Institute of Plant Pathology and Plant Protection of
the Georg-August-Universität Göttingen
Director: Prof. Dr. S. Vidal

The induced resistance against fire blight by the plant activator BTH (BION®) or extract of *Hedera helix* leaves and studies on the mode of action

Thesis
Submitted for the degree of Doctor of Agricultural Sciences of
the Faculty of Agricultural Sciences
Georg-August-Universität Göttingen (Germany)

by
Ömür Baysal, born in Antalya, Turkey
Göttingen, May, 2001

D 7
1st examiner: Prof. Dr. H.-H. Hoppe
2nd examiner: Prof. Dr. W. Zeller
Date of oral examination: 18/05/2001

Dedicated to my wife and my parents…

TABLE OF CONTENTS PAGE

1. INTRODUCTION 1

1.1 Description of the pathogen, its distribution and control 1
1.2 Alternatives to antibiotic compounds 1
1.2.1 Bacterial antagonists 1
.2 Induced resistance 2
1.2.2.1 Plant extracts 2
.2 Etheric oils 3
.3 Synthetic compounds 3
1.3 Aim of this study 4

2. MATERIALS AND METHODS 5

2.1 Plant material 5
2.2. Greenhouse conditions 5
2.3 Bacterial strains and culture medium 5
2.4 Application of BTH (BION®) 6
2.5 Preparation and application of plant extract from *Hedera helix* 7
2.6 Inoculation 7
2.7 Determination of induction time 7
2.8 Symptom development 7
2.9 Determination of bacterial multiplication *in planta* by dilution plating 8
2.10 Determination of bacterial population *in planta* by PCR 9
2.10.1 Testing of phenol inhibitory substances for PCR 9
.2 PCR protocol 10
.3 Preparation of agarose gel 11
.4 Loading of samples in agarose gel 11
2.11 Physiological investigations 11
2.11.1 Preparation of samples for determining enzyme activities 12
.2 Protein assay 12
.3 Total phenol content 12
2.12 Flavonoids 13
2.12.1 Inhibitory substances 13
.2 Inhibitory effect of the detected substance compared with phenolic acids 14
2.13 High-performance liquid chromatography (HPLC) analysis 14
2.14 Peroxidase (PO) 16
2.14.1 Peroxidase activity on SDS-PAGE gel electrophoresis 17
.2 Determination of peroxidase activity with staining solution 17
2.15 Protein patterns 17
2.15.1 Preparation of samples 18
.2 Electrophoresis and staining 18
.3 Staining of gel with Coomassie brilliant blue 18
2.16 Phenylalanine ammonia-lyase (PAL) activity 19
2.17 Polyphenoloxidase (PPO) activity 19
2.18 β-Glucosidase (β-GL) Activity 20
2.19 Activities of chitinase and β-1,3-glucanase 20
2.20 Glutathione-S-transferase (GST) 21
2.21 Salicylic acid (SA) 21
2.22 Statistical analysis 21

3. RESULTS 22

3.1 Determination of the virulence of different strains of *Erwinia amylovora* 22
3.2 Symptom development in untreated, BTH or plant extract treated shoots 22
3.3 Influence of BTH on symptom development and determination of its optimum application time 25
3.4 Influence of the *Hedera helix* plant extract on symptom development and determination of its optimum application time 26
3.5 Bacterial growth *in planta* after resistance induction with BTH or plant extract 27
3.6 Evaluation of bacterial populations *in planta* by PCR 28
3.7 Physiological changes 32
3.7.1 Total phenol content 32
.2 Peroxidase (PO) activity 34
3.7.2.1 Specific peroxidases detected by electrophoresis 36
3.7.3 Protein bands detected by electrophoresis 37
.4 Phenylalanine ammonia-lyase (PAL) activity 38
.5 Polyphenoloxidase (PPO) activity 39
.6 Flavonoids determined by thin layer chromatography (TLC) 41
.7 Inhibitory substances in BTH and plant extract treated shoots 45
3.7.7.1 Inhibitory effect of the substance in liquid culture and comparison to phenolic acids 45
3.7.8 High performance liquid chromatography (HPLC) analysis 46
.9 β-Glucosidase (β-Gl) activity 61
.10 Pathogenesis related (PR) proteins 62
3.7.10.1 Chitinase activity 63
.2 β-1,3-Glucanase activity 64
3.7.11 Glutathione-S-transferase (GST) activity 66
.12 Salicylic acid 68
.13 Physiological changes and symptom development in shoots 70

4. DISCUSSION 73

4.1 Induction time in BTH and plant extract (PE) treated plants 73
4.2 Symptom development and bacterial multiplication in plants after resistance induction 74
4.2.1 Studies on PCR and assessment of changes in signal of PCR product in plants 75
4.3 Physiological changes 76
4.3.1 Phenols, flavonoids and inhibitory substances in treated plant tissue 76
.2 Changes in enzymatic activities 79
4.3.2.1 Peroxidase (PO) activity 79
.2 Phenylalanine ammonia-lyase (PAL) activity 81
.3 Polyphenoloxidase (PPO) activity 83
.4 β-Glucosidase activity 84
4.4 Pathogenesis-related (PR) proteins 85
4.5 Glutathione-S-transferase (GST) 87
4.6 Changes in salicylic acid content 89

4.7 Study hypothesis 91
4.7.1 Mode of action of resistance induction by BTH 91
.2 Mode of action of resistance induction by plant extract from *Hedera helix* 92

5. SUMMARY 94

5.1 Symptom development and growth of bacterial multiplication in BTH and PE treated plants 94
5.2 Physiological changes 94
5.2.1 Phenols, flavonoids and inhibitory substances in treated plants 94
.2 Enzymatic activities, PR proteins and salicylic acid (SA) 95
.3 Mode of action of BTH and PE 96

6. REFERENCES 97

List of Abbreviations

bp	=	base pairs
β-Gl	=	β-glucosidase
BSA	=	bovinem serum albumine
BTH	=	Benzothiadiazole (= Acibenzolar-S-methyl, BION®)
CFU	=	colony forming units
CM	=	carboxymethyl
d	=	day(s)
DCINA	=	2,6-dichloroisonicotinic acid
DNA	=	deoxyribonucleic acid
dNTP	=	dinuclo tri-phosphat
DTT	=	dithiothreitol
EDTA	=	ethylendiamine tetra-acetic acid
et al.	=	and others
etc.	=	et cetera (and others)
Fig.	=	figure
g	=	gram
GSH	=	glutathione
GST	=	glutathione-S-transferase
h	=	hour(s)
H. helix	=	*Hedera helix*
HPLC	=	high performance liquid chromatography
HR	=	hypersensitive reaction
INA	=	isonicotinic acid
Kb	=	kilobase
kD	=	kilo dalton
µl	=	microlitre
mg	=	milligram
min	=	minute(s)
ml	=	millilitre
mM	=	millimolar
mmol	=	millimole(s)
mRNA	=	messenger ribonucleic acid

O.D.	=	optical density
p.i.	=	post inoculation
PAGE	=	polyacrylamide gel electrophoresis
PAL	=	phenylalanine ammonia-lyase
PCR	=	polymerase chain reaction
PE	=	plant extract
pH	=	a measure of acidity
PMSF	=	phenylmethansulfanylfluorid
PO	=	peroxidase
pp.	=	page(s)
PPO	=	polyphenoloxidase
PR	=	pathogenesis related
PVPP	=	polyvinylpolypyrolidone
SA	=	salicylic acid
SAR	=	systemic acquired resistance
SDS	=	sodium dodecyl sulfate
sp.	=	species (single)
spp.	=	species (plural)
TLC	=	thin layer chromatography
Tris	=	Tris-(hydroxymethyl-) aminomethane
UV	=	ultraviolet
v/v	=	volume per volume
w/v	=	weight per volume

1. INTRODUCTION

1.1 Description of the pathogen, its distribution and control

The Gram-negative bacterium *Erwinia amylovora* ((Burril) Winslow *et al*.) is the causal agent of fire blight, a dangerous disease of pome fruits and several ornamentals of rosaceous plants. Fire blight was first described in North America about 200 years ago (VAN DER ZWET and BEER, 1992). After its first occurrence in 1957 in Europe (LELLIOTT, 1959) through its introduction by contaminated fruits and budwood from the North American continent, the disease has been distributed over whole Europe (VAN DER ZWET and BONN, 1999).

Fire blight control measures are mostly restricted to the early eradication of attacked host plants, as in most countries the use of effective antibiotics is prohibited by law. In countries where the antibiotic streptomycin has been allowed, the problem of streptomycin resistant strains of the pathogen occurred (MANULIS *et al*., 1999).

Several chemical copper compounds, Flumequine, Aliette and Oxolinic acid, have been tested as alternatives to the use of antibiotics. They either revealed insufficient efficacy under field conditions, or showed negative side effects (VANNESTE, 2000). Thus, an effective compound against the disease is urgently needed by the growers. Biocontrol agents have been tested as alternatives to antibiotics.

1.2 Alternatives to antibiotic compounds

1.2.1 Bacterial antagonists

A biocontrol method based on antagonism against the disease has already been developed about 30 years ago (BEER and RUNDLE, 1987). Especially with strains of *Erwinia herbicola,* the so-called yellow bacteria, first positive results have been achieved. Later on, a significant reduction of fire blight was achieved by the application of *Bacillus subtilis*, *Pseudomonas fluorescens*, *Pantoea agglomerans* or *Rahnella aquatilis* strains, respectively (BEER and NORELLI, 1986; ZELLER and WOLF, 1996). In recent studies, application of *Pseudomonas fluorescens* caused 40-60% reduction of fire blight symptoms, and for *Pantoea agglomerans* a control of 50-80% was observed (LAUX *et al.*, 1999). Based on the strains *Pseudomonas fluorescens* A506 and *Pantoea agglomerans* C9-1, the product Blight Ban

(Plant Health Systems) was released on the US-market. Another antagonistic strain of *Bacillus subtilis* is the active ingredient of the commercial formulation "Serenade" (Serenade Product Information). However, in most cases the field efficacy of these products was significantly lower than that of antibiotics, and not constant from year to year (FRIED *et al.*, 1998). Therefore, no registration of these products in Europe has been achieved until now.

1.2.2 Induced resistance

Several biocontrol agents have been shown to induce resistance in the plant. Biotic and chemical agents are reported as resistance inducers, although only a few of them are real plant activating agents. Three criteria need to be fullfilled before an agent can be classified as systemic acquired resistance (=SAR) inducing compound:

- The treated plants are resistant to the same spectrum of disease as those in which SAR is induced biologically.
- A lack of direct antimicrobial activity and no conversion of the compound *in planta* into antimicrobial metabolites.
- Induction of the same pre-infectional biochemical processes as observed in plant tissue after biological induction of SAR (KESSMANN *et al.*, 1994a).

1.2.2.1 Plant extracts

As a further alternative to antibiotics several plant extracts have been tested against *E. amylovora in vitro* and *in vivo*. MOSCH *et al.* (1989) reported an antibacterial activity for 24 out of 139 plant extracts tested in an agar diffusion test. The antibacterial activity against *E. amylovora* could also be observed with leaf extracts from *Rhus typhina*, *Berberis vulgaris*, and *Mahonia aquifolium* in field experiments. Extracts from these plants, applied as protective spray showed a high disease control (MENDE *et al.*, 1993). Moreover, a high activity against the disease was reported for plant extracts from *Reynoutria sachalinensis*, *Hedera helix*, *Viscum album* and *Alchemilla vulgaris*. These extracts induced resistance in the high susceptible host plant *Cotoneaster waterei*, causing a slower multiplication of the bacterium and a reduction in disease severity. The same results have been achieved with extracts from *Hedera helix* and *Viscum album* on detached leaves of *Cydonia ablonga* (MOSCH *et al.,* 1993). In field experiments with the apple variety `James Grieve`, an extract from *Hedera helix* showed an efficacy similar to that of streptomycin (MOSCH *et al*., 1996).

The active components of the plant extract from *Hedera helix* which induce resistance have not been elucidated. However, chemical analysis of *Hedera folium* leaves revealed the presence of the following compounds: Hederagenin, oleanolacid, bayogenin (=2β-hydroxyhederagenin) as aglykon and hederasaponin (=hederacosid) (WICHTL, 1997). It is yet unknown whether these compounds occur in the methanolic plant extract from *Hedera helix*. In another study, an inhibitory effect against the disease was also reported for Bactosan, an extract from *Pingania piñata,* by PSALLIDAS and TSIANTOS (2000).

A plant extract from *Hedera helix*, an inducer of resistance, was shown to cause physiological changes. Thus, enhanced levels of PR proteins (chitinase, ß-1,3 glucanase) and enzymes of phenol metabolism, which can be regarded as a marker of resistance induction was observed (MOSCH *et al*., 1996). These enzymes have been reported as markers for induced resistance by several authors (HAMMERSCHMIDT *et al.*, 1982; BINDER *et al.*, 1989; MÈTRAUX *et al.*, 1989; WARD *et al.*, 1991).

1.2.2.2 Etheric oils

Besides plant extracts also etheric oils have been tested against *E. amylovora in vivo* and *in vitro*. SCORTICHINI and ROSSI (1989, 1991, 1993) reported an antibacterial effect against *E. amylovora* with essential oils from origanum, thyme, savory, cinnamon and garlic. In their studies an influence of essential oil constituents on bacterial growth was determined, as for instance by the terpenoids geraniol and citrollenol. In addition, an etheric oil from *Thymbra spicata* was reported as an induction agent of systemic acquired resistance (SAR) **(**BASIM *et al*., 2000).

1.2.2.3 Synthetic compounds

Benzo-(1,2,3)-thiadiazole-7-carbothioic-S-methyl-ester (BTH=Acibenzolar-S-methyl), which is capable of inducing SAR, has been tested against several pathogens (KESSMANN *et al.*, 1994b; RUESS *et al.,* 1995; OOSTENDORP *et al.,* 1996). In 1996, BTH was introduced in Germany and is available as the commercial product BION®. Resistance inducing effects of this product have been demonstrated in plants against *Erysiphe graminis*, *Septoria* spp., *Pyricularia oryzae*, *Peronospora tabacina, Phytophthora* spp., *Didymella bryonia*, (RUESS *et al.*, 1995; KESSMANN *et al.,* 1996; GÖRLACH *et al.*, 1996) CMV-Y (cucumber mosaic

virus) (ANFOKA, 2000) and against *Erwinia amylovora* in recent studies (BRISSET *et al*., 2000; ZELLER and ZELLER, 1998).

The growth regulator Prohexadione-Ca has also been tested as an alternative compound against fire blight. However, sufficient efficacy in field experiments could not be obtained. Recently, the compound harpin was released on the US-market as resistance inducer, but it has not yet been tested against fire blight in the field (PSALLIDAS and TSIANTOS, 2000).

1.3 Aim of this study

Until now not much information is available regarding physiological changes in apple tissue against *E. amylovora* during induced resistance. In the here reported studies, two different resistance inducers were compared for their potential effect against fire blight (*Erwinia amylovora*): the synthetic inducer BTH (BION®), and as a biotic agent a plant extract from *Hedera helix.* Experiments were primarily performed to characterize the physiology of induced resistance, besides the direct efficacy of these inducers against the disease. In particular, alterations of the phenol metabolism and enzymatic activities were followed, since earlier studies indicated a decisive of these reactions in the resistance respons against *Erwinia amylovora* (ZELLER and BRULEZ, 1987).

2. MATERIALS AND METHODS

2.1 Plant material

M26 apple rootstocks obtained from Rheinau (Deutsche Marken-Baumschule) were used as host plants for the studies. These rootstocks are highly susceptible to fire blight caused by *Erwinia amylovora*.

2.2 Greenhouse conditions

Three-month old rootstocks were grown in pots of 20 cm x 15 cm x 15 cm size, filled with 8 kg soil in the greenhouse, with temperatures of 25 ± 5 ^{o}C, humidity of 68 - 80%, and light intensity of 5000- 14000 lux. The plants were used 4 weeks after planting (young shoots were 10-12 cm long with 6-8 leaves per shoot). This environment was maintained during the entire period of the experiment.

Fig. 1: Experimental set-up in greenhouse

2.3 Bacterial strains and culture medium

Bacterial strains of *Erwinia amylovora* (Ea7/74, Ea385, Ea6/98) were obtained from the Federal Biological Research Centre (BBA), Darmstadt (collection of Prof. Dr. W. Zeller). The virulence was tested on M26 rootstocks. Ea7/74 showed the highest virulence and was used in all following experiments. The inoculation method is described in the following.

Stock cultures were preserved on the modified Miller-Schroth medium (MSM, Table 1) (ZELLER and BRULEZ, 1987) in glass flasks at 4 °C in a refrigerator. The bacteria were transferred every 3 months to new flasks.

Table 1: Miller-Schroth medium (MSM) (MILLER and SCHROTH, 1972)

Nutrient Broth (Difco Co. 23400)	8 g
Saccharose (Carl-Roth Co. 4621.2)	50 g
Difco Bacto Agar (Carl-Roth Co. 5210.2)	20 g
0.5 % Bromothymolblue solution (Merck Co. 1.59103.0001)	9 ml
0.5 % Neutralred solution (Merck Co. 1.01369.0025)	2.5 ml
Actidion (Fungicide) (Carl-Roth Co. 8682.3)	50 mg /l
H_2O	1000 ml
pH was adjusted to 7.4 with 2 N NaOH	

2.4 Application of BTH (BION®)

Benzothiadiazole (BTH (BION®)) was used at a concentration of 0.02% (diluted with tap water) as inducing agent by spraying on leaves at 48, 72, 96 and 120 h before inoculation to determine the induction interval. Control plants were treated similarly with water.

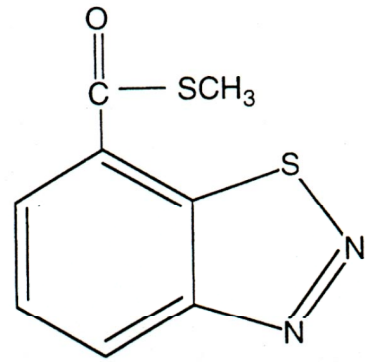


Fig. 2: Formula of BTH (BION®)

2.5 Preparation and application of plant extract from *Hedera helix*

Dried and ground leaf material (powder and ground leaves of about 3 mm size) supplied by Galke Company (Gittelde / Harz) was used for the preparation of plant extract. The plant material was extracted in 80% methanol with a soxhlet–apparatus. The extract was boiled in 80% methanol for 30 min after cooling with a reflux condenser for 4 hours. After 15 min the extract was filtered and dissolved in methanol/water and then again boiled for another 15 min. This treatment was repeated three times. Then, the methanol was evaporated from the extract by a rotation-evaporator (according to MOSCH and ZELLER, 1997). The plant extract was stored in a refrigerator in 30% ethanol solution until use. Before application, the extracts were diluted with water to 3% extract concentration. The extracts were applied by spraying on leaves until run off.

2.6 Inoculation

The two youngest leaves of the shoots were cut at the tip and inoculated by dipping into a suspension of $1x10^8$ CFU/ ml of strain Ea7/74 (ZELLER and MEYER, 1975).

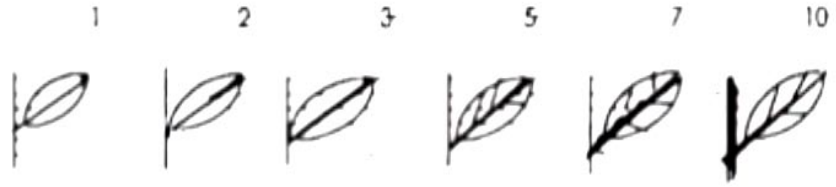
2.7 Determination of induction time

To determine the most efficient induction interval, experiments were conducted at four induction times (48, 72, 96, and 120 h) with BTH application on M26 rootstocks before inoculation. Control plants were sprayed with water at the same intervals. To determine the disease index, eight plants were used for each treatment in greenhouse experiments. These experiments revealed that 48 h were the most efficient induction time. Therefore, this application time was used for BTH and plant extract.

2.8 Symptom development

Symptom development was evaluated at 4, 6, 7, 11, and 14 days after inoculation according to a rating system from 0-10 as given below (Fig. 3). From these data, the disease index (%) was calculated.

Fig. 3: Rating system (class of symptoms)



0- no obvious symptom*

1- main leaf vein turned to brown from cutting point, few mm (3-5)

2- main leaf vein turned to brown from cutting point, several mm (>5 mm, not total leaf length)

3- main leaf vein turned to brown from cutting point , total leaf length

5- main and side leaf veins turned to brown and / or necrosis from cutting point, half leaf length

7- total leaf turned to brown and / or necrosis up to leaf stem

10- infection of shoot, often together turning black, and shoot curved

* minimum necrosis at cutting point was assessed as symptom, as it sometimes also occurred in control plants.

Index of disease (DI %) was calculated as follows:

$$DI\ \% = \frac{\sum (\text{number of leaves X class of symptom})}{\text{Total number of leaves}} \times 100$$

2.9 Determination of bacterial multiplication *in planta* by dilution plating

For the determination of bacterial multiplication, the samples of inoculated shoot tips (ca. 1 g plant material) were collected and homogenized in 0.06% NaCl solution (1:1). From each homogenate, dilution plating (from 10^{-1} to 10^{-6}) was performed on the modified Miller-Schroth medium (ZELLER and BRULEZ, 1987) and incubated for 2 days at 27 °C, (Fig. 4).

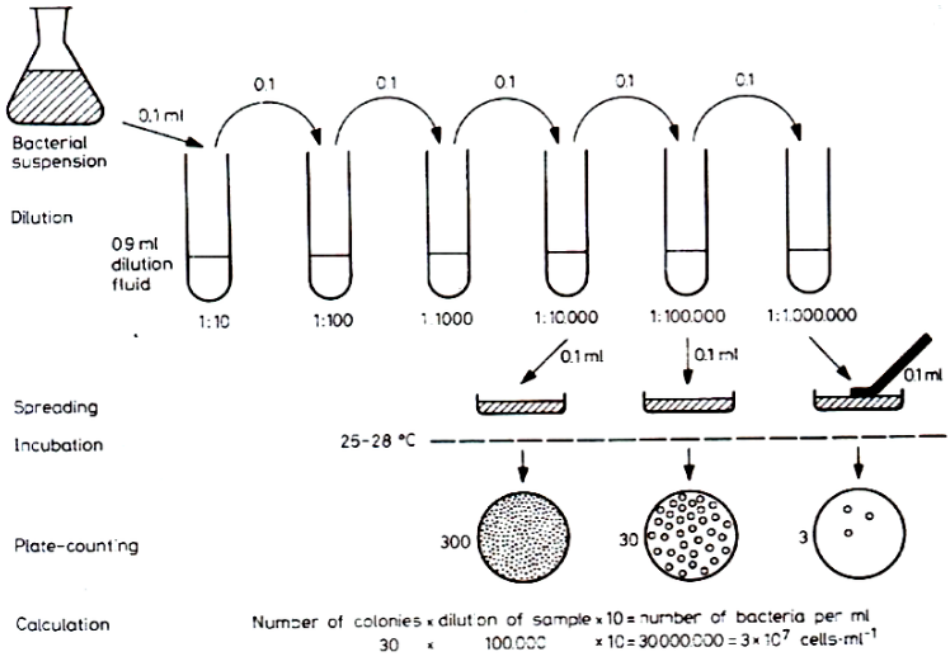


Fig. 4: Plate counting technique: Scheme of the dilution row and calculation of living bacterial cells (KLEMENT *et al*., 1990).

2.10 Determination of bacterial population *in planta* by PCR

In this experiment, in addition to quantitative determination of bacterial population it was tested whether the PCR method allowed to detect any changes in the bacterial population after inoculation or treatment with BTH or plant extract. The samples were collected at the same time of the experimental period as samples used to determine enzyme activities.

2.10.1 Testing of phenol inhibitory substances for PCR

For determination of bacterial populations in plants, bacterial suspensions were adjusted to an absorbance of 0.2 (≅ 10^8 CFU / ml) and homogenized 1:1 v/w with 1 g healthy plant material in order to detect possible negative effects of plant phenolics on sensitivity of PCR. The homogenate was separated from plant residues by filtration through a sieve followed by centrifugation at 15000 X g for 10 min. To 1000 µl of the resulting bacterial suspension 250 µl prepared solution consisting of 1% (v/v) PVPP, 5% (v/v) glycerol and 0.1 M DTT were added and gently shaken to inhibit probable complications, due to phenolic substances in the PCR reaction (LLOP *et al.*, 2000). The bacterial suspension was centrifuged at 13000 X g for 10 min. The bacterial pellet was suspended in 1 ml distilled water and again centrifuged at 13000 X g for 10 min. The pellet was suspended in 1 ml distilled water serially diluted with 1% Tween 20 and incubated at 60 ^oC for 10 min. Aliquots of 10 µl were taken for PCR. Plant

samples of 1 g each without bacteria collected from the greenhouse were homogenized 1:1 (w/v) in distilled water and then the same procedure as above was carried out.

2.10.2 PCR protocol (after JOCK *et al*., 2000)

A specific PCR core kit (Qiagen GmBH) was used to detect the PCR signal. The composition of the reaction mixture and thermal cycle conditions, are listed in the following tables (A-B).

A. Composition of reaction mixture

10X Qiagen PCR buffer	5 µl
dNTP	2 µl
Primer A	1 µl (pEA 29 A)
Primer B	1 µl (pEA 29 B)
Taq DNA polymerase	1 µl
Distilled water	31.5 µl
Sample	10 µl
Total volume	**50 µl**

B. Conditions of the thermal cycler

Initial denaturation	3 min	94 ^{o}C
3-step cycling		
Denaturation:	1 min	94 ^{o}C
Annealing:	1 min	52 ^{o}C
Extension:	1 min	72 ^{o}C
Number of cycles:	28	
Final extension:	10 min	72 ^{o}C

For detection of *Erwinia amylovora* by PCR the specific plasmid pEA29 A-B (BERESWILL *et al*., 1992) was used. As a marker 0.9 Kb AluI pBR 322 (MBI Fermentas Co. # SM0123) was applied.

2.10.3 Preparation of agarose gel

The agarose gel was prepared by dissolving 1.5 g agarose in 100 ml of *Tris-buffer (see below) by microwave. After cooling down in a water bath (60 ^{o}C) the agarose was slowly poured into the gel electrophoresis tank.

Composition of *Tris-buffer

Tris	107.8 g
Boric acid	55.0 g
EDTA	7.44 g
Distilled water	1000 ml
pH adjusted to	7.4

For dissolving the agarose the Tris-buffer was diluted 1:10.

2.10.4 Loading of samples in agarose gel

After preparation of the agarose gel, each sample was applied together with 5 µl ready loading buffer solution, using AluI pBR 322 as a marker. Electrophoresis was performed at 5-6 V/ m for 3 hours. After electrophoresis, the gel was stained with ethidium bromide for 15 min and photographed under UV light (JOCK *et al*., 2000).

2.11 Physiological investigations

A considerable suppression of symptom development and bacterial multiplication was observed in BTH and plant extract treated plants. This effect was supposed to be an induced resistance reaction caused by physiological changes in the plant. Therefore, several typical biochemical and physiological parameters, which are activated by resistance inducing agents, were estimated at different time intervals (1-11 days) after induction and inoculation. At each time interval, four samples (2 shoot tips from each of two seedlings) were taken for each treatment. The following methods were used:

2.11.1 Preparation of samples for determining enzyme activities

For determining enzyme activities of peroxidase (PO), polyphenoloxidase (PPO), phenylalanine ammonia-lyase (PAL) and β-glucosidase (β-Gl) shoots measuring 5 cm from the tip were collected, immersed in liquid N_2 and homogenized with 0.1 M Na-acetate buffer (pH 5.2) (1 g plant material in 10 ml). The homogenate was centrifuged at 15000 g for 30 min at 4 ^{o}C, and the enzyme activities were determined in the supernatants.

For determining pathogenesis related (PR) proteins (chitinase, β-1,3-glucanase) and glutathione-S-transferase, harvested shoots were immersed in liquid N_2 and homogenized in 2 ml 0,1 M Na-acetate buffer of pH 5.2 consisting of 1% (v/v) PVPP (polyvinylpolypyrolidone), 5% (v/v) glycerol, 0.1 M phenylmethansulfanylfluorid, and 0.1 M DTT (dithiothreitol). Homogenates were centrifuged at 15000 g for 30 min at 4 ^{o}C. Supernatants were used to determine enzymatic activities.

2.11.2 Protein assay

Protein content of samples was determined by the Bradford reagent (BRADFORD, 1976) prepared as follows:

100 mg Coomassie Brilliant Blue G-250 (Carl-Roth Co. 3862.1) were gently dissolved in 50 ml ethanol (95%), added to 100 ml 85% H_3PO_4, mixed with 1000 ml water, filtered, left for 24 h, and preserved in a refrigerator (+4 ^{o}C).
Assay: 100 µl homogenate were gently shaken with 1.5 ml Bradford reagent and incubated for 15 min at room temperature. Protein content was determined spectrophotometrically at 595 nm with bovine serum albumine (BSA) (0-5.0 mg/ml) as standard. Na-acetate buffer was used as blank.

2.11.3 Total phenol content

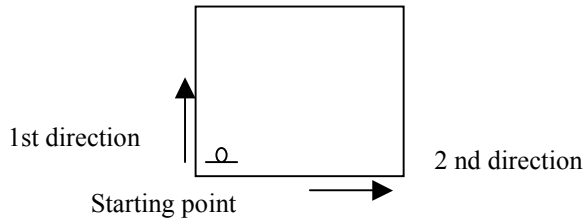
Shoots measuring 5 cm from the tip were immersed in liquid N_2, homogenized in 80% methanol (1 g plant material in 10 ml) and stored in the deep-freeze (-20 ^{o}C). Later, the homogenate was centrifuged at 15000 g for 30 min at 4 ^{o}C. The pellet was discarded. After addition of ascorbic acid (0.1 g to 5 ml) the homogenate was evaporated in a rotary evaporator at 65 ^{o}C 3 times for 5 min. The residues were dissolved in 5 ml of 80% methanol. For the

assay, 0.02 ml methanol extract were incubated for 1 h with 0.5 ml folin ciocalteau phenol reagent and 0.75 ml of 20% Na_2CO_3 solution. The total phenol content was determined spectrophotometrically at 767 nm with gallic acid (0-5 mg) as a standard. Methanol was used as blank. The results were expressed as mg gallic acid / g plant material (RAPP und ZIEGLER, 1973; ZELLER, 1985).

2.12 Flavonoids

Shoots immersed in liquid N_2 were homogenized in 80% methanol (1g plant material in 10 ml methanol), protected from oxidation by replacing oxygen with nitrogen and eliminating light for 48 h, extracted with ethyl acetate (1:1 v/v), and evaporated for 3 times (10 min) at 65 ^oC in a rotary evaporator. The residues were separated by thin layer chromatography (TLC) two-dimensionally on micropolyamide plates of 20 X 20 cm (RAPP und ZIEGLER, 1973). The solvent systems for both directions are shown below. The dried plates were sprayed with ``Naturreagenz A``(Carl-Roth Co. 99201) (1:100 dissolved in methanol). The spots were identified according to hrf values and specific colours (NEU, 1957; ZELLER, 1985; SCHULZ, 1987; HEIMLER and BODDI, 1989).

*Methylpropyl-ketone (pentanon-2)/water/formic acid 2: 12: 6 (v /v) **Dimension 2** (30 min)
*Methylpropyl-ketone (pentanon-2)/butyl format/formic acid 4: 13: 3 (v/v) **Dimension 1** (1 h)



2.12.1 Inhibitory substances

The aim was to characterize phenolic compounds which may play a role as antibacterial substances after application of BTH and plant extract. 10 µl residue (see 2.12) were spotted on silica gel thin layer chromatography plates (Silica gel 60F-254, Merck), and developed with chloroform and acetic acid (95:5 v/v). After drying for 4 h, a bacterial suspension of ca. 10^8 cells / ml of Ea7/74 in liquid culture medium [0.8% Nutrient Broth (Difco Co. 23400), 5%

saccharose (Carl-Roth Co. 4621.2), 1% glycerol, 0,05% actidion (Carl-Roth Co. 8682.3)] was sprayed on the plates, followed by incubation for 24 h at 27 ^{o}C. Plates were dried until opaque appearence, and immediately sprayed with aesculin spray [0.2% w/v aesculin (Carl-Roth Co. 8704.1), 0.1% w/v ammonium ferric citrate; 0.5% w/v yeast extract, in distilled water]. The plates were again incubated at 27 ^{o}C for 24 h in boxes to allow hydrolysis of aesculin to occur. Inhibition zones appeared as white spots against a yellow background (LUND and LYON, 1975).

2.12.2 Inhibitory effect of the detected substance compared with phenolic acids

In recent studies, pH decreases due to increase of phenolic acids in resistant plants have been reported by which the growth of the pathogen was inhibited (MATERN and KNEUSEL, 1988). Also, the present study revealed an increase of the phenol content and PO activity, and the appearance of a yet uncharacterized substance in BTH treated and plant extract treated plants.

The effect of the detected inhibitory substance and phenolic acids as reference substances were tested in liquid shaking cultures against *E. amylovora.* The reference substances (gallic acid, cinnamic acid, chlorogenic acid, and phloretin), which were reported as phytoalexin-like compounds in apple tissue (GOODMAN *et al.*, 1986), were dissolved in 60% acetone. Final concentrations of 0.01 M of the reference substances were added to 50 ml nutrient saccharose (NS, see Table 1.) medium. Parallelly, 30 g plant sample were extracted. 2 ml of each extract were developed on TLC thick layer plates (PSI Merck silica – gel 60F-254). Later on, inhibitory substance was scraped off the dried plates, dissolved in 50% acetone, and added to 50 ml NS. Substance free NS was used as control. At the start of experiment, 250 µl bacterial suspension of 10^{8} CFU/ml of Ea7/74 was applied. Changes of the bacterial concentration in shaking culture were recorded spectrophotometrically at 660 nm for 3 days.

2.13 High-performance liquid chromatography (HPLC) analysis

After determination of the flavonoid content through TLC and testing of inhibitory substances, HPLC analyses were performed in order to observe quantitative changes in phenolic acids and phloretin content in BTH, plant extract treated and untreated plants. Thirty gram plant sample was used and prepared as described above (see 2.12). Samples were

analysed by HPLC in the laboratory of Dr. Treutter (Technical University of München, Institut für Pflanzenbau) according to the method of TREUTTER *et al.* (1994) with dimethylaminocinnamaldehyde (DMACA)-reagent.

The HPLC equipment consisted of two pumps T-414 (Kontron) and the gradient programmer 205 (Kontron). The column (250 x 4 mm I.D.) as prepacked in the laboratory with Shandon Hypersil ODS, 3 µm. The solvents were 5% formic acid (A) and gradient grade methanol (B) with a flow-rate of 0.5ml/ min. The gradient profile used was: 0-5 min. isocratic, 5% B in A; 5-15 min, 5-10% B in A; 15-30 min, isocratic, 10% B in A; 30-50 min, 10-15% B in A; 50-70 min, isocratic, 15% B in A; 70-85 min, 15-20% B in A; 85-95 min, isocratic, 20% B in A; 95-110 min, 20-25% Bin A; 110-140 min, 25-30% B in A; 140-160 min, 30-40% B in A; 175-190 min, 50-90 % B in A.

Directly behind the column a Kontron filter detector (Uvikon 740 LC) was used for detection at 280 nm. Thereafter the eluent containing the phenols was mixed with the reagent in a simple T-connection. A Gynkotek HPLC pump (Model 300-C) moved the reagent at a flow-rate of 0.5 ml /min. For both the T-connection and the pumps stainless steel heads were used. The reactors were knitted PTFE capillaries (0.5 mm I.D) with different lengths. The PTFE capillaries have to be replaced after 4-5 months due to the occurrence of insoluble, blue to violet precipitations which can absorb phenolic compounds leading to peak tailing. The blue reaction products were measured at 640 nm by VIS-detector (Model SP6V, Gynkotek, Germany). The data of both chromatograms were evaluated simultaneously by a computer equipped with Gynkosoft chromatography software (Gynkotek).

For the heating experiments, a stainless-steel capillary (50 cm X 0.5 mm I.D.) was inserted between the T-connection and the PTFE-reactor. This short capillary was clamped between the open ends of the secondary coil of a laboratory-made low voltage/high current transformer and heated directly by an alternating current of approximately 20-30 A. The temperature was controlled electronically using a micro temperature probe attached to the capillary.

The reference compounds were commercially available (catechin, epigallocatechin, epicatechin-3-O-gallate, epigallocatechin-3-O-gallate, epicatechin, procyanidins B2, B5, A2, C1, epicatechin $(4\beta \rightarrow 8)$,-epicatechin $(4\beta \rightarrow 6)$,epicatechin, epicatechin, ent-epicatechin-

(4 $\beta \rightarrow$ 8;2 $\beta \rightarrow$ 7). The number of the peaks and common names of flavonoids are presented in Table.2.

Common name	**Structure**	**Ratio 40/280 Reaction time 2 min**	**Retention time without CRD**	**Elution order (peak number)**
2,3-*cis Series*				
Epigallocatechin		98.8	31.3	4
Epigallocatechin-3-O-gallate		3.2	49.5	6
Epicatechin-3-O-gallate		2.8	85.4	11
Epicatechin		20.9	55.6	7
Procyanidin B2	E(4 $\beta \rightarrow$ 8)E	10.9	41.2	5
Procyanidin C1	E(4 $\beta \rightarrow$ 8)E(4 $\beta \rightarrow$ 8)E	7.7	63.6	9
	E(4 $\beta \rightarrow$ 8)E(4 $\beta \rightarrow$ 8)E(4 $\beta \rightarrow$ 8)E	3.8	67.9	
Procyanidin B5	E(4 $\beta \rightarrow$ 6)E	14.5	112.0	14
	E(4 $\beta \rightarrow$ 8)E(4 $\beta \rightarrow$ 6)E	14.3	126.9	15
2,3-*trans Series*				
Catechin		12.4	28.5	3
Procyanidin B3	C(4 $\alpha \rightarrow$ 8) C	5.5	20.7	1
	C(4 $\alpha \rightarrow$ 8)C(4 $\alpha \rightarrow$ 8)C	3.4	20.7	1
	C(4 $\alpha \rightarrow$ 6)C	6.3	31.3	4
A-*types*				
Procyanidin A2	E(4 $\beta \rightarrow$ 8;2-0$\rightarrow$ 7)E	2.9	100.5	13
	entE(4 $\alpha \rightarrow$ 8;2 α -0$\rightarrow$ 7)E	2.0	94.2	12
	E(4 α 8;2 $\beta \rightarrow$ 0$\rightarrow$ 7)E(4 $\beta \rightarrow$ 8)entE	1.9	49.5	6
		1.8	70.9	10
Sterochemically mixed procyanidins				
Procyanidin B1	E(4 $\beta \rightarrow$ 8)C	9.7	24.0	2
Procyanidin B7	E(4 $\beta \rightarrow$ 6)C	11.9	57.5	8

Table 2: Influence of the structure on peak area ratio (CRD/UV) and retention time for determination of flavonoids (from TREUTTER *et al.*, 1994).

2.14 Peroxidase (PO)

Peroxidase activity was determined spectrophotometrically using guaiacol as a common substrate for peroxidases. The homogenate (see 2.11.1) of 0.2 ml was incubated with 0.1 ml

0.1 M Na-acetate-buffer (pH 5.2), 0.2 ml 1% guaiacol and 0.2 ml 1% H_2O_2 at 25 ^{o}C for 5 min and measured at 436 nm (PUTTER, 1974). Na-acetate buffer was used as blank. Enzyme activity was calculated from the change in absorbance and was expressed as:

Peroxidase activity = $OD_{436nm.}$ / mg protein

2.14.1 Peroxidase activity on SDS-PAGE gel electrophoresis

After the determination of PO activity spectrophotometrically, it was investigated if there were any differences in peroxidase isoenzyme patterns between the treatments. The protein patterns of the samples were observed in the following electrophoresis studies.

2.14.2 Determination of peroxidase activity with staining solution

The gel was incubated in *staining solution in the dark at room temperature until red-brown bands appeared (VALLEJOS, 1983). The gel was washed in water, fixed in 50% glycerol and photographed.

***Staining solution**	
50 mM sodium acetate buffer, pH 5.0	100 ml
3-amino-9-ethyl-carbazole (dissolved in a few drops of acetone)	50 mg
3% H_2O_2 (freshly prepared)	0.75 ml

2.15 Protein patterns

Preserved samples were treated with acetone 1:4 v/v and kept at -20 ^{o}C overnight. These samples were centrifuged (18000 X g) at –1 ^{o}C for 15 min, the supernatant was dried at room temperature and suspended in the *sample buffer (see below)

***Sample buffer**

100 mM tris / HCl
10% glycerol
10% mercaptoethanol
5% SDS
0.01% bromophenolblue
pH 6.8

2.15.1 Preparation of samples

One gram plant material was immersed in liquid nitrogen, stored in deep-freeze (-20 ^{o}C) and homogenized with sea sand, 2 ml *Tris-buffer, 0.05 g polyclar and 0.05 g dowex.

***Tris-buffer**
0.2 M Tris / HCl pH 7.2
14 mM 2- mercaptoethanol

Homogenized plant material was centrifuged (18000 X g) at 1 ^{o}C for 30 min; the supernatant was centrifuged (20000 X g) at 1 ^{o}C for 10 min, and preserved at -20 ^{o}C for SDS-PAGE.

2.15.2 Electrophoresis and staining

Prepared SDS Gel (Fa. BIO-Rad, 10 wells) was used for determination of specific peroxidase activity and protein patterns. The electrophoresis tank was filled with 1% SDS puffer (Fa. Roth). Combs were removed from stacking gel, taking care not to disturb the well dividers. The samples (5 µl) and standard protein as marker (low molecular weight–marker, Pharmacia calibration Kit; 14.4, 20.1, 30, 43, 67, and 94 kD) were applied in 1:1 (v/v) sample buffer in well dividers. Electrophoresis was performed at 60 V in the first 2 h, then at 110 V for 1 h more, after which the gel was removed from the electrophoresis tank.

2.15.3 Staining of gel with Coomassie brilliant blue

After the run, the gel was placed into *Coomassie solution since proteins are not directly visible and incubated for 1 h at room temperature. Coomassie blue dye binds to protein non-specifically. Corresponding protein bands can be detected as blue bands on a clear background (WILSON, 1983). Thereafter, the gel was placed in the *destaining solution under gentle shaking for 4 h to remove the background, prior to evaluation and photography. Protein electropherograms of samples were compared visually with the marker. After removing the solution, the bands of visible protein bands were fixed with 50% glycerol solution and stored in a refrigerator.

***Coomassie solution**
0.25 g Coomassie – blue
400 ml methanol
70 ml glacial acetic acid
1 l distilled water

***Destaining solution**

100 ml isopropanol
100 ml glacial acetic acid
800 ml distilled water

2.16 Phenylalanine ammonia-lyase (PAL) activity

0.5 ml homogenate (see 2.11.1) were incubated with 2 ml 50 mM *Na-borate buffer / HCl (pH 8.8) with 1 ml 60 mM phenylalanine, in 50 mM *Na-borate-buffer at 37 ^{o}C for 2 h. PAL activity was calculated at $OD_{290\ nm}$. Cinnamic acid (0- 5.0 mg) was used as standard. Activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (ZUCKER, 1968).

*Preparation of Na-Borate buffer: 25 ml NaOH (1 N) 3.09 g H_3BO_3 and 349 µl mercaptoethanol, dissolved in 1000 ml distilled water, and pH adjusted to 8.8.

PAL activity = mM cinnamic acid /mg protein

2.17 Polyphenoloxidase (PPO) activity

0.5 ml homogenate, which was used for determination enzymatic activities (see subchapter 2.11.1) were incubated with 2 ml 50 mM *Sörensen phosphate buffer and 0.5 ml substrate Brenzcatechol (Merck Co. 1.59614.0005) at 37 °C for 2 hours and measured at 410 nm (BATRA and KUHN, 1975).

*Preparation of Sörensen phosphate buffer: 6.8 g KH_2PO_4 and 8.99 g Na_2HPO_4 X $2H_20$ are dissolved in 1000 ml water, after addition of 0.372 g /l EDTA the pH is adjusted to 6.5.

PPO activity = $OD_{410\ nm.}$ / mg protein

2.18 β-Glucosidase (β-GL) activity

From the same homogenate, as prepared for other enzyme determinations (PO, PAL, etc.), 0.5 ml were mixed with 1.5 ml Sörensen phosphate buffer (pH 6.5) and 0.5 ml 5 mM p-nitrophenylglucopyranosid and incubated for 5 min at 30 ^{o}C. The O.D at 400 nm with nitrophenol (0-5.0 mg) as standard corresponded to the enzyme activity (ZELLER, 1985).

β-Glucosidase activity = mM p-nitrophenol / mg protein

2.19 Activities of chitinase and β-1,3-glucanase

Chitinase activity was determined by the method of WIRTH and WOLF (1990 and 1992). High polymeric carboxymethyl-substituted chitin, labelled covalently with Remazol Brilliant Violet 5R (CM–Chitin–RBV, Fa. Loewe Biochemica) was used as substrate for chitinase activity, while polymeric carboxymethyl-substituted curdlan, labelled covalently with Remazol Brilliant Violet 5R (CM–Curdlan–RBV, Fa. Loewe Biochemica), was used as substrate for β-1,3-glucanase activity.

Test sample was prepared as follows;

0.50 ml 0.01 M Na-acetate buffer pH 5.2 with 5% (v/v) glycerol
0.25 ml plant extract
0.25 ml dye labelled substrate CM–*RBV solution (2 mg /ml)

Test samples were kept in a water bath incubated at 37 ^{o}C for 120 min for chitinase determination or 60 min for β-1,3-glucanase determination. The enzyme reaction was terminated by adding 0.25 ml 2 N HCl. After centrifugation (14000 X g for 5 min), supernatants containing soluble, dye labelled degradation products were transferred into another cuvette (1 ml). Absorbance was measured spectrophotometrically at 550 nm for chitinase or 600 nm for β-1,3-glucanase. Blanks were prepared similarly with Na-acetate buffer instead of the homogenate. Enzyme activity was expressed as:

OD_{550} / mg protein for chitinase activity
OD_{600} / mg protein for β-1,3-glucanase activity

2.20 Glutathione-S- transferase (GST)

Glutathione-S-transferase activity was determined spectrophotometrically using reduced glutathione as substrate. For the assay, 600 µl homogenate (see 2.11.1) were mixed with 1200 µl Na-phosphate buffer (pH 6,5), 12 µl chloro-dinitro-benzene (CDNB), 12 µl 0.1 M GSH (reduced glutathione) and incubated at 30 ^{o}C for 10 min. Samples were left on ice for 5 min and enzyme activity was determined by measuring O.D. at 340 nm (HABIG *et al.*, 1974).

GST activity = $O.D._{340\ nm}$ / mg protein

2.21 Salicylic acid

500 µl homogenate (see 2.11.3) were mixed with 250 µl HCl (10 N) and 1000 µl methanol. Samples were incubated in a water bath at 80 ^{o}C for 2 h, neutralised with 4-5 drops 1 M $NaHCO_3$, and 1000 µl methanol were added. The O.D. at 254 nm was measured (modified from DAT *et al*., 1998) to calculate the content of salicylic acid and expressed as:

Amount of total salicylic acid = µg / g plant material

2.22 Statistical analysis

For determination of disease index, standard deviations of the mean values of two different experiments were calculated with the statistical analysis programme MSTAT (version 1.41) (RUSSELL, 1989) and expressed as ± standard deviation ($\perp$).

In the physiological studies (enzyme activities and total phenol content) standard deviations of data obtained were calculated from four different samples (2 shoot tips from each of two seedlings) with MSTAT and expressed as ± standard deviation ($\perp$).

3. RESULTS

3.1 Determination of the virulence of different strains of *Erwinia amylovora*

In order to work with a highly virulent strain in the following studies, three strains of *Erwinia amylovora* (Ea385, Ea6/98 and Ea7/74) were tested. Apple rootstocks of M26 were inoculated with a dose of 10^8 CFU/ml. Seven days after inoculation, the disease index of the shoots was determined. Strain Ea7/74 showed highest virulence, according to symptom development. Seven days after inoculation, the strains Ea7/74, Ea385 and Ea6/98 caused 72%, 52% and 41% infection, respectively (Fig. 5). According to these results, strain Ea7/74 was selected for the following experiments.

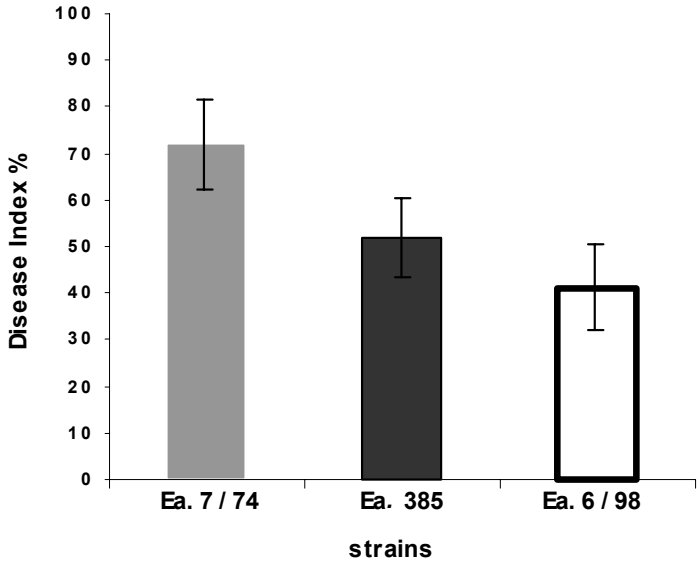


Fig. 5: Virulence of different strains of *E. amylovora* on M26 rootstocks.
⊥ Standard deviations of two replicates (8 plants per variant).

3.2 Symptom development in untreated, BTH or plant extract treated shoots

In the following experiments, control seedlings showed a faster symptom development than BTH, and plant extract treated ones. The first symptoms in **control seedlings** were observed at the main leaf vein after 4 days p.i. At this time, leaves were brownish coloured until 0.5-1 cm distance from infection site. Seven days p.i., symptoms reached half of the main and side leaf vein. Eleven days after inoculation, infection covered the whole leaf and stem (Figs. 6 a, d, g).

In **BTH treated** seedlings, the symptom development was slower than in plant extract treated ones, which in turn, showed significantly slower symptom development than control seedlings. Considerable differences in symptom development were observed at 4 d after inoculation. The symptom development was observed at the main leaf vein, covering 1-5 mm. At 7 d p.i., symptoms occured around 1 cm from the infection site. Very low symptom development was observed during the whole course of the experiment (Figs. 6 b, e, h).

In **plant extract treated** seedlings, first symptoms were observed 4 d after inoculation at the main leaf vein covering 1 cm. Seven days p.i., contrary to BTH treated seedlings the whole leaf showed necrotization (Figs. 6 c, f, i).

Fig. 6: Course of infection in untreated control plants, in comparison to BTH, and plant extract treatment.

a) 4 d p.i. **control** shoots

b) 4 d p.i. **BTH treated** shoots

Generally, in BTH treated and plant extract treated seedlings, infection did **not proceed systemically**, compared to untreated seedlings. Thus, suppression of symptom development was clearly visible in BTH, and plant extract treated seedlings, during course of the infection.

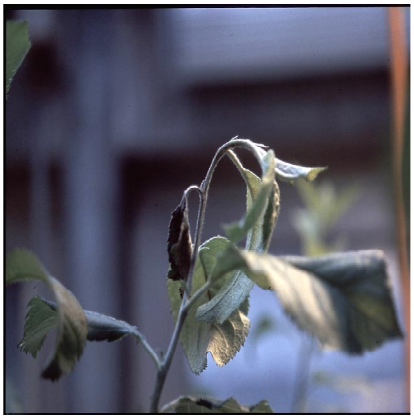
c) 4 d p.i. **plant extract** treated shoots

d) 7 d p.i. **control** shoots

Contrary to untreated seedlings, in BTH and plant extract treated seedlings the infection stopped in the middle of the leaf, at a maximum. BTH treated seedlings did not show any necrotization. However, in the plant extract treated seedlings necrotization of infected leaves was observed.

e) 7 d p.i. **BTH** treated shoots

f) 7 d p.i. and **plant extract** treated shoots



g) 11 d p.i. **control** shoots

h) 11 d p.i. **BTH** treated shoots

i) 11 d p.i. **plant extract** treated shoots

Untreated shoots were heavily diseased. From the upper part of the stem, typical cracking and dark brown-clinging leaves were observed. In BTH treated seedlings, a high disease severity in shoots was never observed compared to untreated and plant extract treated seedlings. In plant extract treated shoots, high necrotization was recorded only in the inoculated leaves. Thus, at the end of the experimental period development of disease was weaker in plant extract treated than in untreated seedlings.

3.3 Influence of BTH on symptom development and determination of its optimum application time

After application of BTH a remarkable reduction in the disease index of fire blight occurred (Fig. 7). When different time intervals between BTH treatment and inoculation (48, 72, 96 and 120 h) were compared the greatest suppression of disease was caused by BTH treatment 48 h before inoculation with *Erwinia amylovora*; but also BTH application at 72 h-120 h before inoculation reduced disease index significantly. The resistance against *E. amylovora* induced by the BTH-treatment was firstly detected 6 days after inoculation and lasted for the entire experimental period until two weeks after inoculation. Untreated plants showed a significantly faster symptom development during this period. Thus, at 7 days p.i. disease index was reduced by 62% in BTH treated seedlings and at 11 and 12 days p.i. up to 67 and 70 %, respectively. At 14 days p.i. the disease indices of control seedlings were 82% whereas those of BTH treated seedlings were only 12% - 35%.

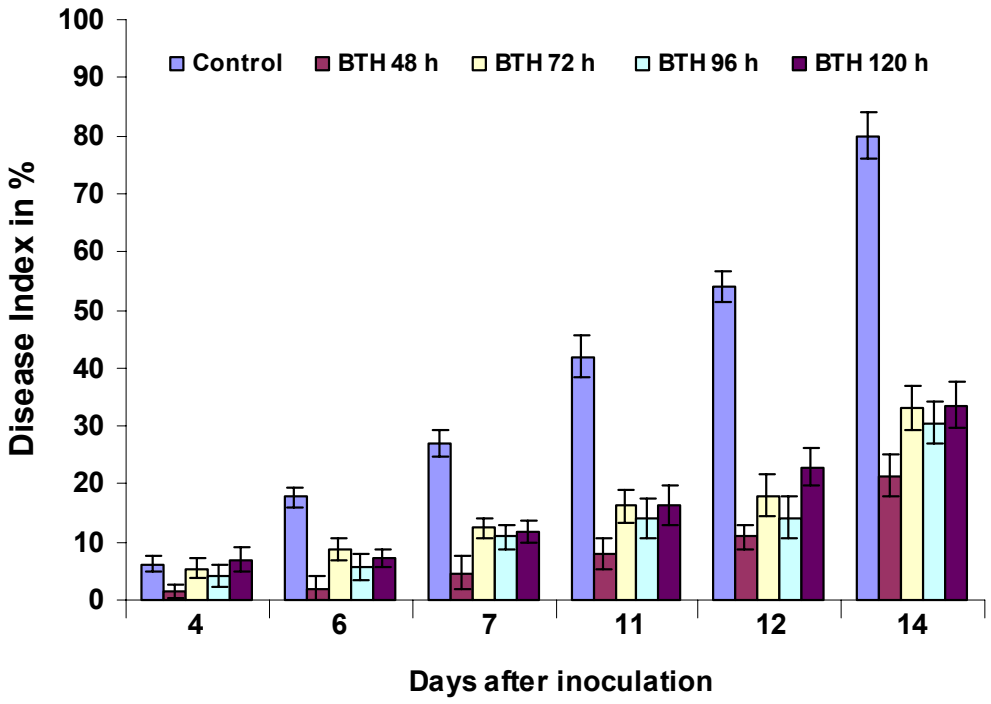


Fig. 7: Disease index of fire blight on M26 rootstocks after different time intervals between BTH-treatment and inoculation (48 –120 h). ⊥ Standard deviations of disease index for two replicates (10 plants per variant).

Since the highest effect was observed at a time interval of 48 h between treatment and inoculation, this application of BTH time was practised in all further experiments.

3.4 Influence of the *Hedera helix* plant extract on symptom development and determination of its optimum application time

Also, application of the *Hedera helix* plant extract significantly reduced symptom development. However, stable differences between different time intervals did not show up. Therefore, the same induction time as for BTH-treatment was also chosen for application of plant extracts (48 h before inoculation). At 4 days p.i., treatment with BTH and plant extract reduced the disease index by 40%, although not statistically significant (Fig. 8). Six days after inoculation, the plant extract and BTH treated seedlings showed 50 and 58% reduction in symptom development, respectively. At all the following dates, from 7–14 days post

inoculation, BTH–treatment always reduced disease indices stronger than treatment with plant extracts (Fig. 8 and Table 3).

Table 3: Disease index of fire blight on M26 rootstock after resistance induction with BTH or plant extract. ⊥ Standard deviations of disease index of two replicates (8 plants per variant).

Control	BTH	Plant extract	Days after inoculation
5 ± 2.4	3 ± 1.8	3 ± 2.1	**4**
12 ± 3.8	6 ± 3.4	7 ± 3.5	**6**
29 ± 4.2	14 ± 3.7	16 ± 5.2	**7**
48 ± 5.6	19 ± 5.2	33 ± 5.3	**11**
54 ± 4.5	22 ± 5.5	40 ± 5.4	**12**
78 ± 5.2	24 ± 5.4	54 ± 5.2	**14**

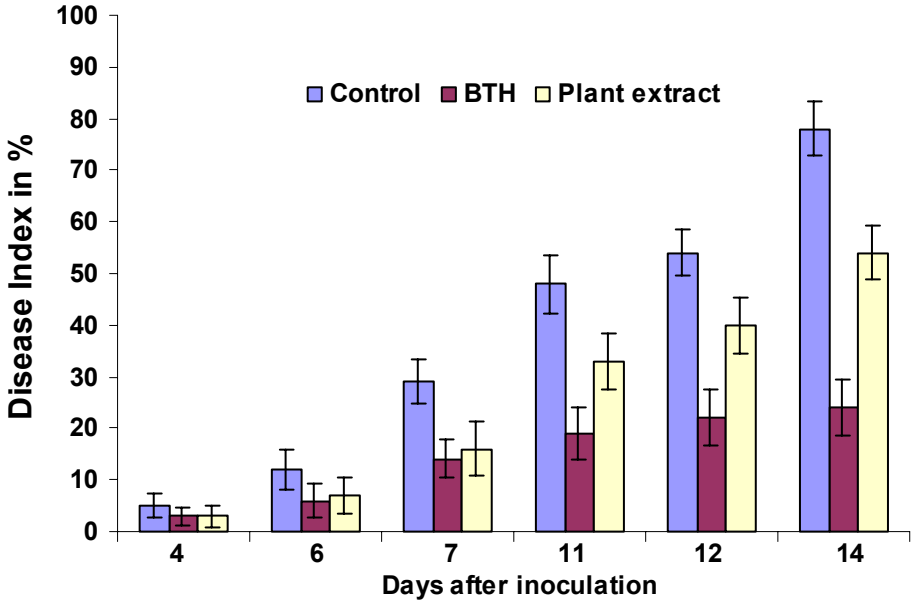


Fig. 8: Disease index of fire blight on M26 rootstock after resistance induction with BTH or plant extract. ⊥ Standard deviations of disease index of two replicates (8 plants per variant).

3.5 Bacterial growth *in planta* after resistance induction with BTH or plant extract

The growth of *E. amylovora* was markedly reduced in BTH and plant extract treated seedlings compared to the untreated control (Fig. 9). This inhibitory effect was firstly observed at 2 d after inoculation and determined until 7 d after inoculation. Also, in these experiments BTH-

treatment caused a stronger reduction of bacterial growth than treatment with plant extract. Thus, the bacterial population was reduced by 80 and 65% by BTH, compared to only 72 and 58% by plant extracts at 4 and 7 d after inoculation, respectively (Fig. 9). At 11 d p.i. no significant differences between treated and non-treated seedlings were detected. This may be due to necrotization of the plant tissue at the end of the infection period.

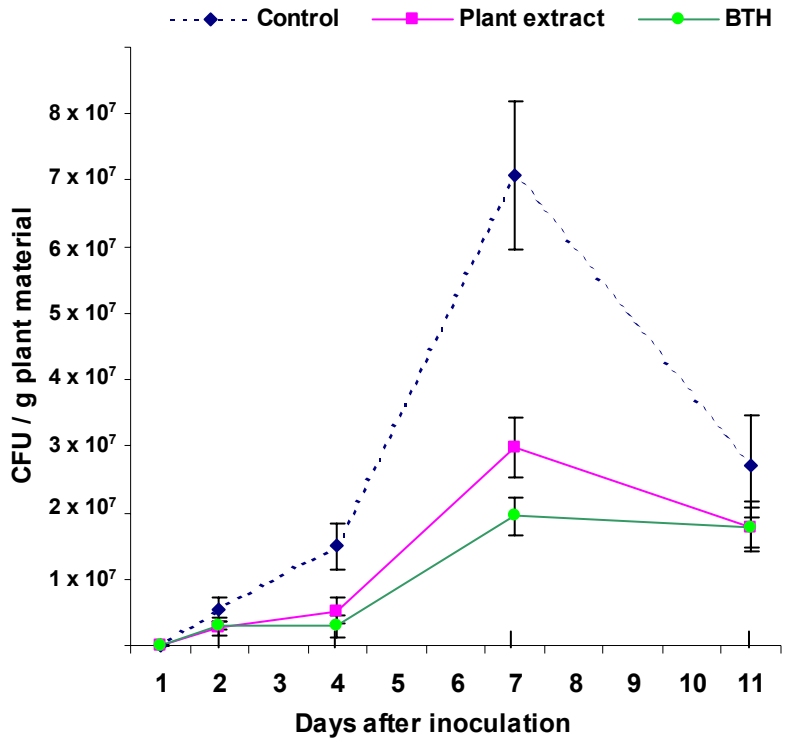


Fig. 9: Growth of *Erwinia amylovora* in shoots of M26 rootstock after treatment with BTH or plant extract. ⊥ Standard deviations of CFU/g for 2 replicates (1 g plant material per variant).

3.6 Evaluation of bacterial populations *in planta* by PCR

For the PCR studies, suspensions of pure bacterial cultures with approximately 10^8 CFU /ml were prepared. When a plant homogenate was added to these bacterial suspensions, the bacterial concentration was reduced to 10^6 CFU /ml as shown by serial dilution and plating (Table 4). By PCR no signal could be achieved below the 10^4 CFU /ml (Fig. 10). However, with addition of inhibitory substances against phenolics (see 2.10.1), the PCR-sensitivity was increased to one step more and a signal could be observed down to 10^3 CFU/ml (Fig. 11).

Table 4: A comparison of bacterial suspensions of $O.D._{660\ nm}$ = 0.2 with and without addition of plant homogenate (* no bacteria grew on Petri plates).

Dilution series	Plant homogenate (CFU/ml)	No plant homogenate (CFU/ ml)
10^{-8}	*	$1.7X10^{8}$
10^{-7}	*	$1.3X10^{8}$
10^{-6}	$4X10^{6}$	$1.2X10^{8}$
10^{-5}	$3.2x10^{6}$	Uncountable
10^{-4}	$3.7X10^{6}$	Uncountable
10^{-3}	Uncountable	Uncountable

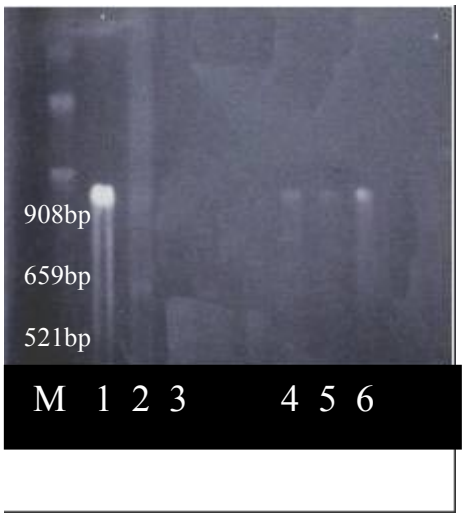


Fig. 10: PCR from dilution series of pure bacterial culture (Ea7/74 from 10^{4} to 10^{6}) mixed with plant homogenate containing no inhibitory substances against phenolics.

1. marker pBR 322 DNA / AluI marker,20

2. positive control (high density bacteria)

3. negative control (without bacteria only plant homogenate)

4. 10^{4} CFU /ml

5.10^{5} CFU /ml

6.10^{6} CFU / ml

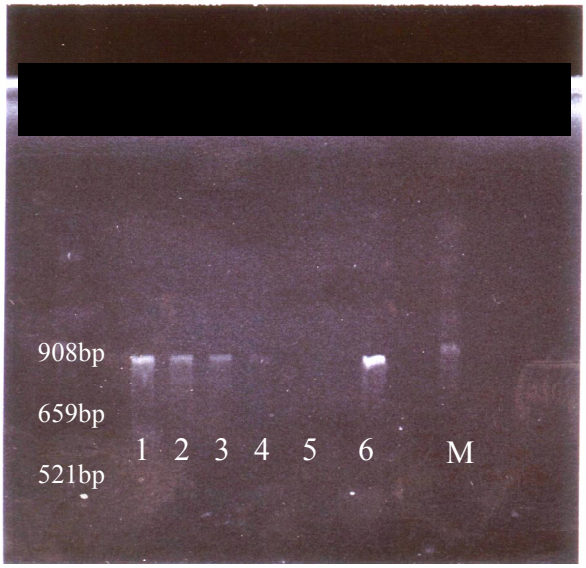


Fig. 11: PCR from dilution series of pure bacterial cultures (Ea7/74 from 10^6 to 10^1) mixed with plant homogenate containing inhibitory substances against phenolics (PVPP and DTT).
1. 10^5 **2.** 10^4 **3.** 10^3 **4.** 10^2 **5.** 10^1 **6.** 10^6
M=pBR 322 DNA/ AluI Marker,20

In a greenhouse experiment, 1 day after inoculation no PCR signal was received from all variants. Two days after inoculation, from control and plant extract treated seedlings very faint signals were obtained. A significant difference was found in BTH treated seedlings 4 days after inoculation (Figs. 12. 4A, 4B) which was maintained up to 7 d p.i., compared to control seedlings. At the end of the experimental period (11 d p.i.) the signal was similar in untreated and BTH treated seedlings. In plant extract treated seedlings (Figs. 12. 2A, 2C), the differences between treated and non-treated seedlings were rather weak from 4 - 11 d after inoculation.

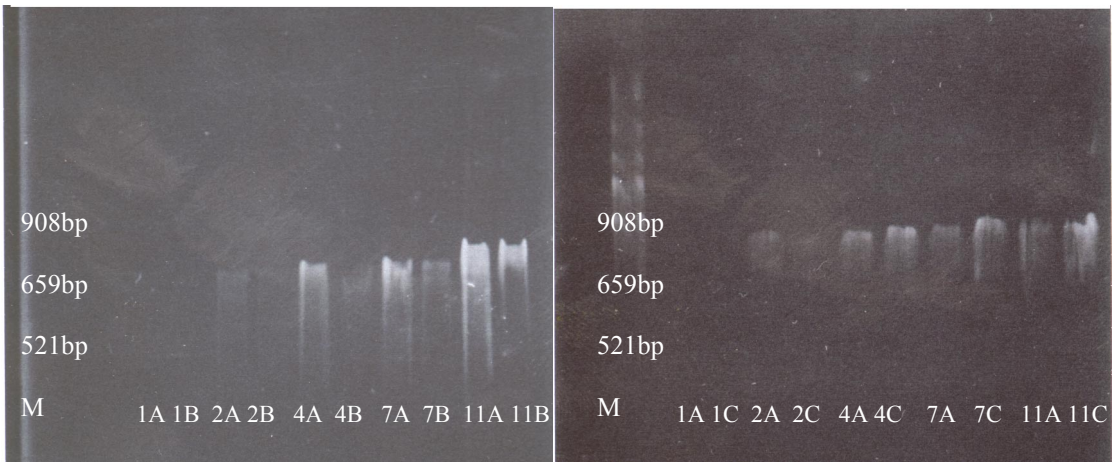


Fig. 12: The signal differences on PCR in plant during the experimental period.

	Days after inoculation				
Treatments	**1 d**	**2 d**	**4 d**	**7 d**	**11 d**
Control	0	(+)	++	+++	++++
BTH treated plants	0	0	(+)	+	+++
Plant extract treated plants	0	(+)	+	++	+++++

0 No signal, + very low, ++ low, ++ medium, +++ high, ++++ very high

M pBR 322 DNA/AluI Marker, 20

1A. Control shoots 1 d p.i. **1B.** BTH treated 1 d p.i. **1C.** PE treated 1 d p.i.

2A. Control shoots 2 d p.i. **2B.** BTH treated 2 d p.i. **2C.** PE treated 2 d p.i.

4A. Control shoots 4 d p.i. **4B.** BTH treated 4 d p.i. **4C.** PE treated 4 d p.i.

7A. Control shoots 7 d p.i. **7B.** BTH treated 7 d p.i. **7C.** PE treated 7 d p.i.

11A. Control shoots 11 d p.i. **11B.** BTH treated 11 d p.i. **11C.** PE treated 11 d p.i.

3.7 Physiological changes

3.7.1 Total phenol content

In general, the BTH and plant extract treated shoots showed a higher phenol content than untreated shoots. This effect was most significant between 2 – 4 d after treatment. In the following these changes are characterized in more detail.

a) Non-inoculated shoots

The total phenol content differed significantly between all three variants, BTH, plant extract treated and non-treated shoots (Fig. 13a). One day after treatment, the total phenol content gradually increased more rapidly in the BTH and plant extract treated shoots (14 and 31%, respectively) than in the untreated shoots. The plant extract treated shoots reached their highest value 2 d after treatment, when the phenol content was 60% higher than in non-treated shoots, and also significantly higher than after BTH treatment (31%). From 4 d to 11 d p.i. the total phenol content decreased gradually after both treatments and nearly reached the level of non-treated shoots 11 d p.i..

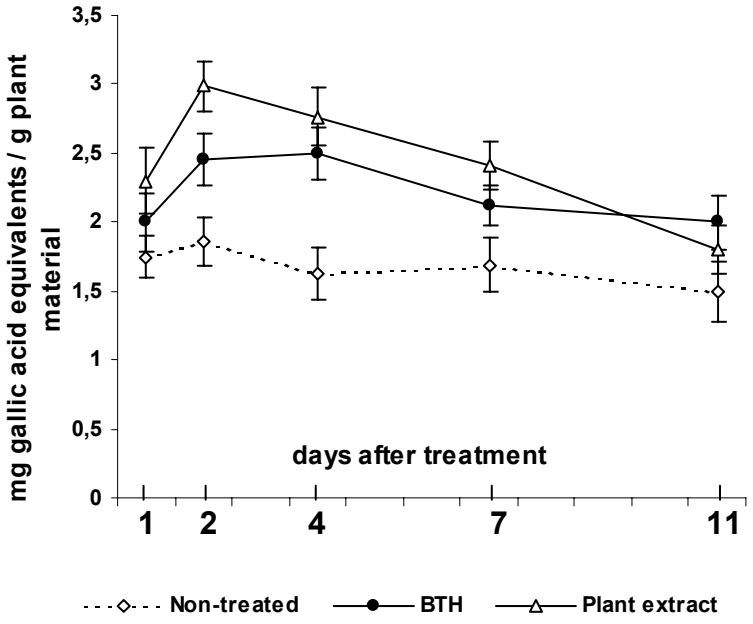


Fig. 13a: Total phenol content in non-inoculated shoots, which were treated or non-treated. ⊥ Standard deviations of measurements in 4 samples (2 plants per sample).

b) Inoculated shoots

The trend of phenol content showed a decrease for all variants post inoculation. At the beginning of the experiment, the inoculated, untreated seedlings had a higher phenol content than non-inoculated seedlings.

One day after inoculation, the treatment with plant extract caused a slight (12%) but significant increase of total phenols compared to BTH treated and control seedlings (Fig. 13b). Thereafter, the content of total phenolics gradually decreased in all variants and reached a similar value 7d p.i..

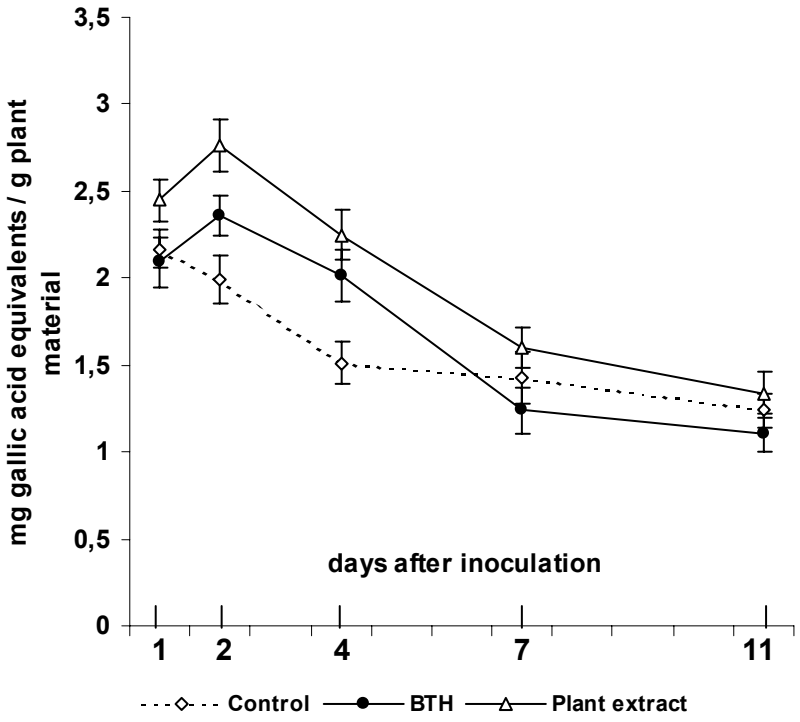


Fig. 13b: Total phenol content in inoculated shoots, and after treatment with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 plants per sample).

3.7.2 Peroxidase (PO) activity

a) Non-inoculated shoots

In the non-inoculated shoots, over the entire experimental period, PO activity was markedly increased in BTH (42%) and plant extract (54%) treated tissue (Fig. 14a), starting 2 d after induction with a significantly higher activity. At 4 d after induction, the plant extract treated shoots showed a significantly higher activity than BTH treated ones (67%). In plant extract treated shoots, the highest increase was up to 135%. Afterwards, the PO activity gradually decreased and reached the normal level at 11 d.p.i..

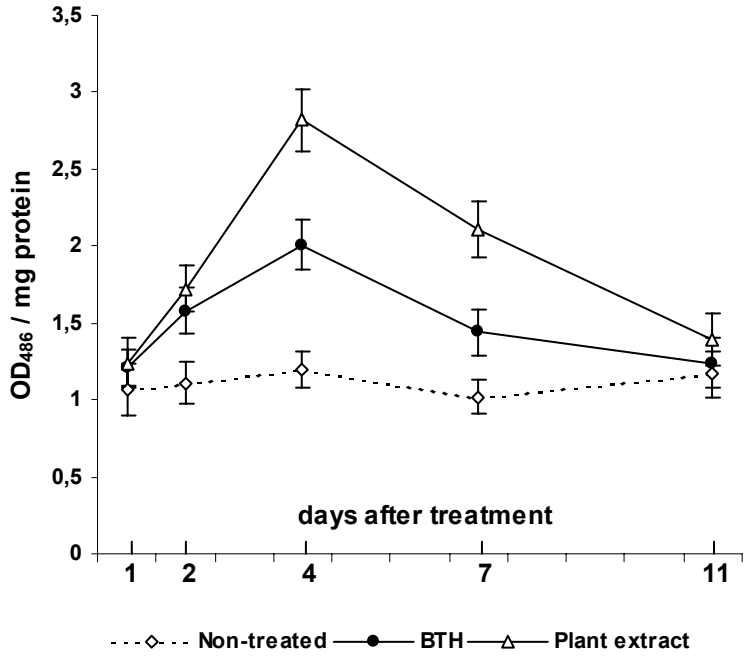


Fig. 14a: Peroxidase activity in non-inoculated shoots, which had been treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 plants per sample).

b) Inoculated shoots

The course of PO activity in inoculated plants was different from non-inoculated ones (Fig. 14b). One day after inoculation, PO activity was significantly lower in BTH treated plants than in plant extract treated and control plants. The activity increased in plant extract treated plants to 50% at 2 d after inoculation, and was significantly higher than in BTH treated and control plants. The activity in control plants remained on the same level up to 4 d after inoculation. In plant extract treated plants, the highest activity (70% more than the control) was observed at 4 d after inoculation, and also BTH treated plants showed a similar activity (80% of control) at the same time. At the end of the experimental period (11 d p.i.) the activity was similar in all treatments.

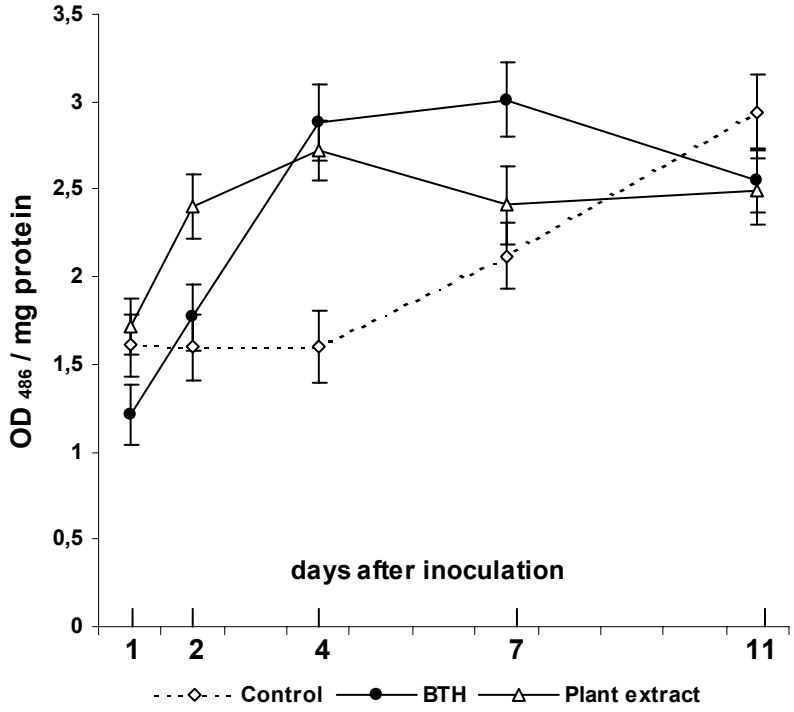


Fig. 14b: Peroxidase activity in inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 plants per sample).

3.7.2.1 Specific peroxidases detected by electrophoresis

To characterize specific changes in PO, the period of highest activity (4 d after treatment and inoculation) was examined by SDS- PAGE.

As demonstrated in Fig. 15, PO activities were found at protein sizes of 20.1 and 43 kD. The plant extract treated seedlings showed high PO activities in 3 bands, and BTH treated seedlings in 2 bands 4 d p.i. These results correlated well to the spectrophotometrical measurements of PO activity.

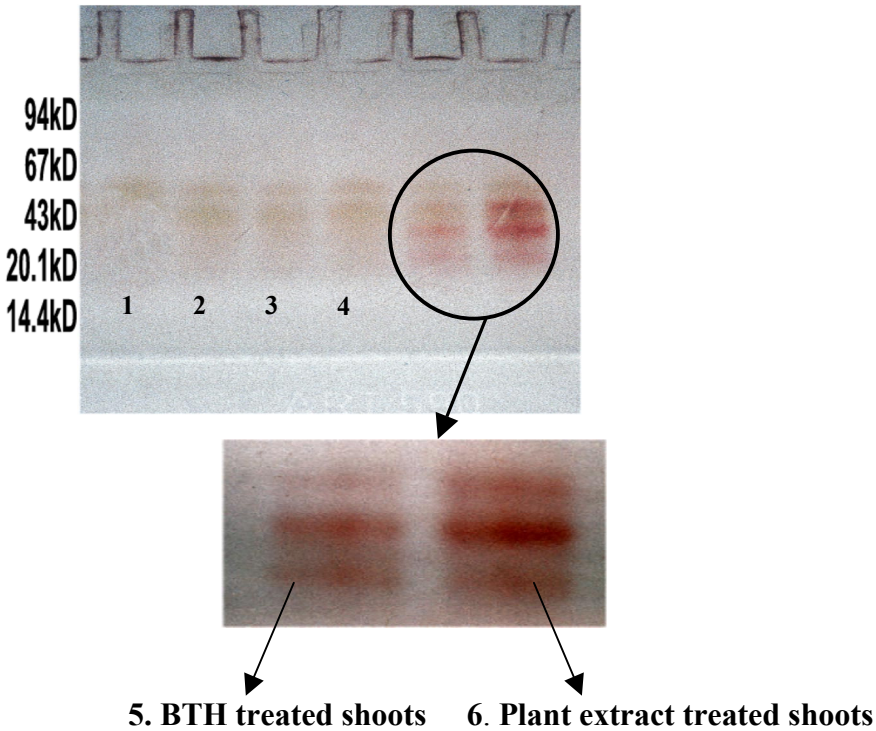


Fig. 15: Specific peroxidase activities in BTH or plant extract treated shoots 4 d after induction and inoculation (1 g plant material per sample).

1. Untreated shoots.

2. BTH treated non-inoculated shoots.

3. Plant extract treated non-inoculated shoots.

4. Control inoculated shoots.

5. BTH treated inoculated shoots.

6. Plant extract treated and inoculated shoots.

3.7.3 Protein bands detected by electrophoresis

For determination of bands corresponding to PO activity (see 3.7.2.1), total proteins were extracted and denaturised. Protein bands were recorded 4 d after treatment and inoculation. In the samples of BTH (B) and plant extract treated seedlings (A), a protein band of 42 kD appeared to increase (see Fig. 16). However, this protein band seemed to be stronger in the untreated inoculated plants. In the homogenate of BTH treated plants (C), additional protein bands at 43–20.1 kD were observed (Fig. 16) which were not present in the non-treated control. On the contrary, the treatment with BTH or plant extracts induced increase of specific protein bands similar to the pattern after artificial inoculation.

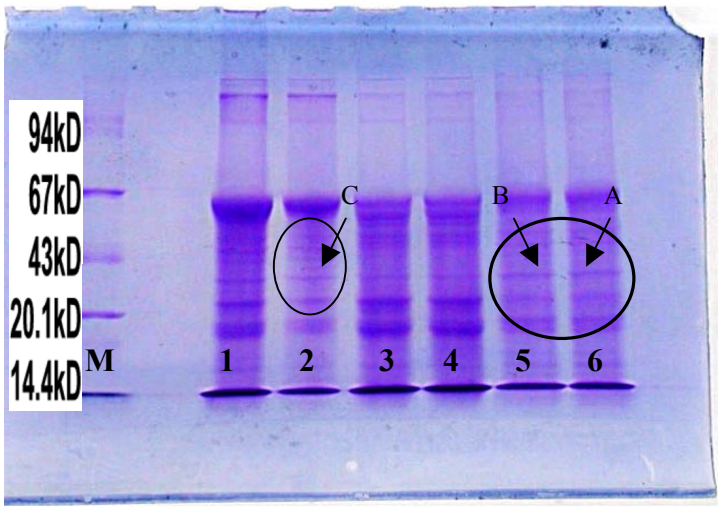


Fig. 16: Protein bands of untreated and treated shoots 4 d after treatment and inoculation (1 g plant material per sample).

1. Non-treated shoots.

2. BTH treated non-inoculated shoots.

3. Plant extract treated non-inoculated shoots.

4. Control inoculated shoots.

5. BTH treated inoculated shoots.

6. Plant extract treated and inoculated shoots.

M. Marker

3.7.4 Phenylalanine ammonia- lyase (PAL) activity

a) Non-inoculated shoots

After treatment of plants with BTH or plant extract, an increased PAL activity was observed. Generally, activation by plant extract was significantly higher than by BTH (Fig. 17a). The PAL activity increased to the highest level at 4 d after treatment with plant extract (93%), and with a delay 7 d after treatment with BTH. On the other hand, at the end of the experimental period (11 d.p.i.) both treatments showed the same enzyme level as untreated plants.

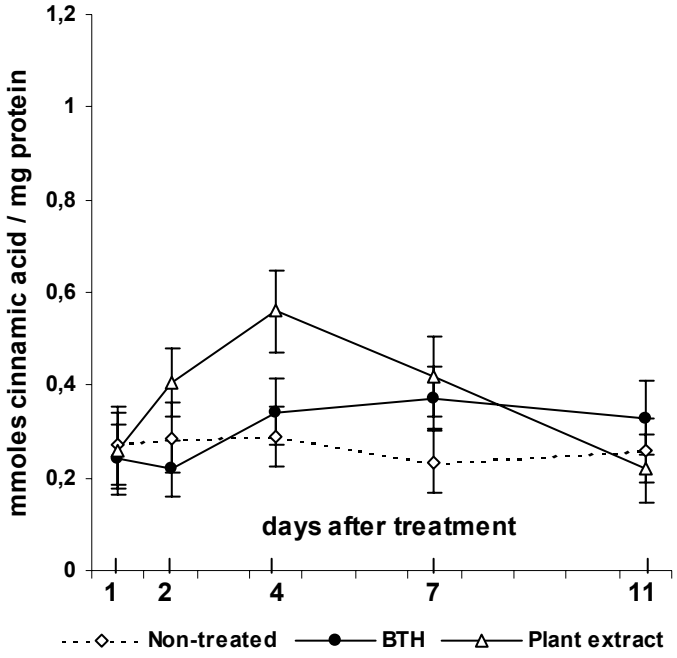


Fig. 17a: PAL activity in non-inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 plants per sample).

b) Inoculated shoots

Generally, a higher PAL activity was observed in all variants after inoculation. The activity in BTH treated seedlings was significantly lower than in plant extract treated ones, but nearly comparable with that of the control (Fig. 17b). On the other hand, the plant extract treated seedlings showed a high activity nearly over the whole experimental period with a peak at 4 d p.i. BTH treatment enhanced PAL activity only weakly with a delay compared to the control.

At the end of the experimental period, all variants showed a decreasing PAL activity with comparably low activity.

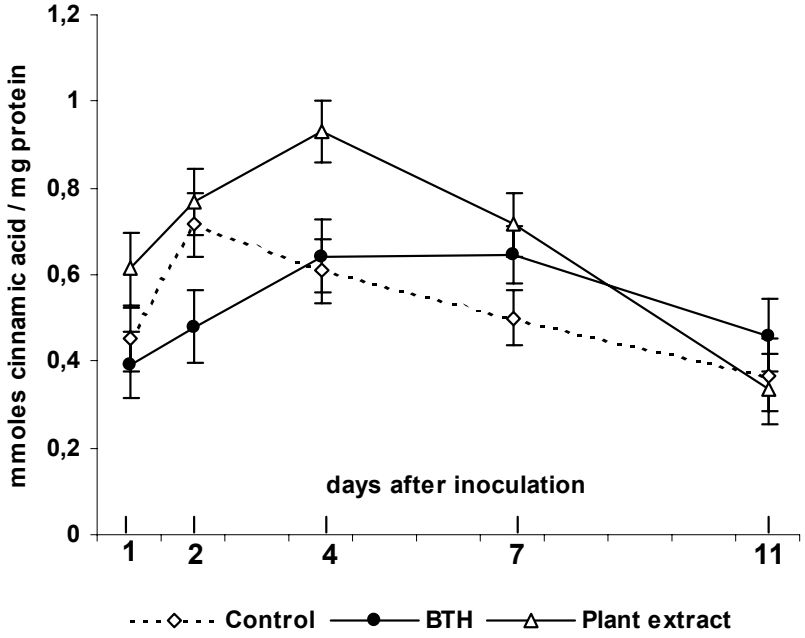


Fig. 17b: PAL activity in inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurement in 4 samples (2 shoots per sample).

3.7.5 Polyphenoloxidase (PPO) activity

a) Non-inoculated shoots

The PPO activity was higher in seedlings treated by BTH or plant extract, compared to the non-treated control over the whole experimental period (Fig. 18a), especially after treatment with plant extract 7 d p.i. (72% increase).

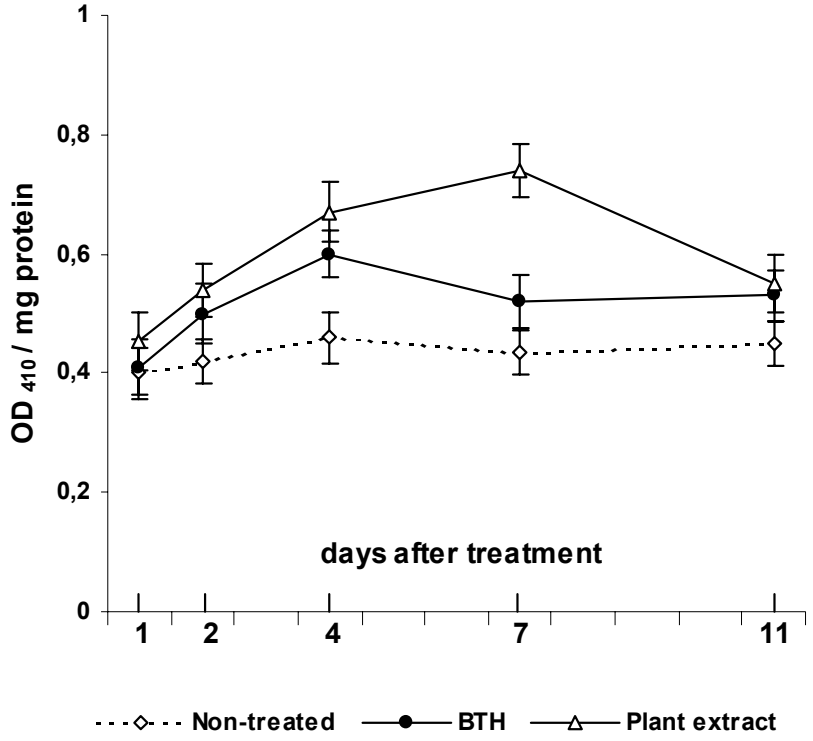


Fig. 18a: PPO activity in non-inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

b) Inoculated shoots

Inoculated shoots showed a gradual increase of PPO activity in the later stages of infection (7 and 11 d p.i.) (Fig. 18b). In contrast, the treatments caused a faster increase of PPO-activity from 2 - 4 d p.i., especially after treatment with plant extract. Later on (11 d p.i.) PPO-activity decreased below the level of inoculated plants without treatments (Fig. 18b).

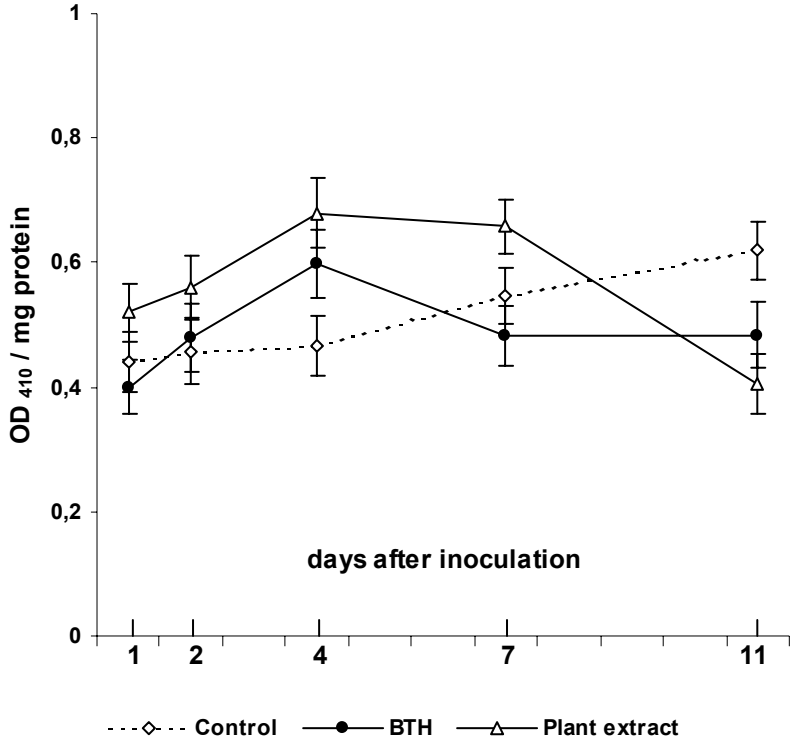


Fig. 18b: PPO activity in inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

3.7.6 Flavonoids determined by thin layer chromatography (TLC)

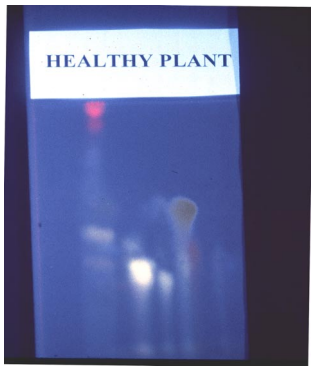
An enhanced total phenol content and increased enzymatic activities (PO, PPO, PAL etc.) were observed in BTH or plant extract treated seedlings, with or without inoculation. Synthesis of phenolic acids and flavonoids is often related to the synthesis of secondary metabolites, and inhibitory substances against several plant pathogens after application of plant protection agents (TOREL *et al*., 1986; MATERN and KNEUSEL, 1988; MAYR *et al*., 1995). The quantitative changes of the flavonoids quercetin, kampferol, procyanidin, and their isoforms, were followed in course of the experiment and the reduction of the disease was often correlated with increases in the flavonoids (FEUCHT and TREUTTER, 1989; MAYR, 1995).

Regarding the flavonoid changes, reduction in monomer flavonoids and increase in oligomer flavonoids 6 days after application of the plant extract from *H. helix* has already been demonstrated before (MOSCH and ZELLER, 1997). Therefore, flavonoid changes in plants were also examined in these experiments. Due to increased enzymatic activities and phenol content 4 d after treatment and inoculation, this time was selected to determine changes in the content of flavonoids, which could be characterized only qualitatively. Flavonoids were determined on basis of hrf values and the colour of substance spots (NEU, 1957; ZELLER, 1985; SCHULZ, 1987; HEIMLER and BODDI, 1989). However, a few flavonoids showed the same colours at different hrf values. Therefore, these flavonoids were assumed to be isoforms.

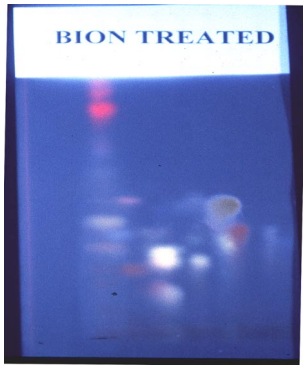
TLC analysis revealed a higher flavonoid content in BTH treated seedlings than in non-treated ones (Fig. 19.2). In the plant extract treated seedlings the flavonoid pattern appeared to be changed (Fig. 19.6). However, the different pattern may have been caused by the fact that all substances travelled higher during TLC in this variant. After inoculation, the BTH treated seedlings (Fig. 19.5) showed a similar flavonoid content as non-treated ones (Fig. 19.4), and a lower content in plant extract treated seedlings (Fig. 19.6).

According to hrf values and the colour of the flavonoids outlined in Table 5 several substances were assumed. Generally, it was observed that in BTH treated seedlings the kampferol isoforms increased more than in plant extract treated and non-treated seedlings. Changes in colours of flavonoids could only be observed after treatments with the plant extract, which were assumed to be flavonoid oligomers. These may play a role as inhibitory compounds against *E. amylovora* after inoculation.

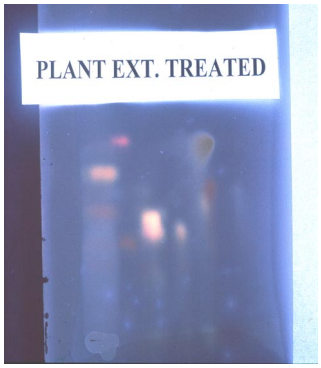
Non-inoculated shoots



1.



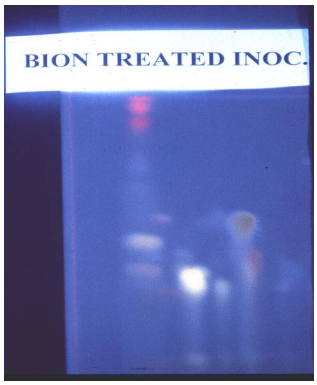
2.



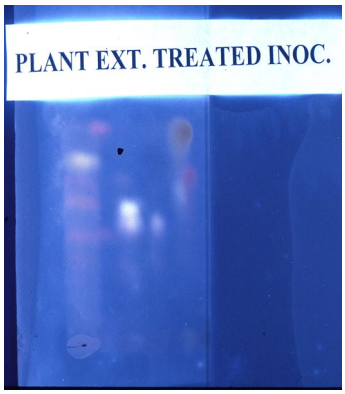
3.

Inoculated shoots

4.



5.



6.

Fig. 19: The flavonoid content in seedlings, and examination of these compounds on TLC.

1. Non-treated shoots.
2. BTH treated non-inoculated shoots.
3. Plant extract treated non-inoculated shoots.
4. Control inoculated shoots.
5. BTH treated and inoculated shoots.
6. Plant extract treated and inoculated shoots.

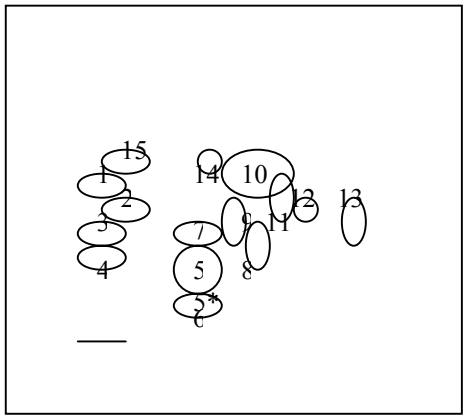


Fig. 20: The place of assumed substances on the TLC plate.

Nr.	**Substance**	**Hrf values** I II	**Colour**
1,3,4	Quercetin isoforms	0.37- 052 and different places	Light green
10	Quercetin	0.34- 0.57	Light-yellow green
5	Kampferol	0.23- 0.49	Light-yellow green
14,6,7,8,9	Kampferol isoforms	0.41- 0.52 and different places	White-blue
12,11	Phloridzin-Phloretin	0.51- 0.47	Dirty- yellow
2	Chlorogenic acid	0.19- 0.21	White -blue
5*	Myricetin	0.51- 0.23	Orange
13	Iso- chlorogenic acid	0.61- 0.70	White-blue

Table 5: Assumed flavonoids and phenolic compounds according to hrf values and colours of spots.

3.7.7 Inhibitory substances in BTH and plant extract treated shoots

After the examination of flavanoid changes in TLC plates several flavanoids were assumed to play a role as inhibitory substances against *Erwinia amylovora*. After one-dimensional TLC spraying the plate with a suspension of *E. amylovora* and aesculin revealed the presence of an inhibitory substance in BTH treated seedlings 4 d after inoculation (Fig. 21, B). Identification of this substance involved scraping of the corresponding spot from the TLC plate, extraction with methanol and analysis. Due to the low amount obtained, the substance could not be characterized completely. Contrary to this result, in plant extract treated seedlings no inhibitory substance was found.

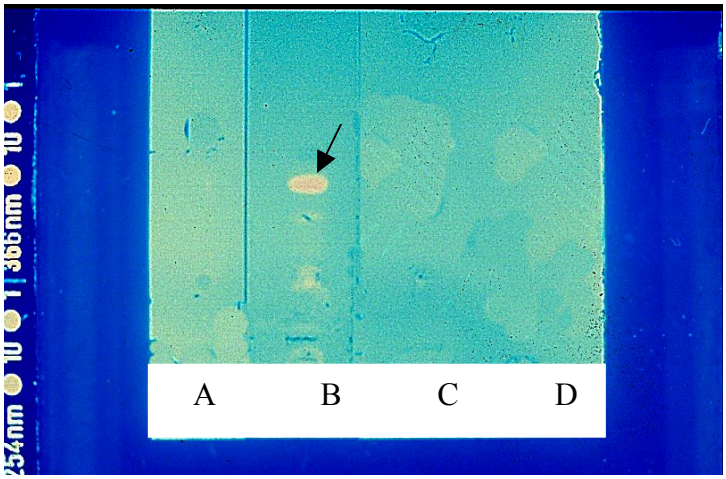


Fig. 21: Inhibitory substance in BTH treated and inoculated shoots 4 d after inoculation **A.** BTH treated uninoculated shoots (4 d after treatment). **B.** BTH treated inoculated shoots (4 d after inoculation). **C.** Untreated inoculated shoots **D.** Untreated non-inoculated shoots.

3.7.7.1 Inhibitory effect of the substance in liquid culture and comparison to phenolic acids

The inhibitory substance from the TLC plate could not be chemically identified. However, a phenolic structure appeared to be likely. Several substances were tested on their effect on *Erwinia amylovora in vitro* in comparison to the substance detected. Cultures containing phloretin, or the tested substance showed the strongest reduction of bacterial growth. The growth reduction at 24 h p.i. was determined with 46 and 35%, respectively (Fig. 22). Phloretin is known as an inhibitory substance in several host parasite interactions (GOODMAN *et al.*, 1986). This could also be assumed for the here detected substance after TLC.

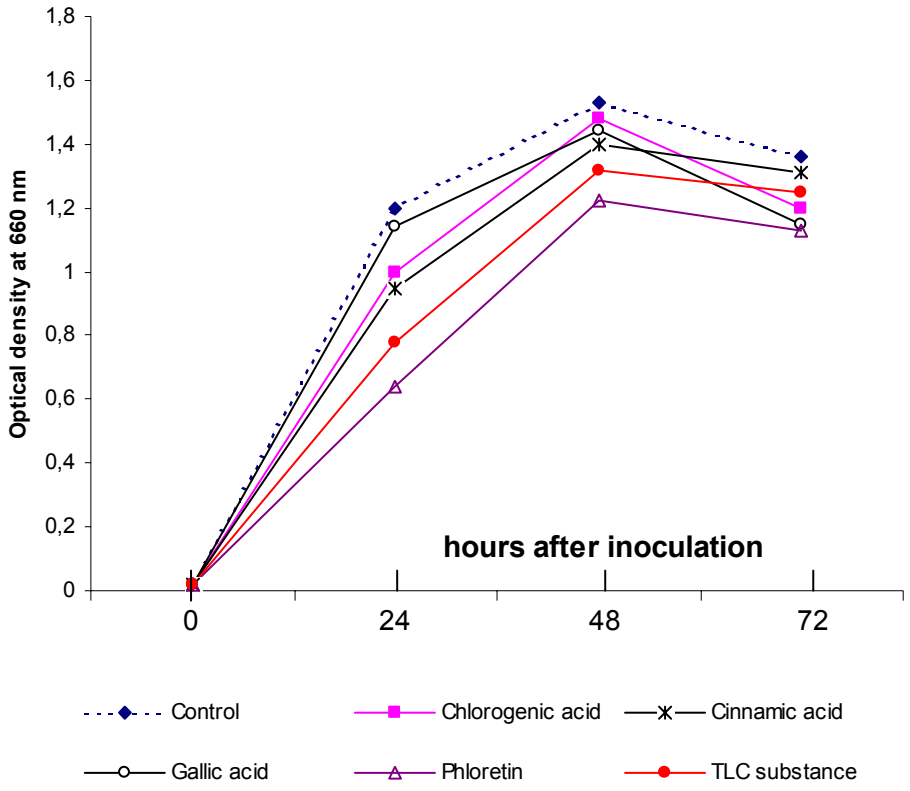


Fig. 22: The effect of some phenolic compounds and the inhibitory substance from TLC on the growth of *Erwinia amylovora* in liquid culture.

3.7.8 High performance liquid chromatography (HPLC) analyses

As described above (Fig. 22) some phenolic compounds reduced the growth of *E. amylovora* in liquid culture. Therefore, changes in concentration of phenolic acids were followed in both, BTH treated and plant extract treated shoots, by HPLC analysis. Enzymatic activities had shown a significant difference in BTH and plant extract treated seedlings compared to untreated ones 4 d after treatment. Therefore, this date was selected for analysis by HPLC. When inoculated seedlings were analysed, the samples were taken 6 d after treatment and 4 d after inoculation.

The HPLC-analyses revealed some changes in the content of flavonoids, flavonols, phenolic acids and phloretin derivates in inoculated seedlings when treated with BTH or plant extract (Figs. 23-34). All the data, obtained on the different compounds in all experimental variants are presented in Table 5 for all treatments. In those cases were the treatment with BTH or PE

caused an increase in phenolic acids, flavonoids, phloretin derivates or other components the data obtained have been marked by an asterix.

The **flavonoids** in general (total content) increased stronger after BTH-treatment than after PE-treatment in inoculated plants. Especially the not yet identified peak (P) 8 showed as strong increase after BTH-treatment, but not after PE-treatment. On the other hand, B5 procyanidin and E-B5 increased stronger after PE-treatment.

The total content of **phenolic acids** increased (doubled) at similar rate in both treatments. These results correlated well with the spectrophotometrical measurements of the phenol content (see 3.7.1). A few phenolic compounds, such as caffeic acid, p-cumaric-glucoside and p-cumaric acid D increased a little stronger after PE-treatment (Table 6). **Phloretin derivates** did not change very much. If at all, they increased after BTH-treatment. Interestingly, infection alone caused a decrease of phloretin derivates.

FLAVONOIDS		**mg/ml**	Buckel	Catechin	B2 Procyanidin	Epicatechin	B5 Procyanidin	Peak 6	E-B5	Peak 8	Peak 9	**Total Flavonoids**			Flavonol	Flavonol	Flavonol	Flavonol	Flavonol	Flavonol		Total		**Total phenolic acids**	**Total phloretin derivatives**
	1	**Untreated inoculated plant**	0.04	0.01	0.03	0.06	0.05	0.04	0.04	0.08	0.03	0.38			0.04	0.17	0.05	0.70	0.17	0.17		**1.3**			
	2	**BTH treated and inoculated plant**	0.02	0.01	0.00	0.07	**0.13***	0.02	0.07	**1.20***	0.09	**1.61***			0.10	0.09	0.07	0.67	0.04	0.18		**1.15**			
	3	**PE treated and inoculated plant**	0.02	0.00	0.00	0.09	**0.26***	0.06	**0.26***	0.09	0.01	**0.79***			0.21	0.13	0.03	1.04	0.12	0.19		**1.72**			
	4	**Untreated and non-inoculated plant**	0.09	0.03	0.01	0.07	0.38	0.06	0.26*	0.09	0.25	1.24			0.30	0.50	0.00	1.22	0.14	0.17		**2.33**			
	5	**BTH treated and non-inoculated plant**	0.09	0.09	0.06	0.14	0.30	0.05	0.34	0.05	0.35	1.47			0.67	0.52	0.05	1.18	0.19	0.13		**2.74**			
	6	**PE treated and non-inoculated plants**	0.04	0.04	0.01	0.06	**0.64***	0.08	0.36	0.05	0.05	1.33			0.49	0.42	0.07	0.72	0.11	0.00		**1.81**			
PHENOLIC ACIDS			P1	P2	P3	P 5	P6	P7	P8	P9	p-Cum-glu	P11	P12	P13	P14	Caffeic acid	Caffeic-D	P17	pCum-D	P19	pCuma	P21		**Total phenolic acids**	
	1	**Untreated inoculated plant**	0.50	0.60	0.20	0.10	0.10	0.20	0.20	0.70	1.40	0.50	0.20	0.10	0.30	2.70	0.30	0.40	0.40	0.10	1.30	0.10		**10.3**	
	2	**BTH treated and inoculated plant**	0.80	1.10	0.20	0.30	0.10	0.40	0.70	0.50	**3.20***	0.20	0.20	0.10	0.50	**4.20***	**1.30***	0.40	**1.20***	0.00	**4.20***	0.00		**19.4***	
	3	**PE treated and inoculated plant**	0.50	0.90	0.50	0.60	0.10	0.30	0.60	0.40	**3.80***	0.40	0.20	0.20	0.30	**3.40***	**0.90***	0.80	0.30	0.30	**3.50***	0.20		**18.2***	
	4	**Untreated and non-inoculated plant**	0.40	0.40	0.40	0.40	0.20	0.40	0.50	0.50	3.20	0.20	0.40	0.20	0.40	2.00	1.60*	0.70	1.30	0.20	2.00*	0.40		**15.8**	
	5	**BTH treated and non inoculated plant**	0.60	0.60	0.70	0.30	0.30	0.60	0.30	0.20	**4.10***	0.40	0.30	0.20	0.60	**3.00***	0.60	0.80	**3.50***	0.20	1.50	0.20		**18.3***	
	6	**PE treated and non-inoculated plants**	0.70	0.50	0.60	0.50	0.40	0.60	0.90	0.60	3.70	0.30	0.20	0.40	0.50	**4.80***	0.90	0.80	**2.80***	0.60	2.50	0.10		**22.5***	
PHLORETIN DERIVATIVES			P22	P23	P24	Phloretin Derivates	P26	P27	Phlorizin	P29	P30	P33	Phloretin-D	Phloretin-D	Phloretin	P44	P45	P46	P47	p-Cumaryl-phlo	p-Cumaryl-phlo	P50	P51	P52	**Total phloretin derivatives**
	1	**Untreated inoculated plant**	0.02	0.01	0.01	0.07	0.01	0.01	0.09.	0.02	0.00	0.02	0.08	0.00	0.05	0.00	0.00	0.02	0.06	0.04	0.05	0.02	0.01	0.00	**0.59**
	2	**BTH treated and inoculated plant**	0.02	0.00	0.00	0.09	0.00	0.01	0.10	0.07	0.02	0.06	0.09	**0.05***	**0.20***	0.02	0.02	0.00	0.01	**0.10***	**0.10***	0.00	0.00	0.00	**0.96***
	3	**PE treated and inoculated plant**	0.01	0.00	0.00	0.05	0.01	0.02	0.08	0.06	0.01	0.04	0.10	0.01	**0.10***	0.05	0.00	0.02	0.02	0.05	0.02	0.00	0.00	0.00	**0.65***
	4	**Untreated and non-inoculated plant**	0.01	0.00	0.00	0.07	0.01	0.01	0.10	0.08	0.00	0.01	0.05	0.01	0.10	0.04	0.03	0.05	0.05	0.05	0.09	0.00	0.01	0.01	**0.78**
	5	**BTH treated and non inoculated plant**	0.01	0.01	0.00	0.10	0.05	0.00	0.10	0.05	0.00	0.01	**0.10***	0.00	**0.20***	0.00	0.00	0.01	0.00	**0.09***	0.07	0.00	0.00	0.00	**0.80**
	6	**PE treated and non-inoculated plants**	0.01	0.01	0.01	0.05	0.00	0.05	0.05	0.04	0.01	0.03	0.07	0.00	0.08	0.01	0.04	0.04	0.04	0.06	0.04	0.00	0.03	0.01	**0.68**

Table 6: Flavonoids, phenolic acids and phloretin derivates in shoots.

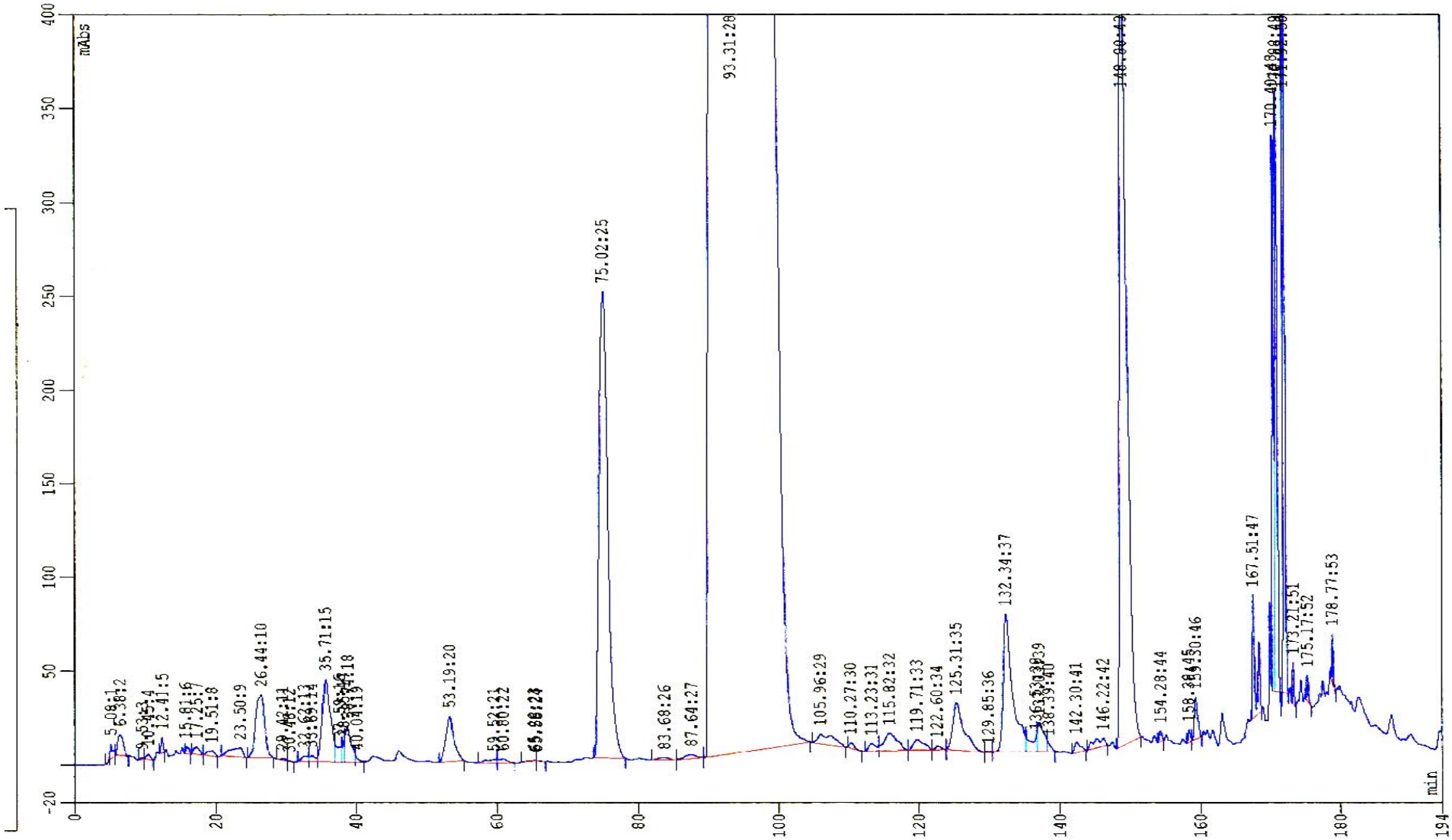


Fig. 23: Phenolic acids in non-treated inoculated plants.

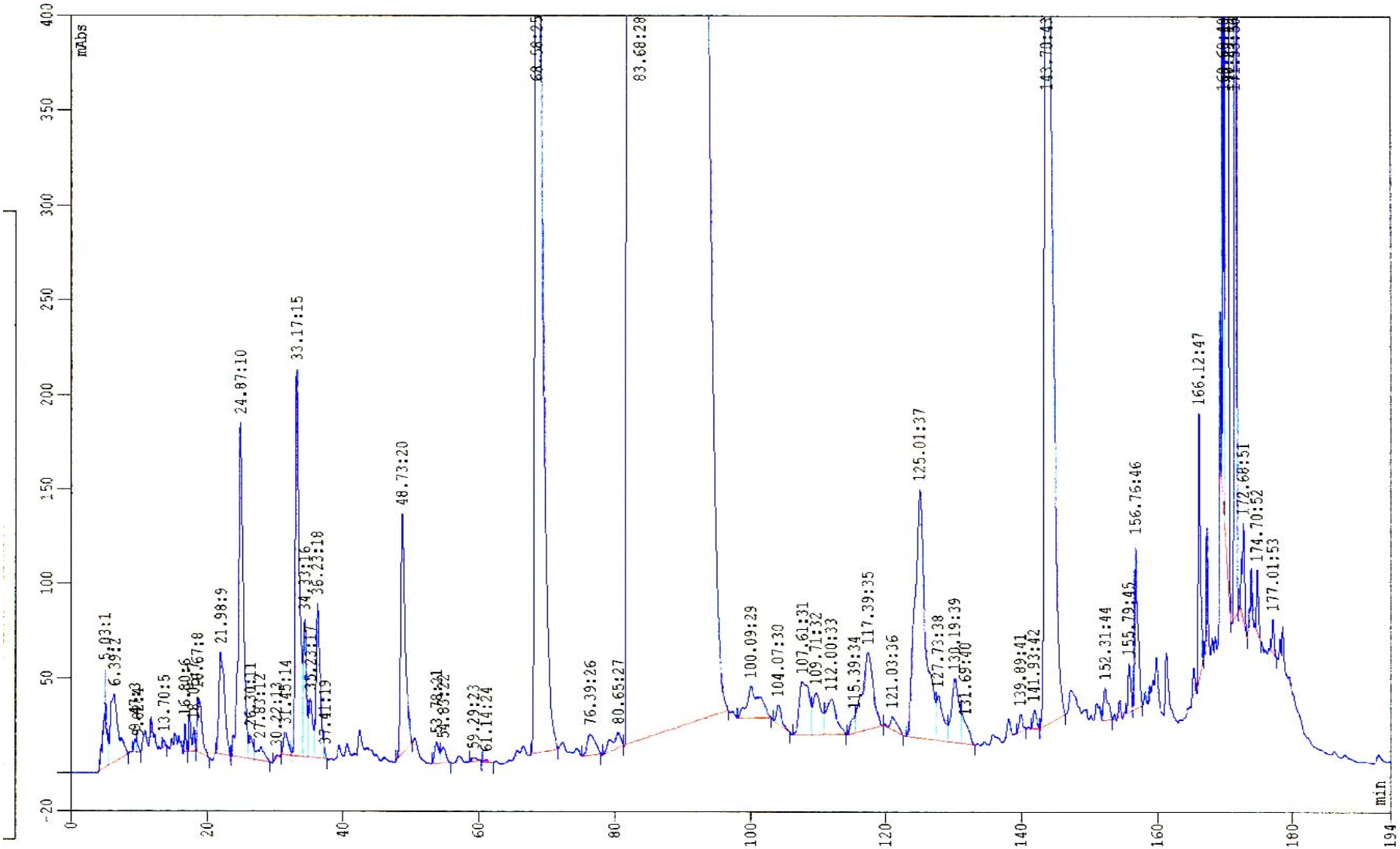


Fig. 24: Phenolic acids in BTH treated and inoculated plants.

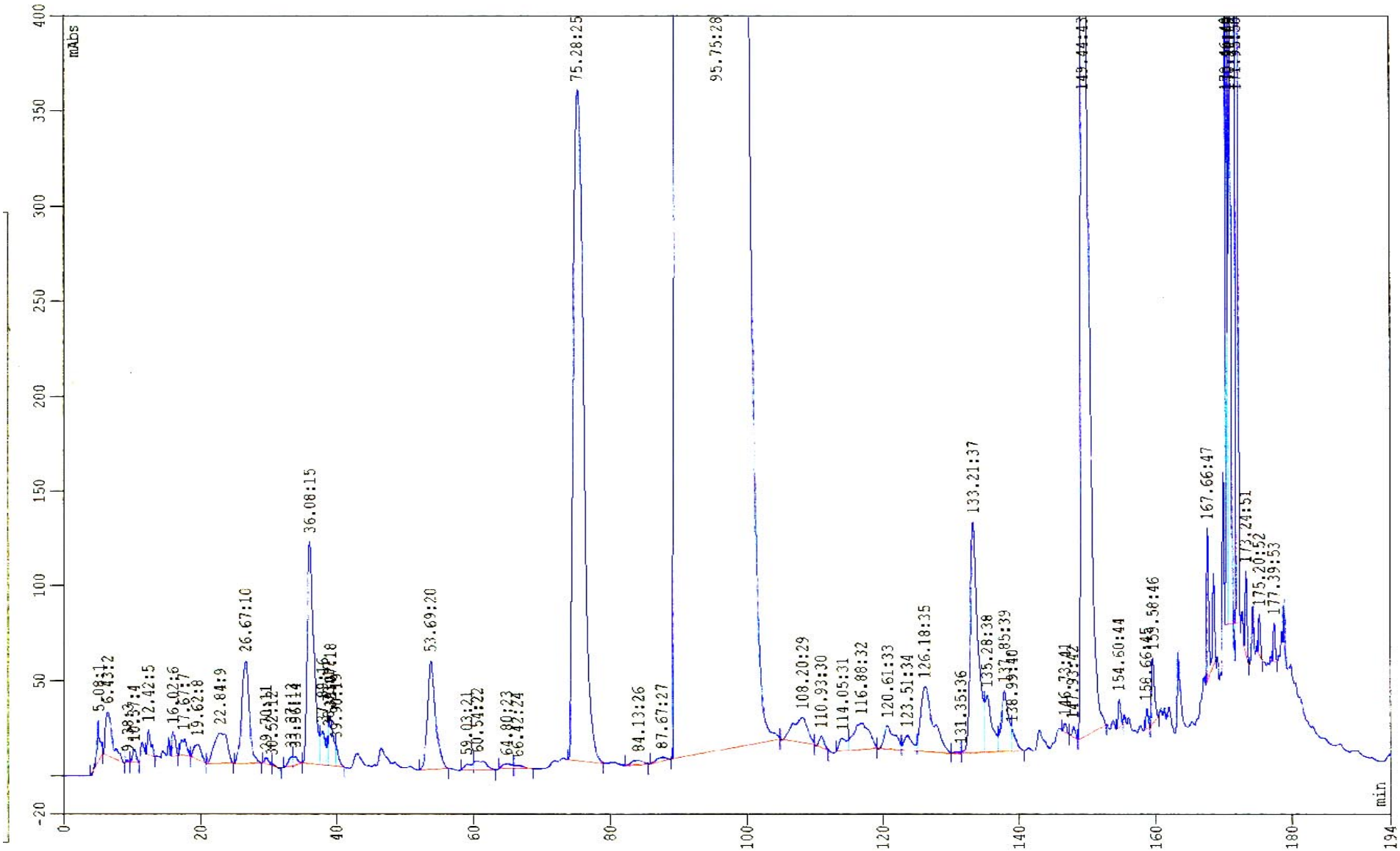


Fig. 25: Phenolic acids in PE treated and inoculated plants.

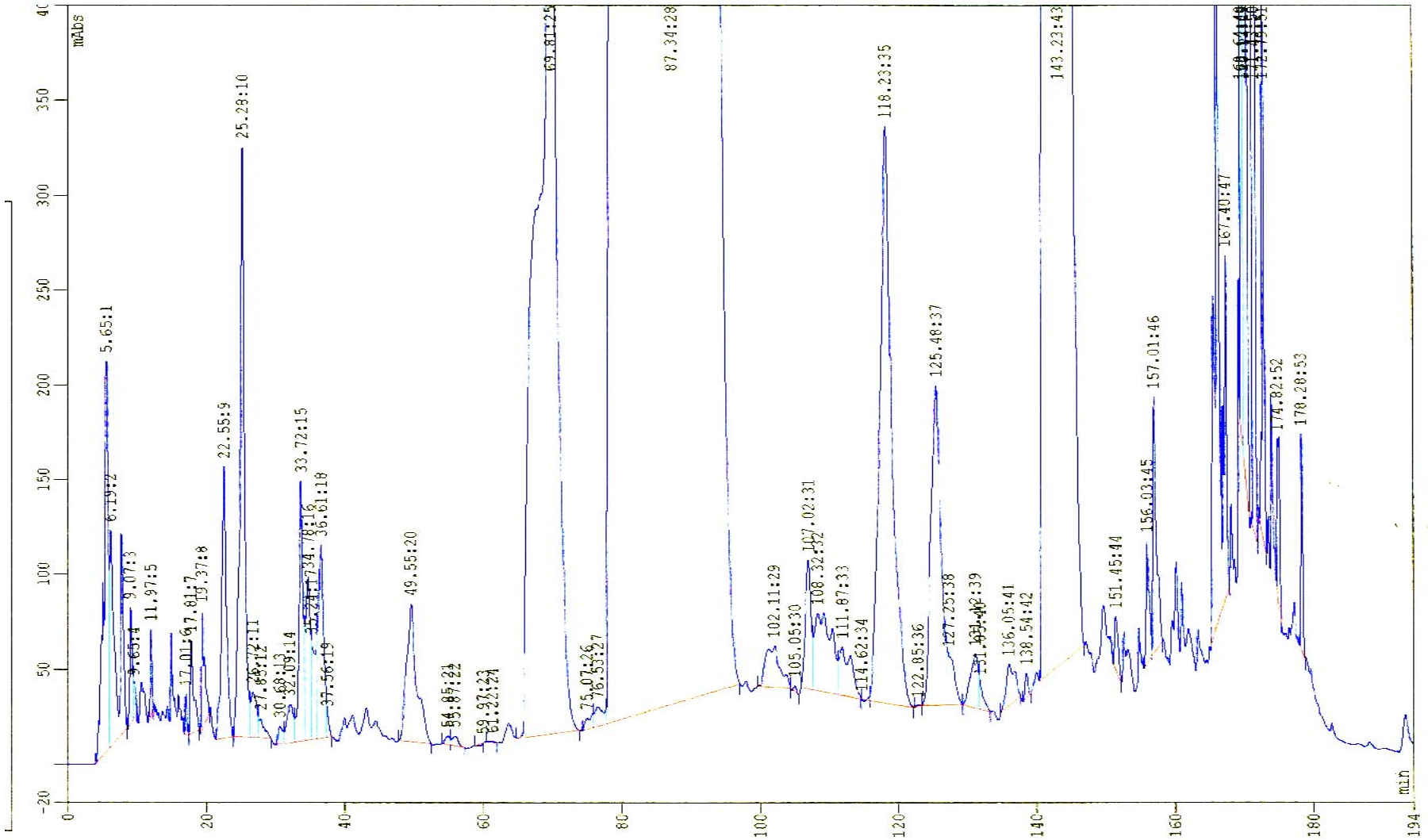


Fig. 26: Phenolic acids in non-treated and non-inoculated plants.

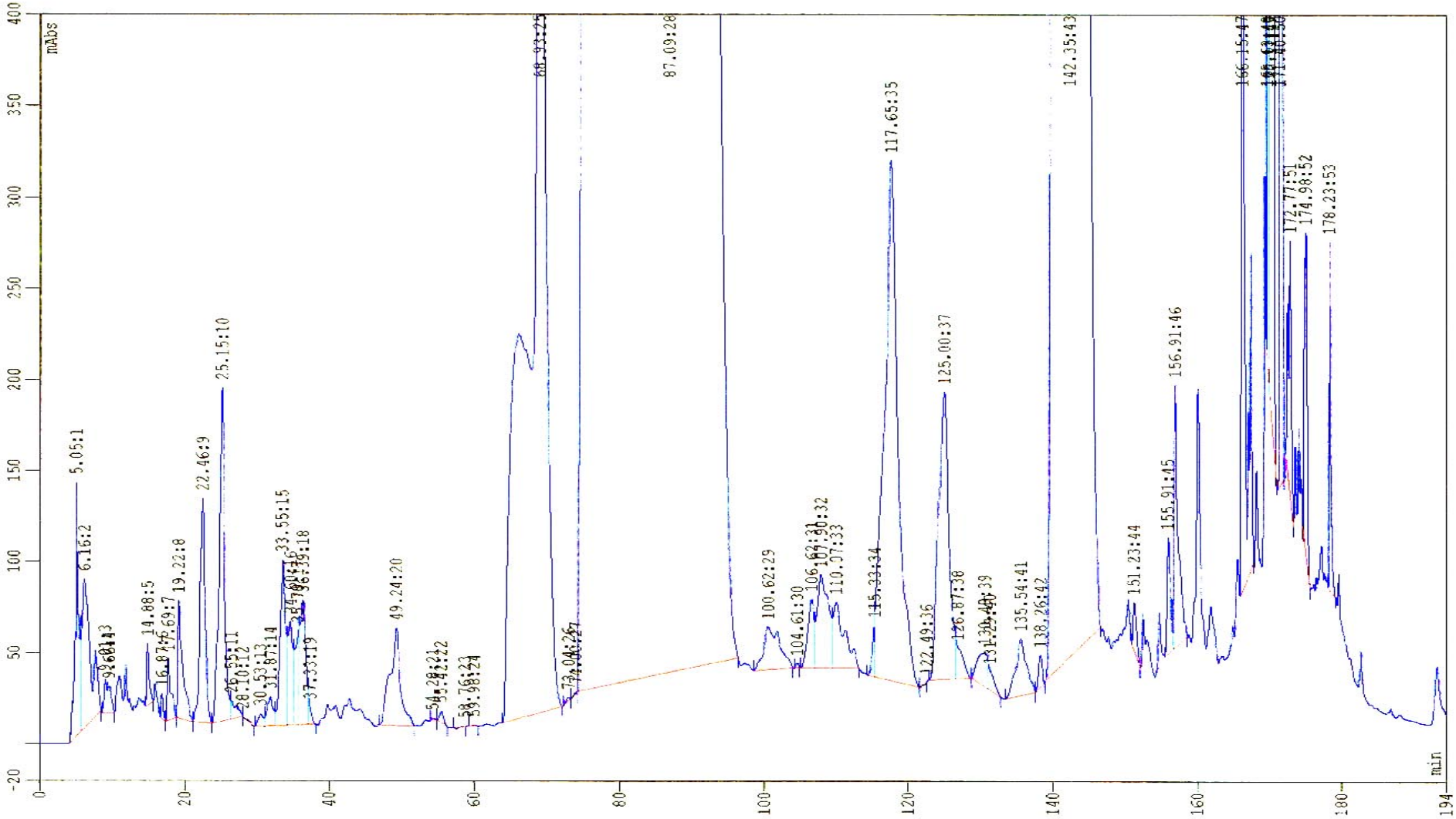


Fig. 27: Phenolic acids in BTH-treated and non-inoculated plants.

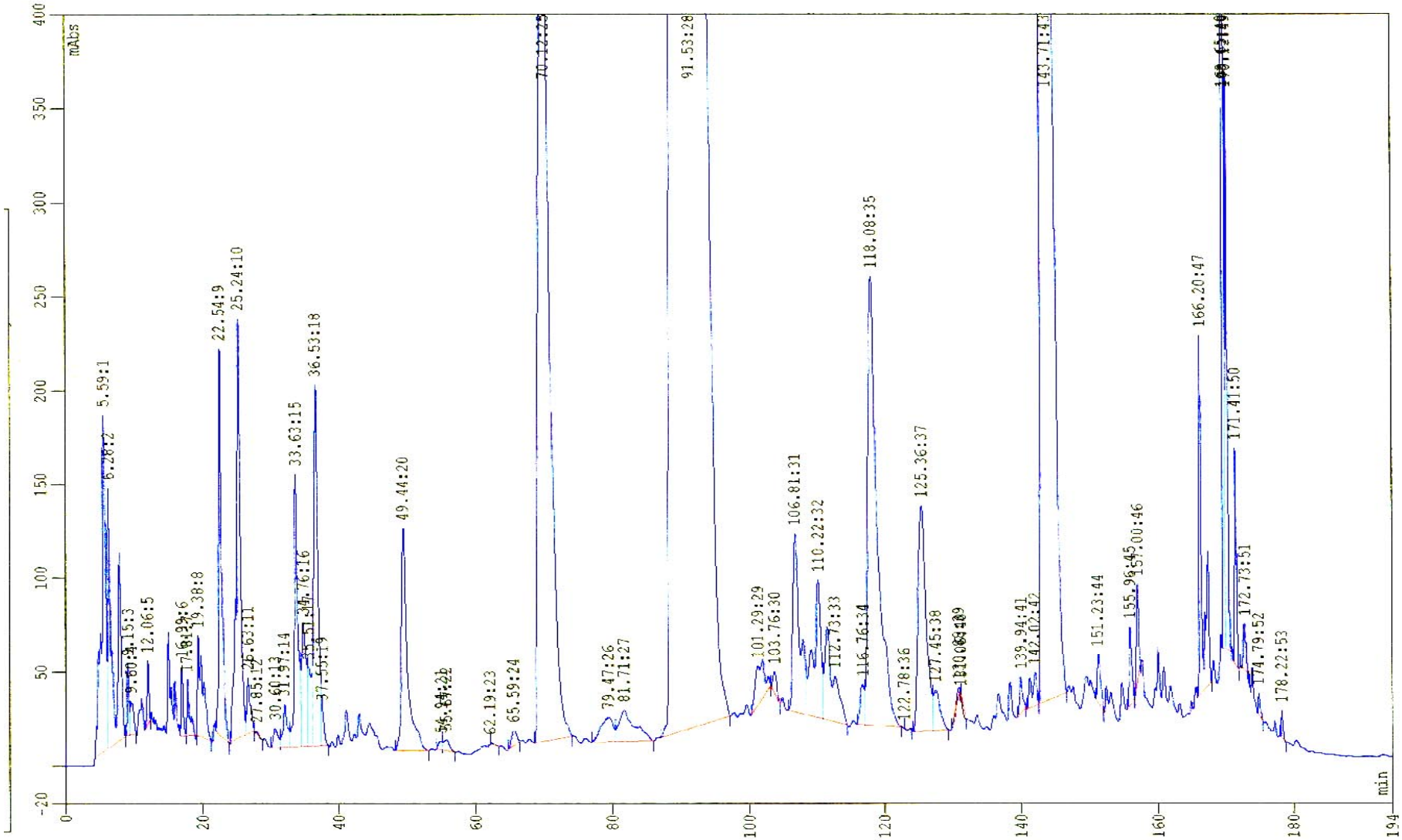


Fig. 28: Phenolic acids in PE treated and non-inoculated plants.

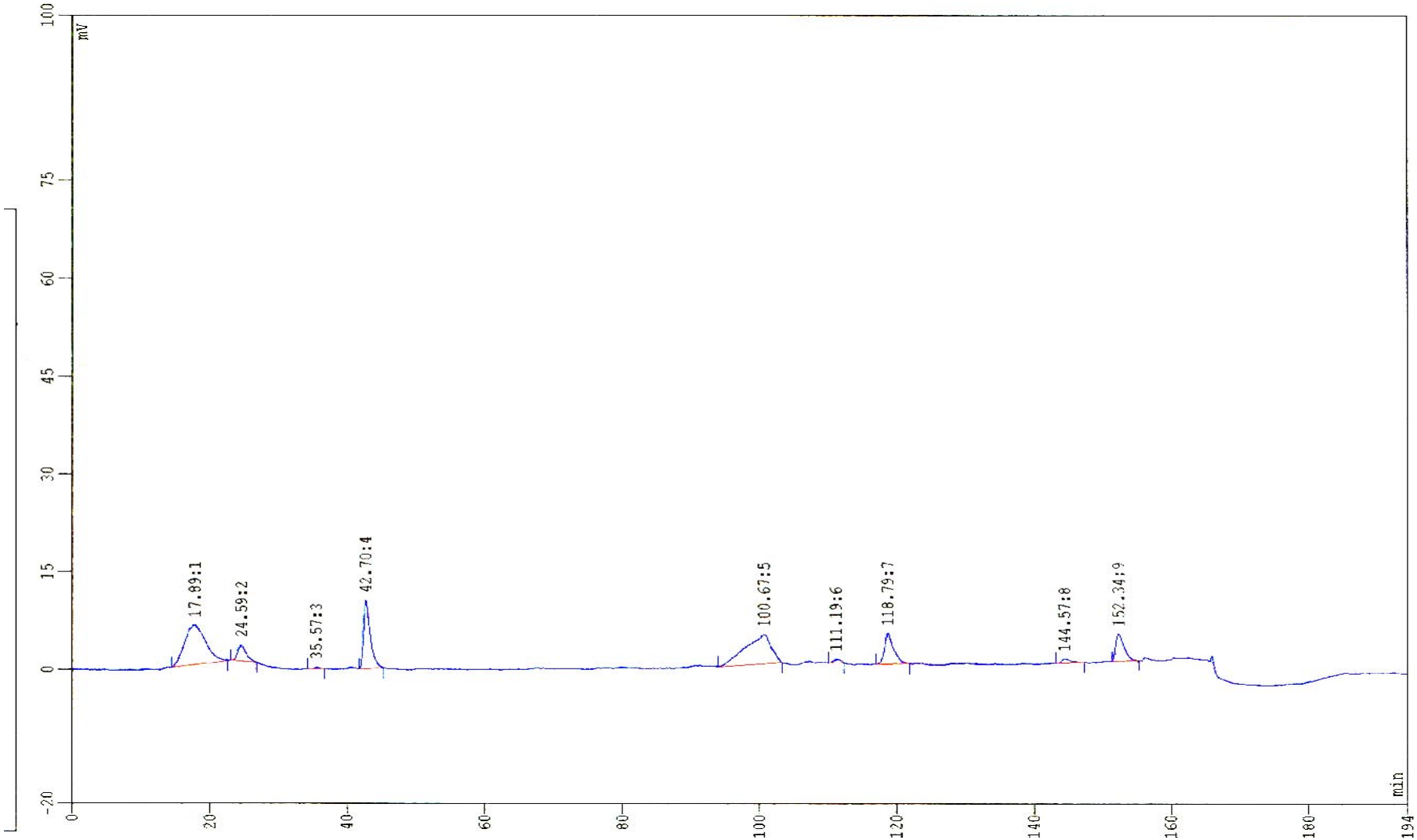


Fig. 29: Flavonoids in non-treated inoculated plants.

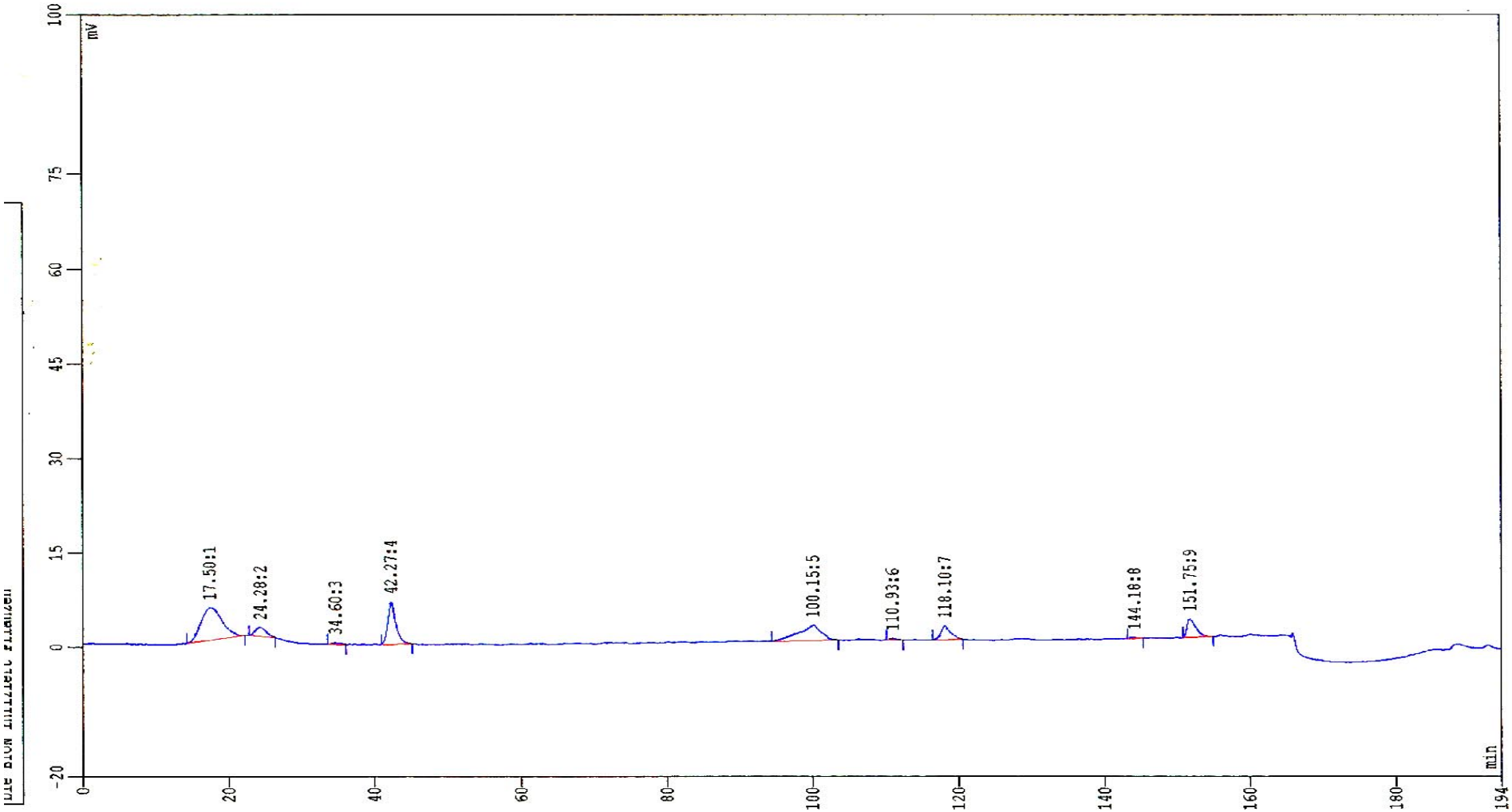


Fig. 30: Flavonoids in BTH treated and inoculated plants.

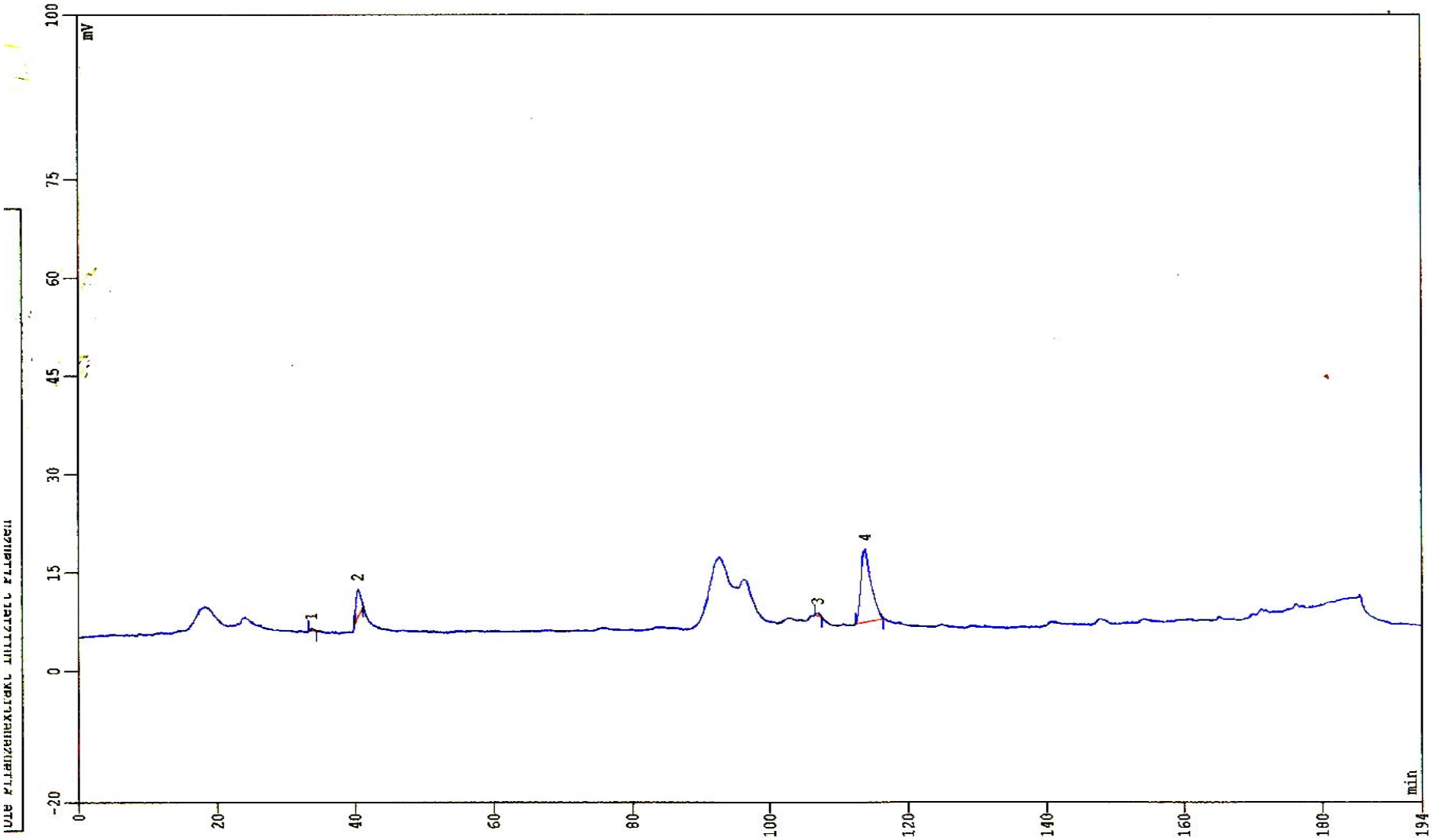


Fig. 31: Flavonoids in PE treated and inoculated plants.

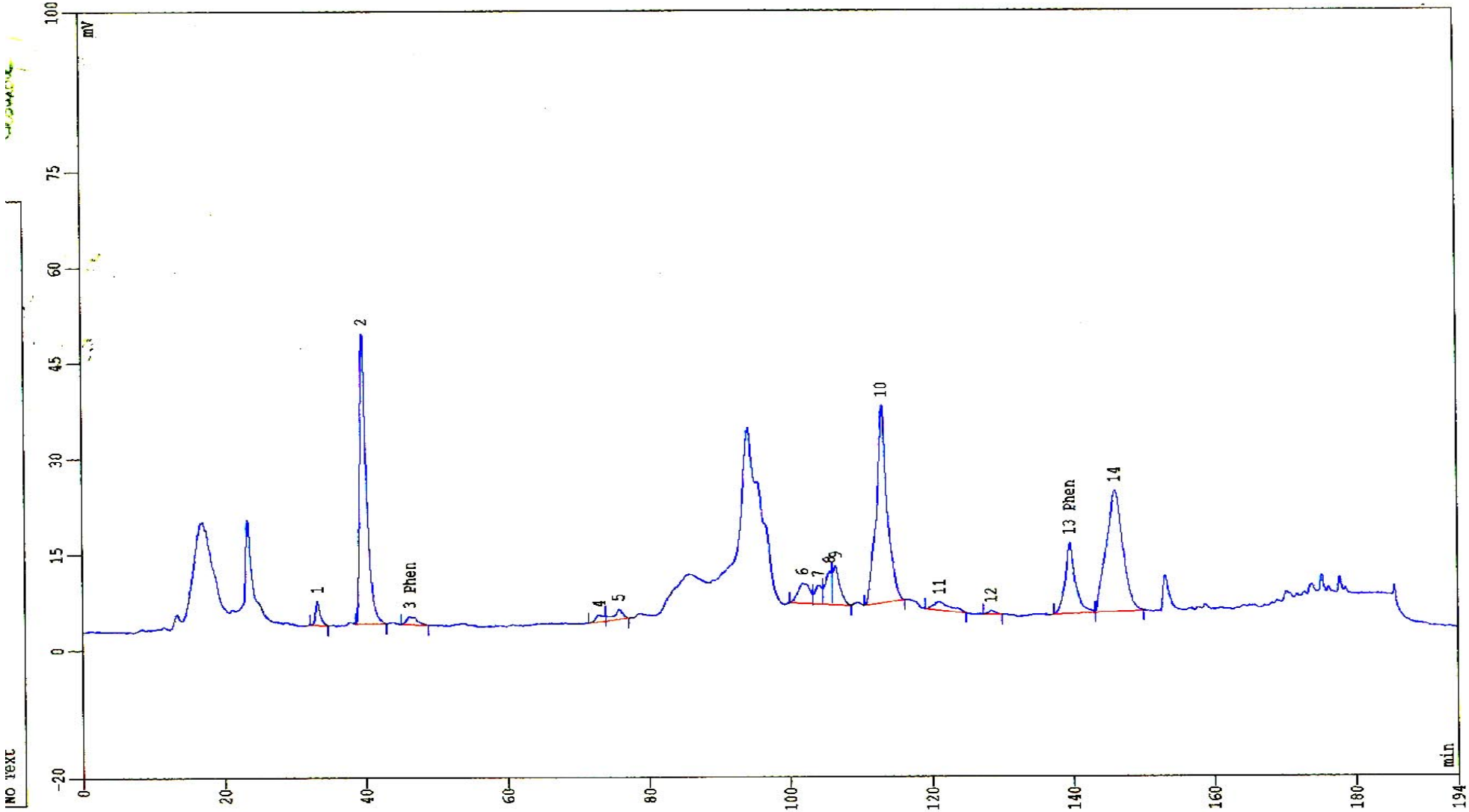


Fig. 32: Flavonoids in non-treated and non-inoculated plants.

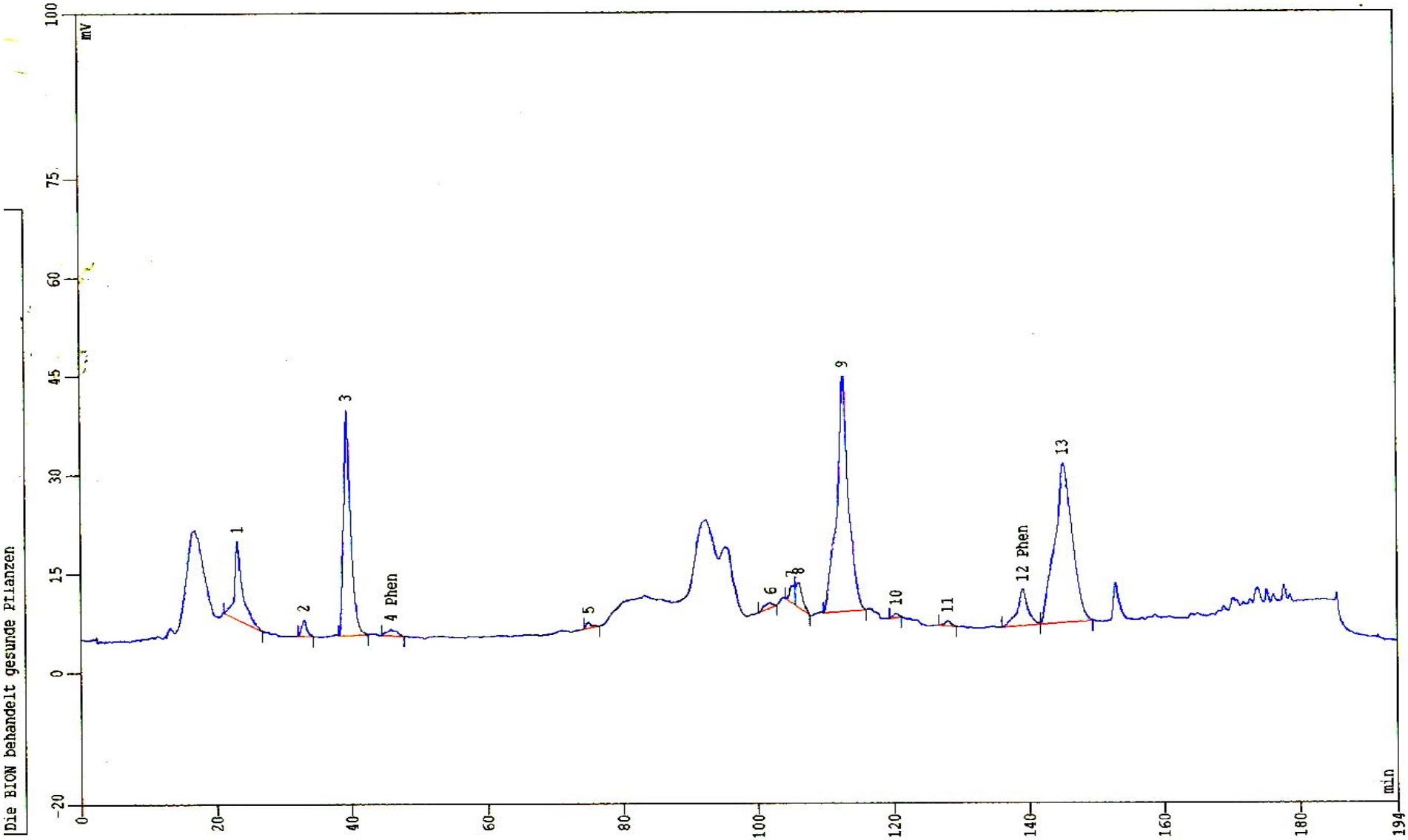


Fig. 33: Flavonoids in BTH-treated and non-inoculated plants.

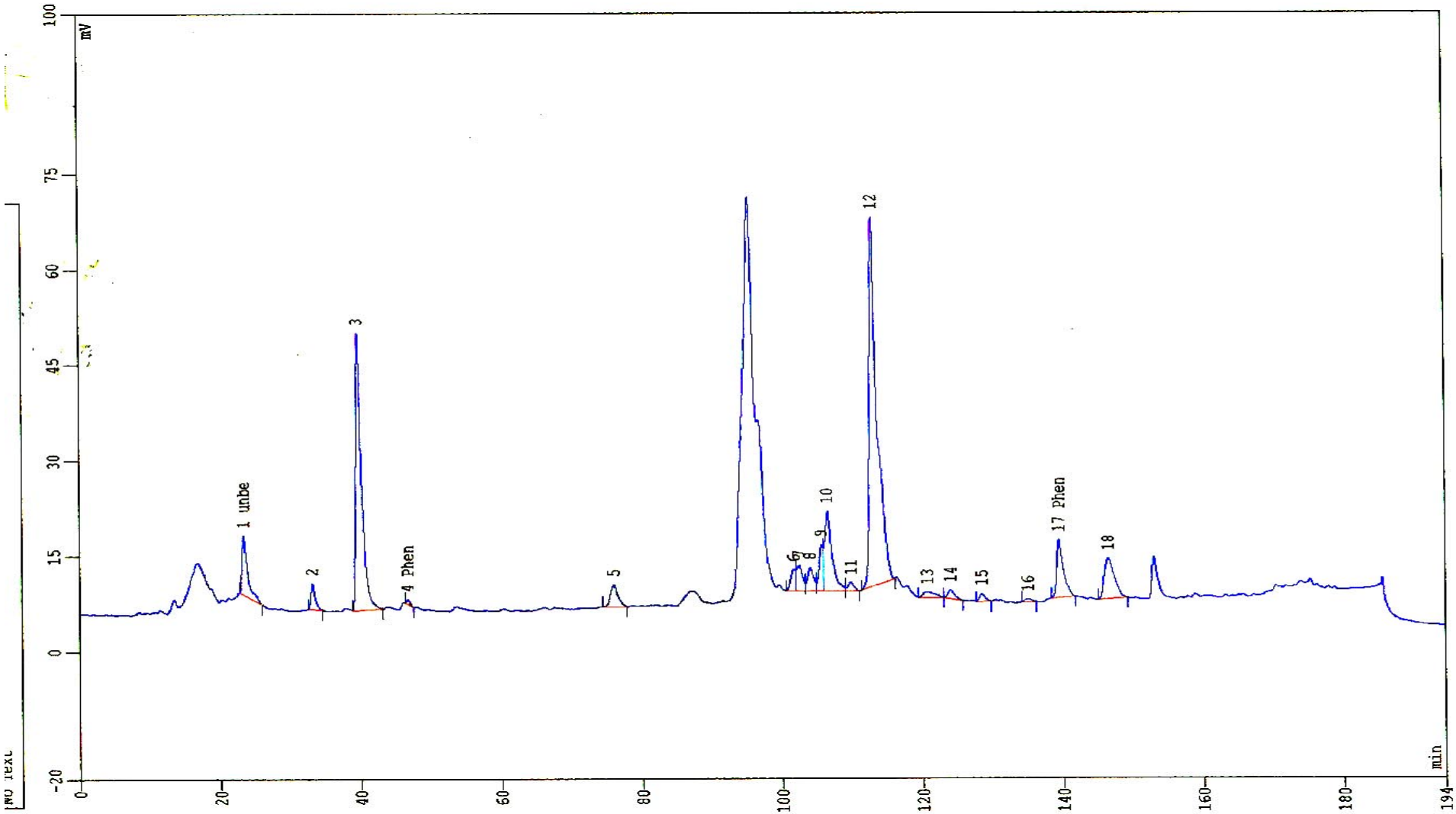


Fig. 34: Flavonoids in PE-treated and non-inoculated plants.

3.7.9 β-Glucosidase activity

a) Non-inoculated shoots

The β-Gl activity increased markedly 4 d p.i. in BTH and plant extract treated shoots up to 110 and 150%, respectively. Thereafter, the activity decreased (Fig. 35a) and reached similar values as the control 11 days after inoculation. The plant extract treated shoots showed a significantly higher activity than BTH treated shoots in the early phase between 1 and 4 d p.i..

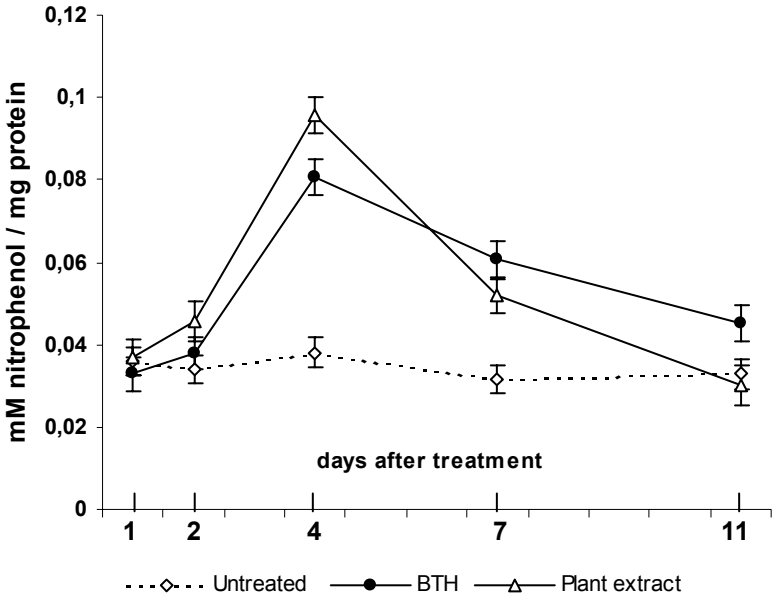


Fig. 35a: β-Glucosidase activity in non-inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviation of measurements in 4 samples (2 shoots per sample).

b) Inoculated shoots

In inoculated plants, the β-Gl activity was similar to that of untreated plants (Fig. 35b). Treatment with BTH or plant extract caused nearly the same changes as in non-inoculated shoots. The only difference was that the first significant increase in β-Gl activity occurred 2 d p.i. in BTH (43% of control) and plant extract treated shoots (93% of control).

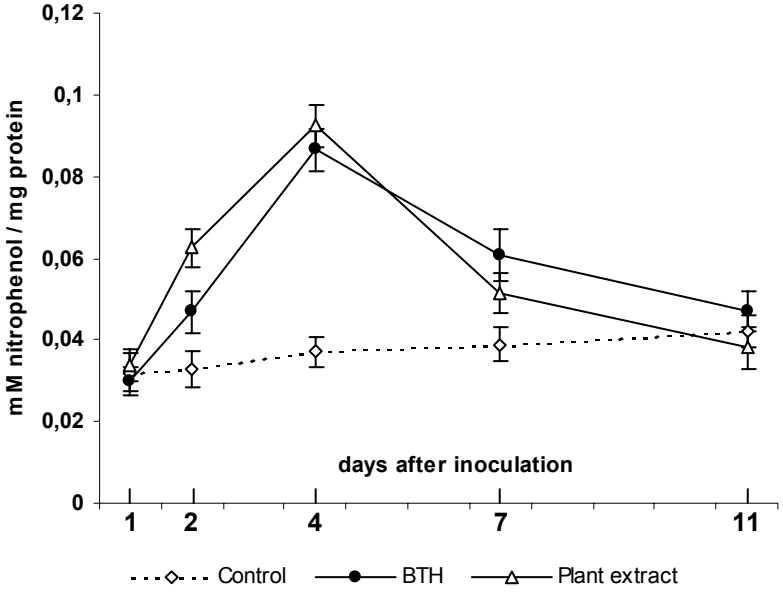


Fig. 35b: β-Glucosidase activity in infected shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

3.7.10 Pathogenesis related (PR) proteins

As physiological markers of induced resistance, PR protein activities were examined in several studies (TATA *et al.*, 1983; HAMMERSCHMIDT and KUĆ, 1995). Therefore, PR protein activities were also determined in BTH and plant extract treated seedlings.

3.10.1 Chitinase activity

a) Non-inoculated shoots

The chitinase activity in BTH treated shoots was higher, compared to control and plant extract treated shoots with exception of the first stage after treatment (1 and 2 d p.i.) (Fig. 36a). The activity increased from 1 d to 4 d after treatment in BTH treated shoots up to 138% and in plant extract treated shoots up to 88% compared to the control. While in BTH treated shoots the activity was considerably higher than in untreated shoots during the whole experimental period, a sharp decrease was recorded in plant extract treated shoots 4 d after induction in control shoots. At the end of the experiment (11 d p.i.) there was only a marginal difference in the activity between all variants.

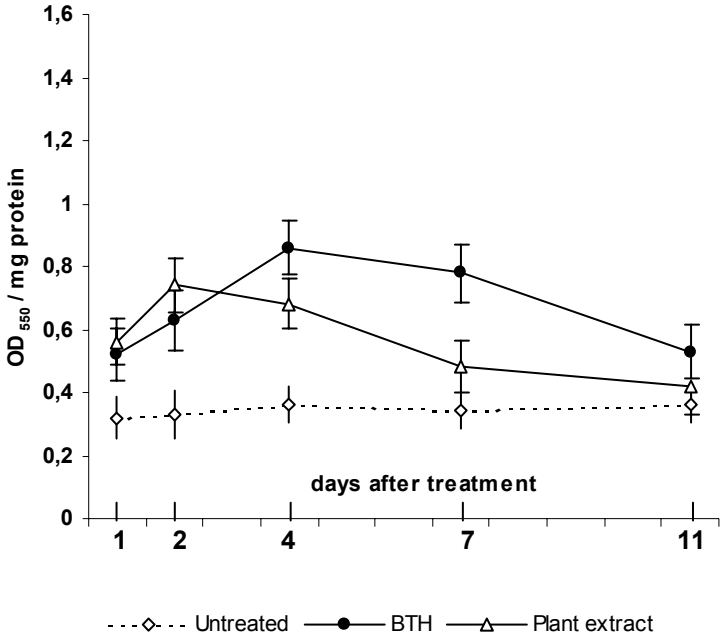


Fig. 36a: Chitinase activity in non-inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

b) Inoculated shoots

In inoculated shoots, the chitinase activity was generally higher than in non-inoculated shoots (Fig. 36b). The enzyme activity increased in BTH and plant extract treated shoots from 1-4 d

p.i. and was higher in BTH treated (214% of the control) than in plant extract treated (151% of the control) shoots. The highest activity was observed in BTH and plant extract treated shoots at 4 d p.i. In all three variants, there was a drop in chitinase activity after 4 d p.i. until the end of the experiment with a similar level. The chitinase activity in control shoots remained at the same level up to 4 d p.i. with a slight increase at end of the experiment.

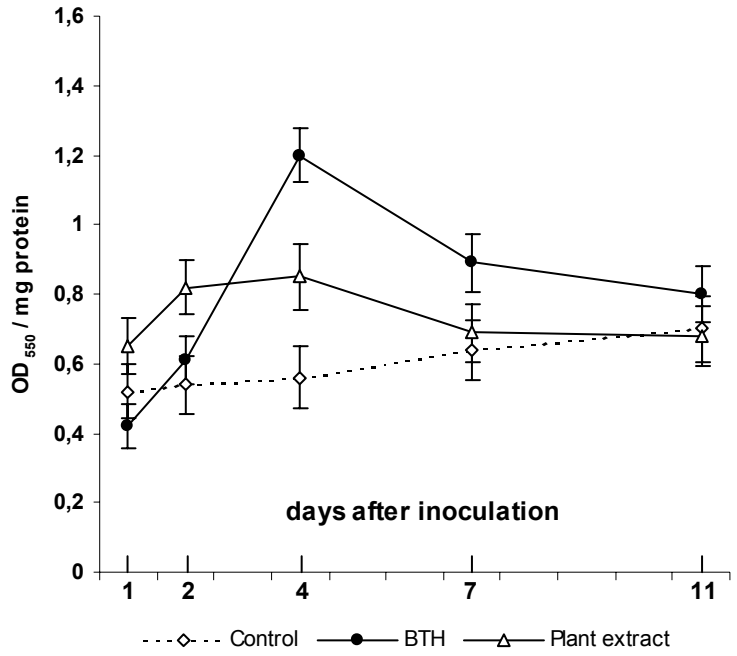


Fig. 36b: Chitinase activity in inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

3.7.10 β-1,3-Glucanase activity

a) Non-inoculated shoots

Similar to chitinase activity, also β-1,3-glucanase activity increased in BTH and plant extract treated shoots over the whole experimental period (Fig. 37a). The activity increased from 1 to 2 d p.i. up to 147% in plant extract treated, and up to 73% BTH treated shoots. In the second experimental period up to 4 d p.i., the plant extract treated shoots showed a significantly higher activity (128% of control) than BTH treated shoots (100% of control). After 4 d p.i.,

the activity decreased until the end of the experiment, when the activity in all variants was nearly the same.

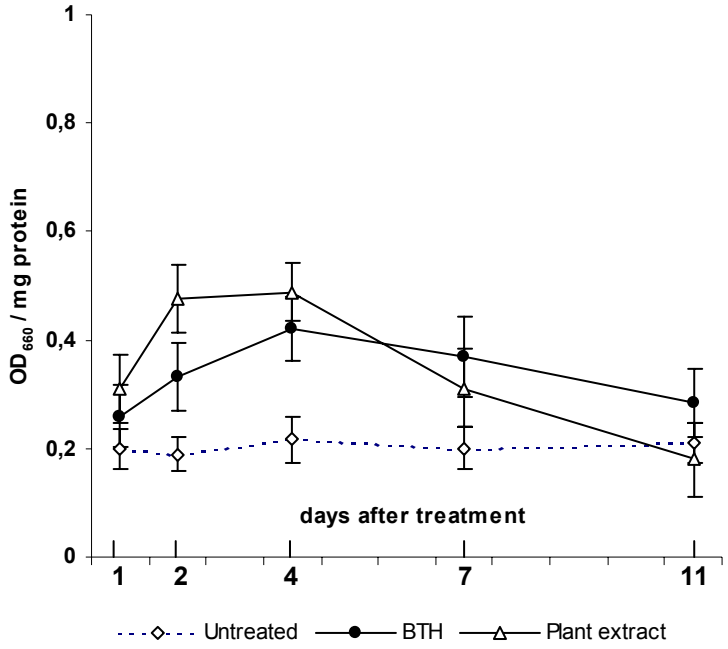


Fig. 37a: β-1,3-Glucanase activity in non-inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

b) Inoculated shoots

Also after inoculation β-1,3-glucanase activity was higher in plant extract treated and in BTH treated shoots (Fig. 37b), especially from 1 to 2 d p.i. Highest increase occurred at 2 d p.i. with 83 and 114% of the control shoots in BTH and plant extract treated shoots, respectively. Afterwards, the activities decreased and showed the same level at the end of experiment. The activity in control plants was nearly on the same level with a slight increase at the end of experiment.

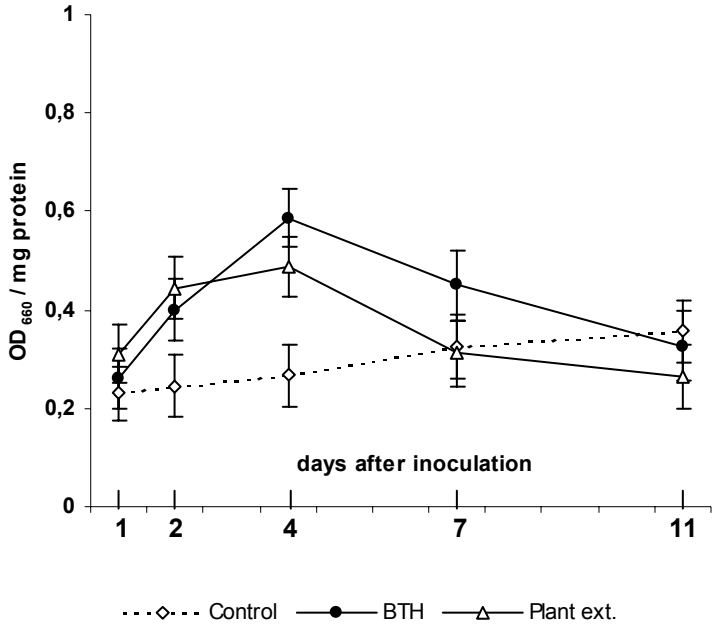


Fig. 37b: β-1,3-Glucanase activity in inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

3.7.11 Glutathione-S-transferase (GST) activity

The glutathione–S-transferase is representing an important enzyme of the glutathione cycle and is part of the antioxidative protection system of plants (MAUCH and DUDLER, 1993). In recent studies, GST activity was reported as an indicator of induced resistance. This was associated with an inhibition of necrotization and a reduced multiplication of the pathogen *in planta* (WINGATE *et al.,* 1988). Therefore, in the present study, this activity was also examined.

a) Non-inoculated shoots

With exception of the first stage of the experiment, BTH and plant extract treated shoots showed a higher GST activity (Fig. 38a). The highest increase occurred after BTH treatment (186%, of the control) 4 d p.i. After this peak, the activity decreased to the level of the control shoots until the end of the experiment (11 d p.i.).

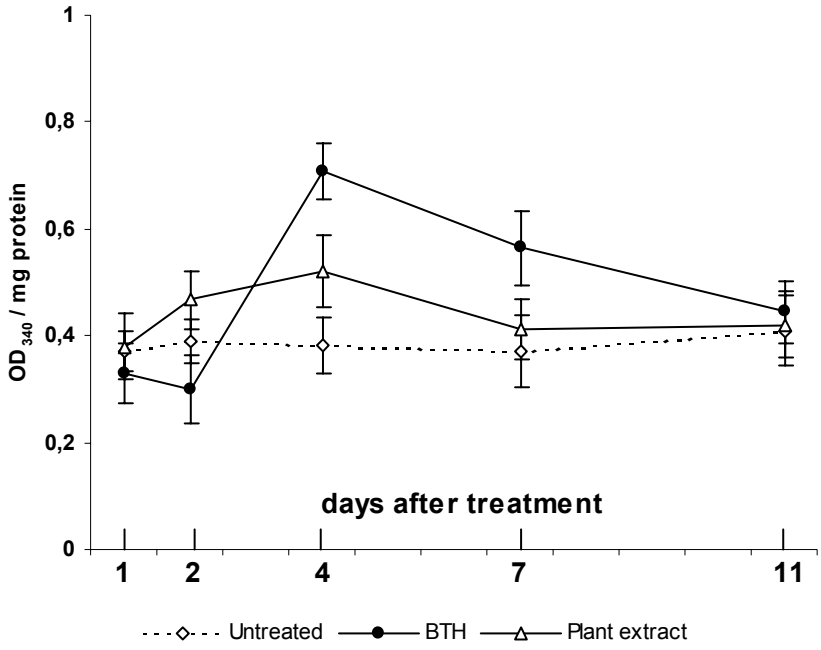


Fig. 38a: Glutathion-S-transferase activity in non-inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

b) Inoculated shoots

In inoculated shoots, the strong increase of GST activity after BTH treatment was also very pronounced with 132% of the control shoot at 4 d p.i. (Fig. 38b), whereas the enzyme increased only slightly in plant extract treated shoots. Until 7 d p.i. the GST activity in BTH treated shoots decreased (51% of control), but was still significantly higher than in the control and in plant extract treated shoots. At the end of experiment, there was no significant difference in GST activity between the three variants.

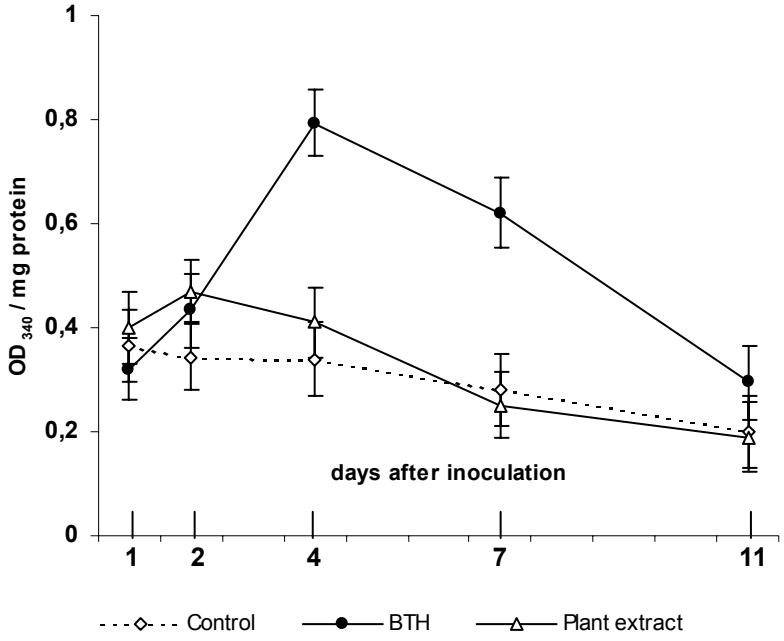


Fig. 38b: Glutathion-S-transferase activity in inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

3.12 Salicylic acid (SA)

Salicylic acid is regarded as a signal transductor in plant defence mechanisms and is essential for the induction of SAR (KESSMANN *et al.*, 1994a). Therefore, a correlation between activation of the investigated enzymatic activities and changes in concentration of salicylic acid was expected for BTH and plant extract treated apple shoots.

a) Non-inoculated shoots

A remarkable SA increase up to 135% of the control was caused by the treatment with plant extract 4 d p.i. Significantly higher activities were also observed at 2 and 7 d p.i. (Fig. 39a). On the contrary, no differences in SA concentration were found between BTH treated shoots and the control shoots.

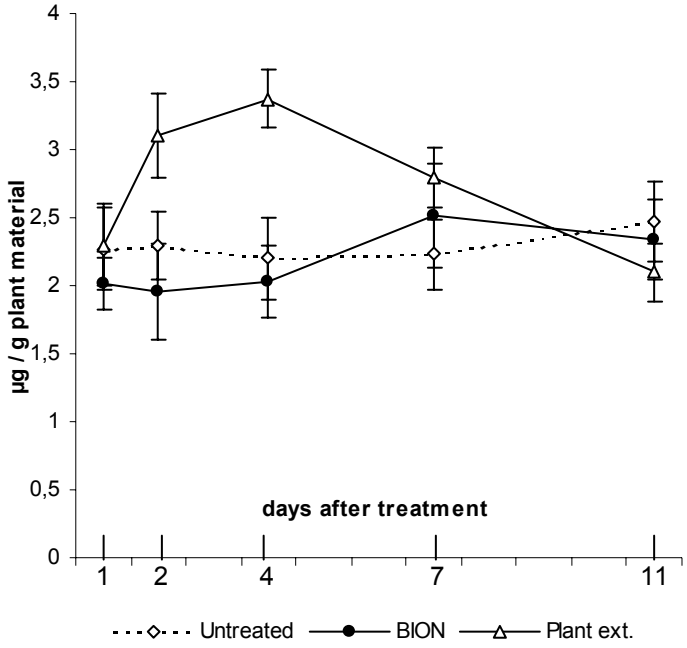


Fig. 39a: Concentration of salicylic acid in non-inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 plants per sample).

b) Inoculated shoots

A similar increase of salicylic acid content was found in inoculated shoots after treatment with plant extract (Fig. 39b). Two days p.i., salicylic acid content in plant extract treated shoots was significantly higher than in BTH treated ones (34% of control), and even more 4 d p.i. (51% of control). Later on, no significant difference between the treatments was observed.

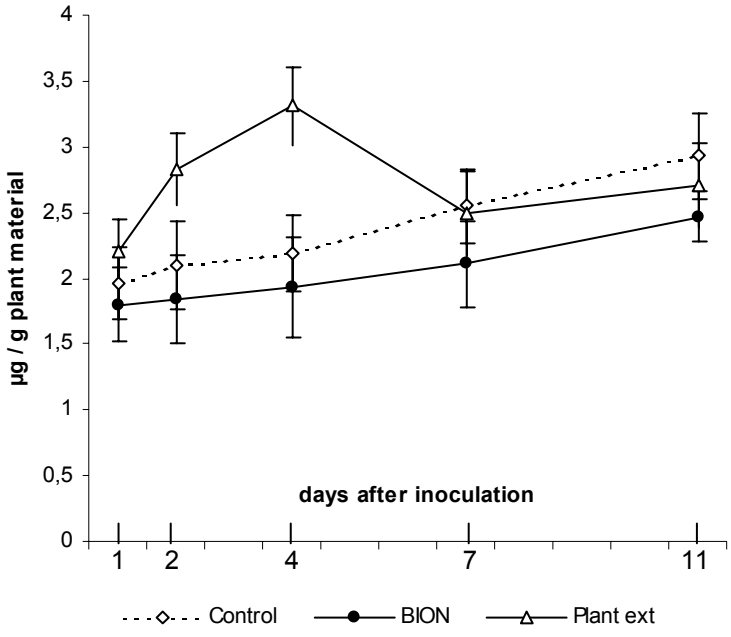


Fig. 39b: Concentration of salicylic acid in inoculated shoots, which were treated with BTH or plant extract. ⊥ Standard deviations of measurements in 4 samples (2 shoots per sample).

3.7.13 Physiological changes and symptom development in shoots

The main results are summarized in Table 7. All the biological studies showed very clearly that the resistance induction by BTH was stronger than by the plant extract (= PE) (Table 7). Thus, after BTH treatment symptoms development slower, and the reduction of symptoms was more effective. Also bacterial growth *in planta* was more reduced by BTH than by PE, at 4 as well as at 7 days after treatment. Only for BTH an optimum induction time, two days before inoculation, was established whereas for PE an optimum induction time could not be detected within two to five days before inoculation. This finding may be explained by the fact that the plant extract from ephew leaves is certainly a mixture of several biologically active substances. These may affect the resistance induction by different mode of actions, which need different time intervals. BTH, on the other hand, is a single synthetic compound so that it can be assumed that a dominant or primary biochemical affect is mainly responsible for resistance induction.

Table 7: The effects of BTH and plant extract (PE) on fire blight pathogenesis and physiological or biochemical activities in apple shoots.

Character	BTH	PE
Optimum induction time	2 d	no optimum from 2–5 d p.i.
Speed of symptom development	**very slow**	slow
Reduction of disease symptoms (maximum)	**70%**	50%
Reduction of bacterial growth in planta (4 d p.i.) (maximum)	**80%**	72%
Reduction of bacterial growth in planta (7 d p.i.)	**65%**	58%
Increase of total phenol content (maximum)	53%	**60%**
Flavonoids	**increase**	no clear increase
Bacteriostatic substance	**yes**	no
Increase of peroxidase activity 2–7 d p.i.	42%	**54%**
Increase of peroxidase activity 4 d p.i. (maximum)	67%	**135%**
Electrophoresis of peroxidase	2 bands increase	**3 bands increase**
Changes in protein banding pattern after electrophoresis	similar to effect of inoculation	
Increase of phenylalanine ammonia - lyase activity	ca. 30% (delay to PE)	**93%**
Increase of polyphenol oxidase activity	30%	**72%**
Increase of glutathione-S-transferese activity	**186%** (4 and 7 d p.i.)	ca. 80%
Necrotization	**0**	+ + (7 d p.i.)
Increase of β-glucosidase activity	110%	**150%**
Increase of chitinase activity	**214%**	151% (earlier)
Increase of β-1,3-glucanase activity	83%	**114%** (earlier)
Increase of salicylic acid	0	**135%**

Although resistance induction was more effective by BTH than by PE it was unexpected that all reactions related to phenol metabolism were stronger after treatment with PE. Thus, PE caused a higher increase of total phenol content, a higher increase of peroxidase activity during 2 - 7 days d p.i. and especially at the maximum of 4 d p.i. Electrophoresis revealed 3 increasing peroxidase bands after PE treatment, but only two after BTH treatment. Even higher were the differences in the increases of enzyme activities for phenylalanine ammonia-lyase (PAL) and polyphenol oxidase, both of which increased much more after treatment with PE. In addition, the increase of PAL by BTH-treatment was delayed in comparison to the PE-treatment. Another striking difference was that only PE induced a strong increase of salicylic

acid, the concentration of which was not significantly affected by BTH- treatment. Therefore, a comparison of the biological and biochemical effects of BTH and PE indicates that the phenol metabolism in general may not be the primary mode of action of BTH.

The data obtained in these studies show, however, that a few reactions were stronger after BTH treatment than after PE treatment. Thus, the activity of glutathione-S-transferase activity was much more enhanced by BTH than by PE. This finding may explain why an application of the resistance inducer together with inoculation caused necrotization only after PE-treatment but not after BTH treatment. Secondly, the synthesis of a bacteriostatic compound was only detected after BTH treatment, as well as increases in certain flavonoids. Thirdly, chitinase increased stronger after BTH treatment.

The possible effects of these different biochemical reactions are dealt with in more detail in the discussion.

4. DISCUSSION

Induced resistance is **defined** as an increase in the level of resistance without any changes in the basic genetic constitution. The plants utilise their own defence mechanism for restriction of pathogen development. As markers of resistance, physiological changes always appear in certain intervals after application of the biotic and abiotic inducers against pathogens (SCHÖNBECK *et al.*, 1980).

The benzo-(1,2,3)-thiadiazole-7-carbothioic-S-methyl ester **(BTH)** which was used in our studies, has been tested against several pathogens (KESSMAN *et al.*, 1994a; RUESS *et al.*, 1995; OOSTENDROP *et al.*, 1996). A resistance induction of BTH was reported against **fungi**, such as *Erysiphe graminis*, *Septoria* spp., *Pyricularia oryzae*, *Peronospora tabacina, Phytophthora* spp., (RUESS *et al.*, 1995; KESSMANN *et al.*, 1996; FRITZ, 1996; GÖRLACH *et al.*, 1996) *Didymella bryonia* (ISHII *et al.*, 1999), against **bacteria**, for instance *Erwinia amylovora* (ZELLER and ZELLER, 1998, ZELLER, 1998) or *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* (LOUWS *et al*., 2001), and against **viruses**, e.g. CMV-Y (cucumber mosaic virus) (ANFOKA, 2000).

The **plant extract** (PE) of *Hedera helix* which has been tested since 10 years showed the highest suppression effect against fire blight in field experiments when compared to other plant extracts (MOSCH and ZELLER, 1989; MOSCH *et al.*, 1989, 1993).

In the present study, BTH and plant extract (PE) from *Hedera helix* were tested against fire blight. The induced resistance was characterized by studying biochemical markers. Thus, in PE as well as in BTH treated apple seedlings the enzymatic activities showed a significant increase up to 4-7 days after inoculation.

4. 1 Induction time in BTH and plant extract (PE) treated plants

For the development of resistance plants need an interval after application of the inducing agent. In most cases this interval was reported between 2 and 7 days. The pre-inoculation of avirulent or virulent biotic agents (pathogens), and of abiotic agents (chemical compounds) was tested for resistance induction against several plant diseases. For instance, an acquired resistance could be observed in cucumber cultivars (KUĆ, 1987). Regarding the determination of the best induction time in other studies, the pathogens were evaluated as

elicitor agents by formation of necrosis around the infection site after application. This effect was observed 1 to 3 days after application (OUCHI *et al*., 1976; KUNOH *et al.*, 1985; SCHWEIZER *et al.*, 1989). Research on plant growth–promoting rhizobacteria (PGPR) showed that these organisms have beneficial effects in controlling plant diseases by inducing host resistance (TUZUN *et al*., 1989; KLOEPPER *et al*., 1992). In other studies using inoculation with TNV, resistance could be detected on the second leaves 7 days after treatment of the first leaf against *Colletotrichum lagenarium*, or *Pseudomonas lachrymans* (JENNS *et al*., 1979).

Besides biotic agents treatment with synthetic chemicals, for instance 2,6-dichloroisonicotinic acid derivates, were tested against fungal diseases, and protection was achieved against *Collectotrichum lagenarium* two days after induction, and against *Cercospora beticola* five days after induction (MÉTRAUX *et al.*, 1990). In addition to these compounds oxalates (DOUBRAVA *et al.*, 1988) and sodium phosphates (GOTTSTEIN and KUĆ, 1989; MUCHARROMAH and KUĆ, 1991) were tested against plant diseases. The stimulation of resistance to bacterial and fungal diseases was observed in many cultivars **1-2 days** after application.

In this study, although different induction times in BTH treated plants showed a low disease index, the best induction time against the fire blight pathogen *E. amylovora* was found to be **two days** before inoculation.

The resistance inducing effect of **plant extracts** and elicitor like substances tested at different application times against several plant pathogens has been reported in several studies (SALT *et al.*, 1986; DOUBRAVA *et al.*, 1988; YAMADA *et al.*, 1990). Regarding the control of *Erwinia amylovora* with plant extracts, it was reported that *Hedera helix*, *Viscum album*, and *Alchemilla vulgaris* caused induced resistance against *E. amylovora* when applied two days before inoculation in *Cotonaster waterei* (RIECK 1992; MENDE *et al.*, 1993).

4.2 Symptom development and bacterial multiplication in plants after resistance induction

During the here reported greenhouse experiments in BTH and PE treated plants, the slower symptom development correlated with a considerable lower bacterial populations compared to

the control. These data indicate, that the resistance inducers did not only suppress symptoms but directly or indirectly **inhibited the pathogen`s multiplication** *in planta*. The reduction of bacterial multiplication in BTH and PE treated plants was also accompanied by physiological changes in the plant`s tissue after induction.

Also in other host/parasite-interactions a correlation between the reduction of bacterial populations and development of resistance in BTH treated plants has been reported (LAWTON *et al*., 1996; SIEGRIST *et al.*, 1997). These results are in accordance with other studies on resistance mechanism where the development of symptoms was delayed and the multiplication of bacteria was on a low, but constant rate (LOZANO and SEQUEIRA, 1970; GOODMAN *et al.*, 1986). A low nutrient concentration in the intercellular space can be a limiting factor for the growth of bacteria. But also apoplastic compounds, or antimicrobial metabolic components of plants with bacteriostatic properties may be involved in the control of bacterial growth in BTH treated plants (SIEGRIST *et al.*, 1997). In the here reported studies with PE and BTH treated apple seedlings a significantly lower bacterial population was observed up to 96 h after inoculation compared to untreated plants. This effect may be caused by an accumulation of anthocyanins hindering bacterial pathogenic enzymes as a physiological barrier in xylem parachymas (GEHRISCH *et al.*, 1996; ZELLER, 1998).

However, 11 days after inoculation the bacterial population in BTH and PE treated plants did no longer show a considerable difference to untreated plants. From this observation it may be concluded that the used resistance inducers have an effect only up to 7 days p i. However, the main reason for the approximation of bacterial populations at 11 d p.i. was that the number of bacteria in the control plants also showed a strong reduction, probably due to the necrotization of the plant tissue. An increase of secondary metabolites due to necrotization in plant tissue can exert a negative effect on the bacterial population even in untreated inoculated plants (ADDY and GOODMAN, 1974).

4.2.1 Studies on PCR and assessment of changes in signal of PCR product in plants

In the PCR studies a positive signal was obtained from the dilution series from 10^6 to 10^3. The reason for obtaining no signal below a population of 10^3 bacteria per ml can be due to a low copy plasmid (BERESWILL *et al.*, 1992). An inhibitory effect of phenolic compounds to the PCR assay was also reported by LLOP *et al.* (2000). From cloned fragments of the multicopy

plasmid pEA29 only a 5 kb fragment is specific for *E. amylovora*. Therefore, it can be assumed that this small specific fragment will not always be integrated by the template DNA. The pEA29 PCR plasmid has been used successfully in other studies to determine populations of *E. amylovora* in plants. However, the infected plant material was shaken in liquid culture media for 8 h to allow a rapid multiplication of bacteria before performing PCR (BERGER *et al.*, 1995). In the have reported studies, the infected plant material was not shaken in liquid culture media but was directly applied to PCR. Moreover, the smearing of PCR products after electrophoresis may have been caused by the use of phenol inhibitors (DTT, PVPP). Maybe the used inhibitors disturbed the structure of DNA and created a deformation of the PCR product. In the test, these inhibitory compounds were added to tubes with the marker, and the same smearing effect could also be observed in the marker. Nevertheless, the PCR studies showed that in BTH treated and PE treated plants the bacterial population was reduced.

4.3 Physiological changes

4.3.1 Phenols, flavonoids and inhibitory substances in treated plant tissue

Between 2 – 4 d after induction and inoculation, BTH and PE treated plants showed a higher content of phenolic compounds than the untreated plants. It can be assumed that these compounds caused some inhibition of the pathogen. In another study, the commercial product MILSANA (a plant extract from *Reynoutria sachalinensis*) stimulated synthesis of phenolic compounds (DAAFY *et al.*, 1995). Also, the application of salicylic acid and dichloroisonicotinic acid (DCINA) (analog of SA) led to increasing phenols in the cell wall (SIEGRIST *et al.*, 1997). Therefore, because of the chemical structure of BTH a similar effect can be suggested which may have reduced the bacterial growth. Thus an accumulation of phenolic compounds at the infection site has been correlated with the restriction of pathogen development (HEATH, 1980). Toxic effect of phenols can kill bacteria and other microorganisms in plant tissue. As components of physical barriers in the form of lignin, they prevent pathogens from penetrating. In form of tannin substances, phenols act as chemical barriers in cell walls (TREUTTER, 1996).

In several studies, an increase in phenolic compounds and development of resistance has been reported (GOODMAN *et al.*, 1986). The resistance may be further increased by phenol

esterification in the cell wall (NICHOLSON, 1992). Changes of the cytoplasmic pH (OJALVO *et al.*, 1987) in plant tissue, due to increased phenolic acid content, and a resulting inhibition of pathogen development have also been proposed. Gallic acid, due to its monomer structure, can easily be decomposed by phenolic oxidation (FEUCHT and TREUTTER, 1989). Similar process may occur in plant tissue after inoculation, since in these studies a slight decrease in the total phenol content after inoculation was recorded. In other studies, oxidation of monomer phenolic compounds, and accumulation near to the infection site were observed (BONHOFF *et al.*, 1987). Generally, many resistance reactions of the plant against pathogens are characterized by very rapid synthesis of phenolics and their polymerisation at the cell wall (MATERN and KNEUSEL, 1988). In order to verify, whether phenolic substances participated in the resistance mechanism induced by BTH and PE in these studies, more substantiated studies appear necessary. Especially the results, that content of total phenolics increased stronger after PE-treatment then after BTH treatment, the latter causing a higher degree of resistance, needs an explanation.

Concerning the flavonoids their content appeared to be higher in BTH treated plants 4 days after treatment when analysed by TLC. BTH can cause accumulation of monomer flavonoids without a pathogenic elicitor (STADNIK and BUCHENAUER, 1998). But activation of the same signal pathways as by pathogenic elicitors can be assumed for BTH in plant tissue. On the other hand, in plant extract treated shoots a significant increase in flavonoids was **not** detected. However, changes in the **pattern of flavonoids** in PE treated plants may occur, since a significant increase in oligomer flavonoids 6 days p.i. in plant extract treated shoots has been reported (MOSCH and ZELLER, 1997). The flavonoid content is often connected with **phytoalexin** production and activation of resistance mechanisms in the plant. Thus, after inoculation of incompatible phytopathogenic pseudomonads in soybean leaves accumulation of several isoflavonoids was demonstrated (INGHAM *et al.*, 1981). An earlier study showed that - as a physiological marker of resistance – phenylalanine ammonia-lyase (PAL) activity correlated with accumulation of oligomer flavonoids and an increase in resistance (KEEN and KENNEDY, 1974). In the here analysed PE treated shoots, the high (93%) increase of PAL activity may be involved in the synthesis of oligomer flavonoids. Especially, anthocyanidin and proanthocyanidin were suggested to play a role in the resistance of apple cultivars against *Venturia inaequalis* (MAYR, 1995).

From our TLC experiments it was assumed that quercetin derivates increased in BTH treated plants. Because an inhibitory effect of quercetin on polyphenol oxidase (PPO) was reported (MAYER and HAREL, 1979; MAYR, 1995) the high content of quercetin in BTH treated plants may be responsible for the low PPO activity. On the other hand, a lower content of quercetin may be the reason for high level of PPO activity in PE treated plants. In BTH treated plants an increase in quercetin content can be accompanied by the production of inhibitory phloretin derivatives. The HPLC analyses indicated that phloretin derivates and procyanidin derivates increased after BTH and plant extract treatment. Quercetin derivatives were also related to an increase in phloridzin and kampferol derivatives (DICK *et al.*, 1985; BILLOT, 1986) and showed an antibacterial and antiviral effect (KÖNIG and DUSTMANN, 1985; 1988). The high level of quercetin and kampferol contents was accompanied by lipid peroxidase activity and antioxidants in chloroplasts and mitochondria (TAKAHAMA *et al.*, 1984; SORATA *et al.*, 1984; PINCEMAIL *et al.*, 1986; TOREL *et al.*, 1986). The quercetin and kampferol contents in BTH treated plants appeared to be higher than in PE treated plants. An increase in activity of antioxidative protection pathways in BTH treated plants can be related to an enhancement of the content of quercetin and kampferol.

Quercetin	3-galactosidase 3-glucoside 3-rhamnoside
Kampferol	3-arabinoside 3-rhamnoglucoside 3-xyloside

Table 8: Different forms of quercetin and kampferol compounds in apple (WILLIAMS, 1982)

On TLC plates, an increase in the derivates of similar compounds could be observed at different places. This observation may indicate the production of different forms of similar flavonoids (Table. 8) (WILLIAMS, 1955; WILLIAMS, 1982; SCHULTZ, 1987) or derivates of flavonoids (TREUTTER *et al.,* 1994). Since BTH induced an increase in kampferol compounds, it may be suggested that for production of these flavanoids pathways occurred independently from salicylic acid and without accumulation of oligomer flavonoids. In PE treated plants, synthesis of quercetin derivates suggests formation of oligomer flavonoids such as myrecitin. For myrecitin additional inhibitory effects were reported, such as detoxification of fatty acids and increase in lipoxygenases (LARSON, 1988). If the lipoxygenase acts as an antibacterial substance, the induction of myrecitin may thus enhance the resistance reaction.

In PE treated plants myrcetin may also play a role as antimicrobial metabolite, instead of quercetin.

Although all the aspects discussed here on the possible role of flavonoids in resistance induced by BTH and PE appear very interesting, many more data on the concentration of chemically identified flavonoids at different time intervals are urgently needed, before their actual role in induced resistance can be elucidated.

4.3.2 Changes in enzymatic activities

4.3.2.1 Peroxidase (PO) activity

Treatment of apple shoots with resistance inducers caused a general increase of peroxidase activity, especially after PE application. Peroxidase activity has been associated with induced resistance after inoculation with several pathogens (HAMMERSCHMIDT *et al.*, 1982), especially acidic peroxidase in the cell wall (SMITH and HAMMERSCHMIDT, 1998). It is long known that peroxidases play a role in resistance of plants against leaf spot causing bacteria (RUDOLPH, 1970; 1995). An enhancement in PO activity was related to several functions, one of them being oxidative polymerisation of hydroxycinnamyl alcohol and formation of cell barriers against pathogen invasion (VANCE *et al.*, 1980). In other studies it was noticed that hydroxycinnamyl alcohols were related to polymerisation under action of a peroxidase to lignin formation (HAMMERSCHMIDT and KUĆ, 1982). Increase in PO activity can be involved in the formation of lignin and inhibition of the pathogen`s spread in xylem **(**ZELLER, 1985). Peroxidase activity was also implicated in oxidative defence mechanisms after elicitor treatment.

The here reported increase of PO activity in BTH and PE treated plants may cause oxidative cross-linking of pre-existing hydroxyproline–rich structural proteins to increase resistance against degradation by microbial enzymes and protect the cell wall against pathogen invasion. The oxidative cross-linking pathways and the synthesis of salicylic acid by the phenylpropanoid pathway were correlated with enhanced PO activities by STERMER (1995) and BRADLEY *et al.* (1992). Peroxidase–generated compounds and hydrogen peroxide have a direct function as antimicrobial agents. In peroxidase, H_2O_2 (hydrogen peroxide) plays an important role in inducing subsequent defence responses in infected plants (APOSTOL *et al.*, 1989; PENG and KUĆ, 1992; CHEN *et al.*, 1993). Therefore, similar to elicitors, BTH and

PE may trigger the defence mechanism in the plant and also affect the production of some antibacterial substances by increasing the PO activity. An increased specific PO activity and transmission of H_2O_2 can also be correlated with the expression of glutathione transferase and glutathione peroxidase genes (LEVINE *et al.*, 1994). However, the knowledge about the signals and function of peroxidase and the biochemical processes underlying these cytological changes is very poor. It has been demonstrated that in some cases specific peroxidase isoenzymes increase in the host tissues as response to pathogen attack (YE *et al.*, 1990).

Most plants contain a number of different **peroxidase isoforms** (LAGRIMINI and ROTHSTEIN, 1987). HAMMERSCHMIDT *et al.* (1982) showed at least three peroxidase isoforms which were associated with induced resistance. A similar set of acidic peroxidases was shown in watermelon and muskmelon (SMITH and HAMMERSCHMIDT, 1988). These isoforms showed a similar charge and molecular weight of 30 to 33 kD. Later, RASMUSSEN *et al.* (1991) reported a 33 kD apoplastic peroxidase in systemic induced resistance.

In our studies, an increase in three acidic peroxidases was found after inoculation in BTH and PE treated shoots, so that the detected PO isoenzymes in BTH and PE treated seedlings appear to be associated with induced resistance. However, the number of PO bands after SDS-PAGE differed. Therefore, it is suggested that activation of isoenzymes and the mechanism of PO activity is different between BTH treated and PE treated apple seedlings. In other studies, induced resistance was correlated to an increased PO activity and enhancement of the PR protein levels (chitinase and β-1,3-glucanase) (BINDER *et al.*, 1989; MÉTRAUX *et al.*, 1989). Also in our greenhouse experiments with BTH or PE treated plants, PO activity and PR protein activities increased (see 3.7.2 and 3.7.10).

Differences were also observed in the **protein bands** of different treatments. In PE treated shoots, it appeared as if the same proteins were expressed as after artificial inoculation. However, in the BTH treated shoots different protein bands showed a high expression compared to control and PE treated shoots. Therefore, during development of induced resistance different mechanisms can be assumed. Further studies are necessary to understand the role of these proteins and their relation to different PO isoenzymes in BTH and PE treated plants.

4.3.2.2 Phenylalanine ammonia-lyase (PAL) activity

PAL activity was considerably higher in PE treated shoots than in BTH treated and control shoots. The inoculated non-treated shoots also showed an increase of PAL up to 2 d after inoculation (Fig. 17 b). Later a decrease in activity occurred. The increased PAL activity in untreated plants has been reported to induce elicitors which affect the adjacent unchallenged areas (ELLISTON *et al.*, 1977). In another study with susceptible rice plants inoculated with *Xanthomonas oryzae* pv. *oryzae*, the PAL activity reached its maximum 2 d p.i. and afterwards declined (LI *et al.*, 1999). BTH treated plants showed a gradual increase in PAL activity compared to untreated plants, but the activity was not at a high level.

In PE treated plants PAL activity increased more than in BTH treated plants. PAL activity and enhancement of PO activity can participate in the production of antibacterial metabolites. Thus, the synthesis of phytoalexins and activation of the enzyme chalcone synthase (CHS) and the triggering of plant defence mechanisms depended on PAL activity in phenylpropanoid pathways (SEKIZAVA and WATANABE, 1981). The regulation of phenylpropanoid–biosynthetic genes is complex. PAL gene regulation and biochemical specialization of the encoded isopolypeptides includes highly diverse biological functions of phenylpropanoid natural products (DIXON and LAMB, 1990).

In this study, PAL activity in BTH treated apple seedlings did not change very much. Therefore instead of synthesis of salicylic acid the phenylpropanoid pathway may be important for synthesis of flavonoids and antimicrobial compounds. In systemically protected potato tissue, however, no sufficient evidence was obtained that either PAL or other enzymes significantly increased and are important for the synthesis of phytoalexins (NICHOLSON, 1992). In other studies with tobacco plants a considerable increase in PAL activity was correlated to the formation of salicylic acid from cinnamic acid (1) (RASKIN, 1992) (Fig. 40).

PAL is a crucial enzyme involved in activation of phenol metabolism in response to infection (HAHLBROCK and SCHEEL, 1989). In PE treated plants the enhancement of PAL activity participates in the biosynthesis of lignin and accumulation of 4-hydrobenzoic, caffeic and ferulic acids (KUROSAKI *et al.*, 1986).

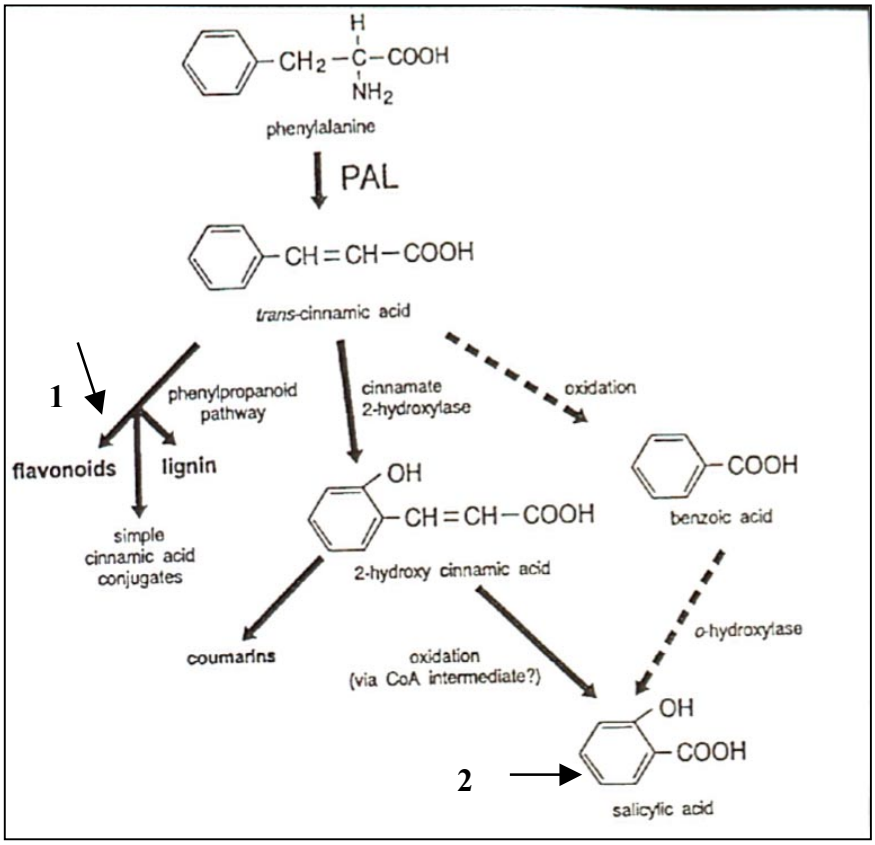


Fig. 40: Proposed pathways for salicylic acid biosynthesis in plants (RASKIN, 1992).

Although PAL activity increased much more after BTH–treatment than after PE-treatment, salicylic acid increased in PE treated shoots but not in BTH treated ones. (Table 7). Therefore, if PAL plays a role in resistance induction by BTH, this cannot occur *via* synthesis of salicylic acid (Fig. 40). The function of PAL may be flavonoid synthesis and production of substrate for GST activity. Thus, the antioxidative protection system can be enhanced by PAL activity. MAUCH and DUDLER (1993) showed that cinnamic acid serves as a substrate for glutathione-S-transferase (GST) activity. The low activity increase of PAL after PE-treatment concomitant with a strong increase of SA indicates, that PAL may not be the limiting factor for SA synthesis in apple shoots. Obviously, different defence mechanisms are likely to occur for different resistance inducing agents.

4.3.2.3 Polyphenoloxidase (PPO) activity

Even more than peroxidase the PPO activity increased much more in PE treated than in BTH treated shoots, which in turn had a higher activity than the untreated shoots at different time intervals. PPO not only contributes to synthesis of phytoalexins (ZINKERNAGEL, 1984) but can also cause enhanced concentration of quinones which are cytotoxicants that can inhibit the growth of bacteria in plants (FARKAS *et al.*, 1959). This may explain why, compared to BTH treated plants, a high necrotization occurred in PE treated plants. But also mechanisms of necrotization are possible. GEHRISCH *et al.* (1996) tested the efficacy of PE (*H. helix)* against *Xanthomonas campestris* pv. *campestris* and showed a high content of anthocyanin in the plant extract. It was suggested that this component may increase the efficacy of PE as resistance inducer.

In other systems, the rapid collapse of plant tissues, so-called HR (hypersensitive response), is caused by biotic or abiotic inducers or "elicitors". However, the resistance inducing activity of different pathogens or plant extracts should be differentiated from elicitors of HR (SEQUEIRA, 1983). Substances which cause a rapid tissue collapse after application cannot be applied as plant protection measure, because the necrotization of many leaves would certainly *per se* lead to significant yield losses.

In BTH treated plants a significant and permanent increase of PPO activity did not occur on the high level as in PE treated shoots. Therefore an increase of PPO-activity may not be the decisive mode of action in BTH treated plants. However, in PE-treated shoots the enhanced PPO activity may lead to production of antimicrobial metabolites restricting the pathogen, and a high level of oxidative burst can be suggested in collapsed cells (VAUGHN and DUKE, 1984; HAMMERSCHMIDT and KUĆ, 1995).

Therefore, it is suggested that the modes of action of BTH and PE are dissimilar. It can be assumed that PE activated a different pattern of enzymes and genes in the plant than BTH. In another study, SCHWEIZER *et al.* (1989) showed differences in induced genes between plants treated with a non-host pathogen agent or INA (analogue of SA).

4.3.2.4 β-Glucosidase (β-Gl) activity

The β-Gl activity increased nearly by the same factor in BTH and PE treated plants up to 4 d after induction. In general the β-Gl activity was a little bit higher in PE treated than in BTH treated shoots. An enhanced level of β-Gl can decompose the binding of glucose to inhibitory substances so that the growth of the pathogen in infected tissues and cell walls is restricted. The enhanced antibacterial activity of the aglycone phloretin over the glycoside phloridzin in tissue of apple shoots was recorded (HILDEBRAND and SCHROTH, 1964). In other studies, abiotic compounds, such as benzoquinone and napthoquinone, were involved in fungitoxic activity in plants and the presence of aglycones correlated with phytoalexin formation (GOODMAN *et al*., 1986).

In the here described experiments BTH and PE induced not only higher PO activity, but also higher β-Gl activity. Enhancement of β-Gl activity may contribute to higher synthesis of phytoalexins. In BTH and PE treated plants, the decomposition of glucosides and the release of phloretin in the cell wall may therefore be maintained longer than in control plants. The reaction chains in formation of antibacterial substances show some differences between β-glucosidase and PPO as shown in Fig. 41. While in PE treated plants the reaction may be dominated by the PPO activity steps, this cannot be proposed for BTH treated plants. Since also the β-glucosidase was a little bit higher in PE-treated shoots it is assumed that in BTH-treated shoots β-glucosidase does not play a major role in induction of resistance.

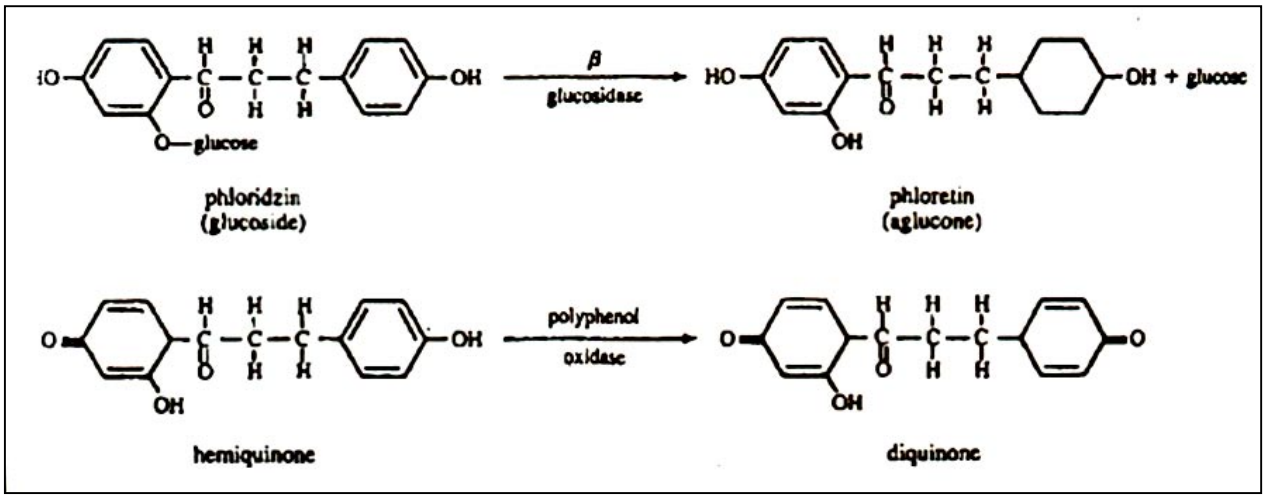


Fig. 41: Comparison β-glucosidase activity with PPO activity (GOODMAN *et al.,* 1986)

4.4 Pathogenesis-related (PR) proteins

In many studies an accumulation of PR proteins has been reported after inoculation of different pathogens (viruses, bacteria, fungi) (DE TAPIA *et al.*, 1986; REDOLFI *et al.*, 1989; AWADE *et al.*, 1989; BOL *et al*., 1990; KESSMANN *et al.*, 1994a). Some PR proteins and the corresponding enzyme activities showed a significant increase after application of biotic and abiotic agents of induced resistance (FISCHER *et al.*, 1988; MÉTRAUX *et al.*, 1989; IRVING and KUĆ, 1990; SMITH *et al.*, 1991; UKNES *et al.*, 1992).

In these experiments, the chitinase activity was higher in BTH treated plants, when compared to the control and PE treated plants. PR proteins` activities (chitinase and β-1,3-glucanase) increased earlier in PE treated than BTH treated shoots. BRISSET *et al.* (2000) also tested BTH against *Erwinia amylovora* and determined an enhanced β-1,3-glucanase activity in *Golden Delicious* seedlings under greenhouse conditions. The protection against fire blight was found to be around 69%. Accumulation of β-1,3-glucanase was determined systematically in all parts of the plants by BRISSET *et al.* (2000). Induced activity was mostly found to be associated with an acidic chitinase (MÉTRAUX and BOLLER, 1986).

Class	Possible role in defence
Peroxidase	Strengthen cell walls, generate toxic free radicals
PR-1	Functions unknown
Chitinases	Antifungal, some of them acidic chitinase
β-1,3-glucanases	Enhance activity of chitinase
PR-4	Unknown
Thaumatin-like proteins	Antifungal; α-amylase / protease inhibitors
Systemic acquired resistance	Unknown
Glycine–rich proteins	Strengthen cell walls

Table 9: Classes of proteins accumulating systemically in plants after local infection (STERMER, 1995).

Subsequent work by MÉTRAUX *et al.* (1988) revealed that the acidic chitinase has a considerable inhibition effect on the pathogen development in plant tissue. The studies demonstrated that several PR proteins, appearing in intercellular fluids of infected leaves, are acidic forms of chitinase and glucanase. These forms were secreted, or released into the intercellular space (DIXON and LAMB, 1990). Some of the encoded mRNA regions which induced PR proteins (chitinase and glucanase) are known and cause high enzymatic activities (KAUFFMANN *et al.*, 1987; LEGRAND *et al.*, 1987).

It is possible that BTH and PE are recognised by the plant as elicitors, which induce similar signalling pathways. In this way, the encoded specific regions of mRNA may cause expression of these enzyme activities. However further studies are necessary to understand the recognition of PE or BTH by the plant. Regarding BTH, another study showed that it has an effect on eliciting of PR proteins (chitinase and β-1,3-glucanase) and activates the SAR signal transduction pathways after application (FRIEDRICH *et al.*, 1996).

Several metabolites which increase during systemic acquired resistance (SAR) have been suggested to possess antimicrobial activity, or are related to classes of antimicrobial proteins (KESSMANN *et al.*, 1994a), for instance, β-1,3 glucanase, chitinase, cysteine-rich proteins related to thaumatin and the PR-1 proteins (see Table 9). Moreover, the PR proteins (chitinase and β-1,3 glucanase) are described as hydrolytic enzymes. The enzymes catalyse the hydrolysis of the main carbohydrate components of most fungal cell walls (MÉTRAUX *et al.,* 1989). BOLLER *et al.* (1983) studied the effect of PR proteins on fungal pathogens and determined that chitinase is able to decompose cell walls of bacteria with lysozyme activity. Besides the direct effect of these enzymes, chitinase and β-1,3-glucanase are capable of hydrolyzing pathogen cell walls by releasing oligosaccharide molecules, having elicitor properties for the recognizing process in infection (KEEN and YOSHIKAWA, 1983; DARVILL and ALBERSHEIM, 1984). In another study, YOSHIKAWA *et al.* (1990) explained resistance induced by ethylene by an increase of glucanase activity which released oligosaccharides acting as elicitors of phytoalexin synthesis at the beginning of infection.

It can be suggested that BTH and PE treatment trigger defence mechanisms and phytoalexin production in plants. In this case, an increase in chitinase activity may be accompanied by lysozyme activity against pathogenic bacteria. After infection, rapid response with

phytoalexins accumulation, hydroxyproline rich proteins, and lignin at the site of infection can be caused by the enhanced level of the chitinase, β-1,3-glucanase and other PR–proteins and peroxidases (KUĆ, 1995). Following this concept, it can be assumed that the BTH and PE treated apple seedlings respond more rapidly against bacteria than untreated seedlings. This response may include both, phytoalexin synthesis and the direct effect of hydrolytic activities. In SDS-PAGE a few protein bands were detected which increased after application of the elicitors. It may be that these protein bands represent specific PR proteins. However, further studies are necessary to characterize these proteins.

4.5 Glutathione-S-Transferase (GST)

It is long known that glutathione (GSH) plays a major role in induction of defence related products and causes a massive selective transcription of defence related genes coding for enzymes synthesizing phytoalexins and hydroxyproline–rich cell wall glycoproteins (WINGATE *et al.*, 1988). Dependend on GSH, GST activity is responsible for the glutathione cycle and subsequent specific pathways (DUDLER *et al.*, 1991). GST is a component of the antioxidative protection system in the glutathione cycle by which oxidative products induced by pathogens are diminished. Thus, GST counteracts against necrotization and collapse of cell walls and release of toxicants or causes herbicide detoxification in plant cells (MAUCH and DUDLER, 1993).

The GST activity increased after application of the resistance inducers. The antioxidative activity of GST may be responsible for the finding that in BTH treated seedlings necrotization occurred at a considerably lower level than in PE treated plants. Therefore, it can be assumed that toxic oxygen species resulting from the enhanced peroxidases were detoxified involving the glutathione system. DALTON *et al.* (1986) studied the elimination of oxygen toxicity originating from special enhanced peroxidases. These are components of scavenger mechanisms concerned with ascorbate, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase.

In another study, DCINA and SA were shown to activate antioxidants (catalase) and PR-2 and PR-3 proteins (chitinases and β-1,3-glucanases) (CONRATH *et al.*, 1995;). In BTH treated shoots a considerable increase in glutathione transferase activity, but not in PAL activity was

recorded, whereas in PE treated shoots PAL activity increased an GST activity increased only for a very short time. Glutathione (GSH) is involved in activation and regulation of biosynthetic processes in plant defence (BOLTER *et al.*, 1993). For instance, when resistance to powdery mildew was induced in wheat tissue by pre-inoculation with an incompatible mildew pathogen, one of the putative genes shown to be activated was homologous to GST in its sequence (SCHWEIZER *et al.*, 1989). On the other hand, an accumulation of phytoalexins and an increased level of PAL activity was elicited by infiltration of GSH in *Lotus corniculatus* (ROBBINS *et al.*, 1991). In the here reported experiments, GST activity is considered as an indicator for enhanced level of GSH, so that it can be assumed that BTH was involved in GSH production itself.

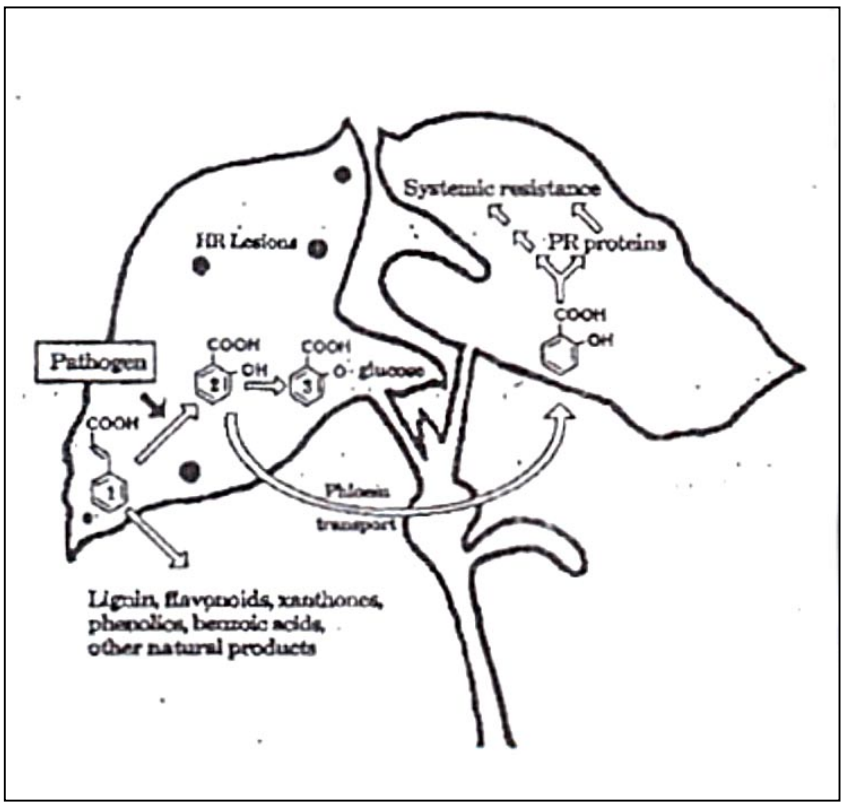


Fig. 42: Proposed role of salicylic acid in systemic acquired resistance (Enyedi *et al.* 1992).

4.6 Changes in salicylic acid content

In PE treated seedlings total salicylic acid content increased early and significantly but not all in BTH-treated seedlings. Salicylic acid is essential for SAR and activation of plant defence responses (MALAMY *et al.,*1990; MÉTRAUX *et al.*, 1990; RASSMUSSEN *et al.*, 1991).

Although little is known about the signal transduction pathway leading to Systemic Acquired Resistance (SAR), one step is apparently involved in the synthesis of salicylic acid (MÉTRAUX *et al.* 1990). In other studies, application of exogenous SA induced resistance against plant pathogens (MALAMY *et al.,* 1990). It was also shown that exogenous application of SA induced the same set of mRNAs as after pathogen infection (ANTONIW and WHITE, 1983; WARD *et al.*, 1991; UKNES *et al.*, 1992). Several data suggest that SA increase is required for SAR, and that SA induced protection is accompained by transcriptional activation of PR protein genes (Fig. 42) (CARR and CLESSIG, 1989) and induced peroxidase, superoxidase dismutase, and glycine-rich cell wall proteins (BOLWEL *et al*., 1985; VAN DE RHEE *et al.*, 1990). Exogeneous applied SA inhibited the biosynthesis of the plant hormone ethylene, stomatal closure and root ion uptake (RASKIN, 1992).

SA was described as signal substance for elicitors causing an activation of receptors in the cell wall (FRITZ, 1996). Systemic resistance needs signal transduction, and it was suggested that SA is transported from infected tissues to uninfected areas (SHULAEV *et al.*, 1995).

In the present studies, SA did not increase in BTH-treated but only in PE treated seedlings. Obviously, BTH treated plants developed resistance without SA increase, and even stronger than in the PE-treated seedlings. Nevertheless, PR proteins and peroxidase activity also increased after BTH treatment. Therefore, it appears possible that BTH induced a downstream of SA instead of SA increase. Recent studies showed that SA binding proteins play a role in signal transmission during defence response. These may lead to changes of certain biochemical and physiological states of plant cells (CHEN *et al.*, 1993). According to other studies, application of the inductor INA activated a component of SAR causing a signalling pathway with suppression of SA accumulation. DCINA induced the same mechanism, however, activated the antioxidants and suppressed the PR-1 proteins (CONRATH *et al.*, 1995). The BTH treated plants did not show significant changes in SA content after application in the here reported as well as in other studies (FRIEDRICH *et al.*, 1996; LAWTON *et al.*, 1996). Activation of antioxidative mechanisms is suggested for BTH treated plants (see subchapter 4.5). Contrary to BTH treated plants the PE treatment caused a strong increase (135% of control) of SA. This accumulation may explain activation of PR-1 proteins and a suppressive effect on antioxidants. Accumulation of SA induced SAR genes. Establishment of the resistance state was correlated with PR-1, PR-2, PR-3 gene expression (WARD *et al.*, 1991; VERNOOIJ *et al.*, 1994;). Consequently, it is suggested that BTH exerts similar effects than SA due to a similar structure (analogue). Because of the nevertheless differing structure of BTH, slightly differing defence responses and expression of different defence related genes are induced than in PE treated apple seedlings.

4.7 Study hypothesis

4.7.1 Mode of action of resistance induction by BTH

Different modes of action have to be assumed for BTH and PE. BTH is thought to act as signalling compound. This signal may lead to an activation of different membrane-bound enzymes resulting in higher levels of oxygen, oxygen radicals, superoxides, hydroxyl radicals and the formation of H_2O_2 (HAHLBROCK *et al.*, 1995). The signal transmission and resistance induction may be similar to that of applied elicitors (WOJTASZEK *et al.*, 1995). Expression of mRNA may be regulated by SA binding proteins which can be assumed as further steps of signal transmission (HAHLBROCK *et al.*, 1995). In another study, a defence response was triggered by several mRNA regions (HAMDAN and DIXON, 1987). It is hypothesised that the SA binding proteins (PR-1-PR-3 etc.) are activated independently to SA (Fig. 43). It is also assumed that PAL activity is responsible for flavonoid biosynthesis (phenylpropanoid pathways) and synthesis of cinnamic acid as a substrate for the glutathione cycles. The synthesis of a bacteriostatic compound in BTH treated apple seedlings may be the reason for the stronger resistance induction of BTH compared to PE. In the present studies, it could be confirmed that BTH treatment suppressed SA accumulation in the plant. (FRIEDRICH *et al.*, 1996; LAWTON *et al.*, 1996). Enhanced PO activity is regarded as a secondary transmission of defence responses. The increased PO activity may serve as elicitor of other defence mechanisms of the plant. The outlined mechanisms can lead to a suppression of the fire blight pathogen up to 7 days after inoculation.

Consequently, BTH is suggested to act as an analogue of SA during resistance reaction in apple seedlings (Fig. 43).

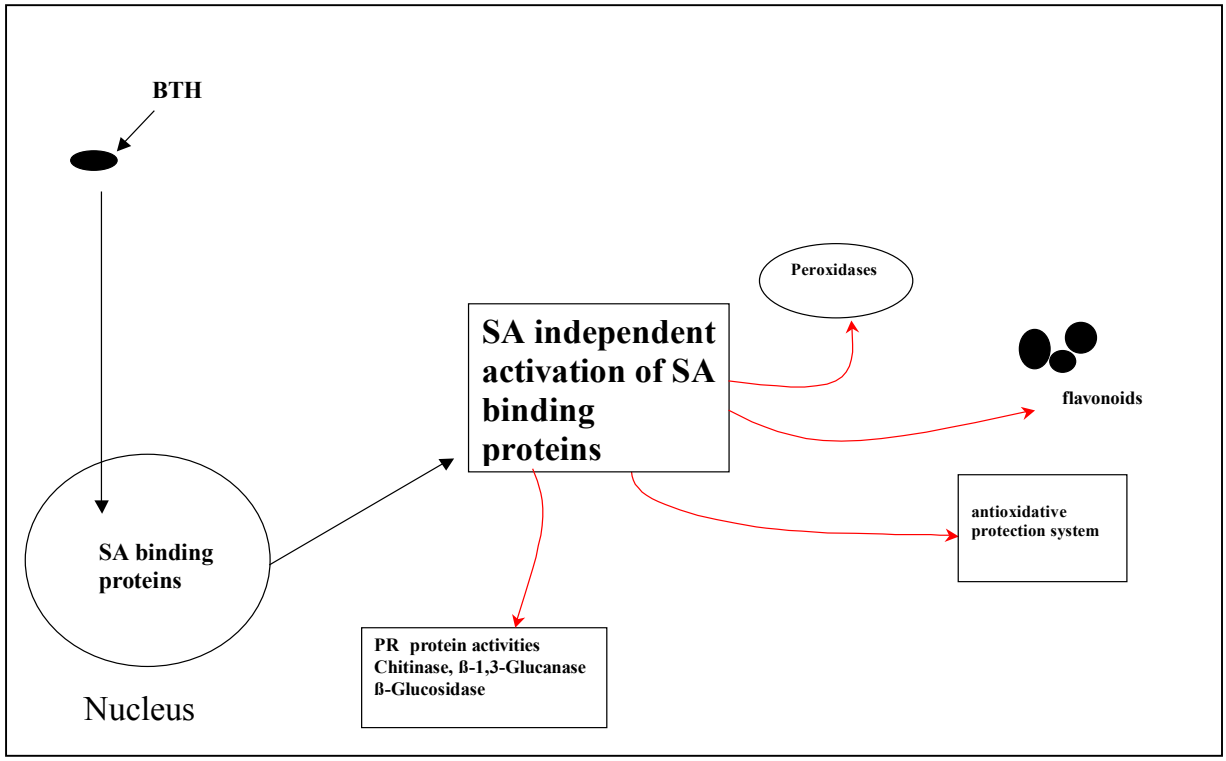


Fig. 43: Assumed mechanism of induced resistance in BTH treated apple seedlings.

4.7.2 Mode of action of resistance induction by plant extract from *Hedera helix*

In contrast to BTH for the PE a direct effect on phenylpropanoid pathways and SA is assumed which has similar consequences as the indirect effect of BTH (Fig. 44). In PE treated plants, ethylene metabolism may play an important role in signal transmission in the plasma membrane (SCHNEIDER and ULRICH, 1994). In addition, an increase of peroxidase activity and appearance of different PO isoenzymes was observed. Therefore, another signal transmission is suggested leading to an enhancement of total SA content, which was not observed in BTH treated seedlings. Contrary to BTH, in PE treated plants there was no significant increase in enzymes of the antioxidative protection system, allowing necrotization of the inoculated tissue with a high level of polyphenoloxidase and possible synthesis of oligomer flavonoids.

Consequently, contrary to BTH, PE is suggested to act *via* increase of SA during the resistance reaction of the plant.

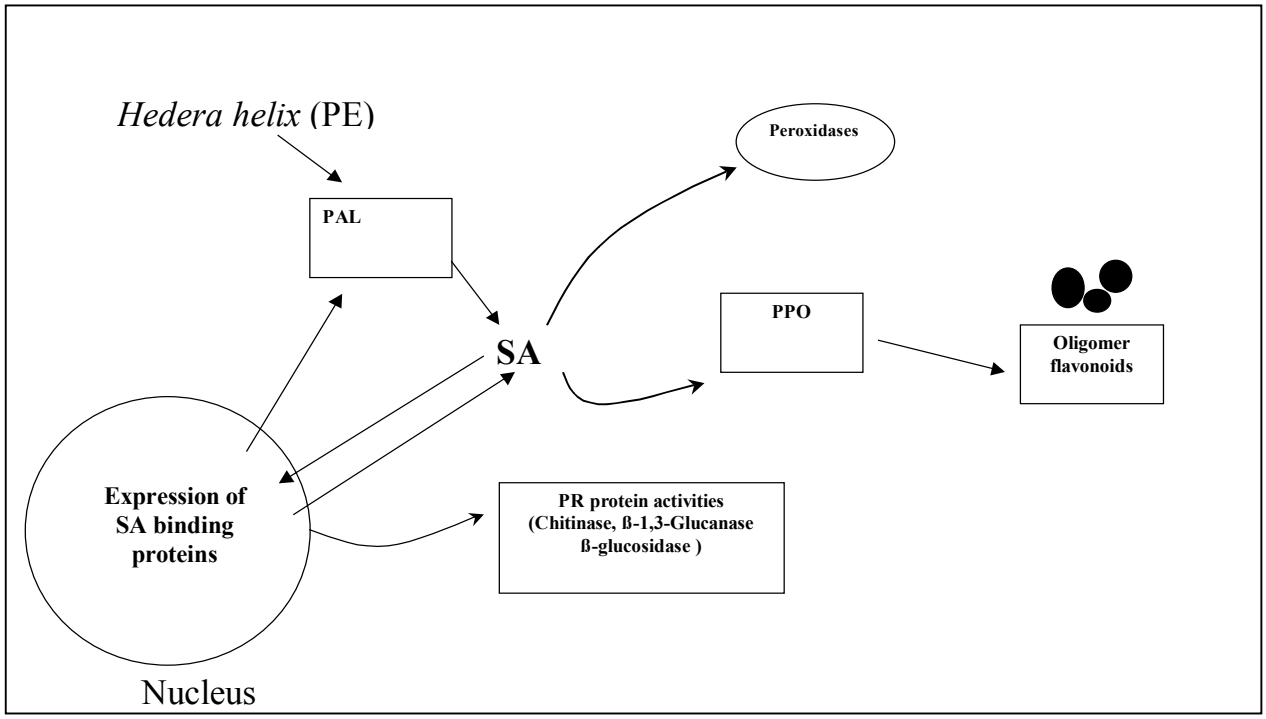


Fig. 44: Assumed mechanism of induced resistance in plant extract treated apple seedlings.

5. SUMMARY

In the present study the plant activator BTH (= benzothiadiazole) (BION®) and an extract from leaves of *Hedera helix* were assayed on resistance inducing effects against fire blight caused by *Erwinia amylovora*, strain Ea7/74 on the highly susceptible apple rootstock M26. The experiments were carried out under controlled climatic conditions in the greenhouse.

5.1 Symptom development and growth of bacterial multiplication in BTH and PE treated plants

Pre-inoculation application of BTH and plant extract to the foliage of rootstock M26 reduced severity of disease as well as the multiplication of bacteria in the tissue. The greatest effect of BTH was achieved by an induction interval of 48 hours before inoculation. For the plant extract an optimum induction interval could not be determined. Therefore, the same induction period of 48 h was used for both treatments. In the treated apple shoots the development of systemic symptoms was suppressed. Reduction of disease severity in BTH treated shoots amounted up to 70% and up to 31% in plant extract treated shoots. In plant extract treated shoots high necrotization was recorded in the inoculated leaves. The reduction of disease severity was correlated with a suppressive effect on multiplication of bacteria *in planta* up to 80% and 72% in BTH and PE treated shoots, respectively. The reduction of bacterial multiplication *in planta* was also demonstrated by PCR with the use of the specific primer pEA29.

5.2 Physiological changes

Several indicators of resistance reactions in plants were examined, such as total phenol content, pattern of flavonoids, bacteriostatic compounds, peroxidase (PO), phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), pathogenesis related (PR) proteins (chitinase, β-1,3-glucanase), β-glucosidase, glutathione-S-transferase (GST), and total salicylic acid (SA) content.

5.2.1 Phenols, flavonoids and inhibitory substances in treated plants

In general, BTH and plant extract treated shoots showed a higher phenol content than untreated shoots. This effect was most significant 2-4 d after treatment. Chromatographic

(TLC) examinations indicated that some flavonoids (quercetin and kampferol isoforms) increased after treatment with BTH and plant extract. An inhibitory substance was found in BTH treated plants which could, however, not be fully characterized. From the data obtained the detected inhibitory substance was assumed to be a phloretin derivate that showed considerable reduction of bacterial multiplication *in vitro*.

5.2.2 Enzymatic activities, PR proteins and salicylic acid (SA)

Peroxidase activity showed a significant increase in BTH and PE treated shoots from 2–7 d p.i. with the highest activity 4 d after treatment. After SDS-PAGE the main PO activity was found in molecular sizes of 20.1 and 43 kD. The plant extract treated shoots showed a high expression in 3 bands and BTH treated shoots in 2 bands.

Treatment with BTH or plant extracts induced an **increase of specific protein bands**, similar to the pattern after artificial inoculation.

Also, **phenylalanine-ammonia lyase** (PAL) activity was increased in BTH and plant extract treated shoots. Generally, the activation by PE was significantly higher than by BTH treatment.

Polyphenoloxidase (PPO): Only treatments with plant extracts caused a remarkable enhancement. This result was correlated to high necrotisation in PE treated plants. In contrast, low PPO activity correlated with low necrotization in BTH treated plants. Infection increased PPO activity much later (11 d p.i.) than PE treatment.

β**-Glucosidase** activity (β-Gl) was considerably higher after both treatments, especially 4 d p.i. In BTH treated plants a significant increase of β-Gl correlated to production of an antimicrobial substance.

Glutathione-S-transferase (GST) was strongly increased in BTH treated shoots 4 and 7 d p.i. This result was correlated to low necrotization after BTH treatment in contrast to PE treatment with more necrotization but less GST activity in the shoots.

Two enzymes, often found in the so-called **PR-proteins** also increased after the treatment: chitinase and β-1,3-glucanase. **Chitinase** activity was higher in BTH treated shoots from 4–11 d p.i. than in plant extract treated shoots, although the increase started earlier in plant extract treated shoots. Also, the activity of **β-1,3-glucanase** increased earlier after PE treatment than in BTH treated shoots.

A remarkable increase in concentration of **salicylic acid** (SA) occurred only in plant extract treated plants, whereas no difference was found between BTH treated and control plants. For a role in signal transduction and defence mechanism BTH was assumed to replace SA, independently. Contrary to BTH, a direct effect of plant extract on SA was assumed.

5.2.3 Mode of action of BTH and PE

Summarizing the above mentioned results on the effects of the resistance-inducing agents BTH and PE on symptom and bacterial development as well as the physiological changes in the plant the following modes of action are proposed:

For BTH, it is hypothesised that the SA binding proteins are activated independently from SA. It was shown that BTH treatment did not increase but enhanced peroxidase activity. These reactions are regarded as secondary transmission of defence responses. Therefore, BTH is suggested to act as an analogue of SA during the resistance reaction of the plant.

For PE a direct effect on the phenylpropanoid pathway and SA accumulation is assumed. A different signal transmission is suggested with peroxidase activity and enhancement of total SA content. PE treated plants led to an increased level of PPO activity, which is related to the formation of oligomer flavonoids. However, in PE treated plants there was no significant increase in enzymes of the antioxidative protection system. So PE is suggested to act directly *via* SA accumulation during the resistance reaction of the plant.

6. REFERENCES

Addy, S.K. and Goodman, R.N., 1974. Phenols in relation to pathogenesis induced by avirulent and virulent strains of *Erwinia amylovora*. Acta Phytopathol. Acad. Sci. Hung. 9, 277-286.

Anfoka, G.H., 2000. Benzo-(1,2,3)–thiadiazole-7-carbothioic acid-S-methyl ester induces systemic resistance in tomato (*Lycopersicon esculentum*. Mill cv. Vollendung) to cucumber mosaic virus. Crop Protect. 19, 401-405.

Antoniw, J.F. and White, R.F., 1983. Biochemical properties of the pathogenesis-related proteins from tobacco. Netherland J. Plant Pathol. 89, 255-264.

Apostol, I., Heinstein, P.F. and Low, P.S., 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol. 90, 109-116.

Awade, A., De Tapia, M., Didierjean, L. and Burkhard, G., 1989. Biological function of bean pathogenesis-related (PR3 and PR4) proteins. Plant Science, 63, 121-130.

Basim, H., Yegen, O. and Zeller, W., 2000. Antibacterial effect of essential oil of *Thymbra spicata* L. var. *spicata* on some plant pathogenic bacteria. J. Plant Dis. and Protect. 107, 279-284.

Batra, G.K. and Kuhn, C.W., 1975. Polyphenoloxidase and peroxidase activities associated with acquired resistance and its inhibition by 2-thiouracil in virus infected soybean. Physiol. Plant Pathol. 5, 239-248.

Beer, S.V. and Norelli, J.L., 1986**.** Newsletter international working group of fire blight research. 1987, 32-40.

Beer, A. and Rundle, I.R., 1987. Disinfectants for treating pruning shears used for fire blight control. Acta Horticult., 217, 243.

Bereswill, S., Bugert, P., Bruchmüller, I. and Geider, K., 1995. Identification of *Erwinia amylovora* by PCR with chromosomal DNA. Appl. Environm. Microbiol. 61, 2636-2642.

Bereswill, S., Pahl, A., Bellemann, P., Zeller, W. and Geider, K., 1992. Sensitive and species–specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. Appl. Environm. Microbiol. 58, 3522-3526.

Berger, F., Bereswill, S., Geider, K. and Zeller, W., 1995. Diagnose des Feuerbranderregers (*Erwinia amylovora*) mit Hilfe der Polymerase-Kettenreaktion (PCR) und erste Ergebnisse zum Monitoring. Nachrichtenbl. Deut. Pflanzenschutzd. 47, 105-108.

Billot, J., 1986. Variations des monophenols et des orthodiphenols au cours de la croissance et de la maturation de la piore `Passe Crassane`. In: ´´Groupe Polyphenols``Bulletin de Liaison; 13. Montpellier, France, 428-430.

Binder, A., Baer, G., Hofmann, C., and Kovats, K., 1989. Mechanisms in systemic induced disease resistance. In: Lugtenberg, B. J. J. (ed.): Signal Molecules in Plants and Plant-Microbe Interactions. Nato ASI Series vol. 36, 267.

Bol, J.F., Linthorst, H.J.M. and Cornellissen, B.J.C., 1990. Plant pathogenesis-related proteins induced by virus infection. Ann. Rev. Phytopathol. 28, 113-138.

Boller, T., Gehri, A., Mauch, F, and Vogeli, U., 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta, 157, 22-31.

Bolter, O., Brammall, A., Cohen, R. and Lazarovits, G., 1993. Glutathione alterations in melon and tomato roots following treatment with chemicals which induce disease resistance to *Fusarium* wilt. Physiol. Mol. Plant Pathol. 42, 321-336.

Bolwell, G.P., Robbins, M.P. and Dixon, R.A., 1985. Metabolic changes in elicitor treated bean cells. Enzymic responses associated with rapid changes in cell wall components. Europ. J. Biochem. 148, 571-578.

Bonhoff, A., Rieth, B., Golecki, J. and Grisebach, H., 1987. Race cultivar-specific differences in callose deposition in soybean roots following infection with *Phytophthora megaperma f.* sp. *glycinea*. Planta (Berl.) 172, 101-105.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-250.

Bradley, D.J., Kjellbom, P. and Lamb, C.J., 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defence response. Cell, 70, 21-30.

Brisset, M.N., Cesbron, S., Thomson, S.W. and Paulin, C.P., 2000. Acibenzolar-S-methyl induces the accumulation of defence–related enzymes in apple and protects from fire blight. Europ. J. Plant Pathol. 106, 529-536.

Carr, J.P. and Klessig, D.F., 1989. The pathogenesis related protein of plants. In: J.K. Setlow, ed., Genetic Engineering, Principles and Methods. Plenum Press, N.Y., 65-109.

Chen, Z., Ricigliano, J.W. and Klessig, D.F., 1993. Purification and characterization of a soluble salicylic-acid binding protein from tobacco. Proc. Natl. Acad. Sci. USA, 90, 9533-9537.

Cohen, Y., Gisi, U. and Mösinger, E., 1991. Systemic resistance of potato plants against *Phytophthora infestans* induced by unsaturated fatty acids. Physiol. Mol. Plant Pathol. 38, 255-263.

Conrath, U., Chen, Z., Ricigliano, J.W. and Klessig, D.F., 1995. Two inducers of plant defense responses, 2,6-dichloroisonicotinic acid and salicylic acid, inhibit catalase activity in tobacco. Proc. Natl. Acad. Sci. USA, 92, 7143-7147.

Daafy, F., Schmit, A. and Belanger, R.R., 1995. The effects of plant extract of *Reynoutria sachalinensis* on powdery mildew development and leaf physiology of long English cucumber. Plant Dis. 79, 577-580.

Dalton, A.D., Russell, S.A., Hanus, F.J., Pascoe, G.A. and Evans, J.H., 1986. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. Proc. Natl. Acad. Sci. USA, 83, 3811-3815.

Darvill, A.G., and Albersheim P., 1984. Phytoalexins and their elicitors - a defense against microbial infection in plants. Ann. Rev. Plant Physiol. 35, 243-275.

Dat, J.F., Lopez-Delgado, H., Foyer, C.H. and Scott, I.M., 2000. Effects of salicylic acid on oxidative stress and thermotolerance in tobacco. J. Plant Physiol. 156, 659-665.

De Tapia, M., Bergemann, V., Awade, A. and Burkar, D.G., 1986. Analysis of acid extractable bean leaf proteins induced by mercuric chloride treatment and alfalfa mosaic virus infection. Partial purification and characterization. Plant Science 45, 167-177.

Dick, A.J., Williams, R., Bearne, S.L., and Lidster, P.D., 1985. Quercetin glycosides and chlorogenic acid: Inhibitors of apple β-galactosidase and apple softening. J. Agr. Food Chem. 33, 798 -800.

Dixon, A.R. and Lamb, C.J., 1990. Molecular communication in interaction between plants and microbial pathogens. Ann. Rev. Plant Physiol. Plant Mol. Biol. 41, 339-367.

Doubrava, N.S., Dean R.A. and Kuć, J., 1988. Induction of systemic resistance to antracnosis caused by *Collectotrichum lagenarium* in cucumber by oxalate and extracts of spinach and rhubarb leaves. Physiol. Mol. Plant Pathol. 33, 69-79.

Dudler, R., Hertig, C., Rebmann, G., Bull, J., and Mauch, F., 1991. A pathogen-induced wheat gene encodes a protein homologues to glutathione-S-transferases. Mol. Plant Microbe Interac. 4, 14-18.

Elliston, J., Kuć, J., Williams, E.B. and Rahe, J.E., 1977. Relationship of phytoalexin accumulation to local and systemic protection of bean against anthracnose. Phytopathology. Z. 88, 114 -130.

Enyedi, A.J., Yalpani, N., Silverman, P., and Raskin, I., 1992. Signal molecules in systemic plant resistance to pathogens and pests. Cell, 70, 879-886.

Farkas, G.L. and Ledingman G.A., 1959. Studies on the polyphenol-oxidase system of wheat stem rust uredospores. Can. J. Microbiol. 5, 37-46.

Feucht, W. and Treutter, D., 1989. Phenolische Naturstoffe. Obst und Gartenbauverlag, München.

Feucht, W., Treutter, D., and Christ, E., 1992. The precise localization of catechins and proanthocyanidins in protective layers around fungal infections. Z. Pflkrankh. Pflschutz. 99, 404-413.

Fischer, W., Schweitzer, P., Christ, U., Mosinger, E., Kovats, K., Baer, G., and Binder, A., 1988. Mechanisms in systemic induced disease resistance. Phytoparasitica, 16, 211.

Fried, A., 1999. Feuerbrand-Bekämpfungsversuche 1998. Obstbau 2/ 99 71-74.

Fried, A., Moltmann, E. and Jelkmann, W., 1998. Feuerbrand-Bekämpfung in Feldversuchen 97/98. Prüfung einiger alternativer Mittel zu Plantomycin. Mitt. Biol. Bundesanstalt Land- und Forstwirtschaft, 322, 213-214.

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

Fritz, I.G., 1996. Physiologische Wirkungen von Resistenzinduktoren, primäre und sekundäre Reaktionen im Wirt-Pathogen-System Gurke/ echter Gurkenmehltau. Dissertation, TH, Darmstadt.

Gehrisch, S.H., Mosch, J. and Zeller, W., 1996. Untersuchungen zur induzierten Resistenz von Pflanzenextrakten gegenüber der Adernschwärze des Kohls (*Xanthomonas campestris* pv. *campestris*) Mittl. Biol. Bundesanstalt, 321, 271.

Goodman, R.N., Kiraly, Z., and Wood, K.R., 1986. The Biochemistry and Physiology of Plant Diseases. University of Missouri Press, Columbia, USA.

Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, E., 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. Plant Cell 8, 629-643.

Gottstein, D. and Kuć, J., 1989. Induction of systemic resistance to anthracnose in cucumber by phosphates. Phytopathology 79, 176-179.

Habig W.H., Pabst M.J. and Jakoby W.B., 1974. Glutathione–S-transferase: the first enzymatic step in mercapturic acid formation . J. Biol. Chem. 249, 7130-7139.

Hahlbrock, K., and Scheel, D., 1989. Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant. Physiol. Plant Mol. Biol. 40, 347-364.

Hahlbrock, K., Scheel, D., Logemann, E., Nürnberger, T., Parniske, M., Reinnold, S., Sacks, W.R. and Schmelzer, E., 1995. Oligopeptide elicitor- mediated defense gene activation in cultured parsley cells. Proc. Natl. Acad. Sci. USA 92, 4134- 4137.

Hamdan, M.A.M.S. and Dixon, R.A., 1987. Differential patterns of protein synthesis in bean cells exposed to elicitor fractions from *Colletotrichum lindemuthianum*. Physiol. Mol. Plant Pathol. 31, 105-121.

Hammerschmidt, R. and Kuć, J., 1995. Induced Resistance to Disease in Plants. Kluwer Acad. Publ., Dordrecht, Netherlands.

Hammerschmidt, R., Nuckles, E.M. and Kuć. J., 1982**.** Association of enhanced peroxidase activity with induced resistance systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiol. Plant Pathol. 20, 73-82.

Heath, M.C., 1980. Reactions of nonsuscepts to fungal pathogens. Ann. Rev. Phytopathol. 18, 211-236

Heimler, D. and Boddi, V., 1989. Chromatographic separation of 14 different flavonoids-comparison of chromatographic behaviour and substitution pattern. J. Chromatogr. 466, 371.

Hildebrand, D.C. and Schroth, M.N., 1964. Fireblight resistance in *Pyrus*: Localization of arbutin and beta glucosidase. Phytopathology 74, 1534-1539.

Ingham, J.L., Keen, N.T., Mulheiern, L.J. and Lyne, R.L., 1981. Inducibly formed isoflavonoids from leaves of soybean. Phytochem. 20, 795-798.

Irving, H.R. and Kuc, J., 1990. Local and systemic induction of peroxidase, chitinase and resistance in cucumber plants by potassium phosphate monobasic. Physiol. Mol. Plant Pathol. 37, 355-366.

Ishii, H., Tomita, Y., Horio, T., Narusaka, Y., Naksawa, Y., Nishimura, K. and Iwamoto, S., 1999. Induced resistance of acibenzolar-S-methyl (CGA 245704) to cucumber and Japanese pear diseases. Europ. J. Plant Pathol. 105, 77-85.

Jenns, A.E., Caruso, F.L. and Kuć, J., 1979. Non-specific resistance to pathogens induced systemically by local infection of cucumber with tobacco necrosis virus, *Colletotrichum lagenarium* or *Pseudomonas lachrymans*. Phytopathol. Mediter. 18, 129-134.

Jock, S., Rodoni, B., Gillings, M., Kim, W.S., Copes, C., Merriman, P., and Geider, K, 2000. Screening of ornamental plants from botanic gardens of Melbourne and Adelaide for the occurrence of *Erwinia amylovora*. Australasian Plant Pathol. 29, 120-128.

Kauffmann, S., Legrand, M., Geoffroy, P. and Fritig, B., 1987. Biological function of pathogenesis-related proteins. Four PR-proteins of tobacco have β-1,3-glucanase activity. EMBO J. 6, 3209-3212.

Keen, N.T., and Kennedy, B.W., 1974. Hydroxyphaseollin and related isoflavonoids in the hypersensitive resistance reaction of soybeans to *Pseudomonas glycinea*. Physiol. Plant Pathol. 4, 173-185.

Keen, N.T. and Yoshikawa, M., 1983. β-1,3-endoglucanase from soybean releases elicitor-active carbohydrates from fungus cell walls. Plant Physiol., 71, 460-465.

Kessmann, H., Oostendorp, M., Ruess, W., Staub, T., Kunz, W. and Ryals, J., 1996. Systemic activated resistance – a new technology for plant disease control. Pesticide Outlook. June, 1996, 256-257.

Kessmann, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., Uknes, S. and Ryals, J., 1994 a. Induction of systemic acquired resistance in plants by chemicals. Ann. Rev. Phytopathol. 32, 439-459.

Kessmann, H., Staub, T., Ligon, J., Oostendorp, M. and Ryals, J., 1994 b. Activation of systemic acquired disease resistance in plants. Europ. J. Plant Pathol. 100, 359-369.

Klement, Z., Mavridis, A., Rudolph, K., Vidaver, A., Perombelon, M.C.M. and Moore, L.W., 1990. Inoculation of plant tissues. In: Klement, Z., Rudolph, K. and Sands, D.C., edts. : Methods in Phytobacteriology, Akademiai Kiadó, Budapest, Hungary, pp. 95- 124.

Kloepper, J.W., Tuzun, S. and Kuć J., 1992. Proposed definitions related to induced disease resistance. Biocont. Sci. and Tech. 2, 349.

König, B. and Dustmann, J.H., 1985. The caffeolics as a new family of natural antiviral compounds. Naturwiss. Rundschau, 72, 59-61.

König, B. and Dustmann, J.H., 1988. Baumharze, Bienen und antivirale Chemotherapie. Naturwiss. Rundschau, 41, 43-53.

Kuć J., 1987. Plant immunization and its applicability for disease control, in I. Chet (ed.) Innovative Approaches to Plant Disease Control, J. Wiley &Sons, New York, 255-274.

Kuć J., 1995. Phytoalexins, stress metabolism, and disease resistance in plants. Ann. Rev. Phytopathol. 33, 275-297.

Kunoh, H., Kuroda, K., Hayashimoto, A. and Ishizaki, H., 1985. Induced susceptibility and enhanced resistance at the cellular level in barley coleoptiles. II. Timing and localization of induced susceptibility in a single coleoptile cell and its transfer to an adjacent cell. Can. J. Bot. 64, 889-901.

Kurosaki, F., Tashiro, N. and Nishi, A., 1986. Induction of chitinase and phenylalanine ammonia–lyase in cultured carrot cells. Phytochem. 26, 1919-1923.

Lagrimini, L.M. and Rothstein, S., 1987. Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. Plant Physiol. 84, 438-442.

Larson, R.A., 1988. The antioxidants of higher plants. Phytochem. 27, 969-978.

Laux, P., Hofer, K. und Zeller, W., 1999. Untersuchungen im Freiland zur Bekämpfung des Feuerbrandes mit bakteriellen Antagonisten. 2. Symposium Phytomedizin und Pflanzenschutz im Gartenbau, 27.- 30. 09. 1999, Wien, 53-54.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T. and Ryals, J., 1996. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J. 10, 71-82.

Legrand, M., Kauffmann, S., Geoffroy, P. and Fritig, B., 1987. Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. Proc. Nat. Acad. Sci. USA, 84, 6750-6754.

Lelliott, R., 1959. Fire blight of pears in England, Agriculture (London) 65, 564-568.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C., 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell, 79, 1-20.

Li, A., Luo, K. and Liu, Y.J., 1999. Studies on physiological indicators of near-isogenic rice lines in interaction with *Xanthomonas oryzae* pv. *oryzae*. Acta Phytophylacica Sinica, 26, 35-39

Llop, P., Bonterra, A., Pefialver, J. and Lopez, M.M., 2000. Development of a highly nested-PCR procedure using a single closet tube for detection of *Erwinia amylovora* in asymptomatic plant material. Appl. Environm. Microb. 66, 2071-2078.

Louws, F.J., Wilson, M., Campbell, H.L., Cuppels, D.A., Jones, J.B., Shoemaker, P.B., Sahin, F. and Miller, S.A., 2001. Field control of bacterial spot and bacterial speck of tomato using a plant activator. Plant Disease, 85, 481-488.

Lozano, J.C. and Sequeira, L., 1970. Differentiation of races of *Pseudomonas solanacearum* by a leaf infiltration technique. Phytopathology, 60, 834-840.

Lund, B.M. and Lyon, G.D., 1975. Detection of inhibitors of *Erwinia amylovora* and *E. herbicola* on thin-layer chromatograms. J. Chromatogr. 110, 193-196.

Malamy, J., Carr, J.P., Klessing, D.F. and Raskin, I., 1990. Salicylic acid: A likely endogenous signal in the resistance response of tabocco to viral infection. Science, 250, 1002-1003.

Manulis, S., Zutra, D. and Kleitman, F., 1999. Streptomycin resistance of *Erwinia amylovora* in Israel and occurence of Fire Blight in pear orchards in the autumn. Acta Horticult. 489, 85-91.

Matern, U. and Kneusel, R., 1988. Phenolic compounds in plant disease resistance. Phytoparasitica 16, 153-170.

Mauch, F. and Dudler, R., 1993. Differential induction of distinct glutathione-S-transferases of wheat by xenobiotics and by pathogen attack. Plant Physiol. 102, 1192-1201.

Mayer, A.M. and Harel, E., 1979. Polyphenoloxidases in plants. Phytochem. 18, 193-215.

Mayr, U., 1995. Einfluβ des Phenolstoffwechsels beim Apfel (*Malus domestica*) auf das Resistenzpotential gegen Apfelschorf (*Venturia inaequalis*). Dissertation T.U. München.

Mayr, U., Treutter, D., Santos-Buelga, H., Bauer, H. and Feucht, W., 1995. Developmental changes in the phenol concentrations of *Golden Delicious* apple fruits and leaves. Phytochem. 38, 1151-1155.

Mende, A., Mosch, J. and Zeller, W., 1993. Untersuchungen zur Resistenzinduktion durch ausgewählte Pflanzenextrakte gegen den Feuerbrand (*Erwinia amylovora* (Burril) Winslow *et al*.). Z. Pflanzkrankh. Pflschutz. 101, 141.

Métraux, J.P., Ahl Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J. and Ward, E., 1991. Induced resistance in cucumber in response to 2,6–dichlor-isonicotinic acid and pathogens. H. Hennecke & D.P.S. Verma (eds) „*Advances in Molecular Genetics of Plant–Microbe Interactions*". 432-439.

Métraux, J.P. and Boller, T., 1986. Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial and fungal infections. Physiol. Mol. Plant Pathol. 56, 161-169**.**

Métraux, J.P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M. and Ryals, J., 1989. Isolation of complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme / chitinase. Proc. Nat. Acad. Sci. USA. 86, 896-900.

Métraux, J.P., Signer, H., Ryals, J., Word, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. and Inverardi, B., 1990. Increases in salicylic acid at the onset of systemic acquired resistance in cucumber. Science, 250, 1004-1005.

Métraux, J.P., Streit, L. and Staub, T., 1988. A pathogenesis related protein in cucumber is chitinase. Physiol. Mol. Plant Pathol., 33, 1-10.

Miller, T.D. and Schroth, M.N., 1972. Monitoring the epiphytic populations of *Erwinia amylovora* on pear with selective medium. Phytopathology, 62, 1175- 1182.

Mosch, J., Klinghauf, F. and Zeller, W., 1989. On the effect of plant extracts against fire blight (*Erwinia amylovora*). Acta Horticult. 273, 355-361.

Mosch, J., Mende, A., Zeller, W., Rieck, M. and Ulrich, W., 1993. Plant extracts with a resistance induction effect against fire blight *(Erwinia amylovora).* Acta Horticult. 338, 389-395.

Mosch, J. and Zeller, W., 1989**.** Control of fire blight with plant extracts based on resistance induction. Acta Horticult. 489, 577-582.

Mosch, J. and Zeller, W., 1997. „Praxisorientierte Versuche zum Einsatz von Pflanzenextrakten mit resistenzinduzierenden Eigenschaften gegenüber Bakteriosen“. Arbeitsbericht 5. „Biologischer Pflanzenschutz durch Induzierte Resistenz“ Biologische Bundesanstalt für Land und Forstwirtschaft.

Mosch, J., Zeller, W., Rieck, M. and Ulrich, W., 1996. Further studies on plant extracts with a resistance induction effect against *Erwinia amylovora* . Acta Horticult. 411, 361- 366**.**

Mozer, T.J., Tiemeier, D.C. and Jaworski, E.G., 1983. Purification and characterization of corn glutathione-S-transferase. Biochem. 22, 1068-1072.

Mucharromah, E., and Kuć, J., 1991. Oxalate and phosphate induce systemic acquired resistance against disease caused by fungi, bacteria and viruses in cucumber. Crop Protec. 10, 265-270.

Nasuraka, Y., Nasuraka, M., Horio, T. and Ishii, H., 1999. Comparison of local and systemic induction of disease resistance in cucumber plants treated with benzothiadiazoles or salicylic acid. Plant Cell Physiol. 40, 388-395.

Neu, R., 1957. Ein neues Reagenz zum Nachweis und zur Untersheidung von Flavonen im Papierchromotogramm, Naturwiss. 43, 82.

Nicholson, L.R., 1992. Phenolic compounds and their role in disease resistance. Ann. Rev. Phytopathol. 30, 369-389.

Ojalvo, I., Rokem J.S., Navon, G. and Goldberg, I., 1987. ^{31}P-NMR Study of elicitor treated *Phaseolus vulgaris* cell suspension culture. Plant Physiol. 85, 716-719.

Oostendorp, M., Kessmann, H., Friedrich, L., Geissmann, A., Görlach, J., Hengy, G., Nordmeyer, D., Reist, R., Ruess, W., and Staub, T., 1996. Influence of plant activator BION® and of triazole-fungicides on plant defence mechanisms. Gesunde Pflanze, 48, 260-264.

Ouchi, S., Oku, H., Hibino, C. and Akiyama, I., 1976. Localization of induced resistance and susceptibility in barley leaves inoculated with the powdery mildew fungus. Phytopathology, 66, 901.

Peng, M. and Kuć J., 1992. Peroxidase-generated hydrogen peroxide as a source of antifungal activity *in vitro* and on tobacco leaf disks. Phytopathology, 82, 696-699.

Pincemail, J., Deby, Y.C., Lion, P., Braquet, P., Hans D.K. and Goutier, R., 1986. Role of flavonoids in lipoperoxidation and radical reactions. In: "*Flavonoids and Bioflavonoids*" 1985 (Farkas, L.; Gabor, M; Kallay F. eds.) Elsesevier, Amsterdam, 423-436.

Psallidas, P.G. and Tsiantos, J., 2000**.** Chemical control of fire blight. In Fire Blight: the Disease and its Causative Agent, *Erwinia amylovora* (ed. J. L. Vanneste), CAB International, Hamilton, New Zealand.

Putter, J., 1974. Peroxidase, In: Bergmeyer, H.U.(ed), Methoden der enzymatischen Analyse, Verlag Chemie, Weinheim, 725.

Rapp, A. und Ziegler, A., 1973. Bestimmung der Phenolcarbonsäure in Rebblättern, Weintrauben und Wein mittels Polyamid-Dünnschichtchromatographie. Vitis 12, 226-236.

Raskin, I., 1992. Role of salicylic acid in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 43, 439-463.

Rasmussen, J.B., Hammerschmidt, R. and Zook, M.N., 1991. Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. Plant Physiol. 97, 1342-1347.

Redolfi, P., Cantisani, A. and Migheli., Q., 1989. The pathogenesis-related proteins of *Phaseolus* bean. J. Phytopathol. 126, 193-203.

Rieck, M., 1992. Induzierte Resistenz gegen Feuerbrand: Wirkung verschiedener Pflanzenextrakte auf die Krankheitsentwicklung und Aktivierung resistenzbezogener Enzyme bei *Cotoneaster watereri*. Diplomarbeit, Technische Hochschule, Darmstadt.

Robbins, M.P., Hartnoll, J., and Morris, P., 1991. Phenylpropanoid defense responses in transgenic *Lotus corniculatus* 1. Glutathione elicitation of phytoalexins in transformed root cultures. Plant Cell Reports 10, 59-62.

Rudolph, K., 1970. Qualitative und quantitative Enzymveränderungen nach Infektion von Bohnenblättern mit *Pseudomonas phaseolicola* und *Uromyces phaseoli*. Angew. Botanik 44, 347-359.

Rudolph, K.W.E., 1995. Pseudomonas syringae pathovars. In: U.S. Singh, R.P. Singh and K. Kohmoto (edts): Pathogenesis and Host Specificity in Plant Diseases, Vol. I: Prokaryotes, Pergamon/Elsevier Science Ltd., Oxford, New York, Tokyo, 47-138.

Ruess, W., Kunz,W., Staub, T., Poppinger, N., and Ahly-Goy, P., 1995. Plant activator CGA 245704, a new technology for disease management XIII. International Plant Protection Congress, The Hague. The Netherlands, 2/7/July, 1995.

Russell, D.F., 1989. MSTAT (version 1.41). Crop and Soil Science Department. Michigan State University

Ryals, J. Uknes, S. and Ward E., 1994. Update on plant microbe interaction systemic acquired resistance. Plant Physiol. 104, 1109-1112.

Salt, S.D., Tuzun, S. and Kuć J., 1986. Effects of β-ionone and abscisic acid on the growth of tobacco and resistance to blue mold. Mimicry of effects of stem infection by *Peronospora tabacina* Adam. Physiol. and Mol. Plant Pathol. 28, 287-297.

Schneider, S. and Ulrich, R., 1994. Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. Physiol. Mol. Plant Pathol. 45, 291-304.

Schönbeck, F., Dehne, H. W. and Beicht, W., 1980. Activation of unspesific resistance mechanisms in plants. J. Plant Dis. Protect. 87, 654-666.

Schultz, I., 1987**.** Untersuchungen zum Wirkungsmechanismus des extrazellularen Polysaccharids (EPS) von *Erwinia amylovora* auf den Phenolstoffwechsel der Quitte, *Cydonia vulgaris.* Diplomarbeit, Biologische Fakultät der Universität Heidelberg.

Schweizer, P., Hunziker, W. and Mosinger, E., 1989. cDNA cloning *in vitro* transcription and partial sequence analysis of mRNA from winter wheat (*Triticum aestivum* L.) with induced resistance to *Erysiphe graminis* f. sp. *tritici*. Plant Mol. Biol. 12, 643-654.

Scortichini, M. and Rossi, M.P., 1989. *In vitro* activity of some essential oils toward *Erwinia amylovora* (Burril) Winslow *et al.* Acta Phytopathol. et Entomol. Hungarica 4, 423-431.

Scortichini, M. and Rossi, M.P., 1991. Preliminary *in vitro* evaluation of the anti-microbial activity of terpene and terpenoids towards *Erwinia amylovora* (Burrill) Winslow *et al*. J. Appl. Bacteriol. 71, 109-112.

Scortichini, M. and Rossi, M.P., 1993. *In vitro* behaviour of *Erwinia amylovora* towards some natural products showing bactericidal activity. Acta Horticult. 338, 191-198.

Sekizawa, Y. and Watanabe, T., 1981. On the mode of action of probenazole against rice blast. J. Pesticide Sci. 6, 247.

Sequeira, L., 1983. Mechanisms of induced resistance in plants. Annu. Rev. Microbiol. 37, 51-79.

Shulaev, V., Leon, J. and Raskin, I., 1995. Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? The Plant Cell, 7, 1691-1701.

Siegrist, J., Glenewinkel, D., Kolle, C. and Schmidtke, M., 1997. Chemical induced resistance in green bean against bacterial and fungal pathogens. J. Plant Dis. and Protect. 104, 599-610.

Siegrist, J., Jeblick, W. and Kauss, H., 1994. Defence responses in infected and elicited cucumber (*Cucumis sativus* L.) hypocotyl segments exhibiting acquired resistance. Plant Physiol. 105, 1365-1374.

Smith, J.A., and Hammerschmidt, R., 1998. Comparative study of acidic peroxidases associated with induced resistance in cucumber, muskmelon and watermelon. Physiol. Mol. Plant Pathol. 33, 255-261.

Smith, J.A., and Metraux, J.P., 1991. *Pseudomonas syringae* pv. *syringae* induces systemic resistance to *Pyricularia oryzae* in rice. Physiol. Mol. Plant Pathol. 39, 451-457.

Sorata, Y., Takahama, U. and Rimura, M., 1984. Protective effect of quercetin and rutin on photosensitized lysis of human erythrocytes in the presence of hematoporphyrin. Biochem. Biophys. Acta, 799, 313-317.

Stadnik, M.J. and Buchenauer, H., 1998. Histological and physiological changes in wheat plants by a benzothiadiazole derivate. In: Modern Fungicides and Antifungal Compounds II 12th International Reinhardsbrunn Symposium 357-366.

Stermer, B.A., 1995. Molecular regulation of systemic induced resistance In: Hammerschmidt, R. and Kuć, J. eds., Induced Resistance to Disease in Plants. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 111-140.

Takahama, U., Youngman, R.J. and Elstner, E.F., 1984. Transformation of quercetin by singlet oxygen generated by a photosensitised reaction. Phytobiochem. Photobiophys. 7, 175-181.

Tata, S.J., Beintema, J.J. and Balabaskaron, S., 1983. The lysozyme of *Hevea brasiliensis* latex; isolation purification, enzyme kinetics and a partial amino acid sequence. J. Rubb. Res. Inst. Malaysia 31, 34-35.

Torel, J., Cillard, J., and Cillar, P., 1986. Antioxidant activity of flavonoids and reactivity with peroxyradical. Phytochem. 25, 383-385.

Treutter D., Santos-Buelga, C., Gutmann, M. and Kolodziej, H., 1994. Identification of flavan-3-ols and procyanidins by high performance liquid chromatography and chemical reaction detection. J. Chromatogr. 667, 290-297.

Treutter, D., 1996. Phenole sind Pflanzeninhaltsstoffe mit zahlreichen Funktionen. Pflanzenschutz Obstbau 2, 96-98.

Tuzun, S., Rao, M.N., Vogelli, L., Schardle, C., L. and Kuć, J., 1989. Induced systemic acquired resistance to blue mold; early induction and accumulation of chitinase, β-1,3 glucanase and other pathogenesis-related (b-) proteins in immunized tobacco. Phytopathology, 79, 979-983.

Uknes, S., Mauch, M.B., Moyer, M., Potter, S. and Williams, S., 1992. Acquired resistance in *Arabidopsis*. Plant Cell 4, 645-656.

Vallejos, C.E., 1983. Enzyme staining. In: Isoenzymes in Plant Genetics and Breeding. Part A, Tanskley, S. D. and Orton, T. J. eds. Elsevier, Amsterdam, 469.

Van de Rhee, M.D., van Kan, J.A.L., Gonzales- Jaen, M.T. and Bol, J.F., 1990. Analysis of regulatory elements involved in the induction of two tobacco genes by salicylate treatment and virus infection. Plant Cell 2, 357-366.

Van der Zwet, T. and Beer, S.V. 1992**.** Fire Blight - its Nature, Prevention and Control: a Practical Guide to Integrated Disease Management. U.S. Dep. Agric. Inf. Bull. 631.

Van der Zwet, T. and Bonn, W.G., 1999**.** Recent spread and current worlwide distribution of fire blight. Acta Horticult. 489, 167-168.

Vance, C.P., Kirk, T.K. and Sherwood, R.T., 1980. Lignification as a mechanism of disease resistance. Ann. Rev. Phytopathol.18, 259-288.

Vanneste, J.L., 2000. Fire blight, the disease and its causative agent, *Erwinia amylovora*. CABI Publishing Wallingford, UK ISBN 0-85199-293-3.

Vaughn K.C. and Duke, S.O., 1984. Function of polyphenoloxidase in higher plants. Physiol. Plant 60, 106.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals J., 1994. Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell, 6, 959-965.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl Goy, P., Metraux, J.P. and Ryals, J.A., 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell, 3, 1085-1094.

Wichtl, M., 1997. *Hedera folium*. In: Teedrogen und Phytopharmaka: ein Handbuch für die Praxis auf wissenschaftlicher Grundlage pp. 280-282.

Williams, A.H., 1955. Phenolic substances of pear–apple hybrids. Nature, 175, 213.

Williams, A.H., 1982. Chemical evidence from flavonoids relevant to the classification of *Malus* species. Bot. Journ. Linn. Soc. 84, 31-39.

Wilson, C.M., 1983. Staining of proteins on gels: comparison of dyes and procedures. Meth. Enzymol. 91, 263-247.

Wingate, V.P., M., Lawton, M.A. and Lamb, C.J., 1988. Glutathione causes a massive and selective induction of plant defence genes. Plant Physiol. 87, 206-210.

Wirth S.J. and Wolf, G.A., 1990. Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. J. Microbiol. Methods 12, 197-205.

Wirth S.J. and Wolf, G.A., 1992. Micro-plate colorimetric assay for endo-acting cellulase, xylanase, chitinase β-1,3–glucanase and amylase extracted from forest soil horizons. Soil Biol. Biochem. 24, 511-519.

Wojtaszek, P., Trethowan, J., and Bolwell, G.P., 1995. Specificity in the immobilisation of cell wall proteins in response to different elicitor molecules in suspension cultured cells of French bean (*Phaseolus vulgaris*). Plant Mol. Biol. 28, 1075-1087.

Yamada, T., Hiramoto, T., Tobimatsu, R., Shiraishi, T. and Oku, H., 1990. Elicitor like substances present in barley and wheat seeds. J. Phytopathol. 128, 89-98.

Ye, X.S., Pan, S.Q. and Kuć, J., 1990. Activity, isozyme pattern, and cellular localization of peroxidases as related to systemic resistance of tobacco to blue mold (*Peronospora tabacina*) and to tobacco mosaic virus. Physiol. Mol. Plant Pathol. 36, 523-531.

Yoshikawa, M., Takeuchi, Y. and Horino, O., 1990. A mechanism for ethylene-induced disease resistance in soybean: enhanced synthesis of an elicitor-releasing factor, β-1,3-endoglucanase. Physiol. Mol. Plant Pathol. 37, 367-376.

Zeller, W., 1985. Untersuchungen zur Feuerbrandkrankheit in der Bundesrepublik Deutschland. Habilitationsschrift, Universität Hannover.

Zeller, V., 1998. Einsatz von Pflanzenstärkungsmitteln zur biologischen Bekämpfung des Feuerbrandes. Diplomarbeit, Fachhochschule Osnabrück.

Zeller, W. and Brulez, W., 1987. Changes in the phenol metabolism of ornamental shrubs (*Cotoneaster* species) infected with *Erwinia amylovora*. Proc. 6th. Intern. Conf. Plant Path. Bac. Beltsville, Maryland, USA, 686-694.

Zeller, W. und Meyer, J., 1975. Untersuchungen zur Feuerbrandkrankheit in der Bundesrepublik Deutschland. 1. Krankheitsverlauf von Obst-und Ziergehölzen nach natürlichem Befall und künstlicher Infektion. Nachrichtenbl. Dt. Pflschutzd. (Braunschweig) 27, 154-156.

Zeller, W. and Wolf, B., 1996. Studies on biological control of fire blight. Acta Horticult. 411, 341-345.

Zeller, V. and Zeller, W., 1998. Studies on control of fire blight with plant activator BION.® Acta Horticult. 489, 639-645.

Zinkernagel, V., 1986. Untersuchungen zur Anfälligkeit und Resistenz von Kopfsalat (*Lactuca sativa*) gegen falschen Mehltau (*Bremia lactucae*). III Peroxidatische Katalase- und Phenoloxidase-Aktivitäten. J. Phytopatho1. 115, 257-266.

Zucker, M., 1968. Sequential induction of phenylalanine ammonia–lyase and a lyase inactivating system in potato tuber disks. Plant Physiol. 43, 365-374**.**

Danksagung

Vor allem danke ich Herrn Prof. Dr. W. Zeller für die Bereitstellung des Arbeitsplatzes in der Biologischen Bundesanstalt für Land- und Forstwirtschaft (BBA), Institut für biologischen Pflanzenschutz, in Darmstadt und für die freundliche Zusammenarbeit und seine konstruktive Diskussionsbereitschaft.

Den Herren Prof. Dr. H.-H. Hoppe, Prof. Dr. P. L. G. Vlek und Prof. Dr. D. Gradman sage ich dank für die Übernahme des Referates und Korreferates und der mündlichen Prüfung. Vom Institut für Pflanzenpathologie und Pflanzenschutz der Universität Göttingen danke ich besonders herzlich Herrn Dr. K. Rudolph für die kritische Durchsicht und Korrektur der gesamten Arbeit und Herrn Dr. A. Mavridis für die mir erwiesene Hilfsbereitschaft. Herrn Dr. habil. D. Treutter von der TU München möchte ich für die HPLC Analysen und seine Gesprächsbereitschaft danken.

Ein besonderes Dankeschön geht an Herrn Dr. P. Laux für seine über das gewöhnliche Maß hinausgehende Hilfsbereitschaft. Meinem Co-Doktoranden Herrn M. Gulati, Dr. J. Mosch sowie unserem Verwaltungschef Herrn W. Bernius, und Frau S. Schneider möchte ich meinen Dank für ihre Unterstützung und Hilfsbereitschaft aussprechen.

Lebenslauf

Name: Ömür Baysal
Geburtstag und -ort: 17. 06. 1972, Antalya / Türkei
Familienstand: verheiratet

Schulbildung / Weiterbildung

1978 – 1983	Grundschule Antalya
1983 – 1989	Gymnasium Antalya College
1989 – 1993	Agrarwissenschaftliche Fakultät der Akdeniz Universität in Antalya (Diplomabschluss)
1994 – 1997	Akdeniz Universität, Institut für Naturwissenschaftler Abt. Pflanzenpathologie und Pflanzenpathologie (Magisterarbeit)
1998 – 2001	Wissenschaftliche Arbeit in der Biologischen Bundesanstalt für Land- und Forstwirtschaft (BBA), Institut für biologischen Pflanzenschutz, Darmstadt (Promotion, an der Georg-August-Universität Göttingen, Institut für Pflanzenpathologie und Pflanzenschutz)
Seit 1998	Wissenschaftlicher Mitarbeiter am Agrarministerium im Institut für Pflanzenschutz, Izmir/ Türkei