×Sylvaner' had been treated 11 times with copper (0.5 g l^{-1}) (b), Pen (15 g l^{-1}) (c), Pen_{2000} (4 g l^{-1}) (d) or not treated at all (a). Pictures were taken at the 6th of August 2003. The untreated control (a) displays disease symptoms caused by *P. viticola*.

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 including oomycetes, ascomycetes and even bacteria (Thuerig et al., submitted). In contrast, most fungicides have a much narrower range of activity. In conclusion, our data suggest that Pen-mediated protection is mainly based on the activation of host resistance mechanisms. However, it can not be completely excluded that Pen may have minor direct inhibitory effects on certain developmental stages of a particular pathogen.

Pen controlled several diseases not only under controlled greenhouse conditions but also under field conditions. Pen was effective under field conditions on grapevine against powdery (*U. necator*) and downy mildew (*P. viticola*), on apple trees against apple scab (caused by *V. inaequalis*) on onion against downy mildew (*P. destructor*) and against early blight (caused by *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 trees 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, the efficacy of Pen was equal or superior in most plant–pathogen systems to these products. The only exception was cucumber, where Bion performed much better against the two tested pathogens *C. lagenarium* and *P. cubensis*. In particular, 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 (Räz 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 that conform to the guidelines of organic agriculture have yet been found. Pesticides to be applied in organic agriculture have to fulfil several criteria (EU, 1991, 1997; IFOAM, 2000). One criterion is the method 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 these guidelines, in contrast to other inducers such as Bion (containing the synthetic 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 commercial application.

However, phytotoxic side effects have been observed related to the use of Pen extract. Grapevines in particular can suffer from severe symptoms caused by the crude Pen extract, particularly with repeated application as required in practice. In contrast, the crude Pen extract was much less phytotoxic to apple trees. In 2 years (1998 and 1999), apple trees did not develop any symptoms at all. Thus, a commercial application for apple production might be possible. In addition, our data indicate that there are several possibilities for reducing phytotoxicity, e.g. a fraction prepared from the crude Pen extract by dialysis (Pen_{2000}) was much less phytotoxic than the crude extract itself. This result suggests that low molecular weight substances such as salts, free sugars or amino acids might be responsible for the phytotoxic effect of Pen. Pen_{2000} protected grapevines similarly to the crude extract against *P. viticola*, but its efficacy was reduced on apple trees against *V. inaequalis*. However, Pen_{2000} was still as efficient as frequently used fungicides such as sulphur or Myco-San. 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_{2000}, 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. In conclusion, we showed that Pen, the aqueous extract from the mycelium of *P. chrysogenum*, protects several crops against a broad range of pathogens under greenhouse and field conditions probably by inducing resistance in

plants; its effect against downy mildews on grapevine and onion is particularly promising. However, the potential phytotoxic side effects are undesirable, but our data suggest that phytotoxicity can be reduced by appropriate techniques, which have still to be improved.

Acknowledgements

We thank Sandoz GmbH (Kundl, Austria) for financing part of this work, Syngenta AG (Stein, Switzerland) for kindly providing grapevine and apple tree seedlings as well as inoculum of *P. viticola*, *P. infestans*, *C. lagenarium* and *P. cubensis* and Thomas Amsler and Maria Peter for spraying apple trees and grapevines in the field.

References

Abbott WS (1925) A method for computing the effectiveness of an insecticide. Journal of Economic Entomology 18: 265–267.

Agrios GN (1997) Plant Pathology, Academic Press, San Diego

Codex Alimentarius Commission (1999) Guidelines for the production, processing, labelling and marketing of organically produced foods. CAC/GL 32-1999, Rev. 1-2001.

Cohen Y, Gisi U and Niderman T (1993) Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic methyl ester. Phytopathology 83: 1054–1062.

Cohen Y, Niderman T, Mösinger E and Fluhr R (1994) β-Aminobutyric acid induces the accumulation of pathogenesis-related proteins in tomato (*Lycopersicon esculentum* L.) plants and resistance to late blight infection caused by *Phytophthora infestans*. Plant Physiology 104: 59–66.

Daayf F, Schmitt A and Bélanger RR (1997) Evidence of phytoalexins in cucumber leaves infected with powdery mildew following treatment with leaf extracts of *Reynoutria sachalinensis*. Plant Physiology 113: 719–727.

Dong H, Delaney TP, Bauer DW and Beer SV (1999) Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the NIM1 gene. The Plant Journal 20: 207–215.

Emmet RW, Wicks TJ and Magarey PA (1992) Downy mildew of grapes. In: Kumar J, Chanbe HS, Singh US and Mukhopadhyay AN (eds) Diseases of fruit crops, Vol. 3 (pp 90–128) Prentice Hall, NJ.

EU (1991) Council Regulation (EEC) No. 2092/91 of 24 June 1991 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs as amended.

EU (1997) Council Regulation (EEC) No. 1488/97 of 29 July 1997 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs as amended.

European and mediterranean plant protection organization (EPPO) (1999) Guideline for the efficacy evaluation of plant protection products.

Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Gut Rella M, Meier B, Dincher S, Staub T, Uknes S, Métraux J-P, Kessmann H and Ryals J (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. The Plant Journal 10: 61–70.

Gorlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T, Ward E, Kessmann H and Ryals J (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. The Plant Cell 8: 629–643.

Hammerschmidt R (1995) Induced Resistance to Disease in Plants. Kluwer Academic Publishers, Dordrecht.

IFOAM (2000) Basic Standards for Organic Agriculture. D-Tholey-Theley.

Kuc J (1983). Induced systemic resistance in plants to diseases caused by fungi and bacteria. In: Bailey JA and Deverall BJ (eds) The Dynamics of Host Defense (pp 191–221) Academic Press, New York.

Kuc J (2001) Concepts and direction of induced systemic resistance in plants and its application. European Journal of Plant Pathology 107: 7–12.

OMRI (2001) Generic Materials List with the National Organic Program Final Rule Listings. Eugene.

Räz B, Schüepp H and Siegfried W (1987) Hundert Jahre *Plasmopara*-Bekämpfung und Kupfereintrag in die Rebberge. Schweizerische Zeitschrift für Obst- und Weinbau 123: 272–277.

Ross AF (1961) Systemic acquired resistance induced by localized virus infections in plants. Virology 14: 340–358.

Schneider S and Ullrich WR (1994) Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. Physiological and Molecular Plant Pathology 45: 291–304.

Sekizawa Y and Mase S (1980) Recent progress in studies on non-fungicidal controlling agent Probenazole, with reference to the induced resistance mechanisms of rice plants. Review of Plant Protection Research 13: 114–121.

Singh RS (2000) Diseases of Fruit Crops, Science Publishers, Enfield (NH).

Speiser B, Berner A, Häseli A and Tamm L (2000) Control of downy mildew of grapevine with potassium phosphonate: Effectivity and phosphonate residues in wine. Biological Agriculture and Horticulture 17: 305–312.

Speiser B, Tamm L, Maurer V, Berner A and Walkenhorst M (2004) Hilfsstoffliste: Zugelassene und empfohlene Hilfsstoffe für den biologischen Landbau. Research Institute of Organic Agriculture (FiBL), Frick.

Tamm L (2001) Organic agriculture: development and state of the art. Journal of Environmental Monitoring 3: 92–96.

Thuerig B, Felix G, Binder A, Boller T and Tamm L (submitted) An extract of *Penicillium chrysogenum* elicits early defense-related responses and induces resistance in *Arabidopsis thaliana* independently of known signalling pathways.

vanLoon LC, Bakker PAHM and Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36: 453–483.

Ward E, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl Goy P, Métraux JP and Ryals J (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. The Plant Cell 3: 1085–1094.

White RF (1979) Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology 99: 410–412.

Zar JH (1996) Biostatistical Analysis , Prentice-Hall Inc, Upper Saddle River, NJ.