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DETECTION AND EFFECTS OF LATENT CONTAMINATION OF POTATO TUBERS BY SOFT ROT BACTERIA, AND INVESTIGATIONS ON THE EFFECT OF HYDROGEN PEROXIDE ON LIPOPOLYSACCHARIDES OF *ERWINIA CAROTOVORA* IN RELATION TO ACQUIRED RESISTANCE AGAINST BIOCIDES

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LIST OF ABBREVIATIONS

APS	Ammoniumpersulphate
bd	Bidistilled
bidest.	Bidestilllata (=bidistilled)
°C	Degree Celisus
ca.	Circa
cfu	Colony forming units
cm	Centimetre
Co.	Company
COSY	Correlation spectroscopy
cv.	Cultivar
CVP	Crystal violet pectate
CVPB	Crystal violet pectate Bulmer
CVPD	Crystal violet pectate double layer
CVPM	Crystal violet pectate modified
DAS-ELISA	Double antibody sandwich-enzyme-linked immunosorbent assay
Dist.	Distilled
DLCVP	Double layer CVP medium
DNA	Deoxyribonucleic acid
DNTP	Desoxynuceotide-5`- Triphosphate
DSM	Deutsche Sammlung von Mikroorganismen
DTE	Dithioerithrol
Eca	*Erwinia carotovora ssp. atroseptica*
Ecc	*Erwinia carotovora ssp. carotovora*
Ech	*Erwinia chrysanthemi*
EDTA	Ethylenediaminetetraacetic acid
Fig.	Figure
g	Gram
GmbH	Gesellschaft mit beschränkter Haftung
GSPB	Göttinger Sammlung phytopathogener Bakterien
h	Hour (s)
ha	Hectare
IF	Immunofluorescence
IFC	Immunofluorescence colony staining
kg	Kilogram
LPS	Lipopolysaccharides
m	Metre
M	Molar

mA	milliampere
mg	milligram
min	minutes
ml	millilitre
mm	Millimetre
mPas X s	Millipascal times seconds
µg	Microgram
µl	Microlitre
N	North
NA	Nutrient agar
NMR	nuclear magnetic resonance
no.	Number
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
ppm	Parts per million
SDS	Sodium dodecyl sulphate
spp.	Species (plural)
ssp.	Subspecies
Tab.	Table
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
TES	Tris-EDTA-2% SDS
TEMED	N,N,N´,N´-Tetramethylethylenediamine
TOCSY	total correlation spectroscopy
Tris	Tris-(hydroxymethyl)-amino methane
TTC	2,3,5-Triphenyltetrazolium chloride
v/v	Volume per volume
w/w	Weight per weight
YDC	Yeast-dextrose-chalk agar

Dedicated

To my mother, my wife, our children and my late father

CHAPTER I

Detection of *Erwinia carotovora* in potato stocks using a semi-selective medium

1 INTRODUCTION

Seed potato tubers contaminated with *Erwinia carotovora subsp. atroseptica* (*Eca*) (van Hall 1902) Dye 1969 and *Erwinia carotovora subsp. carotovora* (*Ecc*) (Jones, 1901) Bergey, Harrison, Breed, Hammer & Huntoon, 1923, are considered the main source of primary inoculum for blackleg and soft rot of potato crops (LANGERFELD, 1984; HARJU and KANKILA, 1993; PEROMBELON and SALMOND, 1995). In addition *Ecc* can occur in many important horticultural crops (ALIPPI *et al*., 1997; CLARK *et al*., 1998). Both subspecies are commonly associated with potato tuber soft rot, but rot in the basal part of the stem (blackleg disease) is usually caused by *Eca* (PEROMBELON and KELMAN, 1980). *Ecc* occurs in both temperate and warm climates but mostly in storage. Since there exist no effective chemical control measures for the disease during storage or in the field, reduction in yield losses can only be achieved by following good crop husbandry, use of non-contaminated planting materials (TOTH *et al*., 1996) and cultivation of resistant or tolerant varietes (ROBINSON and FOSTER, 1987; DÖPKE and RUDOLPH, 1990; STEFANI *et al*., 1990). The rotting of mother tubers during the growing season has been reported as the major source of inoculum for contaminating progeny tubers, which later in storage, when conditions are favourable, could lead to losses due to soft rot of tubers (PEROMBELON; 1976, 1992).

A variety of different methods for detecting of the latent stage of the disease have been used by several authors (ALLAN and KELMAN, 1977; VRUGGINK and DE BOER, 1978; PEROMBELON *et al*., 1987). Especially, the crystal violet pectate medium (CVP) (CUPPELS & KELMAN, 1974) and several modified selective media (PEROMBELON and BURNETT, 1991; BDLIYA, 1995) are used for detection of potato soft rotting erwinias from potatoes. The dilution plating of samples onto a semi-selective medium to isolate the bacteria has been in use for a long time. Its use has been extended to the quantification and identification of erwinias in mixed populations from plants, when characteristic deep cavities are formed only by pectolytic soft rot erwinias on the semi-selective medium (AHMED *et al*., 2000). Sodium polypectate, the main component of CVPB used in the original formulation, was produced by H. P. Bulmer Ltd. (PEROMBELON and BURNETT, 1991). It is no longer available and preparations from other sources have been found to be unsatisfactory because of poor gelling capacity and because typical cavities were not formed on media containing these pectin preparations.

The purpose of this study was to develop an easy, reliable and sensitive microbiological test for quantifying the latent contamination of potato tubers by erwinias. The suitability of the assay developed was tested on a large scale in 3 vegetation periods in cooperation with Stöver Produktion GmbH to investigate the populations and survival of erwinias on superficially contaminated potato tubers.

2 MATERIAL AND METHODS

2.1 Media for bacteria

All the media used in this study were sterilised by autoclaving at 121 °C for 15 min. The pH of the media was adjusted using NaOH or HCl before autoclaving. Antibiotics were sterile filtered and added to the medium after autoclaving and cooling down to about 45-50 °C.

2.1.1 Yeast- dextrose- chalk agar (YDC) (LELLIOTT and STEAD, 1987)

Yeast extract (Oxoid L 21)	10.0 g
$CaCO_3$	20.0 g
D-Glucose	20.0 g
Agar	15.0 g
Dist. water	1000.0 ml

2.1.2 King's medium B (KING *et al.*, 1954)

Proteose peptone (Oxoid L 46)	20.0 g
K_2HPO_4	1.5 g
$MgSO_4$ X 7 H_2O	1.5 g
Glycerol	10.0 ml
Agar	15.0 g
Dist. water	1000.0 ml

pH 7.2

2.1.3 Crystal violet pectate (Bulmer) medium (CVPB) (PEROMBELON and BURNETT, 1991)

A-	Cold dist.water	500.0 ml
	aqueous crystal violet solution	1.0 ml (0.075% (w/v) solution)
	$CaCl_2$ x 2 H_2O	6.8 ml 10% (freshly prepared solution)
	$NaNO_3$	1.0 g
	tri-Na citrate dihydrate	2.5 g
	Agar	2.0 g
	Tryptone (Oxoid L 42)	0.5 g
B-	Sodium polypectate (Bulmer)	9.0 g

Add sodium polypectate slowly with constant stirring and autoclave at 121 °C for 15 min. Cool the medium to about 45 - 50 °C, add 2 ml of 1% novobiocin (sodium salt, Sigma) and pour into Petri plates.

2.1.4 Double layer CVP medium (DLCVP) (PEROMBELON and BURNETT, 1991)

Basal layer		Overlayer	
$CaCl_2$ x 2 H_2O	5.5 g	Sodium polypectate	25 g
Tryptone (Oxoid L 42)	1.0 g	EDTA (disodium salt)	20 ml 5%
Crystal violet solution	2.0 ml of 0.075%	Dist. water	1000 ml
$NaNO_3$	16.0 g	pH	7.0
Agar	15.0 g		
Dist. Water	1000 ml		
pH	7.0 – 7.5		

Before pouring, the basal layer was melted, supplemented with 40 µg/ml novobiocin, and 15 ml were dispensed into each Petri plate and allowed to set before pouring the 5 ml overlayer in each Petri plate.

2.1.5 Crystal violet pectate double layer medium (BDLIYA, 1995)

The semi-selective medium (CVPD) for isolation of pectolytic *Erwinia spp.* consists of two layers as follows :

Basal layer		Overlayer	
Peptone	2.0 g	Crystal violet solution	1.0 ml, 0.075% (w/v)
Yeast extract	0.6 g	KNO_3	1.0 g
NaCl	1.0 g	tri-Na citrate dihydrate	2.5 g
Agar	3.0 g	Agar	2.0 g
Tryptone	1.5 g	Tryptone	1.5 g
L-Asparagine	0.5 g	$CaCl_2$ X 2 H_2O	6.8 ml (10% solution)
SDS	50 mg	Bromothymol blue	0.5 ml (0.5% solution)
Dist. water	250 ml	Cold dist. water	500.0 ml
		Sodium polypectate "Bulmer" [1)]	9.0 g

1) H. P. Bulmer Ltd., Plough Lane, Hereford HR4 OLE, England.

Add 0.5 ml of filter sterilised 1% solution of 2,3,5-triphenyltetrazolium chloride to the basal medium after autoclaving and cooling down to 50 °C, and add 3.2 µg/ml Polymxin B sulphate solution. Dispense 12 ml of basal medium per plate and allow to set for 10 min. Then add 1.2

µg /ml Polymxin B sulphate to the overlayer solution after autoclaving and cooling down to 45 °C, and pour 15 ml/plate on top of the basal medium.

2.1.6 α-Methyl-d-glucoside medium (SAETTLER *et al.,* 1989)

The medium is made up of 5 parts

A-	KH_2PO_4	2.0	g
	K_2HPO_4	7.0	g
	NH_4Cl	1.0	g
	Dist. Water	500.0	ml
B-	Agar	15.0	g
	Casamino acids (Difco)	1.0	g
	Dist. water	500.0	ml
C-	$MgSO_4$ X 7 H_2O	1.0	ml (10% solution)

D- α-Methyl-d-glucoside

(Sigma M 9376) 50 ml (20% solution)

E - 2,3,5-Triphenyl-tetrazoliumchloride 2.0 ml (1% solution)

Each solution of the 5 parts is autoclaved separately. Mix A and B, cool down to 50 °C, add solutions C, D and E, and pour into Petri plates.

2.1.7 **Phosphatase detection medium (SAETTLER *et al*., 1989)**

Bacto peptone (Difco)	10.0	g
Beef extract	5.0	g
Agar	15.0	g
Dist. water	1000.0	ml

pH 7.0

2.1.8 Medium for indole production (SAETTLER *et al.,* 1989)

Tryptone (Difco)	10.0	g
L-Tryptophane	1.0	g
Dist. water	1000.0	ml

2.1.9 Kovac´s indole reagent (LELLIOTT and STEAD, 1987)

p- Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
conc. HCl	25.0 ml

Dissolve the aldehyde in the alcohol by heating gently in a water bath at 50 °C. Cool down and add the acid to give a straw-brown coloured liquid. Store in a dark glass bottle at 4 °C.

2.1.10 Nutrient agar (NA) (LELLIOTT and STEAD, 1987)

Beef extract	1.0	g
Peptone (Oxoid L34)	5.0	g
Yeast extract (Oxoid L 21)	2.0	g
Agar (Oxoid No.3)	15.0	g
NaCl	5.0	g
Dist. water	1000.0	ml

2.1.11 Gas production from glucose (KELMAN and DICKEY, 1980)

A-	Bacto peptone (Difco)	10.0	g
	Bromocresol purpur	0.7	ml 1.5 % solution
	Dist.water	1000.0	ml
B-	Glucose	10.0	g
	Dist. water	1000.0	ml

Place one Durham-tube upside down into each test tube filled with 4.5 ml of solution A and autoclave. Filter sterilise solution B and add 0.5 ml to each test tube after it has been cooled down to about 45-50 °C.

2.1.12 Logan's medium (FAHY and HAYWARD, 1983)

Nutrient agar	28.0	g
Yeast extract	5.0	g
Glucose	5.0	g
Dist. water	1000.0	ml

After autoclaving and cooling to about 45-50 °C the medium is poured into plates.

2.1.13 Lecithinase activity (Egg yolk test)

Egg yolk emulsion was prepared from a fresh hen egg, which was washed and sterilized in 70% ethanol for 5 min. The egg was flamed, broken aseptically, and the yolk separated into a sterile measuring cylinder and diluted to 1.5 % (v / v) with sterile water. 100 ml of this egg yolk emulsion were added to 900 ml of molten Nutrient agar (NA) (see 2.1.8) medium, cooled to 55 °C and poured into Petri plates.

2.1.14 Salt tolerance

Salt tolerance was determined by inoculating the bacterial strains into Nutrient broth as described in 2.1.8, but without agar. NaCl was added to the liquid medium to obtain the required final concentration of 5% NaCl. After autoclaving and cooling to about 45-50 °C the medium was poured into plates.

2.1.15 Mineral salt-pectin-medium (QUANTICK *et al.,* 1983)

Solution A :

KH_2PO_4	7.8 g
Na_2HPO_4 X H_2O	5.4 g
Dist. water	200 ml
pH 6.9	

Solution B:

$(NH_4)_2SO_4$	2.4 g
$MgSO_4$ X 7 H_2O	0.5 g
H_3BO_3	0.6 mg
$CuSO_4$ X 5 H_2O	1.2 mg
$MnSO_4$ X H_2O	1.2 mg
Na_2MoO_4 X 2 H_2O	1.2 mg
$CaCl_2$	12 mg
$ZnSO_4$ X 7 H_2O	12 mg
Dist. Water	100 ml

Solution C:

$FeCl_3$ X 6 H_2O	12 mg (sterile filtrate)

Solution D:

Dissolve 6 g pectin N in 300 ml water and boil daily one hour at 100 °C for 3 days. Solutions A and B are autoclaved separately and then mixed with solution C after cooling to about 45-50 °C. Solution D is added by mixing, and the resulting mixture is divided into portions of 50 ml in 100 ml flasks and stored at 4 °C.

2.1.16 Antibiotics used in this study

Novobiocin (mono Na-salt)(Sigma chemicals)

Polymyxin B sulphate (Sigma chemicals, USA)

Rifampicin (Merck)

Streptomycin sulphate (Sigma chemicals)

2.2 Characterization of *Erwinia spp.*

2.2.1 Bacterial strains used in these studies

Table 1: Origin and source of bacterial strains used in this study

Strain number	Species/ subspecies	Origin
1401	*Eca (Erwinia carotovora ssp. atroseptica)*	GSPB[1)]
2231	*Eca (Erwinia carotovora ssp. atroseptica)*	GSPB
9201	*Eca (Erwinia carotovora ssp. atroseptica)*	DSM[2)]
9204	*Eca (Erwinia carotovora ssp. atroseptica)*	DSM
2967	*Eca (Erwinia carotovora ssp. atroseptica)*	DSM
426	*Ecc (Erwinia carotovora ssp. carotovora)*	GSPB
427	*Ecc (Erwinia carotovora ssp. carotovora)*	GSPB
429	*Ecc (Erwinia carotovora ssp. carotovora)*	GSPB
436	*Ecc (Erwinia carotovora ssp. carotovora)*	GSPB
8201	*Ecc (Erwinia carotovora ssp. carotovora)*	DSM
8202	*Ecc (Erwinia carotovora ssp. carotovora)*	DSM
8203	*Ecc (Erwinia carotovora ssp. carotovora)*	DSM
Strains A[3)] and Strains B	*Eca (Erwinia carotovora ssp. atroseptica)* *Ecc (Erwinia carotovora ssp. carotovora)*	Field experiments
Eca 2962	*Eca (Erwinia carotovora ssp. atroseptica)*	Japan[4)]

1) GSPB = Göttinger Sammlung phytopathogener Bakterien, Göttingen, Germany

2) DSM = Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

3) = Strains A and B were isolated in these studies from field experiments 2000 and lyophilized under the GSPB numbers of 3050, 3051 and 3052 for *Eca* and 3053, 3054 and 3054 for *Ecc*.

4) Strain obtained from Dr. Fukuoka, Shikoku National Industrial Research Institute, Takamatsu, Japan

2.2.2 Isolation of *Erwinia spp.* from different potato specimens

Sixteen different potato tubers specimens (see 3.5.2) without disease symptoms were peeled with a peelstrip (ca. 2 mm thick) by hand. The peels of each specimen were crushed individually in a sterile mortar and pestle with 4 ml 0.01 M magnesium sulphate buffer and allowed to settle at room temperature for 5 min, after which a serial 1:10 dilution was carried out in seven steps. From each dilution 0.1 ml were pipetted into a Petri dish with the semi-

selective medium and evenly distributed with a Drigalsky spatula. Three plates per each dilution were incubated at 27 °C for 24 h. Those bacteria which had caused colonies with deep cavities within one day were tranferred with a toothpick onto King's medium B and incubated at 27 °C for 24 h. The isolates obtained were characterized on the basis of the following tests: utilisation of α-methyl-d-glucoside, phosphatase activity, growth at different temperatures, indole production, gas production from glucose, growth on Logan's medium, and rotting of potato tuber slices.

2.2.3 Conventional physiological tests

2.2.3.1 Utilisation of α-methyl-d-glucoside

The bacterial isolates to be tested were precultured on plates with King's B medium at 27 °C for 24 h, transferred onto the α-methyl-d-glucoside medium (see 2.1.3) and incubated at 27 °C for 48 h. A positive reaction was indicated by a pink colour of the colony. Only strains of *Erwinia carotovora subsp. atroseptica* (*Eca*) show a positive reaction, but not those of *Erwinia carotovora subsp. carotovora* (*Ecc)* and *Erwinia chrysanthemi* (*Ech*).

2.2.3.2 Phosphatase production

The bacterial isolates to be tested were precultured on plates with King's B medium at 27 °C for 24 h, spot-inoculated onto the phosphatase detection medium (see 2.1.4) and incubated at 27 °C for 48 h. For visualisation of phosphatase activity 1.0 ml of ammonium solution (33%) (KELMAN and DICKEY, 1980) were placed on the open lid of each Petri dish and the plate culture inverted over it. Plates were evaluated after 10 sec. Only strains of *Ech* show a positive reaction for phosphatase.

2.2.3.3 Differentiation of *Erwinia spp.* by growth at different temperatures

The bacterial isolates were streaked onto plates with King's B medium and incubated at three different temperatures (27 °C, 35.5 °C and 37 °C) for 24 h. Triplicate plates were evaluated at each temperature. *Ech* is able to grow and form characteristic cavities at all three temperatures, *Ecc* does so at 27 °C and 35.5 °C only, and *Eca* only at 27 °C (JANSE and SPIT, 1989; PEROMBELON and HYMAN, 1986).

2.2.3.4 Indole production

The bacterial isolates were inoculated into the indole production medium (see 2.1.6) by adding 0.1 ml of a bacterial suspension in 0.01 M $MgSO_4$ from fresh bacterial cultures adjusted to an $O.D_{660}$ of 0.06 (ca.10^8 cfu/ml) to 5 ml medium. The tubes were incubated at 27 °C for 48 h. After incubation, 0.5 ml of Kovac's reagent (see 2.1.11) were added to 5 ml of the culture medium and shaken gently. A positive reaction was indicated by a dark red colour. Only strains of *Ech* show a positive reaction (LELLIOTT and STEAD, 1987).

2.2.3.5 Gas production from glucose

The bacterial isolates were inoculated into test tubes with the gas production medium (see 2.1.8) by transferring 0.1 ml of a bacterial suspension containing 10^8 cfu /ml and incubating at 27 °C for 2-7 days. After incubation only strains of *Ech* show gas production in the Durham tubes (KELMAN and DICKEY, 1980).

2.2.3.6 Logan's medium

The bacterial isolates were transferred onto Logan's medium (see 2.1.9) by spot inoculation with 0.1 ml bacterial suspension of 10^8 cfu/ml, and incubated at 27 °C for 24 h. After incubation *Eca* shows small colonies with a red centre, *Ecc* big light red colonies and *Ech* dark red colonies.

2.2.3.7 Lecithinase activity (Egg yolk test)

The bacterial isolates to be tested were precultured on plates with King's medium B at 27 °C for 24 h, streaked onto egg yolk medium (see 2.1.11) and incubated at 27 °C for 7 days. After incubation colonies of *Ech* are surrounded by a highly turbid zone due to lecithinase activity.

2.2.3.8 Salt tolerance

Tolerance to 5% NaCl was detected by visible turbidity after the bacterial isolates were inoculated into test tubes with the salt tolerance (5% NaCl) medium (see 2.1.12) by transferring 0.1 ml of a bacterial suspension containing 10^8 cfu /ml and incubating at 27 °C for 14 days. After incubation only strains of *Ech* show a visible turbidity.

2.2.4 Biotest on potato tuber slices

The bacterial isolates were grown onto King' s medium B for 24 h. Potato tubers, cultivar Cilena, were washed and sterilised with 96% ethanol and flaming, cut with a sterile knife to slices of 8-10 mm and placed into Petri dishes. Ten milliliter of 0.01 M magnesium sulphate were added to each Petri dish, and each slice was inoculated with a drop of 10 µl of 10^8 cfu/ml of bacteria to be tested and incubated at 27 °C for 24 h. Each bacterial strain was tested threefold. A positive reaction was recorded when the potato slices showed soft rotting within two days.

2.2.5 Maintenance and storage of bacterial cultures

The working strains were maintained in sterile distilled water at room temperature in Eppendorf tubes, some of the strains were maintained on YDC agar slants in test tubes and stored at 4 °C. For long time storage the strains were preserved as lyophilized

cultures.

2.3 Development of a new semi-selective medium for *E. carotovora*

2.3.1 Effect of tryptone in the basal and overlayer on the growth of *Erwinia spp*. on the semi-selective medium

The effect of different concentrations of tryptone (0, 0.3%, 0.6%) in the basal and overlayer on the growth of *Erwinia spp.* was investigated to determine the growth and cavity formation by *Erwinia spp.*. The bacterial strains *Eca* 1401 and *Ecc* 1405 were grown on King' s medium B for 24 h, adjusted to an $O.D._{660}$ of 0.06 (ca. 8 X 10^7 cfu/ml), and serially diluted. 0.1 ml of the bacterial suspensions from the dilution 1:10^6 were plated on the CVPM medium and incubated at 27 °C for 24 h (see table 8).

2.3.2 Effect of tri-sodium citrate dihydrate in the basal and over layer on the growth of *Erwinia spp*. on the semi-selective medium

The effect of tri-sodium citrate dihydrate was studied by addition of different concentrations (0, 0.1%, 0.2%, 0.3% and 0.4%) to both the basal and overlayer, and the bacterial suspensions were plated on the medium as described above.

2.3.3 Comparison of different pectin sources for isolation of *Erwinia spp.* on the semi-selective medium

The bacterial strains were grown on King' s medium B and incubated at 27 °C for 24 h. Bacterial suspensions were adjusted to an OD_{660} of 0.06 (10^8 cfu/ml), and 0.1 ml were plated on the Petri dishes with the following different pectin sources:

Copenhagen pectin A/S (Hercules Slendid® type 440), is a lowly esterified pectin (<10%) extracted from citrus peel

pectin A (Roth Co. no. 9123.1) extracted from apple, medium degree of esterification (ca. 36%);

pectin C (Roth Co. no. 8911.1), extracted from citrus, high degree of esterification (ca. 67-71%);

pectin N (Roth Co. no. 8913.1) pectic acid sodium salt with low degree of esterification (8.0 %);

pectin from citrus fruits (Sigma no. 9135), galacturonic acid content 80%, methoxy content 9%);

pectin from apple (ICN Biomedicals no. 156057), polygalacutronic acid methyl ester);

pectin from citrus fruits (ICN Biomedicals no. 102587), polygalacutronic acid methyl ester);

pectin from citrus (Serva no. 31650), high degree of esterification (ca. 65%);

pectin from citrus peel (Fluka Co. no. 76280), high degree of esterification (63-66%);

polygalacturonic acid (Sigma no. P- 3850) from citrus fruit;

sodium polypectate (Sigma no. P- 1879), sodium salt, minimum 85%;

sodium polypectate “Bulmer” (H. P. Bulmer Ltd., Plough Lane, Hereford HR4 OLE, England);

pectic acid from citrus (Herbstreith & Fox CU-L 023/00), low degree of esterification (7%), lot no. 006645;

pectic acid from apple (Herbstreith & Fox AU-L 011/01), low degree of esterification (5%), lot no. 102576;

pectin from apple (Herbstreith & Fox AU-L 012/01), high degree of esterification (32%), lot no. 102577;

(see also table 5) and incubated at 27 °C for 24 h.

2.3.4 Evaluation of bacterial growth on the semi-selective medium

Evaluation was based on growth (++ = bacteria grew on the medium, - = no growth), and cavity formation was evaluated by a scale of 0 - 3 (0 = no cavity, 1 = shallow cavity, 2 = moderately deep and wide, and 3 = deep and wide cavity).

2.3.5 Recovery of *Erwinia spp.* from artificially inoculated potato extracts on semi-selective media and on King´s medium B

Potato tubers without disease symptoms was peeled with a peelstrip ca. 2 mm thick by hand. The peel was weighed and 1 g was crushed in a sterile mortar and pestle with 4 ml 0.01 M magnesium sulphate solution and allowed to settle at room temperature for 5 min. The bacterial strain *Ecc* 436 (*Erwinia carotovora ssp. carotovora)* was grown on Petri dishes with King' s medium B and incubated at 27 °C for 24 h. The bacterial growth was scraped off and suspended in 0.01 M magnesium sulphate solution. The bacterial suspension was adjusted to an OD_{660} of 0.06 (ca. 10^8 cfu/ml), and 0.1 ml were mixed with potato peel homogenate. The mixture was serially diluted, and from each dilution 0.1 ml were pipetted into a Petri dish and evenly distributed with a Drigalsky spatula. Three plates per each dilution were incubated at 27 °C for 24 h.

2.3.6 Detection limit of *Erwinia spp*. on the semi-selective medium and on King' s medium B

The same method as described above (2.3.5) was used, but suspensions with lower concentrations of bacteria (10^4 and 10^3 cfu/ml) were added to the potato homogenates, serially diluted, and 0.1 ml were plated on both, CVPM and King's medium B, and incubated at 27 °C for 24 h.

2.3.7 Isolation of *Erwinia spp*. on the new semi-selective medium

2.3.7.1 Potato peel extracts

Twenty healthy potato tubers were peeled by a hand-peeler, ca. 2 mm thick and weighed. The peels were comminuted in a double amount of 0.01 M magnesium sulphate solution by a homogenizer (Minipimer contro plus, vario, Braun, Melsungen, Germany). The homogenate was allowed to settle at room temperature for 5 min, serially diluted, and 0.1 ml were plated on the CVPM medium and incubated at 27 °C for 24 h. Triplicate plates were used for each experiment.

2.3.7.2 Soakage of potato tubers

Twenty potato tubers per sample were soaked in 0.01 M magnesium sulphate for 3 h at 4 °C with occasional stirring every 30 min by hand. After incubation, 50 ml from each soakage were centrifuged at 5000 g for 15 min. The pellet was dissolved in 1 ml 0.01 M magnesium sulphate and serially diluted 1:10 and 1:100. From each dilution 0.1 ml were plated on the CVPM medium and incubated at 27 °C for 24 h.

2.4 Evaluation of latent contamination of 232 potato tuber samples from the years 1998, 1999 and 2000

In the three years 1998, 1999 and 2000 232 potato tuber samples were obtained from Stöver Co. for detection of latent contamination by erwinias. Each sample consisted of 60 tubers. The samples were stored at 4 °C and analysed within 4 weeks. Each sample was divided in three groups of 20 tubers each and analysed separately ("experiments" 1, 2 and 3, see tables 15, 16 and 17) according to the method described in 2.3.7.2.

2.5 Field experiments

The field experiments were performed by Stöver Co. on a farm in 27239 Natenstedt near Twistringen to compare potato lots with different degrees of latent contamination as determined in Göttingen. Soil type: (h) SL, pH : 5.7, mg P_2O_5 : 26 (D), mg K_2O : 23 (D), mg Mg : 5 (B), N–min : 0-30 cm 119 kg NO_3–N/ha, N-min : 30-60 cm 33 kg NO_3–N/ha. Potatoes were planted on April 4, 1999 by hand. The soil was dry and warm, temperatures 16 °C. Each variant consisted of 4 blocks, each with two rows of 24 potatoes totally, planted with a space of 37 cm. The usual plant protection measures were performed, including weekly sprays against *Phytophthora infestans*. The summer 1999 was unusually warm with high soil temperatures and rainfall below the yearly average. The potatoes were harvested on September 20, 1999, by hand. Evaluation in each block included : Total number of potato tubers, numbers of tubers with soft rot, total weight of tubers, weight of fractions according to tuber sizes, number of tubers in different fractions per 10 kg.

3 RESULTS

3.1 Preservation of the *Erwinia spp.* used in this study

Three different methods were compared for preservation of viable cultures of *Erwinia spp.*. Lyophilization appeared to be most effective in maintaining the bacterial cultures without losing viability. On the other hand, this method needs special apparates (lyophilizator, ampoule-constrictor) and is time–consuming.

Storage of the cultures on YDC medium slants at 4 °C maintained viability of the *Ecc* cultures for at least 6 months and that of *Eca* for 3 - 4 weeks, whereas the cultures of *Ech* died within 3-4 weeks. On the other hand, storage of *Erwinia spp.*, *Eca* and *Ecc* in sterile distilled water at room temperature maintained the viability of all *Erwinia spp.* for more than 16 months, at 4 °C as well as at room temperature (Tables 2 and 3). Therfore, most of the *Erwinia* strains used in this study were preserved in sterile distilled water at room temperature .

Table 2 : Evaluation of different preservation methods for maintaining the viability of *Erwinia spp.* stored **at 4 °C**. *Eca* (2967, 2231 and 1401), *Ecc* (426, 429 and 436), *Ech* (1229, 1860 and 2236)

Preservation method	Viability after 3-4 weeks			Viability after 6 months			Viability after 12 months			Viability after 16 months		
	Eca	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*
Slants on YDC	++	++	-	-	++	-	-	-	-	-	-	-
Sterile water	++	++	++	++	++	++	++	++	++	++	++	++

Table 3: Evaluation of different preservation methods for maintaining the viability of *Erwinia spp.* stored **at room temperature,** the same strains were tested as in table 2.

Preservation method	Viability after 3-4 weeks			Viability after 6 months			Viability after 12 months			Viability after 16 months		
	Eca	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*
Slants on YDC	-	-	-	-	-	-	-	-	-	-	-	-
Sterile water	++	++	++	++	++	++	++	++	++	++	++	++

++ = all cultures were viable

- = all cultures tested were dead

3.2 Development of a semi-selective medium for isolation of *Erwinia spp*.

3.2.1 Effect of tryptone and tri-sodium citrate dihydrate in the basal and overlayer on the growth of *Erwinia spp*. on the semi-selective medium

In these experiments the growth on the optimal but non-selective medium King's B was compared with the semi-selective medium to which different concentrations of tryptone and citrate were added in the basal and over layer. The results obtained (Table 4) show that without tryptone and citrate bacterial colonies with cavities did not develop on the semi-selective medium, and only few colonies and weak cavities were formed when tryptone and the tri-sodium citrate dihydrate were added at low concentrations to the basal and over layer, respectively. On the other hand, many colonies grew when tryptone (0.6 %) was added only to the basal layer and tri-sodium citrate dihydrate (0.4%) only to the overlayer. The addition of tryptone to the overlayer and of tri-sodium citrate dihydrate to the basal layer was not necessary.

Table 4 : Growth of bacterial strains (*Eca* and *Ecc*) on the semi-selective medium with different concentrations of tryptone and tri-sodium citrate dihydrate

Medium	Colonies/ Petri plate	
	Eca 1401	*Ecc* 1405
King' s medium B	8	9
without tryptone in basal layer	0	0
without tryptone in overlayer	0	0
0.3% tryptone in basal layer	3	4
0.3 % tryptone in overlayer	4	5
0.6 % tryptone in basal layer	8	8
0.6 % tryptone in overlayer	4	3
without Na-citrate in basal layer	0	1
without Na-citrate in overlayer	0	0
0.1 % Na-citrate in basal layer	1	0
0.1% Na-citrate in overlayer	2	2
0.2% Na-citrate in basal layer	0	1
0.2 % Na- citrate in overlayer	4	3
0.3% Na-citrate in basal layer	0	0
0.3% Na-citrate in overlayer	7	6
0.4% Na-citrate in basal layer	1	2
0.4 % Na-citrate in overlayer	8	9

3.2.2 Effect of different pectin preparations on the growth of *Erwinia spp.*

Table 5 and Fig. 1 clearly show that the sodium polypectate from Bulmer Co. was best suited for detection of *Erwinia carotovora* in comparison to 14 other different pectins. Unfortunately, the sodium polypectate from Bulmer (Plough Lane, Hereford HR4 OLE, England) is no longer available and most of the preparations from other sources are unsatisfactory because of poor gelling capacity. Only Copenhagen pectin A/S, pectin N from Roth Co., sodium polypectate from Sigma Co., and two pectin preparations from Herbstreith and Fox were found to be sastisfactory in these experiments.

Table 5 : Detection of *Erwinia spp.* on the CVPM medium containing 15 different pectin sources

Pectin preparation	Source of pectin	Cavity formation*)
1- Copenhagen pectin A/S	Hercules Slendid® type 440	2
2- Pectin A	Roth Nr. 9123.1	0
3- Pectin C	Roth Nr. 8911.1	0
4- Pectin N	Roth Nr. 8913.1	1
5- Pectin from citrus fruits	Sigma P-9135	0
6- Pectin from apple	ICN Biomedicals Nr. 156057	0
7- Pectin from citrus	ICN BiomedicalsNr.102587	0
8- Pectin from citrus	Serva no. 31650	0
9- Pectin	Fluka no. 76280	0
10- Polygalacturonic acid	Sigma P-3850	0
11- Sodium polypectate	Sigma P-1879	2
12- Sodium polypectate	Bulmer	3
13- Pectic acid from citrus	Herbstreith & Fox (CU-L 023/00)	2
14- Pectic acid from apple	Herbstreith & Fox (AU-L 011/01)	2
15- Pectin from apple	Herbstreith & Fox (AU-L 012/01)	0

*) 1 = the cavities were shallow and very small and visible after 24 h.
2 = the cavities were moderately deep and wide and visible after 24 h.
3= the cavities were deep and large and were formed after 13 h.

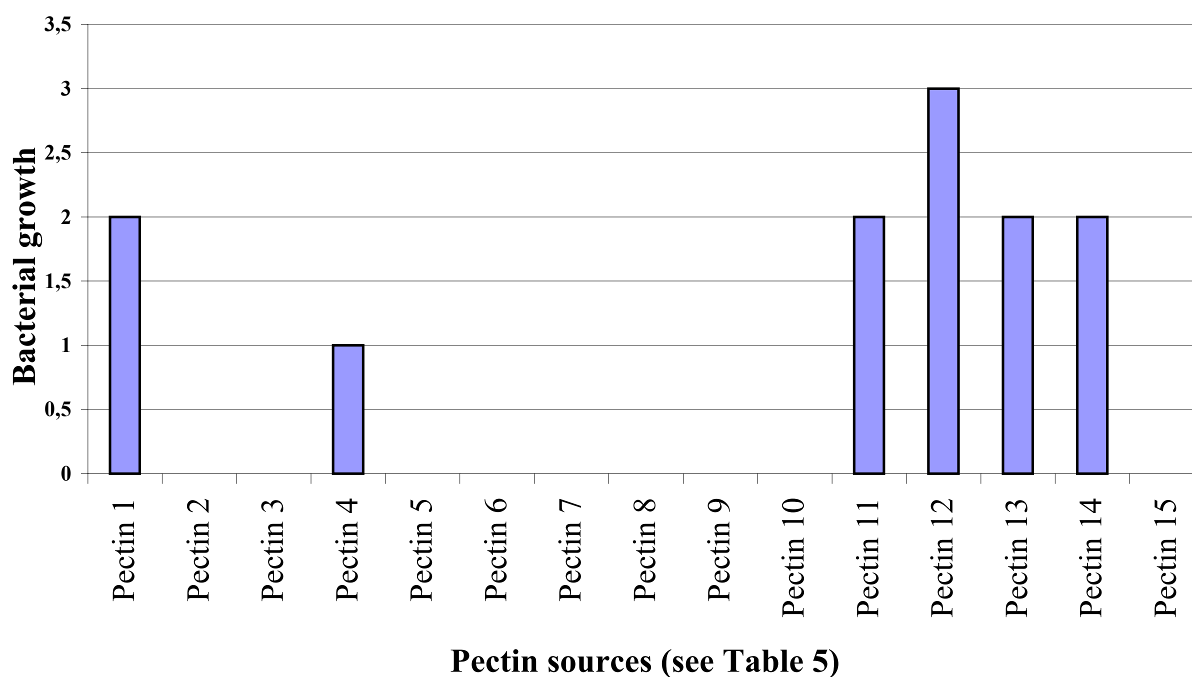

Fig 1: The growth and cavity formation by *Erwinia spp.* on 15 different pectin sources on the semi-selective medium

3.2.3 Composition of the new semi-selective medium (CVPM) for isolation soft rot erwinias

The new semi-selective medium (CVPM) (= crystal violet pectate modified) for isolation of pectolytic *Erwinia spp.* from potato tubers was developed from the medium of BDLIYA (1995) with the following modifications in the overlayer: reduce the amount of sodiumpolypectate from 18 g to 15 g per litre, omit tryptone and bromothymol blue, reduce the amount of $CaCl_2$ X 2 H_2O (10%) from 6.8 to 6.5 ml, and exchange KNO_3 with $NaNO_3$ (PEROMBELON and BURNETT, 1991). With the original amount of 18 g polypectate in the overlayer the medium became too solid and only weak cavities were obtained, omitting of bromothymol blue resulted in more distinctive cavities because the medium of Bdliya (1995) with two indicators (bromothymol blue and crystal violet) became green, so that the appearance of the cavities was not clear. The reduction of the amount of $CaCl_2$ X 2 H_2O (10%) in the overlayer slightly reduced the firmness of this medium and improved the growth and cavity formation of *Erwinia spp.*, whereas without $CaCl_2$ X 2 H_2O (10%) the medium did not solidify.

Basal layer

Peptone from casein (Roth Co. no.8986.2)	2.0 g
Yeast extract (Difco no.0127-01)	0.6 g
NaCl	1.0 g
SDS	0.05 g
L-Asparagine (Merck no.1.00126)	0.5 g
Tryptone (Difco no. 0123-17-3)	1.5 g
Agar	3.0 g
Dist.water	250 ml

pH 7.2

Add 0.5 ml of filter sterilised 1% solution of 2,3,5-triphenyltetrazolium chloride to the basal layer after autoclaving and cooling down to 50 °C, and add 400 µl (3.2 µg/ml) Polymxin B sulphate. Dispense 12 ml of basal medium per plate and allow to set for 10 min.

Overlayer

Crystal violet solution (Sigma no. c-3886)	1.0 ml 0.075% (w/v)
$CaCl_2$ X 2 H_2O	6.5 ml (10% solution)
$NaNO_3$	1.0 g
tri-Na citrate dihydrate	2.5 g
Agar	2.0 g
Na-polypectate (Hercules Slendid® type 440)	7.5 g
Dist.water	500 ml

pH 7.2

Add sodiumpolypectate slowly after all other components are dissolved. Adjust pH to 7.2. After autoclaving and cooling down to 45 °C, add 300 µl (1.2 µg/ml) Polymxin B sulphate to the overlayer solution, and pour 15 ml/plate on top of the basal layer.

3.3 Recovery rate and detection limit of *Erwinia spp.* from artificially contaminated potato extracts

The details for this experiment are described in chapters 2.3.5 and 2.3.6. In these experiments, 0.1 ml of a standardizerd bacterial suspension from a pure bacterial culture were mixed with potato peel homogenate. Then, aliquots of the mixture were plated on different media. In order to determine the recovery rate and detection limit of the *Erwinia spp.*, the concentration in the

original bacterial suspension had to be determined by plating on King's medium B. The results are shown in Table 6.

Several laboratory experiments confirmed that potato tubers from different cultivars, which had been washed by tap water, were nearly free from contamination by erwinias, in contrast to unwashed tubers. It was, therefore, concluded that the bacterial numbers listed in tables 7 and 8 are nearly 100% caused by the artificially added bacterial contamination.

The results obtained in the first experiment show that the **recovery rate** of bacteria (Fig. 2) from artificially inoculated tuber sap was 87% on CVPM, whereas the recovery rate was 49% on the medium of Bdliya (1995) and only 23% on the CVPB medium of Perombelon & Burnett (1991) (Table 7).

The first step in this experiment was to estimate the bacterial concentration in the suspensions adjusted to an OD_{660} of 0.06 = (Table 6). The bacterial concentration in the suspension of the four different *E.c.* strains varied between 3.6 - 6.9 X 10^7 cfu/ml. These bacterial suspensions were diluted 1:10^4, and from each strain 0.1 ml were added to 5 ml potato peel homogenate. For instance, in the case of strain *Eca* 2967, 0.1 ml 1:10^4 diluted suspension containing 6.3 X 10^2 cfu were added to 5 ml potato peel homogenate, resulting in a contamination of 1.26 X 10^2 cfu/ml peel homogenate. When 0.1 ml from this mixture were plated on the semi-selective medium, the maximum number of bacterial colonies obtained on the semi-selective medium should be 1.26 X 10^1, corresponding to 126 cfu per ml potato peel homogenate. Because only 1.20 X 10^1 cfu/ 0.1 ml were recovered on the CVPM medium, the recovery rate (dividing 120 by 126 cfu/ml) was 95% (Table 7).

Since the typical cavities for erwinias became only visible on the semi-selective media it was not surprising that more bacterial colonies were counted on King's medium B which does not allow a differentiation of erwinias and non-erwinias. Therefore, the higher numbers of bacterial colonies on King's medium B do not indicate a higher recovery rate of erwinias.

Table 6 : Concentration of bacterial suspensions of $O.D._{660}$ = 0.06 determined by dilution plating on King's medium B

Strain	Bacterial colonies/plate			cfu/ml of original suspension
	1:10^4	1:10^5	1:10^6	
Eca 2967	uncountable	63	6	6.3 X 10^7
Eca 1401	uncountable	54	5	5.4 X 10^7
Ecc 436	uncountable	69	7	6.9 X 10^7
Ecc 1405	uncountable	36	4	3.6 X 10^7

Table 7: Recovery rate of bacteria from artificially contaminated potato homogenate (bacterial dilution 1:10^4) on four different media. 0.1 ml of each bacterial suspension containing 630, 540, 690 and 360 cfu/ml, respectively, were added to 5 ml potato peel homogenate.

Strain	Determined concentration of bacteria (cfu/ml) on			
	CVPM	Bdliya 1995	CVPB	King's B
Eca 2967	120:126 = 95 %	40 :126 = 32 %	40 :126 = 32 %	120 :126 = 95 %
Eca 1401	90: 108 = 83 %	30 :108 = 28 %	20 :108 = 19 %	100 :108 = 93 %
Ecc 436	120:138 = 87 %	70 :138 = 51 %	20 :138 =14 %	130 :138 = 94 %
Ecc 1405	60 : 72 = 83 %	60 :72 = 83 %	20 :72 = 28 %	70 : 72 = 97 %
Average	87 %	49 %	23 %	(95 %)[1)]

[1)] Since erwinias could not be differentiated from saprophytic bacteria on King's medium B, the calculated 95% do not stand for recovery rate of erwinias.

Table 8 : Detection limit of bacteria (bacterial dilution 1:10^5) on four different media. 0.5 ml of the bacterial dilution 1:10^5 from each bacterial strain containing 315, 270, 345 and 180 cfu/ml, respectively, were added to 5 ml potato peel homogenate.

Strain	Determined concentration of bacteria (cfu/ml potato sap) on			
	CVPM	Bdliya 1995	CVPB	King's B
Eca 2967	60 : 63 = 95 %	20 : 63 = 32 %	0 : 63 = 0 %	60 : 63 = 95 %
Eca 1401	40 : 54 = 74 %	50 : 54 = 93 %	50 : 54 = 93 %	60 : 54 = 111 %
Ecc 436	70 : 69 = 101 %	50 : 69 = 72 %	50 : 69 = 72 %	60 : 69 = 87 %
Ecc 1405	30 : 36 = 83 %	20 : 36 = 56%	0 : 36 = 0 %	50 : 36 = 139 %

It can be concluded from table 8 that the **detection limit** on the new semi-selective medium was at least 3.6 X 10^1 cfu/ml. Theoretically, the detection limit could be lowered to one cfu/ 0.1 ml extract, *i.e.* when the bacterial concentration in the original potato homogenate was 1 X 10^1 cfu/ml. However, in this case the plating of 0.1 ml from the potato extract on the semi-selective medium should be repeated several times. Therefore, it is more realistic to estimate the detection limit with **2 X 10^1 cfu/ml**.

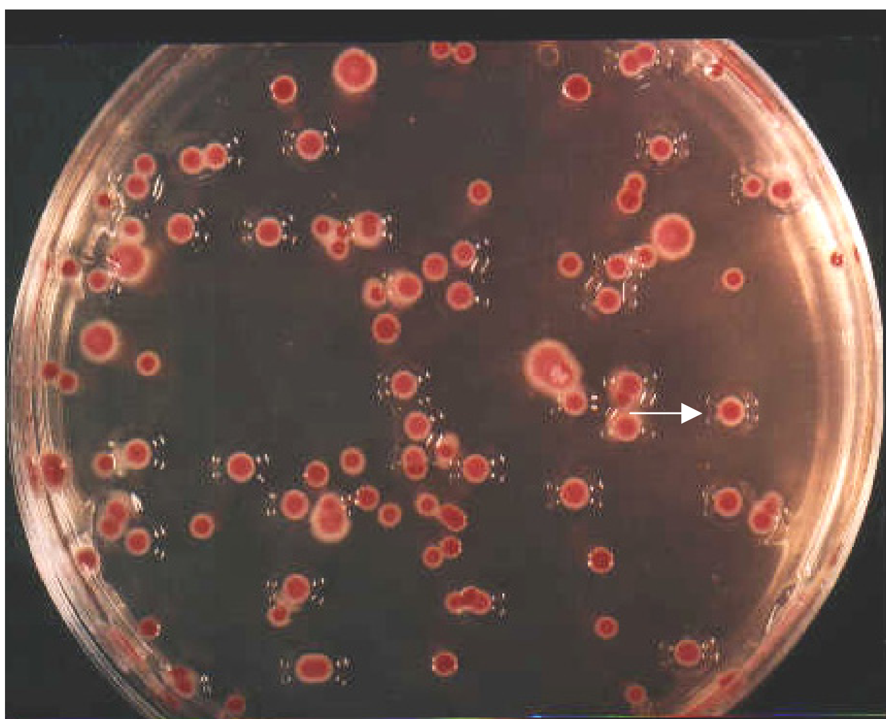

Fig. 2 : Recovery of soft-rot erwinias from artificially inoculated potato peel extract after 24 h

3.4 Application of the new semi-selective medium for detection of *Erwinia spp.* from potato tuber peels using two different methods

The methods for these experiments are described in 2.3.7. The results obtained show that higher numbers of *Erwinia spp.* colonies were obtained from potato tubers by the soaking method compared to potato tuber peel extracts (Table 9). Obviously, soaking of tubers in 0.01 M $MgSO_4$ for 3 h allowed that nearly all *Erwinia spp.* contaminants diffused from the potato tubers into the soaking solution, because tuber contamination by erwinias is usually on the tuber surface. Soaking for only 3 h saved time and did not allow the bacteria to multipliy because the temperature was very low (4 °C). In 10 from 11 samples tested lower numbers of erwinias were obtained from peel homogenates than from soakates (Table 9), and in one case with very low bacterial contamination (no.18) bacterial colonies were only obtained from the soakate. Interestingly, all the bacterial strains isolated from the naturally contaminated potato tubers were *Ecc*.

Table 9: Detection and isolation of *Ewinia spp.* from 12 potato tuber samples using the semi-selective medium

Sample Nr.	Bacterial concentration cfu/ml homogenate (potato tuber peels method)	Bacterial conc. cfu/ml soakage (soaking method)	Identification of *Ewinia spp.* (12 colonies /sample)
8	7.0×10^1	6.0×10^2	
9	1.6×10^4	1.1×10^4	*Ecc*
10	1.2×10^2	5.4×10^3	*Ecc*
11	1.1×10^3	2.7×10^4	*Ecc*
12	1.3×10^3	2.5×10^4	
13	1.9×10^2	3.9×10^4	
14	7.0×10^2	4.3×10^4	
15	2.0×10^1	6.3×10^2	*Ecc*
16	2.0×10^1	3.3×10^2	*Ecc*
17	8.0×10^1	2.4×10^2	
18	0	3.0×10^1	
19	9.0×10^1	1.9×10^3	

3.5 Isolation and identification of erwinias from potato tubers

3.5.1 Identification of soft rot erwinias (*Eca*, *Ecc* and *Ech*) by physiological tests

Table 10 shows the characterization of *Erwinia spp***.** by 10 different tests for grouping pectolytic soft rot erwinias into species and subspecies. When the cavities formed by *Ecc* and *Eca* strains on the semi-selective medium were compared, those of *Ecc* appeared to be broader and deeper than those of *Eca*. The tests confirmed the taxonomic identification of several *Eca*, *Ecc* and *Ech* strains from the GSPB – bacterial collection. All the strains isolated from potato tubers purchased from different shops and supermarkets in this study (no.1-99, see Table 11) were identified as *Ecc*.

Table 10: Identification of *Erwinia* strains from the GSPB (= Göttinger Sammlung phytopathogener Bakterien) and from seed potato tubers by morphological and biochemical tests

Strain No.	T (°C)			∝-Methyl d-glucoside	Phos-phatase	Indole Formation	Logan's medium	NaCl (5 %)	Egg yolk	Gas from glucose
	27	35.5	37							
Eca 1401	+	-	-	red	-	-	s.r.	+	-	-
Eca 2231	+	-	-	red	-	-	s.r.	+	-	-
Ecc 1405	+	+	(+)	white	-	-	l.r.	+	-	-
Ecc 426	+	+	(+)	white	-	-	l.r.	+	-	-
Ech1229	+	+	+	white	+	+	d.r.	-	+	+
Ech1860	+	+	+	white	+	+	d.r.	-	+	+
Ech 2236	+	+	+	white	+	+	d.r.	-	+	+
Eca 2962	+	-	-	red	-	-	s.r.	+	-	-
Strains 1-99	+	+	(+)	white	-	-	l.r.	+	-	-

s.r. = small, red colony, l.r.= big, light red colony, and d.r. = dark red colony
(+) = weak growth

3.5.2 Identification of *Erwinia spp.* from different potato specimens

In the years 1997 and 1998 12 potato specimens were purchased from different supermarkets in Göttingen and tested for the presence of *Ewinia carotovora ssp.* (for method see 2.2.2). In addition, 4 specimens were obtained from freshly harvested field potatoes: Stöver Co.: cultivars Agria and Morene, University farm Reinhausen: cultivars Agata and Forelle. None of the potato tubers tested showed soft rot symptoms. Therefore, it appears very interesting that contamination by *E. carotovora* was detected in 10 of the 16 seed potato samples on the CVPM medium according to physiological reactions and the biotest (Tables 10 and 11). *Erwinia* isolates were identified by their ability to form cavities on the agar due to breakdown of pectate by pectolytic enzymes produced by the bacteria (Fig. 3).
Three different temperatures were used to differentiate between *Eca, Ecc* and *Ech*, because *Ech* is able to grow and form characteristic cavities at all three temperatures, *Ecc* does so at 27 °C and 35.5 °C only, and *Eca* at 27 °C only (Fig. 3). The bacteria from cavities formed after 13 hours on the CVPM-medium were transferred by toothpick to KB plates to obtain pure cultures. Further tests confirmed that all the 99 bacterial isolates were *Ecc*, especially when the growth on ∝-methyl-d-glucoside was evaluated (Fig. 4). These results indicate that *Ecc* is the main soft rotting *Erwinia* occurring on stored potatoes in Germany. Although all the potato

specimens tested looked healthy a latent contamination with *Erwinia carotovora spp.* was determined in $^{2}/_{3}$ (10 from 16) of the samples (Table 11). The cultivars also showed differences in degree of contamination by *Erwinia spp.*. Thus, the cultivars Agria and Morene from Stöver Co. were free from the contamination, whereas bacterial numbers from 5-29 per dilution were recorded for several cultivars from different supermarkets. The cultivar Linda from Aldi supermarket was tested at three different times and found to be free from the contamination on March 5 and May 10, whereas after four months (July 1) erwinias were isolated from this cultivar. These results indicated that in the early stage during storage the tubers were relatively free from erwinias, but later on the bacteria could be detected because the conditions during storage were suitable for the growth of the potato soft rot bacteria. Also, on cultivar Hansa from Löb supermarket the degree of bacterial contamination increased from January 15 to April 3, 1998.

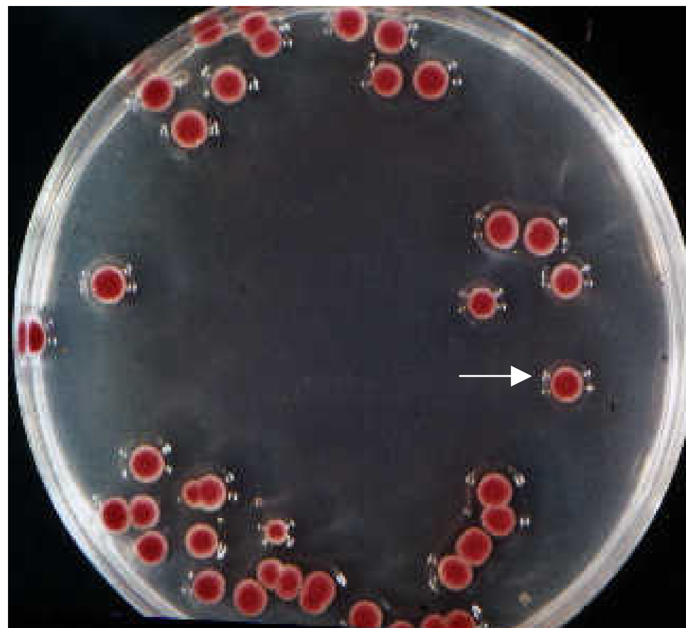

Fig 3: Cavity formation by soft-rot erwinias after incubation at 27 °C for 24 h on the CVPM medium.

Table 11: Source and number of *Erwinia* strains isolated from different potato tuber cultivars

Date	Source	Cultivar	Cavity colonies	Saproph. bacteria	Transferred isolates	Biotest	Identification
25.11.97	Stöver Co.	Agria	-	many	-	-	-
15.1.98	Löb superm.	Hansa	14	many	7	++	*Ecc*
15.2.98	Stöver Co.	Morene	-	many	-	-	-
5.3.98	Aldi superm.	Linda	-	many	-	-	-
21.3.98	Real superm.	Satina	5	many	5	++	*Ecc*
3.4.98	Löb superm.	Hansa	29	many	28	++	*Ecc*
15.4.98	Penny superm.	Nicola	3	many	3	++	*Ecc*
20.4.98	Penny superm.	Spunta	29	many	23	++	*Ecc*
28.4.98	Aldi superm.	Renate	15	many	10	++	*Ecc*
10.5.98	Aldi superm.	Linda	-	many	-	-	-
12.5.98	Löb superm.	Cilena	5	many	5	++	*Ecc*
15.5.98	Löb superm.	Diamant	-	many	-	-	-
1.7.98	Aldi superm.	Linda	15	many	10	++	*Ecc*
10.8.98	Field of university farm	Agata	8	many	4	++	*Ecc*
10.8.98		Forelle	-	many	-	-	-
10.8.98	Real superm.	Bebera	5	many	4	++	*Ecc*
Total number of strains tested					99	++	*Ecc*

(A) **(B)**

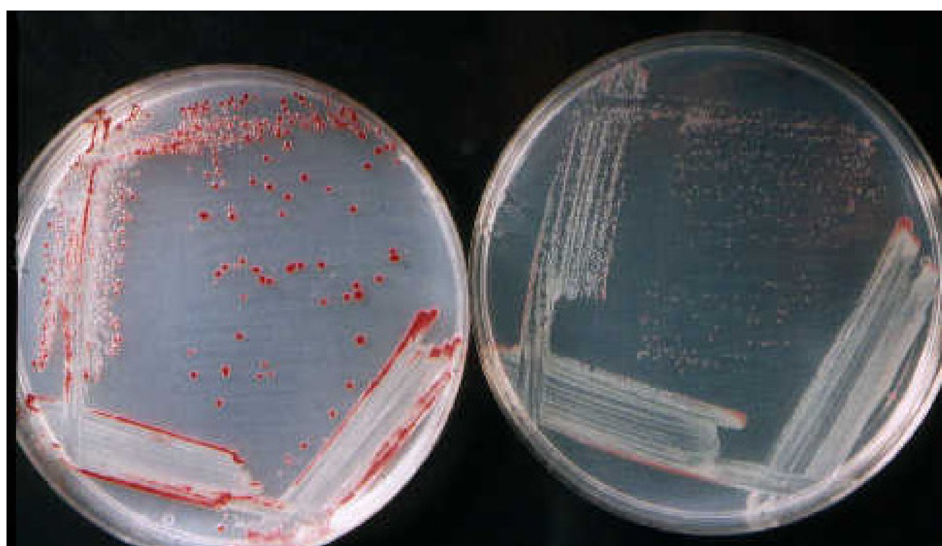

Fig 4: Differentiation between *Erwinia carotovora subsp. atroseptica* (A) and *Erwinia carotovora subsp. carotovora* (B) using the ∝-methyl-d-glucoside medium after incubation at 27 °C for 24 h.

3.6 Detection of pectolytic *Erwinia spp.* on single potato tubers and in groups of potato tubers

It was the aim of these studies to find out, whether latent contamination by erwinias is spread more or less evenly in stored potatoes, or whether only very few potato tubers are contaminated. Only after these experiments it was possible to determine the sample size of the potato specimens to be analysed for latent contamination by erwinias.

As shown in table 12, only three potato tubers from 40 tubers tested were contaminated by *Erwinia spp.*. However, when groups of potato tubers each consisting of 20 tubers were tested, 4 groups from 5 tested were contaminated, but by different quantities of erwinias (Table 13). It was concluded from these experiments that, for quantifying *Erwinia spp.* contamination of a potato stock, a representative sample size should consist of 20 tubers taking randomly. Depending on the size of the potato stock, this test should be repeated at least three times with 20 potato tubers in each repetition.

Table 12: Isolation of *Erwinia spp.* from 40 separately soaked potato tubers using the semi-selective medium

Tuber no.	Bacterial cfu/ml homogenate
2	6.9×10^2
31	3.7×10^3
36	6.3×10^2
X	0

X= 37 potato tubers were free from latent contamination

Table 13: Detection of *Erwinia spp.* from 5 potato tuber groups each consisting of 20 tubers

Group no.	Bacterial cfu/ml homogenate
1	6.8×10^4
2	0
3	2.2×10^3
4	1.1×10^4
5	6.3×10^2

3.7 Detection of latent contamination with *Erwinia carotovora spp.* in different potato tuber samples from Stoever Produktion GmbH & Co. KG

The newly developed semi-selective medium was tested on its practical suitability in the frame of an extensive cooperation with Stoever Co.. In this cooperation 231 potato samples were tested for latent contamination with *Erwinia spp.*. The aim of this investigation was to classify the potato samples according to their grade of contamination with *Erwinia spp.*. On the basis of these studies, heavily contaminated samples should not be planted in the next season in order to avoid high yield losses due to new infections by *Erwinia spp.*.
Because Stoever Co. exclusively supplies all McDonalds restaurants in Germany with potatoes for food processing, fresh potatoes of high quality must be available all around the year. Therefore, we additionally tested a large lot of consumption potato samples on contamination with *Erwinia spp.*. Heavily contaminated potato stocks were processed first while the stocks free from *Erwinia spp.* were processed at the end of the season.

3.7.1 Comparison between laboratory tests and field observations

On October 25, 1998, 12 samples of potato tubers harvested in Septemper 1998 were sent to Göttingen by Stöver Co. In these healthy looking specimens latent contamination with *Erwinia carotovora spp.* was determined as described in chapter 2.3.7.2. The results obtained (Table 14) show a considerable variation in bacterial contamination from “very weak” to “very strong”. These laboratory tests in Göttingen were compared with independent observations by experts from Stoever Produktion Co., as shown in Table 14. The scoring of the potato samples in the 3 grades (weak, moderate and strong) by Stöver Co. was based on the following criteria: visible appearance of potato tubers, knowledge on susceptibility of different cultivars, optical impression of field plants, and conditions on the field, such as soil type and soil humidity. Thus, the grading by Stöver company was completely independent of any estimation of bacterial attack and only based on the experience of the experts.
A comparison of these two independent assessments of the potato specimens from different sources revealed a rather good correspondence in most of the cases. The only two exceptions were nos. 9 and 16 which were found to be very strongly contaminated by erwinias (laboratory tests in Göttingen), but assessed as moderately affected by Stöver Co. experts (Table 14).

Table 14: Comparison of the determined latent contamination with *Erwinia spp.* in 12 potato tuber samples in Göttingen with field data obtained by Stoever Company in 1998.

Sample no.	Laboratory tests, Göttingen	Field observations, Stöver Co.
8	weak	moderate 1)
9	very strong	moderate 2)
10	moderate	moderate
11	very strong	strong
12	strong	strong
13	strong	strong
14	moderate	moderate
15	strong	strong
16	very strong	moderate
17	moderate	weak
18	very weak	weak
19	moderate	moderate

1) = potato tuber sample no. 8 was used as a control and was not treated with Degaclean

2) = potato tuber sample no. 9 was treated with Degaclean (see chapter II)

3.7.2 Evaluation of latent contamination of 232 potato tuber samples from the years 1998, 1999 and 2000

In the years of 1998, 1999 and 2000 232 samples of potato tubers were sent to Göttingen by Stöver Co. for determination of latent contamination. The potato specimens originated from 3 different locations: Carolinensiel (Ca) near Wittmund (Ostfriesland), 18334 Semlow (Se) near Ribnitz–Dammgarten and 23968 Gägelow (Gä.) near Wismar (Mecklemburg).

In each year, the potatoes were planted between April 1 to April 20 and harvested between September 1 to 30. Twenty potato tubers from each sample were analysed separately in triplicates as described (2.3.7.2) using the CVPM medium, and classified according to the bacterial concentration per Petri dish and also per tuber. Tables 15, 16 and 17 summarize the results obtained in the years 1998, 1999 and 2001, respectively. These results reveal considerable differences between the samples. In general, the specimens from the year of 1998 were much stronger contaminated than those of the years 1999 and 2000. The lowest contamination was determined for the potatoes harvested in 2000. Thus, the average degrees of contamination were 2.13, 1.24 and 0.42 for the years 1998, 1999 and 2000, respectively.

Table 15 : Detection of latent contamination with *Erwinia spp.* of potato tubers harvested in the year 1998

	Experiment 1			Experiment 2			Experiment 3				
Sample no.	cfu/ Petri dish	cfu/tuber	Degree of contamination	cfu/ Petri dish	cfu/tuber	Degree of contamination	cfu/ Petri dish	cfu/tuber	Degree of contamination [1)]	Average	Classification
Ca.1	3	45	2	27	405	3	0	0	1	2.0	2
Ca.2	245	3675	4	3	45	2	3	45	2	2.7	3
Ca.3	1	15	1	3	45	2	0	0	1	1.3	1
Ca.4	0	0	1	0	0	1	1	15	1	1.0	1
Ca.5	2	30	2	1	15	1	0	0	1	1.3	1
Ca.6	6	90	2	1	15	1	0	0	1	1.3	1
Ca.7	0	0	1	0	0	1	0	0	1	1.0	1
Ca.8	37	555	3	0	0	1	1	15	1	1.7	2
Ca.9	6	90	2	1	15	1	12	120	2	2.0	2
Ca.10	825	12375	5	3	45	2	7	105	2	3.0	3
Ca.11	0	0	1	0	0	1	0	0	1	1.0	1
Ca.12	6	90	2	0	0	1	2	30	2	1.7	2
Ca.13	5	75	2	12	180	2	9	90	2	2.0	2
Ca.14	10	150	2	8	120	2	1	15	1	1.7	2
Ca.15	25	375	3	1	15	1	0	0	1	1.7	2
Ca.16	7	105	2	0	0	1	0	0	1	1.3	1
Ca.17	450	6750	4	140	2100	4	490	7350	4	4.0	4
Ca.18	500	7500	4	0	0	1	2	30	2	2.3	2
Ca.19	180	2700	4	0	0	1	0	0	1	2.0	2
Ca.20	10	150	2	0	0	1	0	0	1	1.3	1
Ca.21	80	1200	3	0	0	1	0	0	1	1.7	2
Ca.22	685	10275	5	1300	19500	5	2	30	2	4.0	4
Ca.23	370	5550	4	1440	21600	5	45	675	3	4.0	4
Ca.24	80	1200	3	0	0	1	0	0	1	1.7	2
Ca.25	0	0	1	6	90	2	0	0	1	1.3	1
Ca.26	0	0	1	0	0	1	86	1290	3	1.7	2
Ca.27	45	675	3	34	510	3	26	390	3	3.0	3
Ca.28	0	0	1	5	75	2	1	15	1	1.3	1
Ca.29	420	6300	4	370	5550	4	0	0	1	3.0	3
Ca.30	0	0	1	260	3900	4	450	6750	4	3.0	3
Ca.31	320	4800	4	0	0	1	12	180	2	2.3	2
Ca.32	3	45	2	160	2400	4	0	0	1	2.3	2
Ca.33	400	6000	4	0	0	1	0	0	1	2.0	2
Se.1	26	390	3	2	30	2	22	330	3	2.7	3
Se.2	0	0	1	0	0	1	0	0	1	1.0	1
Se.3	1	15	1	0	0	1	0	0	1	1.0	1
Se.4	35	525	3	1	15	1	41	615	3	2.3	2
Se.5	137	2055	4	2	30	2	4	60	2	2.7	3

Se.6	6	90	2	165	2475	4	0	0	1	2.3	2
Se.7	47	705	3	135	2025	4	0	0	1	2.7	3
Se.8	24	360	3	8	120	2	1360	20400	5	3.3	3
Se.9	34	510	3	182	2730	4	0	0	1	2.7	3
Se.10	0	0	1	1	15	1	0	0	1	1.0	1
Se.11	9	135	2	0	0	1	0	0	1	1.3	1
Se.12	0	0	1	1	15	1	0	0	1	1.0	1
Se.13	6	90	2	0	0	1	0	0	1	1.3	1
Se.14	60	900	3	0	0	1	465	6975	4	2.7	3
Se.15	9	135	2	0	0	1	4000	60000	5	2.7	3
Se.16	0	0	1	0	0	1	0	0	1	1.0	1
Se.17	10	150	2	0	0	1	0	0	1	1.3	1
Gä.1	127	1905	3	15	225	3	140	2100	4	3.3	3
Gä.2	16	240	3	121	1815	3	11	165	2	2.7	3
Gä.3	4	60	2	0	0	1	0	0	1	1.3	1
Gä.4	4	60	2	0	0	1	0	0	1	1.3	1
Gä.5	200	3000	4	6	90	2	28	420	3	3.0	3
Gä.6	38	570	3	0	0	1	44	660	3	2.3	2
Gä.7	13	195	2	6	90	2	35	525	3	2.3	2
Gä.8	41	615	3	5	75	2	125	1875	3	2.7	3
Gä.9	1	15	1	0	0	1	3	45	2	1.3	1
Gä.10	19	285	3	0	0	1	15	225	3	2.3	2
Gä.11	118	1770	3	5	75	2	130	1950	3	2.7	3
Gä.12	42	630	3	20	300	3	5	75	2	2.7	3
Gä.13	27	405	3	77	1155	3	92	1380	3	2.7	3
Gä.14	53	795	3	98	1470	3	69	1035	3	2.7	3
Gä.15	357	5355	4	265	3975	4	90	1350	3	3.3	3
Gä.16	262	3930	4	127	1905	3	31	465	3	3.0	3
BA.1	0	0	1	20	300	3	6	90	2	2.0	2
BA.2	6	90	2	0	0	1	0	0	1	1.3	1
BA.3	6	90	2	1	15	1	0	0	1	1.3	1
BA.4	30	450	3	0	0	1	0	0	1	1.7	2
BA.5	30	450	3	149	2235	4	17	255	3	3.3	3
BA.6	30	450	3	2	30	2	0	0	1	2.0	2
BA.7	70	1050	3	4	60	2	2	60	2	2.3	2
BA.8	240	3600	4	30	450	3	34	1020	3	3.3	3
Average degree of contamination										**2.13**	

1) Footnote for table 15:

Degree of contamination	
0	Free of contamination
1	very weak
2	weak
3	moderate
4	strong
5	very strong

Table 16 : Detection of latent contamination with *Erwinia spp.* of potato tubers harvested in the year 1999

Sample no.	Experiment 1			Experiment 2				
	cfu/ Petri dish	cfu/tuber	Degree of contamination	cfu/ Petri dish	cfu/tuber	Degree of contamination	Average	Classification
Ca.1	0	0	1	0	0	1	1.0	1
Ca.2	2700	40500	5	3	45	2	3.5	4
Ca.3	0	0	1	0	0	1	1.0	1
Ca.4	1	15	1	0	0	1	1.0	1
Ca.5	0	0	1	0	0	1	1.0	1
Ca.6	0	0	1	0	0	1	1.0	1
Ca.7	0	0	1	0	0	1	1.0	1
Ca.8	0	0	1	0	0	1	1.0	1
Ca.9	3	45	1	1	15	1	1.0	1
Ca.10	0	0	1	0	0	1	1.0	1
Ca.11	12	180	2	0	0	1	1.5	2
Ca.12	0	0	1	0	0	1	1.0	1
Ca.13	0	0	1	0	0	1	1.0	1
Ca.14	2400	3600	5	425	6375	4	4.5	5
Ca.15	0	0	1	0	1	1	1.0	1
Ca.16	0	0	1	0	1	1	1.0	1
Ca.17	2	30	1	0	1	1	1.0	1
Ca.18	0	0	1	0	1	1	1.0	1
Ca.19	0	0	1	0	1	1	1.0	1
Ca.20	13	195	2	2	30	2	2.0	2
Ca.21	0	0	1	2	30	2	1.5	2
Ca.22	0	0	1	0	0	1	1.0	1
Ca.23	0	0	1	0	0	1	1.0	1
Ca.24	0	0	1	0	0	1	1.0	1
Ca.25	0	0	1	0	0	1	1.0	1
Ca.26	0	0	1	0	0	1	1.0	1
Ca.27	24	360	3	0	0	1	2.0	2
Ca.28	0	0	1	0	0	1	1.0	1
Ca.29	0	0	1	0	0	1	1.0	1
Ca.30	0	0	1	0	0	1	1.0	1
Ca.31	0	0	1	0	0	1	1.0	1
Ca.32	0	0	1	0	0	1	1.0	1
Ca.33	0	0	3	0	0	1	2.0	2
Ca.34	26	390	3	0	0	1	2.0	2
Ca.35	0	0	1	0	0	1	1.0	1
Ca.36	4	60	2	0	0	1	1.0	1
Ca.37	0	0	1	0	0	1	1.0	1
Ca.38	0	0	1	0	0	1	1.0	1
Ca.39	0	0	1	0	0	1	1.0	1
Ca.40	0	0	1	0	0	1	1.0	1
Ca.41	0	0	1	0	0	1	1.0	1

Ca.42	0	0	1	0	0	1	1.0	1
Ca.43	0	0	1	0	0	1	1.0	1
Gä.1	0	0	1	0	0	1	1.0	1
Gä.2	1	15	1	0	0	1	1.0	1
Gä.3	0	0	1	0	0	1	1.0	1
Gä.4	0	0	1	0	0	1	1.0	1
Gä.5	0	0	1	0	0	1	1.0	1
Gä.6	16	240	2	5	75	2	2.0	2
Gä.7	1	15	1	0	0	1	1.0	1
Gä.8	75	405	3	0	0	1	2.0	2
Gä.9	1	15	1	0	0	1	1.0	1
Gä.10	0	0	1	0	0	1	1.0	1
Gä.11	0	0	1	0	0	1	1.0	1
Se.1	0	0	1	0	0	1	1.0	1
Se.2	0	0	1	0	0	1	1.0	1
Se.3	0	0	1	0	0	1	1.0	1
Se.4	0	0	1	0	0	1	1.0	1
Se.5	0	0	1	0	0	1	1.0	1
Se.6	0	1	0	0	1	1	1.0	1
Se.7	1500	325000	5	0	0	1	3.0	3
Se.8	0	0	1	0	0	1	1.0	1
Se.9	0	0	1	0	0	1	1.0	1
Se.10	0	0	1	0	0	1	1.0	1
Se.11	8	120	2	0	0	1	1.5	2
Se.12	0	0	1	0	0	1	1.0	2
Se.13	3	45	2	0	0	1	1.5	2
Se.14	0	0	1	0	0	1	1.0	1
Se.15	28	420	3	0	0	1	2.0	2
Se.16	0	0	1	0	0	1	1.0	1
Se.17	0	0	1	0	0	1	1.0	1
Se.18	27	405	3	8	120	2	2.5	3
Se.19	0	0	1	0	0	1	1.0	1
Se.20	0	0	1	0	0	1	1.0	1
Bradby 1	0	0	1	0	0	1	1.0	1
Bradby 2	0	0	1	0	0	1	1.0	1
Average degree of contamination							**1.24**	

Table 17 : Detection of latent contamination with *Erwinia spp.* of potato tubers harvested in the year 2000

	Experiment 1			Experiment 2			Experiment 3				
Sample no.	cfu/ Petri dish	cfu/tuber	Degree of contamination	cfu/ Petri dish	cfu/tuber	Degree of contamination	cfu/ Petri dish	cfu/tuber	Degree of contamination	Average	Classification
Ca.1	0	0	0	0	0	0	0	0	0	0	0
Ca.2	0	0	0	0	0	0	0	0	0	0	0
Ca.3	0	0	0	0	0	0	0	0	0	0	0
Ca.4	0	0	0	0	0	0	0	0	0	0	0
Ca.5	14	420	3	0	0	0	0	0	0	1.0	1
Ca.6	2	60	2	3	90	2	2	60	2	2.0	2
Ca.7	0	0	0	0	0	0	0	0	0	0	0
Ca.8	0	0	0	0	0	0	0	0	0	0	0
Ca.9	0	0	0	1	30	2	0	0	0	0.66	1
Ca.10	0	0	0	0	0	0	0	0	0	0	0
Ca.11	0	0	0	0	0	0	0	0	0	0	0
Ca.12	0	0	0	0	0	0	0	0	0	0	0
Ca.13	0	0	0	0	0	0	0	0	0	0	0
Ca.14	0	0	0	0	0	0	0	0	0	0	0
Ca.15	0	0	0	0	0	0	0	0	0	0	0
Ca.16	0	0	0	0	0	0	0	0	0	0	0
Ca.17	0	0	0	0	0	0	0	0	0	0	0
Ca.18	0	0	0	0	0	0	0	0	0	0	0
Ca.19	0	0	0	0	0	0	0	0	0	0	0
Ca.20	0	0	0	0	0	0	0	0	0	0	0
Ca.21	0	0	0	0	0	0	0	0	0	0	0
Ca.22	0	0	0	0	0	0	0	0	0	0	0
Ca.23	0	0	0	0	0	0	0	0	0	0	0
Ca.24	0	0	0	0	0	0	0	0	0	0	0
Ca.25	0	0	0	0	0	0	1	30	2	0.66	1
Ca.26	0	0	0	0	0	0	0	0	0	0	0
Ca.27	1	30	2	5	150	2	0	0	0	1.3	1
Ca.28	0	0	0	0	0	0	0	0	0	0	0
Ca.29	0	0	0	0	0	0	0	0	0	0	0
Ca.30	0	0	0	1	30	2	1	30	2	1.3	1
Ca.31	0	0	0	0	0	0	0	0	0	0	0
Ca.32	0	0	0	0	0	0	0	0	0	0	0
Ca.33	0	0	0	3	90	2	2	60	2	1.3	1
Ca.34	0	0	0	1	30	2	1	30	2	1.3	1
Ca.35	0	0	0	0	0	0	0	0	0	0	0
Ca.36	0	0	0	0	0	0	0	0	0	0	0
Ca.37	0	0	0	0	0	0	0	0	0	0	0
Ca.38	0	0	0	0	0	0	0	0	0	0	0
Ca.39	0	0	0	0	0	0	0	0	0	0	0

Ca.40	0	0	0	0	0	0	0	0	0	0	0
Ca.41	70	2100	4	30	900	3	1	30	2	2.6	3
Ca.42	0	0	0	0	0	0	0	0	0	0	0
Ca.43	0	0	0	0	0	0	0	0	0	0	0
Ca.44	0	0	0	0	0	0	0	0	0	0	0
Ca.45	0	0	0	0	0	0	0	0	0	0	0
Ca.46	0	0	0	0	0	0	0	0	0	0	0
Gä.1	1	30	2	0	0	0	0	0	0	0.67	1
Gä.2	0	0	0	0	0	0	0	0	0	0	0
Gä.3	0	0	0	0	0	0	0	0	0	0	0
Gä.4	13	390	3	23	690	3	23	690	3	3.0	3
Gä.5	1	30	2	3	90	2	1	30	2	2.0	2
Gä.6	6	180	2	16	480	3	390	1170	3	2.7	3
Gä.7	0	0	0	0	0	0	0	0	0	0	0
Gä.8	1	30	2	1	30	2	1	30	2	1.0	1
Gä.9	0	0	0	0	0	0	0	0	0	0	0
Gä.10	0	0	0	0	0	0	0	0	0	0	0
Gä.11	0	0	0	0	0	0	0	0	0	0	0
Se.1	0	0	0	0	0	0	0	0	0	0	0
Se.2	0	0	0	0	0	0	0	0	0	0	0
Se.3	0	0	0	0	0	0	0	0	0	0	0
Se.4	3	90	2	3	90	2	1	30	2	2.0	2
Se.5	0	0	0	0	0	0	0	0	0	0	0
Se.6	0	0	0	0	0	0	0	0	0	0	0
Se.7	0	0	0	0	0	0	0	0	0	0	0
Se.8	1	30	2	1	30	2	1	30	2	2.0	2
Se.9	0	0	0	0	0	0	0	0	0	0	0
Se.10	0	0	0	0	0	0	0	0	0	0	0
Se.11	1	30	2	14	420	3	14	420	3	2.7	3
Se.12	0	0	0	0	0	0	0	0	0	0	0
Se.13	0	0	0	0	0	0	0	0	0	0	0
Se.14	0	0	0	0	0	0	0	0	0	0	0
Se.15	0	0	0	0	0	0	0	0	0	0	0
Se.16	0	0	0	0	0	0	0	0	0	0	0
Se.17	0	0	0	0	0	0	0	0	0	0	0
Se.18	0	0	0	0	0	0	0	0	0	0	0
Se.19	1	30	2	0	0	0	12	360	3	1.6	2
Se.20	50	1500	3	13	390	3	10	300	3	3.0	3
B1	1	30	2	0	0	0	0	0	0	0.67	1
B2	0	0	0	0	0	0	0	0	0	0	0
B3	0	0	0	0	0	0	0	0	0	0	0
B4	2	60	2	0	0	0	0	0	0	0.67	1
WE1	0	0	0	0	0	0	0	0	0	0	0
Average degree of contamination										**0.42**	

Footnotes for tables 15 – 17 **:**
Ca = SHG Carolinensiel
Gä = Gägelow
Se = Semlow

For the 82 potato specimens tested in 2000 (see Table 17) an estimation of "suspected" contamination was reported by Stöver Co. on the basis of field observations and other data (see chapter 3.7.1).

A comparison of these two independent scorings of latent contamination is shown by Table 18. Only 6 specimens were suspected to be contaminated by Stöver Co. Five of these were, indeed, found to be contaminated by erwinias in the laboratory tests in Göttingen. In additional 15 specimens, which were suspected to be free of contamination by Stöver Co., erwinias were detected in Göttingen, however with a low degree in most of the cases (5 very weak, 8 weak). All the other specimens were scored as free of contamination in both independent assessments.

Table 18: Comparison of latent contamination determined in Göttingen and suspected contamination estimated by Stöver Co. from field observations of potatoes harvested in 2000

Sample no.	Laboratory tests, Göttingen	Field observations, Stöver Co.
Se. 4	weak	free
Se. 8	weak	free
Se. 11	moderate	moderate
Se. 12	free	moderate
Se. 19	weak	free
Se.20	moderate	moderate
Gä 01	very weak	free
Gä 04	moderate	free
Gä 05	weak	very weak
Gä 06	moderate	moderate
Gä 08	weak	free
Ba 01	very weak	moderate
Ba 04	very weak	free
Ca 05	weak	free
Ca 06	weak	free
Ca 09	very weak	free
Ca 27	weak	free
Ca 30	very weak	free

3.7.3 Evaluation of potato lots with different degrees of latent contamination by field experiments in the following year

The potato lots with degrees of latent contamination by *Ecc* of 1 (weak), 2 (moderate) and 3 (strong) were all cultivar Morene harvested in 1998, but originated from Semlow 18 ha, Gägelow, and Semlow 33 ha, respectively. These potato lots were analysed for latent contamination in Göttingen in 1998. The results can be drawn from Table 15, as follows:

Sample no.	Designation in 1998	Grading in 1998
1	Se 2	1.0
2	Se 4	2.3
3	Se 5	2.7

In the following vegetation period (1999) these potatoes were grown in an experimental design with 4 repetitions (subchapter 2.5) on a farm in Natenstedt. The results (Table 19) clearly show a higher tuber yield and higher tuber size in the variant 1 originating from weakly contaminated seed potatoes. The lowest total tuber yield was recorded in the variant 3 originating from strongly contaminated seed potatoes. Although the differences between variants 1 and 3 were highly significant, the differences between strongly (3) and moderatly (2) contaminated potato tubers were insignificant. Also, the percentage of tubers with soft rot was significantly lower in variant 1 than in variants 2 and 3 (BRUER, Stöver Co., person. communication).

Table 19: Effects of different degrees of latent contamination of seed potato lots with *Ecc*, as determined in laboratory tests in Göttingen, on yield, tuber sizes and tuber numbers in different fractions according to tuber size

Yield and tuber size	Seed potato lots with latent contamination		
Yield in t/ha	1 : weak	2.3 : moderate	2.7 : strong
Fraction 35 – 40	1.82	2.1	2.06
Fraction 40 - 50	17.69	17.2	17.08
Fraction 50 - 55	13.19	15.4	11.58
Fraction 55 - 60	11.94	10.1	10.83
Fraction 60 +	16.32	9.5	11.83
Total	**60.95**	**54.3**	**53.4**
Tuber size			
Number of tubers/10 kg			
Fraction 50 +	54	62	54
Fraction 55 +	44	49	44
Fraction 60 +	36	41	36

4 DISCUSSION

Contamination with *E. carotovora* is very common in potato stocks and often becomes important during long storage periods when soft rot is caused (PEROMBELON *et al*., 1979). Soft rot of tubers in storage can be found in most enviromental conditions as major cause of infection of seed potatoes. It has also been clearly established that daughter tuber contamination may occur in the soil by spread of erwinias from rotting mother tubers and equipment used for harvesting and handling (GRAHAM and HARDIE, 1971). Therefore, the use of roguing has still an important place in controlling potato seed quality. In addition, seed quality can be improved substantially by determining *Erwinia spp.* levels on potato tubers (TOTH *et al*., 1996). Therefore, the most important measure to reduce losses by *Erwinia carotovora* soft rots is to eradicate or reduce the number of contaminated tubers during long-time storage and in seed potatoes. Also, potato genotypes have been screened for tuber resistance to erwinias and differences in resistance have been found (MUNZERT & HUNNIUS, 1980; LAPWOOD *et al*., 1984). However, the degree of resistance is rather low in most of the cases. Several methods have been developed to quantify the number of erwinias on potato tubers. However, the sensitivity of these methods is often not satisfactory. Therefore, these studies aimed to improve the methods for detection of *Erwinia spp.* in potato stocks.

4.1 Identification and differentiation of *Erwinia spp.*

Three different soft rot erwinias are commonly associated with potatoes, *Erwinia carotovora subsp. atroseptica* (*Eca*) (van Hall 1902) Dye 1969, *Erwinia carotovora subsp. carotovora* (*Ecc*) (Jones, 1991) Bergey, Harrison, Breed, Hammer & Huntoon, 1923, and *Erwinia chrysanthemi* (*Ech*) BURKHOLDER *et al*., all of which can cause tuber soft rot (PEROMBELON 1992). Erwinia cells in general are identified by their ability to form cavities on the semi-selective medium due to breakdown of polypectate by pectolytic enzymes. Two methods are available to differentiate between *Eca* and *Ecc*. The older one is based on the incubation at differential temperatures (PEROMBELON and HYMAN, 1986) and evaluation of cavity formation (PEROMBELON, 1992); the second method is the growth on the α-methyl-d-glucoside medium. The results obtained in our studies revealed that *Eca* is able to form characteristic cavities already after 24 h at 27 °C, *Ecc* does so at 27 °C and 35.5 °C, whilst *Ech* forms cavities at 27 °C, 35.5 °C and 37 °C within 24 h. On α-methyl-d-glucoside the bacterial colonies of *Eca* showed a pink colour, whereas weak white colonies were obtained by *Ecc* and *Ech* on the same medium after incubation at 27 °C for 24 h. Both tests gave good results in these studies in differentiation of several strains from the GSPB culture

collection. The test on α-methyl-d-glucoside was very safe to discriminate *Eca* from *Ecc* and *Ech*. However, it was difficult to distinguish *Ecc* and *Ech* by growth at 35.5 and 37 °C, as was also reported by JANSE and SPIT (1989). It appeared easier to differentiate *Ecc* and *Ech* by the phosphatase test, as was also shown by SHEKHAWAT and RUDOLPH (1978) after protein electrophoresis.

4.2 Preservation of *Erwinia spp.* strains used in this study

Several culture preservation methods were tested and evaluated for their suitability in maintaining the viability of *Erwinia spp.*. Our experiments showed that a very suitable method for preservation of *Erwinia spp.*, *Eca* and *Ecc* was incubation in sterile distilled water at room temperature. Thus, the viability of all *Erwinia spp.* was maintained for more than 16 months at 4 °C as well as at room temperature. This method is very simple, and the bacterial strains did not lose virulence during storage. BDLIYA (1995) reported that all strains of *Erwinia spp.* were preserved at 18 °C in calcium carbonate based liquid medium (YPC) in tightly closed screw capped test tubes.

4.3 Development of a semi-selective medium for isolation of soft rot erwinias

Specific media for isolation of *Erwinia spp.* from potato tubers and identification of the pathogen have been in use for a long time. Several selective media containing pectin or sodium polypectate were developed with different degrees of success for detection of soft rot bacteria from potatoes (STEWART, 1962; LOGAN, 1963; BERAHA, 1968; PEROMBELON, 1971; THORNE, 1972; CUPPELS and KELMAN, 1974; COTHER *et al*., 1980; BDLIYA 1995). The isolation of *Erwinia spp.* using the selective media is based on the ability of the pathogen to hydrolyze polypectate and to form typical cup-shaped pits (cavities) in the medium. As was shown by PEROMBELON (1971), other pectolytic bacteria occurring in the soil, especially certain pseudomonads, also form cavities, but these are shallow and quite distinct from those caused by varieties of *E. carotovora,* which are easily recognized even when there is overcrowding by other organisms. CUPPLES and KELMANN (1974) developed the crystal violet pectate based selective medium (CVP) for isolation of erwinias dependent on the quality of the polypectate used (PIERCE & McCAIN, 1992). PEROMBELON and BURNETT (1991) described two crystal violet polypectate media for detection of soft rot erwinias. These were a single layer (CVPB) and a double layer (DLCVP) medium based on the sodium polypectate "Bulmer". The authors found that the growth of *Erwinia spp.* on the CVPB medium was poor and they suggested that the tryptone probably chelates heavy metals present in the "Bulmer"

pectin preparation rather than acting as a nutrient source. The adding of 0.1 or 0.5% tryptone to the selective medium resulted in growth and cavity formation. The effect of increasing the concentration of tryptone on the sensitivity of the selective-medium was therefore evaluated by PEROMBELON and BURNETT (1991). BDLIYA (1995) also modified the double layer crystal violet mediun for isolation of *Erwinia spp.* by using the sodium polypectate "Bulmer" and found that the addition of tryptone to the basal and over layer improved the cavity formation by soft rot erwinias.

Our studies revealed that **without tryptone** and **citrate** bacterial colonies with cavities did not develop, and only few colonies and weak cavities were formed when tryptone and citrate were added at low concentrations to the basal and over layer, respectively. On the other hand, many colonies grew when tryptone (0.6 %) was added only to the basal layer and tri-sodium citrate dihydrate (0.5%) only to the overlayer. It was concluded from these results that tryptone diffusing from the basal layer served as nitrogen source for the bacteria in the over-layer and promoted their growth, whereas concomitant presence of tryptone and pectin in the overlayer did not force the bacteria to utilize pectin as carbon source. Obviously, the additional carbon source citrate enhanced bacterial growth in the overlayer without reducing pectin degradation by the bacteria. Thus, the addition of tryptone to the overlayer and of citrate to the basal layer was not necessary.

With the original amount of 18 g **polypectate** in the overlayer the medium became too solid and only weak cavities were obtained, whereas the reduction to 15 g polypectate per litre allowed very good cavity formation. Omitting of **bromothymol blue** resulted in more distinctive cavities because the medium of BDLIYA with two indicators (bromothymol blue and crystal violet) became green, so that the appearance of the cavities was not clear. The reduction of the amount of **$CaCl_2$** X 2 H_2O (10%) in the overlayer, compared to the medium of BDLIYA (1995), slightly reduced the firmness of this medium and improved the growth and cavity formation of *Erwinia spp.*, whereas without $CaCl_2$ X 2 H_2O (10%) the medium did not solidify. Also *in planta*, the formation of calcium–pectate complexes appears to be responsible for increased resistance, as it may protect the tissue against enzymatic degradation by the pathogen (BATEMAN and MILLAR, 1966; PAGEl and HEITEFUSS, 1989).

4.4 Comparison of different pectin sources for detection of *Erwinia spp.* on the semi-selective medium

Several commerical pectin and polypectate preparations were used in these studies and found to be unsatisfactory for isolation of *Erwinia spp.* from potatoes because some did not solidify

at pH 7.2 or did not allow satisfactory growth of erwinias. This may have been due to high amounts of heavy metals which are toxic to *Erwinia spp.* (PEROMBELON & BURNETT, 1991).

From our studies we concluded that there is a strong relationship between the suitability of the pectins and **degree of esterification**. All the *Erwinia spp***.** produce several pectolytic enzymes two of which play a major role. The first is pectinmethylesterase (PME), and the second pectintranseleminase (pectate lyase) (PL). PL is produced in higher activity than PME by erwinias (MORAN and STARR, 1969; GARDNER and KADO, 1976; CHATTERJEE *et al*., 1979; FAVEY *et al*., 1992). Since PL cleaves internal glycosidic linkages by ß-elimination **after demethylation** of the polymer by pectin methylesterase, PL causes a rapid decrease in the viscosity of polygalacturonic acid (GLENN, 1976; BRAUN and SCHMITZ, 1980; FAVEY *et al*., 1992) but not of pectin. Also, an increase in pectin esterification (more than 20% of galacturonic acid residues methoxylated) leads to less efficient degradation of the substrate by PL (FAVEY *et al*., 1992). PAGEl and HEITEFUSS (1990) found that 14 h after inoculation of potato tubers with *Eca* the main enzyme activity was PG, whereas after 22 h PL played the dominant role. Therefore, the growth of *Erwinia spp.* and cavity formation was good on pectin with a low degree of esterification, such as pectic acids from apple (H.+ C.) and citrus (H.+ C.), Copenhagen (A/S) pectin and pectin N (Roth), that had an esterification degree of 5, 7, <10% and 8%, respectively. On the other hand, pectins with high esterification did not show clear cavities, such as pectin A (Roth), pectin C (Roth), pectin from citrus (Serva), pectin from citrus (Fluka), and pectin from apple (H.+ C.) with degrees of esterification of 36%, 67-71%, 65%, 63-66% and 32%, respectively, (see 2.3.3). It is suggested, therefore, that future tests with pectins suited for detection of erwinias should select preparations with a low esterification degree. It appears as if the content of heavy metals, as suggested by PEROMBELON and BURNETT (1991), does not play a decisive role for the suitability of pectins in the semi-selective medium.

4.5 Recovery rate and detection limit of erwinias in artificially contaminated potato homogenates

The recovery of *Erwinia spp.* strains on CVPM were 87% compared to 49% and 23% on the media of BDLIYA (1995) and PEROMBELON & BURNETT (1991), respectively. As reported by PEROMBELON & BURNETT (1991) 7 from 12 strains showed a recovery rate of <50% on CVPB compared to NA.

From our studies it can be concluded that the detection limit on the new semi-selective medium was 2 X 10^1 cfu/ml. Also BDLIYA (1995) reported that the detection limit on his medium was 10^2 cfu/ml, whereas the dilution platings on a selective medium of PEROMBELON and HYMAN (1986) allowed detection of 1 – 5 X 10^4 cfu/ml peel extract (JONES *et al*., 1994). VAN VUURDE and ROOZEN (1990) reported a detection limit of *Ecc* and *Eca* at levels of 10^3 cfu/ml of slurry by isolation on CVP. The low detection limit determined in our studies may be due to some modifications in the semi-selective medium of Bdliya (1995) as described in subchapter 4.3.

Thus, the modified semi-selective medium (designated CVPM) has **several advantages** compared to other selective media: 1) The detection limit of soft rot erwinias from potatoes was 2 X 10^1 cfu/ml, 2) the ability of *Erwinia spp.* to produce the cavities were distinctive after 13 h of incubation at 27 °C (AHMED *et al*., 2000), 3) erwinias can be detected in the concomitant presence of high populations of other saprophytic bacteria, and 4) the two soft rot erwinias commonly associated with potatoes (*Ecc* and *Eca*) can be differentiated by cultivation at different temperatures and by transferring directly from the cavities to the α-methyl-d-glucoside medium.

Previous work has shown that standard serological methods such as immunofluorescence staining and the double antibody sandwich enzyme linked immunosorbant assay (DAS)(ELISA) are not suitable to quantify *E.c.* contamination of potato tubers, partly because of low sensitivity of 10^5 cfu/ml (JONES *et al*., 1994), and because the quantitative methods do not distinguish clearly between different subspecies (JONES *et al*., 1993). By using the new semi-selective medium (CVPM) the potato seed quality can be improved substantially by determining latent contamination with *E. carotovora* on tubers. However, a number of different methods for enumeration of *E. carotovora* on the tuber stocks are available and are continuously by being improved, such as immunofluorescence and DAS-ELISA (VAN VUURDE & ROOZEM, 1990; TOTH *et al*., 1996; ALLEFS *et al*., 1995; HELIAS *et al*., 2000).

4.6 Occurrence of different erwinias

The morphological and biochemical tests applied to differentiate erwinias in these studies confirmed the taxonomic identification of several *Eca*, *Ecc* and *Ech* strains from the GSPB–bacterial collection. Although all the potato specimens tested looked healthy, a latent contamination with *Erwinia carotovora spp. carotovora* was determined in $^2/_3$ (10 from 16) of the samples purchased from shops. Also, the cultivars showed differences in degree of contamination by *Erwinia spp.* according to the contamination level on the tubers.

Interestingly, all the bacterial strains isolated from potato tubers purchased from different shops and supermarkets in this study (99 isolates) were identified as *Ecc*.

Earlier studies concluded that the tubers obtained from apparently blackleg free tubers were contaminated with both, *Eca* and *Ecc* (PEROMBELON, 1972; PEROMBELON and KELMAN, 1987). Similar results were obtained in Germany (Van den BOOM, 1967; FICKE *et al*., 1973; NAUMANN *et al*., 1976); and in USA (De BOER and KELMAN, 1975; NIELSON, 1978), where the relative proportion of *Eca* and *Ecc* varied widely on the tubers. PEROMBELON (1971) reported that more *Eca* cells (4 X) than *Ecc* were obtained from tubers taken from storage. In contrast, 90% of the isolates from naturally rotting tubers in storage were *Ecc* in studies of LAZAR and BUCUR (1964). None of these workers reported the presence of *Ech*. Although *Eca* is the predominant agent involved in blackleg under cool climates, *Ecc* occurs in both temperate and warm climates but mostly in storage, and *Ech* predominates in warm regions as a storage pathogen (PEROMBELON and KELMAN, 1987; PEROMBELON and HYMAN, 1989; SALMOND, 1992; HELIAS *et al*., 2000). The two pathogens *Ecc* and *Eca* can attack the tubers in the field and after harvest, and *Ech* may also be an important cause of decay of seed potatoes at soil temperatures in the region of 30 °C (LELLIOTT, 1974; LUND, 1979; COTHER, 1980 and SKERMAN *et al*., 1980).

According to our results all isolates obtained from tubers originating from potato stocks were *Ecc*. Although these results confirmed those reported by LAZAR and BUCUR (1964) that 90% from the erwinias were *Ecc*, they differ from several other reports as listed above. The conflicting results may be due to many factors such as anaerobic conditions prevailing during storage, free water covering the tuber surface, temperatures above the minimum required for growth of the pathogen, and physiological factors such as a high water potential, all of which favour infection by *Ecc* (CROMARTY and EASTON, 1973; KELMAN *et al*., 1978).

The non–detection of *Eca* in potato stocks in our studies may be due to the fact that the potato lots were assessed after several months of storage. Thus, SCHOBER and ZADOKS (1999) reported that initial numbers of *Eca*, obtained by artificial infection, decreased rapidly during the first weeks of storage and did not increase significantly later in the storage period. Similarly, Van VUURDE & De VRIES (1994) found a continuous decrease of *Eca* populations on the surface of potato tubers during storage.

Another possibility is that the new semi-selective medium favours the growth of *Ecc* more than that of *Eca*, because EL–KAZAZZ (1984) found that generally *Ecc* strains showed higher activities for pectate lyase (PL) *in vitro* and *in vivo* than *Eca.* It was also observed in our studies with pure cultures that cavities of *Ecc* strains on the semi-selective medium were broader and deeper than those of *Eca* (chapter 3.5.1). Therefore, the possibility can not be

excluded that contaminations by *Eca* were overlooked in some of the samples because the cavities produced were much smaller than those by *Ecc*. On the other hand, field experiments on the effect of Degaclean on infection of potatoes by erwinias clearly showed that also *Eca* contamination was detected by King B medium which was supplemented with rifampicin (chapter 2). Further extensive experiments have to find out whether the sensitivity of the CVPM medium to detect contamination with *Ecc* and *Eca* under natural conditions differs.

4.7 Evaluation of methods to identify and quantify latent contamination of potatoes by erwinias

In our studies two methods were tested for isolation and detection of soft rot causing erwinias from potatoes. The results obtained showed that higher numbers of *Erwinia spp.* colonies were obtained from potato tubers by the soaking method compared to potato tuber peel homogenates (Table 9). It was shown earlier by PEROMBELON (1973) that often the tuber **surfaces** are contaminated by erwinias. The high proportion of latent contaminations in collective tuber samples at harvest can probably be explained by the fact that collective samples have a higher probability of being contaminated by at least one contaminated tuber, but may also reflect the higher contamination rates of small tubers (HELIAS *et al*., 2000). It is assumed that nearly all the bacteria on the potato tuber surfaces were isolated by the soaking method. This method also needed less time than analyzing homogenates from potato peels. Soaking of tubers in 0.01 M $MgSO_4$ for 3 h may allow nearly all *Erwinia spp.* contaminants to diffuse from the potato tubers into the soaking solution, because tuber contamination by erwinias is usually on the tuber surface. Soaking for only 3 h did not allow the bacteria to multiply because the temperature was very low (4 °C). In 10 from 11 samples tested, lower numbers of erwinias were obtained from peel homogenates than from soakates, and in one case with very low contamination bacterial colonies were only obtained from the soakage. Thus, our results confirmed those obtained by PEROMBELON *et al*. (1998) who also explained that tuber contamination by erwinias is usually superficial, having originated from infected rotting mother and progeny tubers in the field and during mechanical handling after harvest (PEROMBELON, 1992).

4.8 Sample size

When quantifying *E. carotovora* contamination of a potato stock, the tuber sample size should be representative, taking into account the variation in tuber contamination level that can be expected. The numbers of bacteria vary from tuber to tuber (PEROMBELON, 1972;

PEROMBELON *et al*., 1998), and it is practice to take between 25 and 70 tubers randomly per stock depending on how the tubers are stored, and the expected level of contamination. According to our results (Tables 12 and 13), for quantifying *Erwinia spp.* contamination of a potato stock a representative sample size should consist of at least 20 tubers taken randomly. Depending on the size of the potato stock, this test should be repeated at least three times with 20 potato tubers in each repetition.

4.9 Comparison of different years

Contamination with *E. carotovora* (*Eca* and *Ecc*) was very common in stored seed potato stocks classified as “Elite” seed or commercial seed (HARJU and KANKILA, 1993). However, contamination levels were highest after a rainy growing season (HARJU and KANKILA, 1993). In our studies, the semi-selective medium (CVPM) was tested on 232 samples from Stoever Co. from three vegetation periods (1998, 1999 and 2000). The results obtained (Tables 15, 16 and 17) showed a considerable variation in bacterial contamination from “very weak” to “very strong”. The lowest contamination was determined for the potatoes harvested in 2000, and the highest contamination occurred in 1998. Thus, the average degrees of contamination were 2.13, 1.24 and 0.42 for the years 1998, 1999 and 2000, respectively.

Table 20: Rainfall in mm from April – Septemper

Location	Year	Months						Total
		April	May	June	July	August	Sept.	
Carolinensiel	00	32.0	74.0	79.0	62.0	53.0	60.0	360
	99	33.0	17.0	51.0	63.0	73.0	65.0	302
	98	107.0	28.0	102.0	96.0	104	86.0	523
Semlow	00	24.0	44.0	93.0	90.0	85.0	59.0	395
	99	55.0	43.0	117.0	39.0	89.0	38.0	381
	98	62.0	49.0	77.0	144.0	95.0	46.0	473
Gägelow	00	27.0	35.0	55.0	116.0	106.0	61.0	400
	99	21.0	87.0	70.0	24.0	47.0	48.0	297
	98	73.0	23.0	140.0	99.0	57.0	24.0	416

Obviously, the high degree of contamination by erwinias in 1998 was due to higher rainfall in this year compared to 1999 and 2000 (Table 20) and to wet conditions during harvest in 1998 (Bruer, Stöver Co., person. communication). The same correlation existed when the potato

samples of the three locations Carolinensiel, Semlow and Gägelow were compared for the year 2000. Thus, rainfall in August with 53, 85 and 106 mm corresponded to average contamination degrees of 0.26, 0.6 and 1.1, respectively (Table 17).

4.10 Comparison of field observations and laboratory tests

Two methods for assessing latent contamination by erwinias were compared, field observations by Stöver Co. and laboratory tests in Göttingen, with 94 samples of potato tubers in two years (1998 and 2000). The scoring of the same potato provenances by experts from Stöver Produktion Co. in the 3 grades (weak, moderate and strong) was based on the following criteria: visible appearance, knowledge on susceptibility of different cultivars, optical impression of field plants, and conditions on the field, such as soil type and soil humidity. Thus, the grading by Stöver company was completely independent from the laboratory tests in Göttingen and only based on the experience of the experts.

In the year of 1998 12 specimens of healthy looking potato tubers were assessed. The laboratory tests in Göttingen (Table 14) showed a considerable variation in bacterial contamination from “very weak” to “very strong”, but these scorings were very similar to those by Stöver Co.. Only in 2 form 12 samples the results differed markedly, in that the potatoes were found to be very strongly contaminated by erwinias in laboratory tests in Göttingen, but assessed as moderately affected by Stöver Co. experts.

In the year of 2000, a comparison of these two independent assessments of latent contamination of 82 specimens showed that only 6 specimens were suspected to be contaminated by Stöver Co., and five of these were, indeed, found to be contaminated by erwinias in the laboratory tests in Göttingen. In additional 15 specimens, which were suspected to be free of contamination by Stöver Co., erwinias were detected in Göttingen, however with a low degree in most of the cases (5 very weak, 8 weak). All the other specimens were scored as free of contamination in both independent assessments.

The comparison of field observations and laboratory tests in the two years, thus, revealed 1) a rather **good agreement** between both assessments, and 2) a **higher sensitivity** of the microbiological tests in Göttingen.

The microbiological assay can be affected by variations from tuber to tuber and also from stock to stock. Thus, if only one strongly contaminated tuber was contained in the sample the whole sample would be scored as contaminated. Also JONES *et al*. (1994) found that the tubers taken from the surface of a stock or box tended to be less contaminated than those from below the surface. Because of the rather good correspondence of the two assessments in most of the cases

Stöver Produktion Co. agreed to continue the cooperation with the Department of Plant Pathology and Plant Protection in Göttingen to classify the potato stocks for contamination level by soft rot causing *Erwinia spp.*. This successful cooperation was only possible by applying the new semi-selective medium which is particularly well suited for detecting *Erwinia carotovora ssp. carotovora*, the major causal agent of potato soft rot during storage.

4. 11 Field experiments with potato lots assessed in different degrees of latent contamination by *Ecc*

These experiments (see Chapter 3.7.3) clearly revealed that potato lots assessed as lowly contaminated by *Ecc* (degree 1) gave higher yields in the following vegetation period with higher tuber sizes and a lower percentage of tubers with soft rot than potato lots assessed in contamination degrees 2.3 (moderate) and 2.7 (strong). As shown in Table 19, the yields from seed potatoes which were graded as moderately or strongly contaminated by *Erwinia carotovora* did not differ significantly with 54.3 and 53.4 t/ha, respectively. This was not surprising because also the gradings for latent contamination with *Erwinia carotovora* showed only slight differences with 2.3 and 2.7, respectively. On the other hand, the seed potatoes graded as weakly contaminated (grade 1.0) gave a higher yield (60.95 t/ha) in the following year than the other two samples.
Therefore, potato lots assessed as lowly or non-contaminated by *Erwinia carotovora* in the microbiological tests appear to be best suited for long storage and should as far as possible be preferred for potato production in the following year, because yield and quality could be improved.
The finding that potatoes originating from the lot with contamination degree 2 (moderate) contained significantly smaller tubers than the other two variants at harvest, may be due to the fact that this was the only potato lot originating from Gägelow and not from Semlow. Some observations indicated that the potatoes grown in Gägelow were stronger affected by virus diseases than those in Semlow (BRUER, person. communication).

5 SUMMARY

1) The **objective** of this study was to detect latent contamination of potato tubers by soft rot causing erwinias *[Erwinia carotovora subsp. atroseptica* (*Eca*), and *Erwinia carotovora subsp. carotovora* (*Ecc*)*]* and to improve the methods for isolation and identification.

2) All the *Erwinia* strains used in this study were clearly **identified and differentiated** by the classical tests, including pectolytic activity on potato tuber slices, utilization of α-methyl-d-glucoside, production of reducing substances from sucrose, phosphatase activity, growth on Logan's medium, and indole production. Virulence of the strains was tested by bioassay on potato tuber slices.

3) Testing several methods for **preservation** of *Erwinia spp*. revealed that the *Erwinia* strains (*Eca*, *Ecc* and *Erwinia chrysanthemi*; *Ech*) maintained viability for at least 16 months when preserved in sterile distilled water at 4 °C as well as at room temperature.

4) For a **sensitive detection** and identification of soft rot erwinias, the semi-selective double layer medium of Bdliya (1995) was modified. The amount of sodium polypectate was reduced from 18 g to 15 g per litre, tryptone and bromothymol blue were omitted from the overlayer, and the amount of $CaCl_2$ X 2 H_2O (10%) was reduced.

5) Most crucial for the modified medium, called CVPM, was the **pectin source**. When 15 pectin preparations were tested, only 5 allowed the formation of typical cavities by erwinias. Obviously, the **degree of esterification** was decisive. Thus, the growth of *Erwinia spp.* and cavity formation was much better on the CVPM medium with pectic acids with a low esterification degree (5 - 9%) than with pectins with a high degree of esterification (32 - 71%).

6) The **recovery rate** of bacteria from artificially inoculated tuber sap on the new semi-selective medium was ca. **90%** in comparison to other semi-selective media, where the recovery rate was 49% for CVPD (BDLIYA, 1995) and only 23% for the medium of PEROMBELON & BURNETT (1991). Therefore, the **detection limit** by using the CVPM

medium was **2 X 10^1 cfu/ml**.

7) In order to detect latent contamination of potatoes with *Erwinia spp.* the tubers were **soaked** in 0.01 M $MgSO_4$ for 3 h at 4 °C. The soakage was concentrated by centrifugation.

8) For quantifying *Erwinia spp.* contamination of potato stocks a representative **sample size** should consist of at least 20 tubers taken randomly. Depending on the size of the potato stock, this test should be repeated at least three times with 20 potato tubers in each repetition.

9) By application of this procedure 99 strains were obtained from different shops, supermarkets and farms. In $^2/_3$ of the specimens erwinias were isolated from healthy looking potato tubers and all of the isolates were **identified as *Ecc***.

10) The semi-selective medium (CVPM) was tested on **232 potato specimens** obtained from Stoever Produktion GmbH & Co. KG and harvested in the years 1998, 1999 and 2000. These tests revealed considerable differences between the samples. In general, the specimens from the year of 1998 were much stronger contaminated than those of the years 1999 and 2000. The lowest contamination was determined for the potatoes harvested in 2000.

11) In two cases (12 and 82 potato specimens harvested in 1998 and 2000, respectively) **a comparison of the results** obtained by isolation of erwinias with the semi-selective medium and field observations from Stoever Co. showed a very good correspondence of the determined and suspected contamination by *Erwinia carotovora*. It was concluded that the CVPM medium is well suited for isolation of *Erwinia spp.* (particularly for *Ecc*) from latently contaminated potatoes.

12) Field experiments proved that seed potato lots assessed as moderatly or strongly contaminated by *Ecc* gave lower yields and less quality than potato lots assessed as weakly contaminated. Therefore, the microbiological assessment of potato lots for latent contamination by *Erwinia carotovora* appears to be very valuable for improving yield, quality and storage capability of potatoes.

CHAPTER II

Effect of Degaclean® 150 on the reduction of soft rot of potato tubers

1 INTRODUCTION

One of the economically most important bacterial pathogens of plants and plant products is *Erwinia carotovora.* Especially during storage of potatoes high losses can occur. In contrast to fungal pathogens, the erwinias, like most other phytopathogenic bacteria, cannot be **controlled** by specific bactericides. Antibiotics are rather expensive and are not allowed in most of the cases, because of the risk that an application of antibiotics on a broad scale may give rise to antibiotic resistant bacterial populations which may transfer the resistance gene(s) to human pathogenic bacteria. Other bactericidal compounds, such as copper or sulphur–containing formulations, are not very effective and cause environmental pollution.

Therefore, several other measures have been developed to reduce bacterial diseases and post–harvest losses (OKON, 1990). In the case of *Erwinia carotovora* these measures include, for instance, crop rotation, optimal fertilization, careful irrigation, and especially several precautions during harvest and storage of potatoes.

Another alternative of directly eradicating erwinias is the application of **oxidizing agents** which are not harmful to the environment. The toxicity of oxygen against microorganisms is long known (GOTTLIEB, 1971). Thus, hydrogen peroxide is known since many years as disinfectant to inactivate or kill microorganisms. Peracetic acid is being used as disinfecting compound in human and veterinary medicine as well as in paper and cellulose industry, bottling industry and in food markets.

GREGORY and FRIDOVICH (1973) studied the **mode of action** and concluded that O^{*---} is one of the active agents of the peroxid. The second active component is the hydroxyl radical (OH^{*}). The extreme reactive radical O^{*---} oxidizes very fast diverse cell components, such as unsaturated fatty acids. Because these reactions occur so fast and unspecifically, allmost all microorganisms are killed after short exposure times (ROTILIO *et al*., 1973). The advantages of using hydrogen peroxide and peracids are twofold. Because of the fast and undirected effect the probability of microorganisms to become resistant is low. Secondly, these substances disintegrate after short reaction-time into non-polluting compounds, such as water, gaseous oxygen and organic acids. Consequently, the environment is hardly endangered by application of peracids and hydrogen peroxide.

Very short reaction-times are always of advantage when the microroganisms have to be killed quickly (PRIOR *et al*., 1998). Therefore, Degaclean® and Clarmarin® were developed by Degussa AG, Frankfurt. **Degaclean® 150** is a mixture of < 5% peracetic acid (CH_3COOOH) and 20 – < 60% hydrogen peroxide. Earlier experiments of MIELKE and HOPPE (1982) revealed that peracetic acid applied to the soil affected several fungal pathogens causing foot rot diseases of cereals.

However, these products are not plant protectants, such as fungicides or bactericides, because due to their unspecificity they also damage living plants. But, they can be used as **disinfectants** and additions to washing processes. Thus, experiments of NIEPOLD (1999b) showed that application of peracids to sewage water of a starch processing factory was very effective in destroying all microorganisms, including the quarantine species *Ralstonia solanacearum* and *Clavibacter michiganensis ssp. sepedonicus*. A concentration of 2000 ppm Clarmarin/catalase inhibitor (1:1) killed all microorganisms while the traditional hydrogen peroxide preparation (“Perhydrol”) affected vitality of the microbial populations only weakly.

Due to these positive effects, **our studies aimed** to find out whether the addition of Degaclean to the washing water of potato tubers or spraying of potato tubers would decrease latent contamination of potatoes by soft rotting erwinias. Therefore, laboratory as well as field experiments were carried out. Additionally, the potential of *Erwinia carotovora* strains to develop Degaclean-tolerance should be investigated and possible modes of action be characterized, such as changes in the LPS structure.

2 MATERIAL AND METHODS

2.1 Selection of antibiotics resistant isolates from *Erwinia spp.*

In order to facilitate the monitoring of *Erwinia carotovora* populations as affected by Degaclean-treatment of potato tubers, antibiotic-resistant strains were generated. This was achieved by subjecting the strains *Erwinia carotovora spp. atroseptica* (*Eca*) (GSPB no. 2967) and *Erwinia carotovora spp. carotovora* (*Ecc*) (GSPB no. 436) to increasing concentrations of the antibiotics Rifampicin (Fluka no. 83907) and Streptomycin (Sigma no. S-6501) (AL SHINAWI, 1996). The experiment started by culturing the bacteria in King's medium B (KB) (see chapter I, 2.1.2) for 24 h. The bacterial growth was scraped off and inoculated into 50 ml King' s B liquid medium and again incubated at 27 °C for 24 h. After that **5 ppm** of sterile filtrated **Rifampicin** (200 mg/10 ml methanol) and **25 ppm Streptomycin sulfate** (1.0 g /10 ml water) were added to separate bacterial cultures and incubated at 27 °C for 24 h. From each flask 0.1 ml were streaked onto King's medium B. After incubation of plates at 27 °C for 24 h, bacterial growth was scraped off and inoculated into new flasks, incubated at 27 °C for 24 h, and **10 ppm Rifampicin** and **50 ppm Streptomycin sulfate** were added. The flasks were incubated for 24 h. In the same way, the concentrations of the antibiotics were increased stepwise until 200 ppm Rifampicin and 800 ppm Streptomycin sulfate. The finally obtained isolates with resistance against the antibiotics were lyophilized and stored in the GSPB culture collection with numbers 2968 (*Eca* resistant against **200 ppm Rifampicin)** and 2970 (*Ecc* resistant against **200 ppm Rifampicin and 800 ppm Streptomycin sulfate)**.

2.2 Selection of Degaclean tolerant isolates of *Erwinia spp.*

The generation of two strains of *Erwinia spp.* (from *Eca* 2967 and *Ecc* 436) with tolerance against Degaclean was performed similar to generation of antibiotic resistant strains (subchapter 2.1, see above) by subjecting the bacteria to increasing concentrations of Degaclean. The bacterial strains were grown on Petri plates with King's medium B and incubated at 27 °C for 24 h, after which the bacterial growth from each strain was scraped off and in the **first step** was inoculated into Erylenmeyer flasks with King's medium B and incubated again at 27 °C for 24 h. Aliquots from the bacterial suspensions were serially diluted and 0.1 ml were plated on King's medium B in order to estimate the bacterial concentration before Degaclean was added. In **the second step** 0.3% Degaclean was added, the Erylenmeyer flasks were incubated at 27 °C for 12 h, and serial dilutions were plated on King's medium B. New Erylenmeyer flasks with 50 ml King's B liquid medium were then inoculated with 0.1 ml

from the flasks with 0.3% Degaclean, incubated at 27 °C for 24 h, after which the Degaclean-concentration was raised to 0.6%, followed by incubation at 27 °C for 12 h, serial dilution and plating of aliquots. The same procedure was continued several times until 1.5% Degaclean was reached. This concentration totally inhibited further bacterial growth.

2.3 Inoculation of potato tubers with *Erwinia spp.* by wounding

The bacterial strains *Erwinia carotovora subsp. atroseptica* (*Eca*) (GSPB nos. 9201, 2967 and 2968) and *Erwinia carotovora subsp. carotovora* (*Ecc*) (GSPB nos. 427, 429 and 436) were grown on King's medium B at 27 °C for 24 h, after which the growth was scraped off in 0.01 M $MgSO_4$ solution. Each strain was adjusted to an $O.D._{660}$ of 0.06 (photometer, Spectronic 20, Bausch & Lomb), corresponding to ca. 2.2 X 10^8 cfu/ml *Eca* and ca. 2.6 X 10^8 cfu/ml for *Ecc*. The three bacterial suspensions of each subspecies were mixed at the ratio of 1:1:1. Potato tubers of cultivars Bintje, Morene and Cilena were wounded by a pinflowerholder ("fakirbed" no. 202, Ø 32 mm, ZÜRICH) which was turned on the tuber surface under slight pressure by hand. Potatoes were wounded before or after soaking in 1.5 litre of two bacterial concentrations (ca. 10^8 and 10^6 cfu/ml) in buckets at room temperature for 3 h. Then the potato tubers were placed on filter paper at room temperature for drying over night. For the laboratory experiments five tubers from each variant were incubated in plastic boxes (with moist paper at the bottom) at 27 °C for 3-6 days to find out which inoculation method was best suited.

2.4 Effect of Degaclean on potato tubers infected by *Erwinia spp.*

2.4.1 Origin of Degaclean

Degaclean was specified as follows: Degaclean® 150, stabilized mixture of 20 - < 60% (w/w) hydrogen peroxide and < 5% (w/w) peroxyacetic acid.

A sample of 1 kg was obtained from
SVR, Lösungen für die Umwelt, Birgit Schneider,
Hanauer Landstr. 2, D-63594 Hasselroth, Tel. and Fax 06055-83873
by order of
Degussa-Hüls AG, Herrn Huss, Abt.: IC-BC, Weissfrauenstr. 9, D-60311 Frankfurt/Main

2.4.2 Laboratory experiments

Potato tubers were inoculated as described above (2.3). Potato samples of 5 tubers each were immersed in 0.5%, 1.0% or 2.0% Degaclean for different time intervals (15, 30 or 60 min). Each variant was repeated twice. Finally, the potato tubers of each variant were placed on wet filter paper in plastic boxes (19 cm X 19 cm X 6 cm) which were covered by a lid, incubated at 23 °C for 3-7 days and scored for soft rotting.

2.4.3 Field experiments

Bacterial inoculation of potato tubers and treatment with Degaclean were performed as described above (2.3 and 2.4) if not stated otherwise.

2.4.3.1 **Field experiments in Göttingen**

Potato tubers were artificially inoculated by a mixture of *Ecc* and *Eca* (3 strains of each subspecies) as described in 2.3, with the following specifications: potato tubers were at first wounded and then immersed in bacterial suspension (ca. 10^8 cfu/ml) for 3 h. In 1999 inoculation was performed 2 days before planting on May, 10. In 2000 the variant E (see Table 1) was inoculated early (Jan. 15), the variants B and F late (May, 24). Treatment of potato tubers with Degaclean included three concentrations in 1999 (0.5, 1.0 and 2.0%) and only one concentration (2.0%) for the experiments of 2000 (in April), as described in 2.4.2.

In the second experiment (2000), two antibiotic-resistant mutants were used, *Eca* no. 2968 resistant against Rifampcin, and *Ecc* no. 2970 resistant against Rifampcin and Streptomycin (see 2.1). The potato tubers, cultivar Agria (obtained from Stöver Co.), were planted in April 2000.

The field experiments were performed in 1999 and 2000 on the experimental field of the Institute of Tropical and Subtropical Agronomy (“Am Wassergraben”) in Göttingen-Weende. The soil type was Typic Hapludalf, according to United States Dept. of Agriculture (USDA). Fertilizer was applied to the soil only in 2000 on June 9 in the form of 100 kg N per hectare of “Kalkammmonsalpeter” (= nitrate of lime and ammonia, *i.e*. mixture of ammonium nitrate and calcium carbonate), containing 27% N. Potatoes were planted on May 12, 1999 and April 26, 2000, by hand. The soil was dry and warm at the time of planting.

In 1999, the potato tubers (cultivar Bintje, obtained from Naturkost Elkershausen GmbH Göttingen) were only treated **lately** (just before planting in the field) with different concentrations of Degaclean, so that the following five variants resulted:

A – Inoculation with bacteria, no treatment with Degaclean

B - Bacteria, and 0.5% Degaclean

C - Bacteria, and 1.0% Degaclean

D - Bacteria, and 2.0% Degaclean

E - Without bacteria, no treatment with Degaclean (control)

Each variant consisted of 3 repetitions (3 rows of 15 m length with 50 potatoes per row), and the distance between the rows was 75 cm and 1.5 m between each variant (Fig. 1). Within the rows the potatoes were planted with spaces of 30 cm.

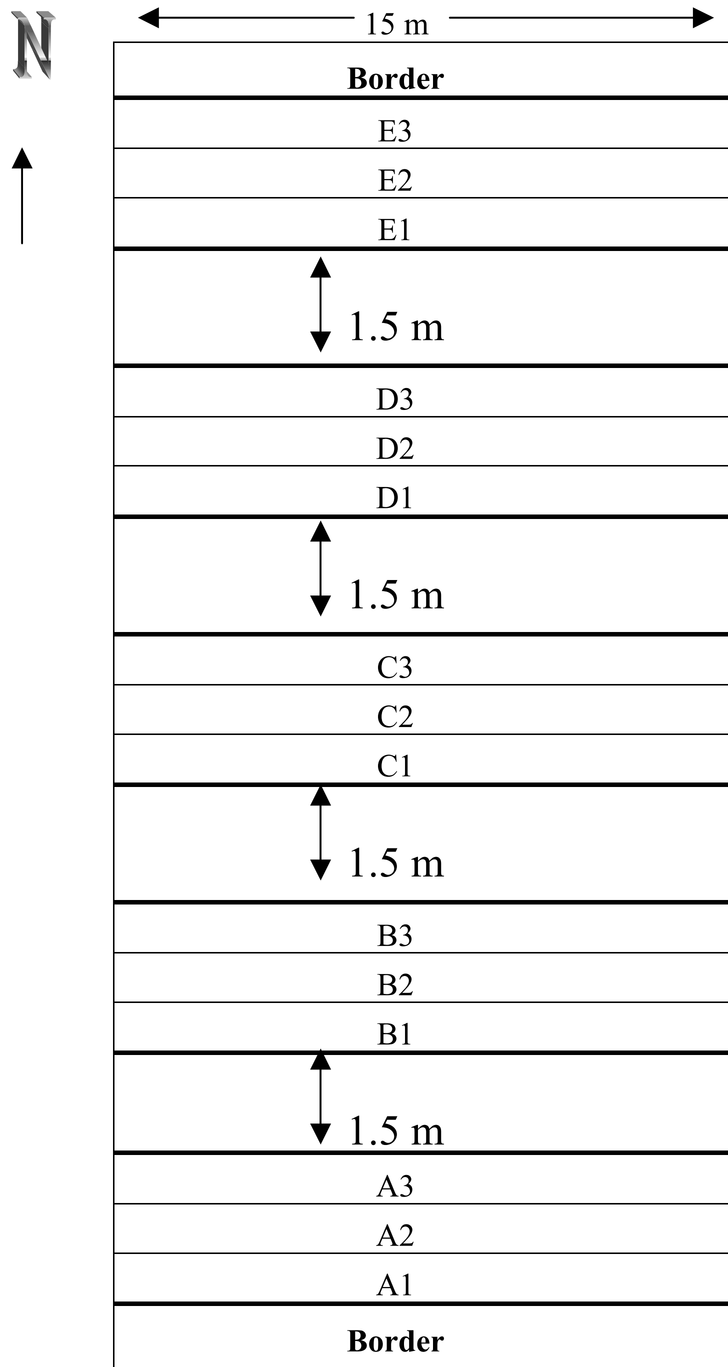

Fig. 1: Design of field experiments 1999

In 2000 the field experiments consisted of 6 variants (Table 1)

Table 1: Variants of field experiments 2000

Designation	Bacterial inoculation	Degaclean treatment
A	-	-
B	late, 24/04/2000	-
C	-	early, 15/01/2000
D	-	late, 25/04/2000
E	early, 14/01/2000	early, 15/01/2000
F	late, 24/04/2000	late, 25/04/2000

In 2000, each variant consisted of 4 repetitions with 20 planted tubers, *i.e* in total 80 planted tubers per variant. The repetitions of the 6 variants were differently mixed on the 6 plots as shown in Fig. 2. Distances of potato plants within the rows and between rows were as described in field experiments 1999.

Weeds were removed by hoe, and dams were produced by mattock when the potato plants were 15 cm high. Chemical plant protection measures were performed only in 2000, that is, fungicide Shirlan (0.3 litre/ha, active substance 500g/l Fluazinam, conc. 0.13%) two months after planting, and fungicide Acrobat plus (1.5-2.0 kg/ha, active substance 9% Dimethomorph + 60% Mancozeb, conc. 0.67%) two weeks later, against *Phytophthora infestans*. In 1999, the potato plants were strongly affected by potato late blight (*Phytophthora infestans*) and had to be harvested early, on August 25 by hand. In 2000 the potatoes were harvested on September 7.

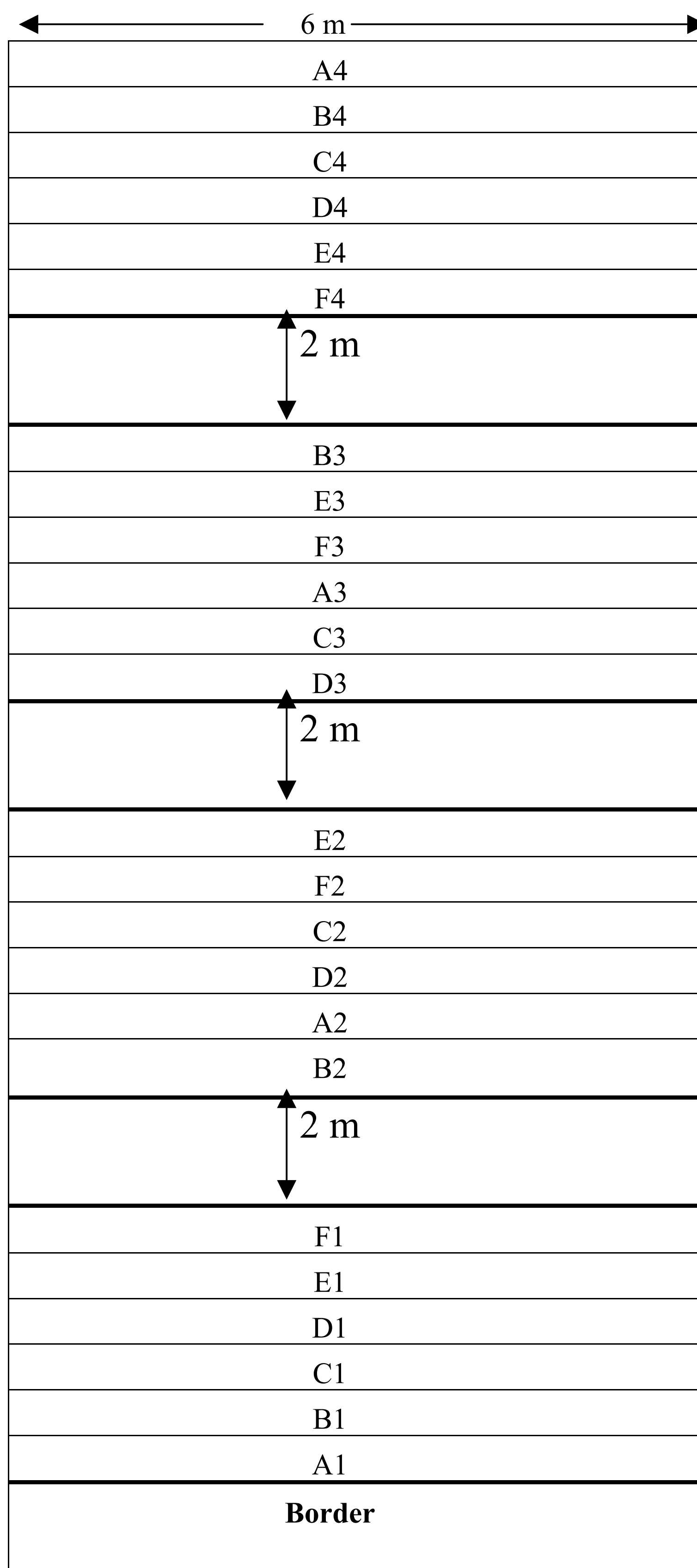

Fig. 2: Design of field experiments 2000

2.4.3.1.1 **Evaluation at harvest**

The potatoes were harvested by hand and the tubers of each row (repetition) were collected in one sample. Rotted potato tubers were counted during harvesting and discarded. The healthy looking tubers were weighed and stored at 4 °C for 2-4 weeks until evaluation of latent contamination.

The potatoes harvested from each plot were divided into six "groups" (repetitions), each of which was analysed separately for latent contamination. Evaluation of each separate "group" 2-4 weeks after harvest included recording the total weight of potato tubers, numbers of tubers with soft rot, and determination of latent contamination by *Erwinia carotovora.* For the latter purpose, each potato sample was immersed in 0.01 M $MgSO_4$ for 3 h at 4 °C with occasional stirring every 30 min by hand. Finally 50 ml were taken and analysed as described in Chapter I, 2.3.7.2.

2.4.3.2 **Field experiments by Stöver Co.**

The field experiments were performed in 1999 on a farm in Natenstedt near Twistringen to study the effect of Degaclean treatment on potato lots which had been found to be latently contaminated by *Ecc* in Göttingen (samples Se 4 and Se 5 listed in Chapter I, Table 15). In September 1998 or in April 1999 the tubers were strongly sprayed with 0.5% Degaclean until the surfaces of the tubers became completely wet. Then the potato tubers were placed on filter paper at room temperature over night for superficial drying. The field experiments were performed and the potato tubers were planted as described earlier (Chapter I, 2.5). Each of the four variants consisted of 4 blocks, each with two rows of 24 potatoes totally, planted with a space of 37 cm (Fig. 3). Potatoes were planted on April 20, 1999 by hand. The four variants of the experiment were:

A – cv. Morene from Semlow (= Se 4), early treatment (in fall 1998) with 0.5% Degaclean,

B – cv. Morene from Semlow (= Se 4), no treatment with Degaclean,

C – cv. Morene from Semlow 33 ha (= Se 5), (strongly contaminated, degree 3), no Degaclean treatment,

D – cv. Morene from Semlow 33 ha (= Se 5), late treatment (in spring 1999) with 0.5% Degaclean.

The potatoes were harvested on September 20, 1999, by hand. Evaluation of these field experiments was performed as described in Chapter I, 2.5.

Block 4	A.	B.	C.	D.					
Block 2	A.	B.	C.	D.	A.	B.	C.	D	**Block 3**
					A.	B.	C.	D.	**Block 1**

Fig. 3: Design of field experiments 1999 by Stöver Co.

2.5 Characterization of lipopolysaccharides from *Erwinia carotovora*

2.5.1 Culture of bacteria and washing of cells

Lipopolysaccharides were isolated from five strains as follows:
Eca GSPB no. 2962 from Japan which did not contain the LPS O-chain,
Eca GSPB no. 2967, highly virulent,
Ecc GSPB no. 436, very highly virulent,
Eca GSPB no. 2967 "a", Degaclean-tolerant and less virulent than the parent strain,
Ecc GSPB no. 436 "a", Degaclean-tolerant and less virulent than the parent strain.
At first, all the strains were grown on King's medium B (agar) as described in 2.3. Then, the bacteria were transferred into a liquid medium containing per Litre 10.0 g peptone from casein (Roth Co., no. 8986.2), 5.0 g glucose and 1.0 g casamino acids (Difco no. 0231-010). The medium was autoclaved at 121 °C for 15 min, the bacterial cultures were shaken at 110 rpm at 28 °C (Clim-O–shake, Firma Kühner, Basel, Switzerland). After 48 h of growth the bacteria were precipitated by centrifugation (10000 X g / 4 °C) and washed three times with EDTA-saline, finally with water. The cells were frozen at –20 °C /24 h and lyophilized.
EDTA-saline
10 g NaCl
10 mM Na_2-EDTA

2.5.2 Extraction of LPS by the phenol-water method

For extracting LPS, the lyophilized cells were suspended in water (4 g/ 100 ml) to give a slurry. The slurry was warmed to 70 °C and mixed with 90% warm phenol (1:1), the mixture was stirred for 20-30 min and cooled down in an ice–bath overnight. Phenol-water mixtures are monophasic at 70 °C, but separate into two phases below that temperature. The two phases were separated by centrifugation (10000 g/ 4 °C), then the upper watery phase was carefully siphoned off. It contains LPS, polysaccharides, RNA, and salts. The lower phase contains

proteins, lipids, phospholipids, pigments, and DNA. The water phase was dialyzed against bidist. water for 4 days and finally lyophilized (Type BETA I, Christ Company).

2.5.3 Polyacrylamide gel electrophoresis (PAGE)

LPS was separated by gel electrophoresis in the Minigel-system (Biometra Company, Göttingen) with 10 X 10 cm glass plates and 1 mm space. Electrophoresis was performed in sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE).

2.5.3.1 Preparation of separation gel

All the constituents, except ammoniumpersulphate (= APS) were put in a 10 ml, side–arm vacuum, conical flask. The flask was closed with a rubber stopper and the suspension was deaerated for 2 min under slight stirring, because oxygen may hinder polymerization. The glass plates of the apparatus were marked ca.1 cm below the lower end of the combs, to ensure that the height of the stacking gel was at least 2 times more than the height of LPS samples to be poured. APS was mixed with the other components, stirred for 1 min, and poured into the sandwich apparatus with a sterile glass pipette. About 1 cm bidest. water was layered over the gel to avoid contact of the gel with air. The gel polymerized within about 45 min.

2.5.3.2 Preparation of stacking gel

After polymerization, the bd-water was poured off from the sandwich, and the stacking gel prepared as described in Table 2 was put into a 10 ml, side–arm vacuum, conical flask, and deaerated for 2 min under slight stirring. APS was mixed for 1 min and the combs were inserted into glass plates and the gel was gradually poured with a sterile glass pipette. All air bubbles were removed by moving the comb up and down. The stacking gel polymerized within 30 min.

Table 2: Receipts of separating and stacking polyacrylamide gels for LPS separation

Ingredients	Separating gel (10%)	Stacking gel (3%)
Acrylamide 30%, 0.8% Bisacrylamide	2.0 ml	250 µl
Separating gel buffer	2.0 ml	
Stacking gel buffer		500 µl
TEMED (10%)	100 µl	50 µl
APS (10%)	15 µl	15 µl
Bidistilled water	3.0 ml	1.750 µl
Total	7.115 ml	2.565 µl

2.5.3.3 Buffers, chemicals and reagents for electrophoretic studies

Electrophoresis reagents of electrophoretic grade were purchased from Roth (Karlsruhe), and Merck (Darmstadt)

1) Acrylamide stock solution — 30% Acrylamide, 0.8% Bisacrylamide

2) Separation gel (3 X) — 1 M Tris
0.3 g SDS
Add 100 ml bd H_2O pH 8.8 with HCl

3) Stacking gel (3 X) — 1 M Tris
0.3 g SDS
Add 100 ml bd H_2O pH 6.8 with HCl

4) TEMED (10%) — 10% in bd H_2O

5) Ammoniumpersulphate APS (10%) — 10% bd H_2O

6) Denaturation buffer — 0.0625 M Tris
2.0% SDS
10% glycerol
0.002% Bromphenolblue pH 6.8 with HCl

7) Electrode buffer (10X)

30.3	g Tris
144	g glycine
10	g SDS
1000	ml bd H_2O

2.5.3.4 Preparation of LPS samples

Each sample was dissolved in a 1 ml Eppendorf tube (0.2-0.5 mg/ml water) and mixed at a ratio of 1:1 with denaturation buffer. The mixture was incubated at 65 °C/ 5 min, after which DTE was added (final concentration 10%), and the mixture was heated at 100 °C for 5 min (MÜLLER, 1998).

2.5.3.5 Detection of LPS in the gel using silver staining (HEUKESHOVEN and DERNICK, 1988)

A few gels were stained with silver by the following procedure:

Step	Component	Concentration
1) Fixing (2 h)	31.25 ml ethanol (96%)	30.0%
	10.00 ml acetic acid (100%)	10.0%
	add 100 ml H_2O bidest	
2) Incubation (2 h)	31.25 ml ethanol (96%)	30.0%
	4.00 g sodium acetate	0.5%
	2.00 g glutardialdehyde	0.5%
	0.31 g sodium thiosulphate X 5 H_2O	0.2%
	add 100 ml H_2O bidest	
2) Washing with bd-water 3 times 10 min		
3) Staining (45 min)	0.1 g $AgNO_3$	0.1%
	28.5 ml formaldehyde (35%)	0.01%
	add 100 ml bd H_2O	
6) Developing (3-10 min)	2.5 g Na_2CO_3	2.5%
	add 100 ml bd H_2O	
7) Stopping (5-10 min)	1.86 g EDTA	0.5 M
	add 100 ml bd H_2O	

8) Washing with bd-water 3 times 10 min

The developing solution was shaken until the LPS bands appeared weakly, and the treatment was stopped when the bands were still clearly visible with a colourless background of the gel. The gel was then preserved in bd-water at 4 °C.

3 RESULTS

3.1 Effect of Degaclean on reduction of potato soft rot in laboratory experiments

3.1.1 Development of a standardized method for inducing soft rot by artificial contamination of potato tubers

Table 3 clearly shows that only a high bacterial concentration (10^8 cfu/ml) of *Erwinia spp.* caused partial rotting of wounded potato tubers. The low bacterial concentration (10^6 cfu/ml) never caused any infection. Tubers which were wounded after treating with a high bacterial concentration showed only very weak rotting. Tubers of cultivar Morene were more often infected than the other two cultivars (Bintje and Cilena). According to these results, in the following experiments the high bacterial concentration (10^8 cfu/ml) was applied, and potato tubers (cv. Morene) were wounded before soaking in bacterial suspension.

Table 3: Number of potato tubers with soft rot 5 days after soakage in bacterial suspension (*Eca* und *Ecc*) before or after wounding

cultivar	Wounding before / after treatment with water		Wounding before / after treatment with 10^8 cfu/ml (*Eca*+*Ecc*)		Wounding before / after treatment with 10^6 cfu/ml (*Eca*+*Ecc*)	
	before	after	before	after	before	after
Bintje	1*)	0	2	1	0	0
Cilena	0	0	2	1	0	0
Morene	0	0	4	1	0	0

*) = 5 potato tubers were used for each experiment

3.1.2 Disease severity caused by *Erwinia spp.* after treatment of potato tubers with different concentrations of Degaclean

Different concentrations of Degaclean reduced soft rotting of potato tubers only very weakly (Table 4) in comparison to the control which was not treated with Degaclean. Unexpectedly, the longest treatment with Degaclean (60 min) was less effective than treatment for 15 or 30 min. Most effective against soft rot by erwinias was a treatment with 2% Degaclean for 30 min (Table 4). Because of these results potato tubers were treated with 2% Degaclean for 30 min in the field experiments of 2000.

Table 4: Number of potato tubers with soft rot after treatment with different concentrations of Degaclean (Dgc.)

Treatment	after 4 days	after 6 days
Only bacteria, without Degaclean	4*)	5
Bacteria + 0.5% Dgc./15 min	2	4
Bacteria + 0.5% Dgc./30 min	2	4
Bacteria + 0.5% Dgc./60 min	2	4
Bacteria + 1.0% Dgc./15 min	2	4
Bacteria + 1.0% Dgc./30 min	2	4
Bacteria + 1.0% Dgc./60 min	3	4
Bacteria + 2.0% Dgc./15 min	2	4
Bacteria + 2.0% Dgc./30 min	1	3
Bacteria + 2.0% Dgc./60 min	3	4

*) = 5 potato tubers were used per experiment

3.2 Effect of Degaclean on reduction of blackleg and potato soft rot in field experiments

3.2.1 Field experiments 1999

3.2.1.1 Field experiments in Göttingen

The results obtained (Table 5) show that even in the control (without bacterial inoculation, and without Degaclean treatment) latent contamination with *Ecc* and soft rotting occurred, but treatment of seed potatoes with **bacteria** decreased the number of germinating potatoes from 95.5% to 82.7%, due to soft rot, and increased latent contamination of harvested potatoes from 3.1 X 10^3 to 4.6 X 10^5 cfu/kg.

However, the late **Degaclean** treatment of seed potatoes (just before planting in the field) caused only a **slight reduction of latent contamination** at harvest from 4.6 X 10^5 to 3.5 X 10^4 cfu/kg at the highest Degaclean concentration (2%). Treatment with 0.5% Degaclean did not cause a significant reduction of latent contamination with *Ecc*.

Unexpectedly, Degaclean treatment significantly reduced the number of germinated potato tubers by 27.6%, 24.4% and 23.7% at the 3 concentrations, compared to potato tubers which were only treated with bacteria (17.3%), and also the percentage of tubers with soft rot increased significantly after Degaclean treatment, especially at the low concentration of 0.5 and

1.0%. Therefore, also the **yields were reduced** by all Degaclean treatments compared to the untreated variants. Potato plants with blackleg symptoms were not observed in the experimental field plots in 1999. All bacterial isolates which were obtained from these experiments were *Ecc*.

Table 5: Field experiments 1999: Effect of Degaclean on the number of latent contamination with *Erwinia carotovora ssp. carotovora* (*Ecc*)

Treatment	Number of non-germinated tubers in %	Number of potato tubers with soft rot at harvest absolute per 10 kg		Colony forming units (cfu) per kg	Yield in kg
Control, without Bacteria, without Degaclean	4.4	31	2.9	3.1×10^3	107. 4
Bacteria, without Degaclean	17.3	27	2.8	4.6×10^5	95. 3
Bacteria, with 0.5% Degaclean	27.6	32	4.1	2.5×10^5	78. 9
Bacteria, with 1.0% Degaclean	24.4	35	4.0	2.9×10^4	87. 2
Bacteria, with 2.0% Degaclean	23.7	28	3.2	3.5×10^4	86. 4

3.2.1.2 Field experiments by Stöver Co.

The field experiments of Stöver Co. aimed to compare Degaclean treatments of seed potatoes in fall and spring. Potato tubers from cultivar Morene, produced in Semlow, were either treated in fall (September 1998) or in spring (April 1999). In 1999 Degaclean treated and untreated potatoes from the same lot were grown in an experimental design with 4 repetitions (subchapter 2.5, page 18) on a farm in Natenstedt.

The results (Table 6) show that **Degaclean–treatment in fall reduced** the percentage of tubers with **soft rot** after harvest in the **following year** from 3.4 to 1.3%. In contrast, treatment in spring did not reduce percentage of tubers with soft rot. Also, the appearance of blackleg symptoms in the field was reduced by Degaclean treatment in fall (BRUER, person. communication). The other parameters – yield and tuber sizes - were not affected by Degaclean–treatments.

Table 6: Field experiments 1999 by Stöver Co. on the effects of Degaclean treatments in fall and spring on latent contamination with *Erwinia spp.*

Soft rotting, yield and tuber size	Treatment in fall		Treatment in spring	
	untreated control	Degaclean treated	untreated control	Degaclean treated
Soft rotted tubers in %	3.4	1.3	5.4	5.4
Yield in t/ha				
Fraction 35-40	1.81	1.69	2.06	1.92
Fraction 40-50	12.69	13.88	17.08	14.25
Fraction 50-55	11.38	11.50	11.58	11.83
Fraction 55-60	13.69	10.69	10.83	13.08
Fraction 60+	17.13	18.44	11.83	12.50
Total	**56.7**	**56.2**	**53.4**	**53.6**
Tuber size Number of tubers/10 kg				
Fraction 50+	52	53	54	56
Fraction 55+	44	44	44	47
Fraction 60+	36	37	36	37

3.2.2 Field experiments 2000

For these experiments, **antibiotic resistant mutants** were generated from two strains, *Eca* 2968 and *Ecc* 436, by exposition to Rifampicin and Streptomycin. Before planting, potato tubers were inoculated with the antibiotic–resistant strains. By applying these strains it was possible to differentiate between erwinias originating from the tubers or from the soil and erwinias which had been applied on the potato tubers. *Eca* 2968 was resistant against 200 ppm Rifampicin and *Ecc* 436 was resistant against 200 ppm Rifampicin and 800 ppm Streptomycin.

The results obtained in these experiments (Table 7) show that the bacterial inoculation was also successful in this year, because the percentage of potato tubers with soft rot at harvest nearly doubled, and because the latent contamination with *E. carotovora* on the harvested potatoes increased tremendously (from 3.2 X 10^3 to 5.6 X 10^6 cfu/kg).

Degaclean-treatment alone, without artificial inoculation of bacteria, **reduced germination** rate of potatoes badly, especially at the **late application** date shortly before planting. Since soft rotting of potatoes was simultaneously enhanced at harvest, Degaclean treatment resulted in **yield reduction** of 25 – 45%. These negative effects were less at the earlier application date.

Table 7: Effect of Degaclean on latent contamination with *Erwinia spp.* (*Eca* und *Ecc*) and on yield in field experiments 2000

Treatment	Number of non-germinated tubers in %	Number of potato tubers with soft rot at harvest		Colony forming units (cfu) per kg	Yield in kg
		absolute	per 10 kg		
Control, without Bacteria, without Degaclean	20	15	1.2	3.2×10^3	126. 5
Without Bacteria, early Degaclean	26.3	16	1.6	2.4×10^4	100. 5
Without Bacteria, late Degaclean	58.8	19	2.5	4.6×10^4	77. 6
Only Bacteria, without Degaclean	23.8	28	2.6	5.0×10^6	106. 8
Bacteria, early Degaclean	40	23	2.4	3.4×10^5	96. 3
Bacteria, late Degaclean	62.5	35	4.8	8.9×10^5	72. 3

A combination of bacterial inoculation and Degaclean treatment showed a **small reduction** of **latent contamination** of potato tubers at harvest. However, this positive effect was strongly exceeded by the negative effects, that is reduction of germination, higher percentage of soft rotted potatoes at harvest and lower yield. Thus, the yield was reduced by 10 and 32% by the early and late Degaclean treatment, respectively.

The results (Table 8) clearly show that the antibiotic-resistant *Erwinia carotovora* strains were isolated only from those experimental variants which had been inoculated by these bacteria (nos. 4, 5 and 6). Spontaneous mutants with resistance against Rifampicin and Streptomycin did never show up in variants no. 1-3, which had not been inoculated with antibiotic-resistant bacteria. Therefore, these experiments allowed to differentiate between latent contamination of harvested potatoes originating

a) from the seed potatoes before treatment or from the soil (natural contamination), or

b) from the artificially inoculated bacteria.

The experiments confirmed that late Degaclean-treatment (no. 6) resulted in higher latent contamination than early Degaclean-treatment (no. 5). But even the early Degaclean-treatment

(no. 5) did not reduce latent contamination resulting from artificially inoculated bacteria (no. 4). Only the non-antibiotic-resistant erwinias were slightly reduced by Degaclean-treatment in nos. 5 and 6 compared to no. 4, as shown on the CVPM-semi-selective medium (see Chapter I, 3.2.3). However, these results were not observed in nos. 2 and 3 when compared to no. 1.

Because the artificially inoculated *Erwinia carotovora* subspecies differed in antibiotic-resistance (*Eca* was resistant against Rifampicin, while *Ecc* was resistant against Rifampicin and Streptomycin) the numbers of *Eca* 2968 isolates (column 5) could be calculated by subtracting the values in column 6 from those in column 4.

On the field, **blackleg symptoms** were observed in three variants two months after planting, that is in each of the following variants two plants with blackleg symptoms occurred: early and late Degaclean treatment without bacterial inoculation and inoculation of bacteria only.

Table 8: Detection and isolation of soft rotting *Erwinia spp.* on potato tubers from field experiments in 2000 on three different media

No.	Treatment	cfu/kg potato tubers			
		CVPM[1]	KB with Rifampicin *Eca* 2968 + *Ecc* 2970	*Eca* 2968	KB with Rifampicin and Streptomycin *Ecc* 2970
1	Control, without Bacteria, without Degaclean	3.8×10^3	0[2]	0	0
2	Without Bacteria, early Degaclean	5.9×10^4	0	0	0
3	Without Bacteria, late Degaclean	5.7×10^4	0	0	0
4	Only Bacteria, without Degaclean	1.1×10^5	3.6×10^3	2.2×10^3	1.4×10^3
5	Bacteria, early Degaclean	5.2×10^4	4.4×10^3	1.2×10^3	3.2×10^3
6	Bacteria, late Degaclean	9.5×10^4	1.1×10^4	4.8×10^3	6.3×10^3

[1] CVPM = crystal violet pectate medium modified (see Chapter I, 3.2.3)

[2] 0 = no growth

3.3 Generation of Degaclean tolerant strains of *Erwinia carotovora ssp. carotovora* and *ssp. atroseptica*

3.3.1 Sensitivity against different concentrations of Degaclean

Cultures of the strains *Eca* 2967 and *Ecc* 436 were consecutively exposed to increasing concentrations of Degaclean (see II, subchapter 2.2) to investigate whether the bacteria could develop tolerance against certain Degaclean concentrations. As shown in Table 9, after exposure to 1.2% Degaclean for 24 h 2.5–3.1 X 10^4 cfu/ml were detected in the nutrient medium, indicating that these bacterial cells were tolerant against 1.2% Degaclean. Thus, from both strains mutants could be selected which were tolerant against 1.2% Degaclean.

However, 1.5% Degaclean completely killed all bacterial cells. From the growth medium with 1.2% Degaclean one isolate each of *Eca* and *Ecc* (Degaclean-tolerant isolates) was selected and stored in sterile water to further investigate other bacterial features, such as virulence and chemical structure of LPS. These strains were also included into the lyophilized bacterial collection (GSPB nos. 447 and 448).

Table 9: Bacterial concentration of two *E. carotovora* strains in King's medium B after exposure to different concentrations of Degaclean after growth for 24 h

Treatment	*Eca* 2967 cfu/ml	*Ecc* 436 cfu/ml
0 % control	6.2 X 10^7	9.8 X 10^7
0.3 % Degaclean	3.2 X 10^7	5.2 X 10^7
0.6 % Degaclean	8.2 X 10^6	6.1 X 10^6
0.9 % Degaclean	6.2 X 10^5	1.1 X 10^5
1.2 % Degaclean	2.5 X 10^4	3.1 X 10^4
1.5 % Degaclean	0	0

3.3.2 Virulence

The virulence of the generated Degaclean-tolerant strains was tested by inoculation of potato tubers slices (see I, subchapter 2.2.4). Results (Table 10) show that the mutant strains from both subspecies were less virulent than the corresponding wild parent strains in all experimental variants. It was also observed that the *Ecc* strains were more virulent than the *Eca* strains in these tests.

Table 10: : Determination of virulence of four strains of *Erwinia spp.* on potato tuber slices by determining diameter of rotted tissue in cm. Designation “a” indicates Degaclean-tolerant strains

Strain	Bacterial concentration 1.2 X 10^8	1.2 X 10^7	1.2 X 10^6	1.2 X 10^4	1.2 X 10^3
Eca 2967	2.0***)**	1.3	1.0	0	0
Eca 2967 “a”	1.3	1.0	0	0	0
Ecc 436	3.0	2.3	1.5	0	0
Ecc 436 “a”	1.7	1.3	0	0	0

***)** = the rotting of slices (diameter of soft rot tissue in cm) was measured after 48 h

3.3.3 Effect of Degaclean-tolerance on LPS

Lipopolysaccharides from *E. carotovora* strains were separated by SDS-PAGE electrophoresis (Fig. 4). The stained gels revealed a characteristic ladder–like pattern for the 4 bacterial strains from GSPB. However, a distinct difference between the wild strains (nos. 2967 and 436) and the corresponding Degaclean-tolerant strains (nos. 2967 ”a” and 436 “a”) was not observed. The LPS from the Japanese strain *Eca* 2962 did not show the typical ladder-like pattern. Obviously, the LPS of *Eca* 2962 did not contain an O-chain, indicating a “rough” LPS.

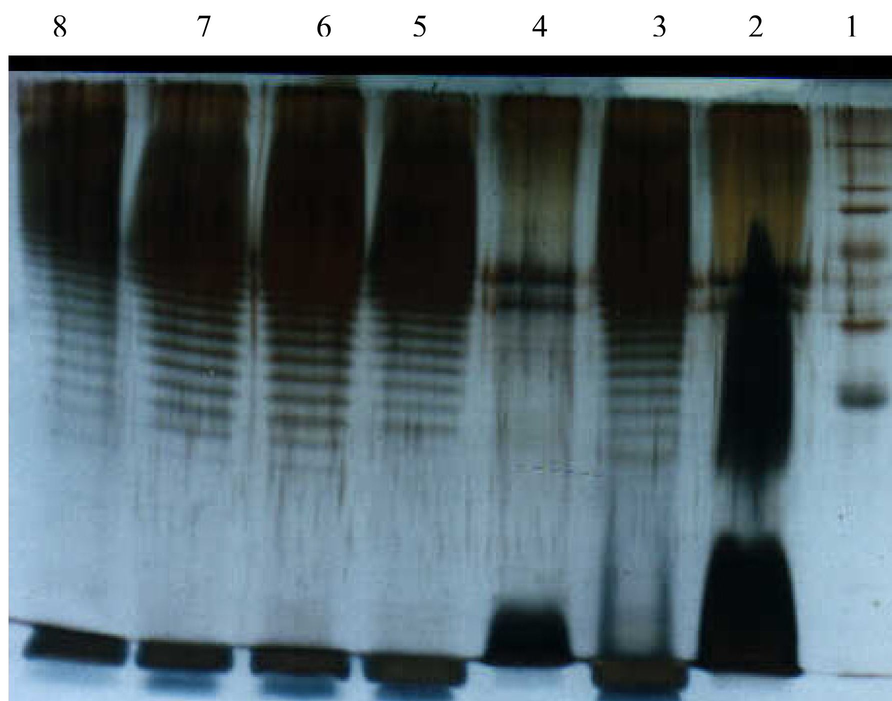

Fig. 4: SDS-PAGE of lipopolysaccharides from 5 *Erwinia carotovora* strains. The samples were run on a 10% acrylamide gel. Lanes: (1) Marker VIII, (2) *Eca* 2962, (3) *Ecc* 436, (4) *Eca* 2962, (5) *Ecc* 436 “a” (Degaclean-tolerant), (6) *Eca* 2967, (7) *Eca* 2967 “a” (Degaclean-tolerant), and (8) *Ecc* 436. Into each slot 10 µl LPS-solution (containing 0.2-0.5 mg/ml water) were added. Only no. 2 contained 15 µl.

4 DISCUSSION

The **disease control** of blackleg (*Erwinia carotovora ssp. atroseptica*) relies primarily on the production of **healthy potato tuber seeds** (PEROMBELON and HYMAN, 1992). To guarantee the health status of the seed stock, seed classification was traditionally based on a visual crop inspection for blackleg affected plants. Blackleg incidence in the field is related to tuber contamination, but can be affected by environmental and cultural factors (DE BOER *et al*., 1996).

Resistant varieties may be part of the solution. The culture of resistant potatoes cultivars could be useful for the control of soft rot *Erwinia* species, as no chemical means of control are available (LAPWOOD & GANS, 1984; DÖPKE and RUDOLPH, 1990; STEFANI *et al.*, 1990). Resistance to soft rot is common within the gene resources of *Solanum tuberosum* (CIAMPI-PANNO and ANDRADE-SOTO, 1984) and of related species (VAN SOEST, 1983). One resistance source correlated with a high level of pectin methylation, increasing resistance of cell walls to enzyme degradation, was identified from the non–tuber–bearing *Solanum brevidens* and transferred to *Solanum tuberosum spp.* (PEROMBELON & SALMOND, 1995). However, resistance as well as other measures, such as seed certification, good crop husbandry and farm hygiene may help, but are **not totally effective** (BOURGEOIS and PRIOU, 1995). Therefore, the **aim** of this study was to investigate the effect of Degaclean-treatment on reduction of blackleg and / or soft rot of potatoes in storage as well as in the field. **The advantage** of Degaclean is that no noxious residues are released, because the active substances are very soon transformed into environmentally friendly compounds. Thus, the amount of H_2O_2 was 0.04% after application, but 15 min later the amount was reduced to 0.006%, and after 2 - 4 h the active oxygen was no longer detectable (STEINKAMP, 1998).

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4.1 Comparison of methods for artificial inoculation

In order to study the effect of Degaclean on potato soft rot and potato blackleg a reliable method for inoculating potato tubers mimicking natural conditions was needed. The results of these studies clearly show that only a high bacterial concentration (10^8 cfu/ml) of *Erwinia spp.* caused partial rotting of wounded potato tubers. The low bacterial concentration (10^6 cfu/ml) never caused any infection. Tubers which were wounded **after** treating with a high bacterial concentration showed only very weak rotting. Tubers of cultivar Morene were more often infected than the other two cultivars (Bintje and Cilene).

Our experiments confirmed results from other workers that many soft rotting bacteria ´may be present **superficially in lenticels** and suberized **wounds** in most of the seed stocks, and that

under favourable conditions the bacteria are transmitted to the lenticels of progeny potatoes during harvesting and grading where **wounds** caused by **mechanical damage** can become infected by bacteria (PEROMBELON and KELMAN, 1980). Also TOTH *et al*. (1996) reported that several sources for contamination can lead to blackleg infection, such as rain, insects, irrigation water via the soil from rotting mother tubers, irrigation water, or from contamination of **wound sites** caused during harvesting. Extensive contamination also occurs during harvesting and grading when erwinias from occasional rotting tubers spread to **fresh wounds**, once the bacteria can persist until planting time (PEROMBELON and HYMAN, 1995).

4.2 Effect of Degaclean on reduction of soft rot in laboratory experiments

NIEPOLD (1999b) reported that in laboratory experiments only 100 to 200 ppm of the Clarmarin/KH10 mixture with 1–2 min of incubation time were required to absolutely destroy a cell suspension of 10^9 cells per ml of two quarantine bacteria (*Ralstonia solanacearum* and *Clavibacter michiganensis ssp. sepedonicus*), suspended in an aqueous solution (Ringer). These results indicated that the possibility now exsists to efficiently interrupt the infection cycle of quarantine bacteria. For killing all bacterial cells of the same concentrations (10^9 cells per ml) of *Ralstonia solanacearum* in sewage water, 1000 ppm of the Clarmarin/KH10 mixture and incubation for 20 min were necessary (NIEPOLD, 1999b). The tenfold higher concentration of Clarmarin/KH10 and the longer incubation time were explained by the presence of other organic material in the sewage water, reacting also with the peroxides.

Also, STEINKAMP (1998) reported that the bacterial concentration of *Erwinia carotovora* was reduced from ca. 1 X 10^8 to ca. 1 X 10^4 cells per potato tuber after washing with 5% Degaclean® 97 for 0.5 min. When the potato tubers were washed with the same concentration for 15 min they were completely free from bacteria. Our results revealed that the different concentrations of Degaclean reduced actual soft rotting of potato tubers only very weak, but most effective was a treatment with 2% Degaclean for 30 min. Unexpectedly, the longest treatment with Degaclean (60 min) was less effective than treatment for 15 or 30 min.

Thus, the results obtained in this study showed that a slight reduction of potato rotting by Degaclean (2%) can be achieved. It appears necessary that experiments are repeated several times with different concentrations of Degaclean to find out whether a Degaclean-treatment may be suitable to protect food potatoes against soft rotting during long storage periods.

4.3 Field experiments

4.3.1 Effect of Degaclean on sprouting of potato tubers

STEINKAMP (1998) studied germination and sprouting of potato tubers cvs. Gloria and Cilene **in storage** within 4 weeks after washing with Degaclean® 97. In these experiments, a negative effect of Degaclean was never observed. In contrast, it appeared as if the Degaclean-treated potatoes had a slightly higher number of sprouts than the untreated control.

On the other hand, our **field experiments** revealed that the newer preparation Degaclean® 150 significantly reduced the number of germinating potatoes by 24-28% at all Degaclean concentrations used. The reason for these conflicting results is still unknown. However, we observed that all the non-germinating potato tubers in the field showed a strong soft rot. Therefore, it was concluded that treatment with Degaclean® 150 may have somehow damaged the potato tubers at the outside or inhibited the natural resistant or warding off reactions, so that these tubers were infected by always present bacterial populations of *Erwinia carotovora.* However, the observations of STEINKAMP (1998) by scanning microscopy did not reveal any visible damage of the potato peel after Degaclean-treatment. Since also these results of STEINKAMP (1998) differed from our findings, it should be studied whether the different Degaclean-preparations (Degaclean® 97 tested by STEINKAMP and Degaclean® 150 in our experiments) may explain these conflicting results. According to the producer (Degussa Co., Mrs. G. Mitschke) Degaclean 150 contains 15% active substance, Degaclean 97 only 5% active substance. In addition, the combination of stabilizing compounds differs in the two preparations.

4.3.2 Effect of Degaclean on soft rotting of potato tubers

The results obtained by Stöver Co. showed that Degaclean–treatment in fall reduced the percentage of tubers with soft rot after harvest in the following year from 3.4 to 1.3%. In contrast, treatment in spring did not reduce percentage of tubers with soft rot. Our results clearly showed that the percentage of tubers with soft rot increased significantly after Degaclean treatment, especially at the low concentration of 0.5 and 1.0%. However, the earliest Degaclean-treatments in our experiments were performed rather late, on January 15, that is 4 months after harvest.

4.3.3 Effect of Degaclean-treatment on potato yields

The results obtained by Stöver Co. show that the other parameters – yield and tuber sizes - were not affected by Degaclean–treatments. Our results revealed that in the year of 1999 the

yields were reduced by all Degaclean treatments compared to the untreated variants. Thus, the yield was reduced by 10 and 32% by the early and late Degaclean treatment, respectively, indicating that the negative effects were less at the earlier application date.

4.3.4 Effect of Degaclean on reduction of latent contamination of potatoes

The results obtained in this study showed that the late Degaclean treatment of seed potatoes (just before planting in the field) caused only a slight reduction of latent contamination at harvest from 4.6 X 10^5 to 3.5 X 10^4 cfu/kg at the highest Degaclean concentration (2%). Other concentrations had no effects. However, this positive effect was strongly exceeded by the negative effects, that is reduction of germination, higher percentage of soft rotted potatoes at harvest and lower yield. Much more positive effects of Degaclean® 97 were reported by STEINKAMP (1998). Treatment of potatoes with 5% Degaclean for 0.5 min reduced the latent contamination from 10^8 to 10^4 cfu/tuber, and treatment with the same concentration for 15 min destroyed nearly all latently occurring *Erwinia carotovora* cells on potato tuber surfaces.

4.3.5 Differentiation of *Eca* and *Ecc*

Our results showed that all bacterial isolates obtained in the 1999 field experiments were *Ecc*. In contrast, in the 2000 field experiments the bacterial isolates were approximately 50% *Eca* and 50% *Ecc.* A possible explaination for these conflicting results is that the new semi-selective medium favours the growth of *Ecc* more than that of *Eca*, so that the small growth cavities of *Eca* may be inhibited by the saprophytic bacteria in the Petri plates. Our studies with pure bacterial cultures indicated that cavities of *Ecc* strains on the semi-selective medium were broader and deeper than those of *Eca.* Therefore, the possibility can not be excluded that contaminations by *Eca* were overlooked in some of the potato samples because the cavities produced by *Eca* were much smaller than those by *Ecc*. On the other hand, in the 2000 field experiments with antibiotic resistant strains of *Ecc* and *Eca*, the survey of the potatoes harvested clearly showed that also *Eca* contamination was detected on King's medium B which was supplemented with rifampicin. Thus both, of *Eca* and *Ecc*, were found together on the potatoes.

Another possibility for the exclusive isolation of *Ecc* in 1999 may be that subcultures were only carried out from the bigger growth cavities on the CVPM medium and, therefore, *Eca* strains were not isolated from latently contaminated potatoes in 1999. Also, the results obtained in the 2000 field experiments showed that *Eca* and *Ecc* were isolated only from the potatoes which were inoculated by the antibiotic-resistant strains but not from non-inoculated

potatoes (Table 8). Further extensive experiments have to find out whether the sensitivity of the CVPM medium to detect contamination with *Ecc* and *Eca* under natural conditions differs because the semi-selective medium may be suited better for *Ecc* than *Eca* due to a faster growth after the standard incubation period.

4.4 Effect of Degaclean-tolerance on LPS structure

The bactericidal effect of Degaclean is due to the relase of hydrogen peroxide. Especially the extrem reactive radical $O^{* ---}$ oxidizes very fast diverse cell compounds, such as **unsaturated fatty acids** (ROTILIO *et al.,* 1973). Therefore, the possibility existed that Degaclean-tolerant strains pocessed altered lipopolysaccharides (LPS). Our results obtained in these studies did not indicate that the LPS of Degaclean-tolerant strains differed in the O chain structure when compared to the wild parent strains which were not treated. However, the lipid LPS component (lipid A) of Degaclean-tolerant strains was not analysed in our studies.

GREGORY and FRIDOVICH (1973) reported that superoxide dismutase is an important component of the defences of *Escherichia coli* against the toxicity of oxygen. Due to these results it can be expected that the Degaclean-tolerant strains may have a higher superoxide dismutase. This aspect has to be investigated by further studies on the levels of superoxide dismutase and its association with a greater tolerance of hyberbaric oxygen. Results obtained in these studies also showed that the mutant strains from both subspecies were less virulent than the corresponding wild parent strains in all experimental variants. Obviously, physiological or biochemical alterations responsible for Degaclean-tolerance led to a decreased virulence compared to the wild parent strains.

5 SUMMARY

1- In **laboratory experiments** treatment of potato tubers with 0.5, 1.0 and 2.0% Degaclean® 150 **weakly reduced soft rotting** of potato tubers by *Erwinia carotovora ssp. carotovora* and *atroseptica.* Most effective was a treatment with 2% Degaclean for 30 min.

2- **In field experiments** treatment of seed potatoes with Degaclean caused a slight reduction of latent contamination of harvested potato tubers. However, Degaclean-treatment also **reduced the germination rate** of seed potatoes and **increased** percentage of tubers with **soft rot**, so that yields after treatment of seed potatoes were reduced.

3- These **negative effects** of Degaclean were strongest at **a late** application, for instance just before planting of seed potatoes. The optimal application date appears to be immediately after harvest of potatoes.

4- Future experiments have to reveal whether the positive effects of Degaclean-treatment at the early application exceed the negative effects on germination.

5- Strains of *Eca* and *Ecc* developed **tolerance** against Degaclean concentrations up to 1.2%. However, the virulence of the tolerant “mutants“ was lower than that of the wild strains.

6- The mechanism of Degaclean-tolerance is unknown. **LPS** appeared to be **unaltered** in the tolerant strains.

CHAPTER III

Chemical structure of the LPS-O-chain of *Erwinia carotovora* and its possible role in virulence

1 INTRODUCTION

All Gram–negative bacteria contain **lipopolysaccharides (LPS)** which are complex molecules containing polysaccharides, fatty acids and phosphates. The LPS molecule consists of three components: lipid A and the sugar moieties, core region and the so-called O-specific chain (RIETSCHEL and BRADE, 1992; KNIREL and ZDOROVENKO, 1997). Thus, LPS forms an asymmetric bilayer with phospholipids such as phosphatidylethanolamine on the bacterial cell surface (SHUKLA *et al.,* 1980). By means of their lipid moiety the LPS are anchored in the outer bacterial membrane and form a major constituent of it.

The **outer membrane** of Gram–negative bacteria acts as an impermeable barrier against hydrophobic molecules, owing to its highly ordered molecular assembly, and protects the cell from or adapts it to environmental changes (COSTERTON *et al*., 1974; NIKAIDO and VAARA, 1985). As LPS contains polysaccharide, fatty acids and phosphates, the formation of vesicle-like particles in water can be expected for this type of amphiphile (HANNECART *et al.,* 1973). Therefore, LPS is also **secreted** into the surrounding medium (RAMM, 1993; SCHRÖDER, 2000; SCHRÖDER *et al*., 2001). According to FUKUOKA *et al*. (1989) also *Erwinia carotovora* produced extracellular LPS.

The LPS antigens have been especially valuable in **serological classification** of enterobacteria (EDWARDS and EWING, 1972), and the serological specificities of LPS have been useful criteria for differentiating several species into serological groups. Also for *Erwinia carotovora*, attempts have been made to characterize serological specificity, fatty acid composition and the structure of the core saccharide of *Erwinia carotovora* using cellular LPS extracted from the cells (DE BOER *et al*., 1979; RAY *et al*., 1987). DE BOER *et al*. (1985) determined the serological specificity and sugar composition of LPS purified from 16 *Erwinia carotovora* strains and differentiated six serogroups. The electrophoretic behaviour of lipopolysaccharides purified from *Erwinia carotovora subsp. atroseptica* serogroups I, XVIII, XX and XXII was investigated to see if there was a correlation between electrophoretic behavior and serogroup (MURRAY *et al*., 1990). Of 20 strains received as serogroup I, 18 gave a pattern identical to an authentic serogroup I strain. The two strains which did not give the same pattern were shown by immunological tests not to be serogroup I. Five *atroseptica* strains of serogroup

XXII gave a distinict pattern characteristic of the serogroup while *atroseptica* strains of serogroup XVIII (four strains) and XX (five strains) gave patterns that could not be distinguished from each other.

Also for **other plant pathogenic bacteria**, especially the *Pseudomonas syringae*-group (OVOD *et al.* 1997; 1999) and the *Xanthomonas campestris*-group (SENCHENKOVA *et al.* 1999) the LPS may serve as a taxonomic character for differentiation between pathovars. In all these cases, the LPS-specificity is due to the structure of the O-specific chain. It is long known that changes in LPS O-chain structure can cause changes in electrophoretic mobility of lipopolysaccharides in SDS-PAGE system (HITCHCOCK *et al*., 1986).

Besides their usefulness for serological diagnosis LPS may also play an important role in **pathogenesis**. In human pathogenic bacteria, LPS exhibit many biological functions: mitogen activity, endotoxicity, Schwartzman reaction and others (LÜDERITZ *et al*., 1982; ALEXANDER and RIETSCHEL, 1999). The pathogenicity of soft rot causing bacteria of the genus *Erwinia* has been studied extensively in regard to excretion of pectinolytic enzymes (BARRAS *et al.,* 1994; COLLMER and BAUER, 1994; PEROMBELON and SALMOND, 1995), whereas not much is known on the role of LPS. The association of LPS with certain enzymes has been studied to elucidate the interactions between lipopolysaccharide from *Salmonella typhimurium* and release of alkaline phosphatase (LINDSAY *et al*., 1973), or the interactions of the lipopolysaccharide from *Escherichia coli* with lysozyme (OHNO and MORRISON, 1989). The role of LPS in **bacterial-plant interactions** is likely to be complex. As well as providing an indispensable **barrier** for the bacteria against toxic plant compounds (NIKAIDO and VAARA, 1985), the interaction of LPS with the plant cell may **promote pathogenesis** or symbiosis (GROLMS, 1996; LAUX, 1998; VENKATESH and RUDOLPH, 2001; DAZZO *et al*., 1991) and/or may trigger **defense-related responses** (MÜLLER, 1998; MÜLLER *et al.,* 1998).

Thus, GROLMS and RUDOLPH (1997) found that when the components originated from a compatible mixture, *i.e*. pectins from leaves susceptible to the *Pseudomonas syringae* pathovars, interactions were characterized by increased viscosity and yield stress. Also, LAUX *et al.* (1998) reported that the LPS from a smooth (s)-strain and a less virulent (r)-mutant of *Pseudomonas syringae pv. phseolicola* reacted differently with pectins from a susceptible bush bean. It was concluded from these studies that a synergistic interaction between plant pectins and bacterial LPS forms a gel-like matrix in the intercellular space and thus allows high multiplication of the bacteria in the susceptible reaction (RUDOLPH, 1999). Although several bacterial virulence factors play a role in host/pathogen-interaction during development of leaf

spots, neither bacterial toxins nor avirulent genes can until now explain the narrow host-specificity of the *Pseudomonas syringae* pathovars on the species level, and it was suggested that interactions between bacterial LPS and host polymers may be decisive (RUDOLPH, 1998). Also, in soft rot diseases caused by *Ecc* or *Ech* a low virulence of the bacterial pathogens has been correlated with defects in the LPS, especially the O-chain (SCHOONEJANS *et al*., 1987; PIRHONEN *et al*., 1991; TOTH *et al*., 1999).

The chemical structure of LPS of *Erwinia carotovora* has been analysed regarding the core region (SANDULACHE and PREHM, 1985; FUKUOKA *et al.,* 1997) and the lipid A (FUKUOKA *et al.,* 1992). However, the chemical structure of the LPS-O-chain of *Eca* and *Ecc* has never been elucidated. Therefore, **structural studies** of *Erwinia* LPS appear to be important for better understanding its role in the bacterium/plant-interaction and in the specific phase behaviour of LPS-containing membranes (FUKUOKA *et al.,* 1990; FUKUOKA *et al.,* 1995).

In **this study** lipopolysaccharides (LPS) from five *Erwinia* strains of the GSPB collection have been investigated extensively for differentiation between *Erwinia carotovora subsp. atroseptica* 9262 without LPS-O-chain (FUKUOKA, 1995; 1997) and other *Erwinia* subspecies. The purpose of this study was to investigate the chemical structure of the LPS-O-side chain and its role in virulence.

2 MATERIAL AND METHODS

2.1 LPS characterization

2.1.1 Extraction and purification

The method was described in chapter II (see subchapter 2.5.2).

2.1.2 SDS polyacrylamide gel electrophoresis (PAGE)

The separation and stacking gels were prepared as described in chapter II (2.5.3.1 and 2.5.3.2). Combs were pulled out carefully, wells were rinsed with electrophoretic buffer 1 x half strength of stock solution (see chapter II, 2.5.3.3). The samples were prepared as described in chapter II (2.5.3.4). The samples were applied with 10 µl LPS-solution in each slot. Only slot no. 2 contained 15 µl LPS-solution. The LPS-solutions contained 0.2-0.5 mg/ml water.
The samples were separated first at 10 mA for 15 min and then changed to 20 mA for 1-1.5 hours. Electrophoresis was stopped when the front of the LPS samples had travelled to about 1 cm above the lower end of the gel. The gel was removed and put into a big Petri dish, then treated with other chemicals as described in chapter II, 2.5.3.3.

2.2 Structure analysis of the LPS-O-chain

These experiments were carried out in the laboratory of Dr. KNIREL (Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospekt 47, Moscow 117913, Russian Federation). The O-chain polysaccharide (OPS) was prepared by degradation of the LPS sample with aq. 2% AcOH for 1.5 h at 100 °C followed by GPC on a columm (70 x 2.6 cm) of Sephadex G-50 using 0.05 M pyridinium acetate buffer (pH 4.5) as eluent and monitoring with a Knauer differential refractometer.

Sugar analysis: The OPS (0.5 mg) was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h); monosaccharides were identified by GLC as their alditol acetates (SAWARDEKER *et al*., 1965) using a Hewlett-Packard 5880 instrument with an Ultra 2 capillary column and a temperature gradient of 150 °C (1 min) to 290 °C at 10 °C/min. The absolute configurations were determined by GLC of acetylated glycosides with (-)-2-octanol by the method of LEONTEIN *et al*. (1978) under the same chromatographic conditions as described above.

Methylation analysis: Methylation was carried out with methyl iodide in Me_2SO in the presence of solid NaOH (CIUCANU and KEREK, 1984). Hydrolysis was performed as in sugar analysis; partially methylated monosaccharides were reduced with $NaBH_4$, acetylated, and analyzed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a DB-5

fused-silica capillary column and a Nermag R10-10L mass spectrometer, using a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min. Identification of partially methylated alditol acetates was performed using the data of JANSSON *et al.* (1976).

NMR spectroscopy: The OPS samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined in a solution of 99.96% D_2O. Spectra were recorded using a Bruker DRX-500 spectrometer at 50 °C. Data were acquired and processed using the XWINNMR version 2.1 software. Mixing times of 200 and 100 ms were used in 2D TOCSY and NOESY experiments, respectively. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_c 31.45).

2.3 Determination of the virulence of two *Eca* - strains

2.3.1 Laboratory experiments

For the laboratory experiments, the potato cultivar Agria was used. Two different strains of *Erwinia carorovora ssp. atroseptica* (*Eca* 2967 and 2962) were compared according to their virulence. The tubers were washed under tap water, dried, immersed in alcohol, flamed and cut with a sterile knife in slices 2.5 mm, thick. The potato slices were placed in Petri dishes.

The two bacterial strains were grown on King's medium B at 27 °C for 24 h. Each strain was adjusted to an $O.D._{660}$ of 0.06 (ca.1.6 X 10^8 cfu/ml) (photometer Spectronic 20, Bausch & Lomb) and serially diluted. Three different concentrations (5 X 10^4, 1 X 10^4 and 5 X 10^3) from each strain were inoculated (5µl) in the middle of the tuber slices, and 5 ml sterile distilled water were poured into Petri dishes to maintain high humidity. The Petri plates were incubated at 27 °C and checked daily for 5 days.

2.3.2 Greenhouse experiments

Potato tubers of cultivar "Agria" were stored at room temperature in the light for two weeks for sprouting, after which they were planted in pots (ca. 20 cm diameter) filled with loamy soil mixed with sand containing one tuber in each pot.

The two bacterial strains were cultured and adjusted to a standardized concentration as described above (2.3.1). Four concentrations (10^8, 10^7, 10^6 and 10^5 cfu/ml) were used for each strain and three replications for each concentration. The plants were inoculated 21 days after planting with 20 µl by a syringe in the **stem** of the potato plants 2 cm above soil surface and incubated at 20 /14°C (day /night). The plants were checked daily and the evaluation was

based on appearance of the **blackleg symptom**.

In addition, the *Eca* strains 2962, 2967 and *Ecc* 436 were tested for pathogenicity on **Chinese cabbage** and **leaves** of tomato, potato and tobacco in two experiments. Chinese cabbage, tomato, potato and tobacco plants were planted in in pots (ca. 20 cm diameter) filled with loamy soil mixed with sand and containing one plant in each pot. The bacterial strains were grown on King's medium B and each strain was adjusted to an O.D $_{660}$ of 0.3 (ca. 10^9 cfu/ml) or 0.06 (ca. 2 X 10^8 cfu/ml) as described above. The leaves of young plants were injected under a slight pressure with a syringe without needle.

In the first experiment, the bacterial suspensions of ca. 10^8, 10^7, 10^6 and 10^5 cfu/ml of *Eca* strains 2962, 2967 and *Ecc* 436 were used to inoculate **leaves** of tobacco, tomato and Chinese cabbage.

In the second experiment, bacterial suspensions of OD 0.3 (ca. 10^9 cfu/ml) and 10^8 cfu/ml of the same bacterial strains as listed above were used to inoculate leaves of tomato, potato and Chinese cabbage plants as well as **thick leaves** of Chinese cabbage heads. Detached thick leaves of Chinese cabbage heads were inoculated with 5 µl from the bacterial suspensions, which were injected under slight pressure with a hypodermic needle, and leaves were incubated in plastic boxes (with moist paper at the bottom) at 27 °C for 5 days to assess symptoms.

2.4 Determination of pectolytic activity of two *Eca* - strains

A) Substrate: **solution (1)**: 0.1 M citric acid (4.2 g in 200 ml H_2O); **solution (2)**: 0.1 M Na-citrate (14.7 g in 500 ml H_2O). Mix 152.5 ml solution (1) and 347.5 ml solution (2), adjust pH to 5.2, after warming the buffer to 80 °C, add 5 g from pectin N (Roth Co.), mix vigorously with magentic stirrer and adjust pH to 5.0. This substrate was used within 2 days.

B) At first, the two bacterial *Eca* strains were precultured on King's medium B (agar) at 27 °C for 24 h. Then, a loopful of bacteria was inoculated into pectin liquid medium, and incubated in a shaker at 27 °C for 24 h. From this 2nd preculture bacteria were pelleted by centrifugation, and the pellet was suspended in 0.01 M $MgSO_4$ and adjusted to an OD_{660} of 0.06. From these bacterial suspensions 0.1 ml were inoculated into Erlenmyer flasks with pectin liquid medium and incubated in the shaker at 110 rpm (Clim-O-shake, Firma Kühner, Basel, Switzerland) for 48 h. At different time intervals (0 h, 6 h, 12 h, 18 h, 24 h, 30 h, 42 h and 48 h) samples of 10 ml were taken, centrifuged at 6000 g/10 min, and the supernatant stored at 4 °C until use. When the last sample had been taken, each supernatant was mixed with 30 ml substrate

solution (A) supplemented by 5 ml 0.01 M $CaCl_2$ and 0.02% sodium azide. The reaction mixtures were incubated for 24 h at 37 °C, after which the viscosity was measured by a viscosimeter.

At the same time intervals (0 - 48 h) bacterial growth was determined. 0.5 ml from the bacterial culture were serially diluted, and 0.1 ml were plated onto King's medium B and incubated at 27 °C for 24 h.
Viscosity of the reaction mixtures was determined with a falling ball viscosimeter (Haake, Germany). For this purpose, 20 ml culture fluid were used. The time in seconds required for the ball to pass through a definite distance in a tilted (100) cylindrical tube was recorded. The readings obtained were converted into the units of mPa X sec by the formular equation (EL-SHOUNY, 1993), where 1 mPa X sec = 1 cP (Centipoise):

$$\text{Viscosity} = F - (S_k - S_f) - K$$

F = falling time in sec

S_k = density of ball (2.397)

S_f = density of culture fluid (ca. 1.002991 g cm^{-3})

K = ball constant (0.0565)

All viscosity measurements were taken at room temperature. The sample was allowed to equilibrate for 2 min in the viscosimeter before the viscosity was determined.

2.4.1 Multiplication of two *Eca* - strains in pectin and King's B liquid medium

The bacterial strains (GSPB *Eca* 2967 and GSPB *Eca* 2962*)* were grown as described in 2.4. The next step was a preculture in King's B or pectin liquid medium, in a shaker at 27 °C at 110 rpm (Clim-O-shake, Firma Kühner, Basel, Switzerland) for 24 h. Bacterial cells (10 ml) were centrifuged at 6000 g/10 min, and the pellet was dissolved in 10 ml 0.01 M $MgSO_4$ to adjust the bacterial concentration to an $O.D._{660}$ of 0.06, corresponding to ca.3.3 $X10^7$ cfu ml (*Eca* 2962) and 3.5 X 10^7 cfu/ml (*Eca* 2967). The suspensions were serially diluted and 0.1 ml from the dilution $1:10^5$ were plated onto King's medium B and incubated at 27 °C for 24 h. 0.1 ml from the same concentration were inoculated into an Erlenmeyer flask and shaken (110 rpm) at 27 °C for different times (0 h, 6 h, 12 h, 24 h, 30 h, 42 h and 48 h). The bacterial growth was determined by dilution plating.

2.4.2 Determination of bacterial growth of two *Eca* strains using the semi-selective medium (CVPM)

The two bacterial strains (2967 and 2962) were grown on King's medium B and incubated at

27 °C for 24 h. The bacterial growth was scraped off in 0.01 M $MgSO_4$, and adjusted to an $O.D._{660}$ of 0.06 (ca. 10^8 cfu/ml). The bacterial suspensions were spot-inoculated onto the semi-selective medium and incubated at 27 °C for 24 h.

3 RESULTS

3.1 Analysis of lipopolysaccarides (LPS)

3.1.1 Characterization by SDS-PAGE

The results of silver staining after SDS-PAGE are shown in **Fig. 1**. Both preparations from *Eca* 2967 and *Ecc* 436 (lane nos. 4, 5 and 6) were found to give typical lipopolysaccharide electrophoretic patterns with the ladder-like appearance due to the LPS-O-chain. In contrast, the LPS extracted from the *Eca* strain 2962 (lane nos. 2 and 3) did not show the typical ladder-patterns, indicating a "rough" LPS.

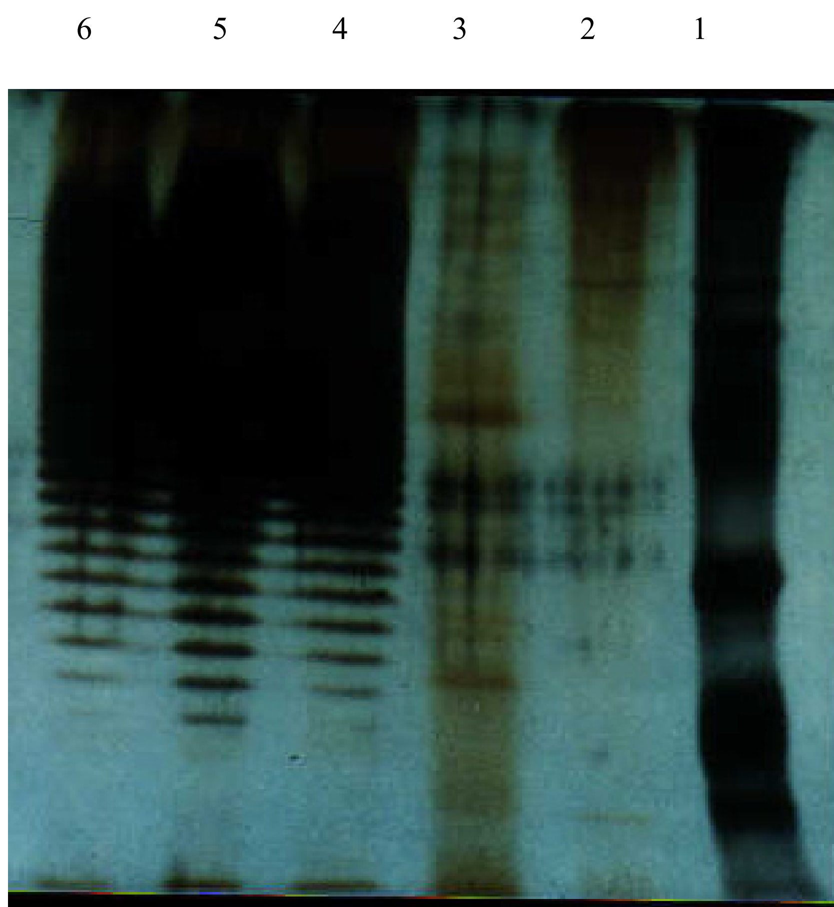

Fig. 1: SDS-PAGE of lipopolysaccharides from *Erwinia spp.*. The samples were run on a 10% acrylamide gel and stained as described in Chapter II, 2.5.3. Lanes: (1) Marker VIII, (2 and 3) *Eca* 2962, (4) *Ecc* 436, (5) *Eca* 2967, (6) *Ecc* 436. Into each slot 10 µl LPS-solution (containing 0.2-0.5 mg/ml water) were added. Only no. 2 contained 5 µl.

3.1.2 Structure of the LPS-O-chain of *Ecc* (GSPB 436)

The O-polysaccharide of *Erwinia carotovora ssp. carotovora* GSPB 436 was studied by sugar and methylation analysis and ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, NOESY and H-detected ^{1}H, ^{13}C heteronuclear multiple-quantum coherence (HMQC) experiments in the laboratory of Dr. Knirel (see subchapter 2.2). The polysaccharide was found to contain L-rhamnose, D-glucose and D-fucose in the ratio 2:1:1 **(Fig. 2)**, and the following structure of the tetrasaccharide repeating unit was established:

→3)-ß-L-Rha*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Fuc*p*-(1→
3
↑
1
α-D-Glc*p*

Fig. 2: Structure of *Ecc* GSPB 436 O-side-chain (after Knirel, with ^{13}C NMR- spectroscopy analysis)

3.1.3 Structure of the LPS-O-chain of *Eca* (GSPB 2967)

The structure of the LPS-O-chain of *E. carotovora ssp. atroseptica* (strain GSPB 2967) was only preliminary determined by Dr. Knirel and coworkers. Accordingly, the O-chain has a pentasaccharide repeating unit containing three galactose residues, one rhamnose and a new higher branched sugar (similar to caryophyllose). The higher branched sugar showed some similarity to caryophyllose from *Pseudomonas caryophylli* (ADINOLFI *et al*., 1995 a and 1995 b), but has less carbons in the side chain.

3.2 Virulence test of two *Erwinia carotovora spp. atroseptica* strains on potato tuber slices and Chinese cabbage

3.2.1 Tests on potato slices in Petri dishes

Results in **Table 1** show that the strain *Eca* 2967 was highly virulent on potato slices, because 3 days after inoculation with 5 X 10^4 cfu/ml and 5 days after inoculation with 1 X 10^4 cfu/ml nearly the whole potato tissue was macerated. On the other hand, strain *Eca* 2962 was very weakly virulent since only the highest concentration of 5 X 10^4 cfu/ml caused medium rotting of potato slices after 4-5 days.

Table 1: Virulence of two *Eca* strains on potato tuber slices

Day	Bacterial concentration *Eca* 2967			Bacterial concentration *Eca* 2962		
	$5\ X10^4$	$1.0\ X10^4$	$5.0\ X10^3$	$5\ X10^4$	$1.0\ X10^4$	$5.0\ X10^3$
1	1.3*	0.5	0	0.2	0	0
2	2.0	0.7	0	0.3	0	0
3	Whole	1.2	0	0.7	0	0
4	Whole	2.0	0	1.0	0	0
5	Whole	2.5	0	1.2	0	0

* = Diameter of rotted tissue in cm

3.2.2 Tests on potato plants in pots in the greenhouse

Fig. 3 shows a strong difference in symptoms caused by the two strains. Typical blackleg symptoms appeared on potato cultivar Agria one week after inoculation with strain *Eca* 2967 at bacterial concentrations of 10^8, 10^7 and 10^6 cfu/ml. In contrast, none of the four bacterial concentrations tested of *Eca* 2962 had caused disease symptoms within one month after inoculation. It was concluded from this experiment that the Japanese strain *Eca* 2962 was nearly avirulent as incitant of potato blackleg.

Fig. 3: Blackleg symptoms on potato plants (cultivar Agria) after inoculation with 10^8 cfu/ml. Only strain *Eca* 2967 (left) caused typical blackleg symptoms while inoculation with strain *Eca* 2962 (right) showed only small necrotic spots due to the wounding by the inoculation precedure.

3.2.3 Tests on leaves of Chinese cabbage, tomato, potato and tobacco and on thick leaves of Chinese cabbage heads

Results **(Fig. 4)** show that in the first experiment, the Japanese strain *Eca* 2962 caused slight necrotic symptoms on leaves of tomato plants only, with a high bacterial concentration (ca.10^8 cfu/ml), whereas the other bacterial concentrations of *Eca* 2967 and *Ecc* 436 did not cause any symptoms on all the plant leaves tested (tomato, potato, tobacco). The results of the second experiment, **(Figs. 5, 6 and 7)** show that the higher concentrations (10^9 cfu/ml) of *Eca* 2962 and *Eca* 2967 caused unspecific necrotic spots on leaves of tomato **(Fig. 5)** and potato **(Fig. 6)** but no symptoms on young leaves of Chinese cabbage **(Fig. 7)** and tobacco (not shown). On tomato leaves, the symptoms caused by the Japanese strain *Eca* 2962 appeared to be a little bit stronger than those caused by *Eca* 2967 **(Fig. 5)**. On thick leaves of Chinese cabbage extended soft rotting was caused by 10^9 cfu/ml of *Eca* 2967, but only limited soft rot spots by *Eca* 2962 **(Fig. 8)**. With the lower concentration of 10^8 cfu/ml, the Japanese strain 2962 caused mostly necrotic spots with a small soft rotting border, while *Eca* 2967 caused large confluent soft rotted areas **(Fig. 9)**. It was concluded from these tests that the Japanese strain was a very weak soft rot pathogen.

Fig. 4: Spot symptoms on tomato leaves after inoculation with 10^8 cfu/ml. Only strain *Eca* 2962 (left) caused necrotic symptoms while no symptoms were observed with *Eca* 2967 and *Ecc* 436, respectively, 5 days after inoculation.

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Fig. 5: Necrotic spots on tomato leaves after inoculation with 10^9 cfu/ml. Strain *Eca* 2962 (right) caused a little bit stronger necrotic spots with more yellowing than that caused by *Eca* strain 2967 (left).

Fig. 6: Necrotic spots on potato leaves after inoculation with 10^9 cfu/ml. Both *Eca* strains (2962 and 2967) showed similar necrotic symptoms 3 days after inoculation.

Fig. 7: Leaves of Chinese cabbage inoculated with 10^9 cfu/ml. Both *Eca* strains (2962 and 2967) had caused no symptoms 3 days after inoculation.

Fig. 8: Soft rot symptoms on Chinese cabbage after inoculation with 10^9 cfu/ml. Strain *Eca* 2967 (left) caused extended soft rotting while strain *Eca* 2962 (right) provoked limited soft rot spots, 2 days after inoculation.

Fig. 9: Soft rot symptoms on Chinese cabbage after inoculation with 10^8 cfu/ml. Strain *Eca* 2967 (left) caused large confluent soft rotted areas while strain *Eca* 2962 (right) caused mostly necrotic spots with a small soft rotting border, 2 days after inoculation.

3.3 Pectolytic activity

As shown in **Fig. 10** and listed in **Tables 2** and **3** the supernatant of the German strain (GSPB 2967) possessed a much higher pectinase activity than that of the Japanese strain (GSPB 2962) from 6 to 30 h after inoculation of the pectin medium. However, when the pectinase activity in the supernatant was calculated per bacterial cell it turned out that the pectolytic activity of the Japanese strain was similar to that of the German strain, the difference between the two strains not being significant. On the other hand, the German strain showed maximum detectable pectinase activity clearly after 18, the Japanese strain only after 42 h. Therefore, in further experiments the **growth** of the two strains was compared in and on different media (chapter 3.4).

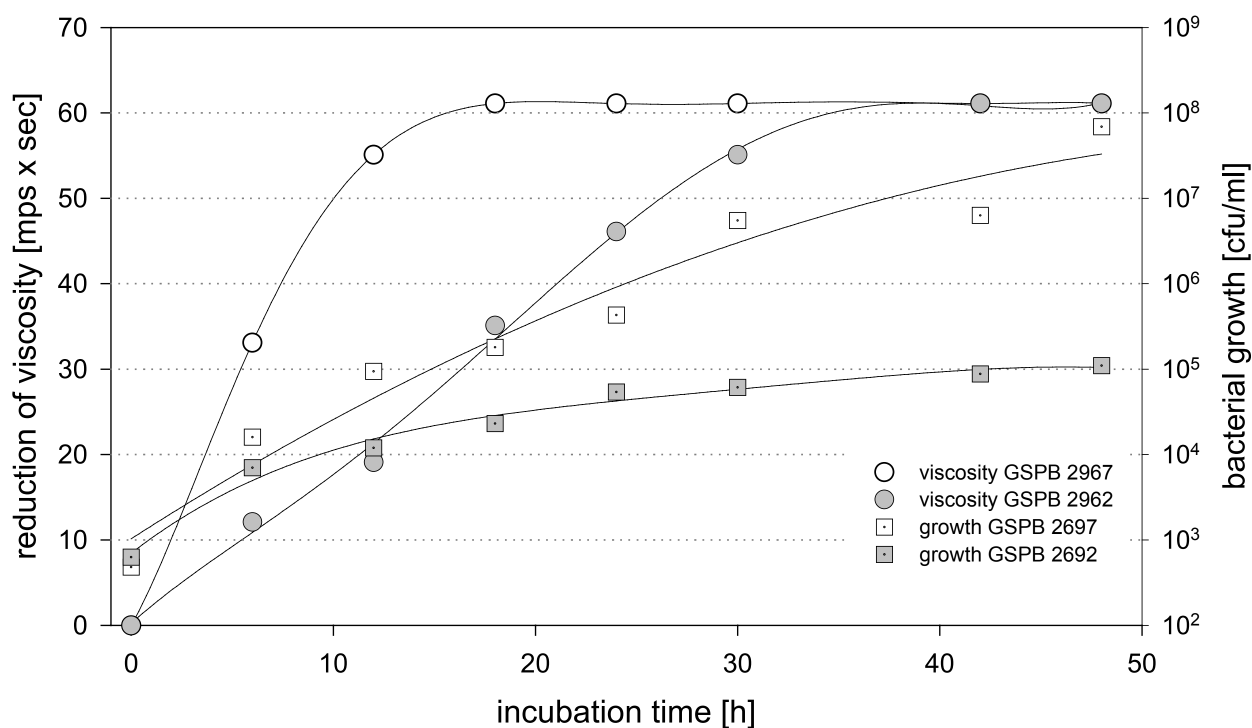


Fig. 10: Pectinase activity and bacterial growth from 0 – 48 h in a pectin liquid medium

Table 2: Pectolytic activity of *Erwinia carotovora ssp. atroseptica*, German strain (GSPB 2967)

Incubation time	Bacterial concentration in the pectin liquid medium (cfu/ml)	Speed of fall in seconds/5 cm	Viscosity (mpa X sec)	Reduction of viscosity in (mpa X sec)	Pectinase activity per bacterial cell (mpa X sec)
0 h	4.8×10^{2}	65	61.6	0	0.0
6 h	1.6×10^{4}	32	28.5	33.1	2.1×10^{-3}
12 h	9.4×10^{4}	10	6.5	55.1	0.59×10^{-3}
18 h	1.8×10^{5}	3.0	0.46	61.1	0.34×10^{-3}
24 h	4.3×10^{5}	3.0	0.46	61.1	0.14×10^{-3}
30 h	5.5×10^{6}	3.0	0.46	61.1	0.01×10^{-3}
42 h	6.3×10^{6}	3.0	0.46	61.1	0.01×10^{-3}
48 h	6.9×10^{7}	3.0	0.46	61.1	0.001×10^{-3}

Table 3: Pectolytic activity of *Erwinia carotovora ssp. atroseptica*, Japanese strain (GSPB2962)

Incubation time	Bacterial concentration in the pectin liquid medium (cfu/ml)	Speed of fall in seconds/5 cm	Viscosity (mpa X sec)	Reduction of viscosity in (mpa X sec)	Pectinase activity per bacterial cell (mpa X sec)
0 h	6.3×10^{2}	65	61.6	0	0.0
6 h	7.0×10^{3}	53	49.5	12.1	1.73×10^{-3}
12 h	1.2×10^{4}	46	42.5	19.1	1.59×10^{-3}
18 h	2.3×10^{4}	30	26.5	35.1	1.53×10^{-3}
24 h	5.4×10^{4}	19	15.5	46.1	0.85×10^{-3}
30 h	6.1×10^{4}	10	6.5	55.1	0.9×10^{-3}
42 h	8.8×10^{4}	3.0	0.46	61.1	0.69×10^{-3}
48 h	1.1×10^{5}	3.0	0.46	61.1	0.56×10^{-3}

3.4 Mutiplication of *Eca*-strains in/on different media

3.4.1 In pectin medium

As shown in **Fig. 11** the German strain *Eca* 2967 multiplied faster in liquid pectin medium than the Japanese strain *Eca* 2962. Thus, GSPB 2967 reached 10^{7} cfu/ml after 48 h, whereas GSPB 2962 reached only 10^{5} cfu/ml after 48 h. It might be concluded from these results that the growth of the German strain was much faster than that of the Japanese strain, because the German strain had a higher pectinase activity. In order to proof this hypothesis, bacterial growth on King's medium B (with glycerol as carbon source) was also determined, see subchapter 3.4.2.

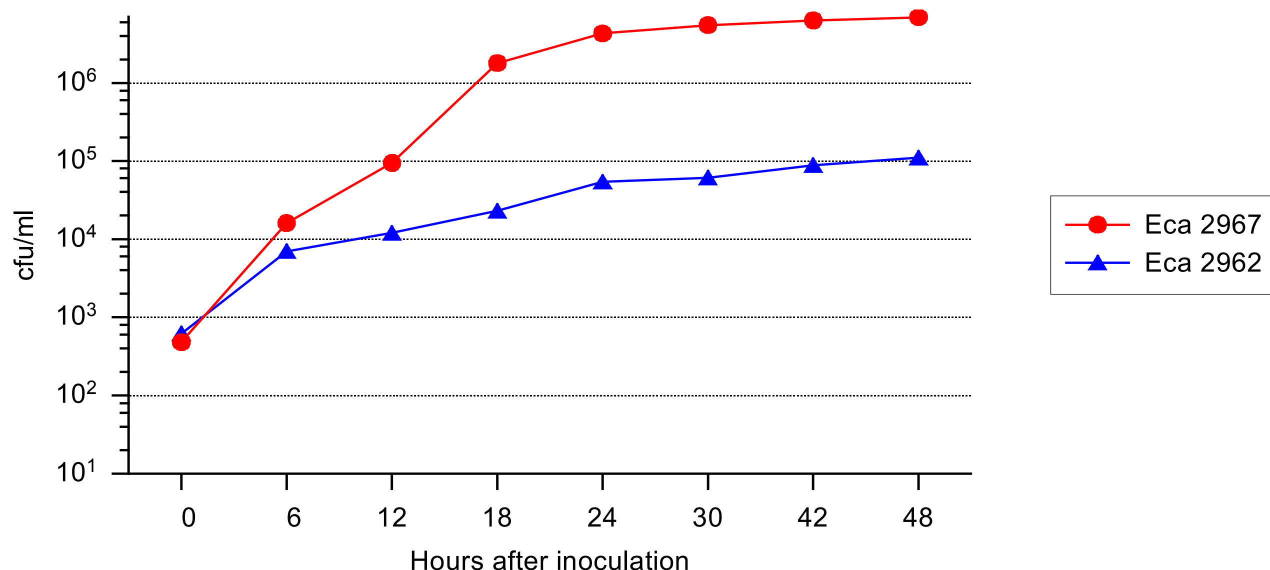


Fig. 11: Growth of the two *Eca* strains 2967 and 2962 in liquid pectin medium.

3.4.2 In KB medium

Unexpectedly, an even higher difference in the multiplication rate of strains 2967 and 2962 was observed on King's medium B than in pectin medium (see **Fig. 12**). For instance, more than 10^9 cfu/ml were determined for the strain 2967 48 h after inoculation, but only 10^6 cfu/ml for strain 2962. It was concluded from these results that the slower growth of the „rough" *Eca* strain 2962 in King's medium B compared to the pectin medium was not due to a low pectinase activity of strain 2967. Obviously, **strain 2962** was characterized by a **slower growth vigour** than strain 2967, and the lower pectinase activity may be a consequence of this partial deficiency. The question whether the complete loss of the LPS-O-chain is in some way connected with the lower growth vigour remains unanswered.

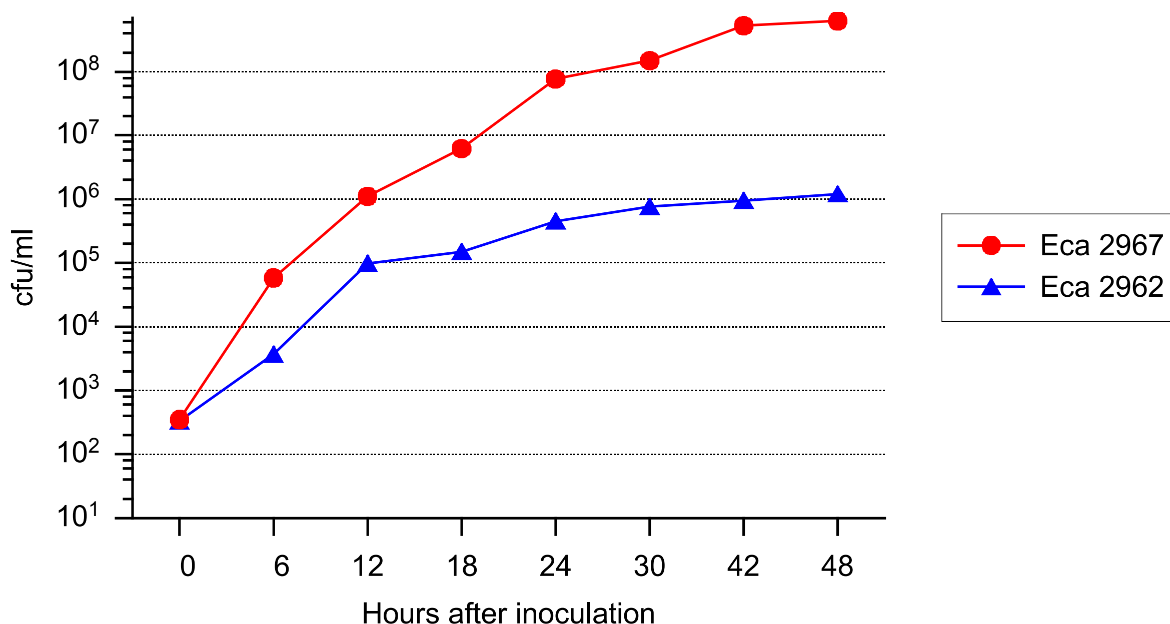


Fig. 12: Growth of the two *Eca* strains 2967 and 2962 in liquid KB medium

3.4.3 Comparison of the growth of two *Eca* strains on the CVPM medium

As shown in **Fig. 13** the cavities formed on CVPM medium by the German strain *Eca* 2967 were broader and deeper compared to those formed by the Japanese strain *Eca* 2962, the cavities of which were shallow and smaller. Also, these results indicated that the German strain multiplied faster on the semi-selective medium than the Japanese strain. Therefore, the cavities were bigger and broader.

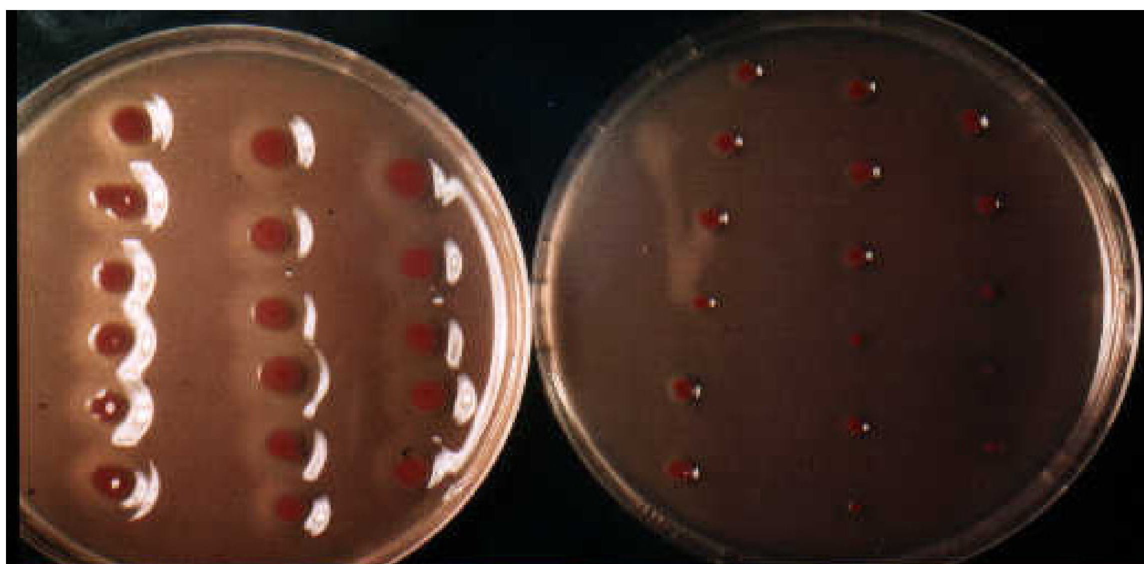


Fig. 13: Cavity formation by the two *Eca* strains on CVPM medium. Strain *Eca* 2967 formed deep and broad cavities (left) while strain *Eca* 2962 showed shallow and small cavities (right).

4 DISCUSSION

It was suggested that bacterial phytopathogenicity is affected by the cell wall lipopolysaccharides (LPS) (CHATTERJEE and STARR, 1980). LPS of Gram-negative bacteria are composed of three genetically and structurally distinct regions; lipid A, core region and O-specific chain. The preparation of largely intact, i.e. biologically active **lipid A** from enterobacteriaceae was possible because of the acid-labile ketosidic linkage between the core oligosaccharide and lipid A (ZÄHRINGER *et al.,* 1998). The lipid A extracted from *Erwinia carotovora* strain GSPB 2962 (=Ferm P-7576) consists of a ß-1',6-linked glucosamine disaccharide substituted with phosphate at position 4, and carries ester- and amide-bound fatty acids similar to the lipid A from other Gram-negative bacteria (FUKUOKA *et al.*, 1992).

It has been assumed that parts of the **core oligosaccharide** may contribute to endotoxic activities and in fact it is now generally accepted that the core oligosaccharide may modulate the biological activity of lipid A (RIETSCHEL *et al*., 1993). DE BOER *et al.* (1985) found that all R-type core LPS structures from 16 *Erwinia carotovora* strains contain the same components as several enterobacteriaceae such as KDO, glucosamine, heptose, glucose and usually galactose. Thus, it appears that *Erwinia carotovora* has an LPS core structure very similar to that of animal and human pathogens classified in genera such as *Salmonella*, *Shigella* and *Escherichia*. FUKUOKA *et al.* (1997) studied the complete structure of R-type lipopolysaccharide of *Erwinia carotovora* strain Ferm P- 7576 and found the structure to differ from that reported for *Erwinia carotovora* strain B374 (SANDULACHE and PREHM, 1985).

The O-antigenic polysaccharides (**O-specific chain**) are present in S-strains of Gram-negative bacteria, which form "smooth" colonies. Therefore, the fine structure of the O-chain is the principal antigenic determinent of the enterobacterial LPS. Also, for the heterogeneous group of *Erwinia carotovora* subspecies and strains it was proposed that serogroup specificity is a function of the O-chain structure (DE BOER & Mc NAUGHTON, 1987), because strains with the same LPS composition differed in serogroup affinity. Thus, the strains in serogroups IX and XVIII contain rhamnose but these strains and their purified LPS do not cross-react serologically (DE BOER *et al*., 1985). These results indicate that *Erwinia carotovora* serogroups probably are based on the LPS-O-chains. It is clear that the *Erwinia spp*. LPS will electrophorese in the same characteristic fashion as LPS from other bacterial species (SIDBERRY *et al*., 1985). Thus, the fastest migrating bands on the silver-stained acrylamide gel in the here reported studies **(Fig. 1)** indicated a clear difference between the two strains (S-

type), which have an O-specific chain in comparison with the other strain (R-type), which does not have an O-specific chain.

Notwithstanding extensive studies on different serogroups of *Erwinia carotovora* which were attributed to different LPS-structures, the chemical structure of the O-chains of *Erwinia carotovora* -LPS has never been elucidated.

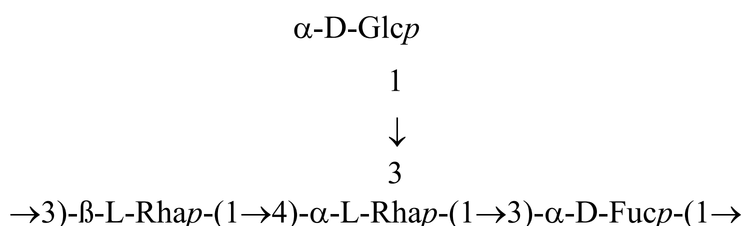
The aim of this study was to elucidate and investigate the chemical structure of LPS-O-chain from *Erwinia* strains, and to evaluate its possible role in virulence of *Eca* strains.

4.1 Structure of the LPS-O-chain of *Erwinia carotovora ssp. carotovora*

The chemical composition of the LPS-O-chain of Gram-negative bacteria has been assumed to be highly diverse and to determine the immunospecificity of bacterial species, subspecies and serotypes (KNIREL and KOCHETKOV, 1994). Each serologically distinguishable S-strain of bacteria produces an O-specific polysaccharide with its own unique structure and intraspecies serological classification. The resulting typing schemes of S-strains of bacteria which are important for epidemology are based on the specificity of the O-chains (KNIREL and KOCHETKOV, 1994; KNIREL and ZDOROVENKO, 1997). For instance, lipopolysaccharides of *Salmonella* T I (a transient variant from rough to smooth form) are characterized by the presence of polysaccharide chains of two types, which are the homopolymers D-ribofuranose and D-galactofuranose (KNIREL and KOCHETKOV, 1994). The O-chains appear to be specific for certain pathovars of *Pseudomonas syringae* or *Xanthomonas campestris* (KAMIUNTEN and FUJITA, 1990; ZDOROVENKO *et al.*, 1992; YAKOVLEVA *et al.,* 1994; OVOD *et al.,* 1996, 1997) and may play a **role in host specificity** (RUDOLPH, 1999).

Our studies revealed for the first time the chemical structure for the LPS-O-chain from *Erwinia carotovora ssp. carotovora* (SENCHENKOVA *et al.*, 2001). Earlier studies elucidated only the chemical structure of the core region and lipid A of *Erwinia carotovora* (FUKUOKA *et al.*, 1992, 1997). These authors analysed the LPS of strain *Eca* 2962 (Ferm P- 7576), which is an R-type and does not contain the O-chain. Our studies confirmed that the LPS of *Eca* 2962 strain **did not** show the typical ladder-like pattern of the LPS-O-chain, and this was similar to those obtained by FUKUOKA *et al.* (1997). Therefore, we selected the highly virulent strains *Eca* 2967 and *Ecc* 436 possessing smooth LPS to elucidate the structures of their LPS-O-chains. The results obtained by SENCHENKOVA *et al.* (2001) indicated that the chemical structure of the LPS-O-chain of *Erwinia carotovora ssp. carotovora* strain GSPB 436 was

similar to that of *Pseudomonas fluorescens* biovar A (strain IMV 472) (KNIREL *et al.*, 1994) as follows:



But, even more interesting, the O-chain of ***Xanthomonas maltophilia*** serogroup O19 (WINN *et al.*, 1993) appeared to be identical with that of *Erwinia carotovora ssp. carotovora*, strain 436. The bacterium *Xanthomonas maltophilia*, earlier named *Pseudomonas maltophilia* (HUGH and RYSCHENKOW, 1961) was recently named *Stenotrophomonas maltophilia* (PALLERONI and BRADBURY, 1993). This species is not a plant pathogen and most of its strains have been isolated from clinical specimens (human faeces, blood, pericardial fluid, ascitic fluid, pleural fluid, spinal fluid, oropharyngeal swabs and contaminated tissue). However, it can be an opportunistic human pathogen, and it is also found in river water, well water, raw milk, frozen food and in the rhizosphere of some cultivated plants. The colonies formed by some *Xanthomonas maltophilia* (phenon 1) strains are umbonate, do not have an even colony margin and do not form slime. *Xanthomonas maltophilia* is a cosmopolitan in distribution and a heterogeneous collection of opportunistic bacteria (SWINGS *et al.*, 1983).

There are some **physiological similarities** between *Erwinia carotovora subsp. carotovora* and *Xanthomonas* (=*Stenotrophomonas*) *maltophilia*. Thus, both species do not produce acid from glucose, indol formation is negative, the optimal temperature for growth is 35 °C, and the following physiological reactions are positive: nitrate reduction, hydrolysis of gelatine, acid produced from maltose and use of cellobiose as carbon source for growth. Both bacterial species can be isolated from the rhizosphere of some cultivated plants, they are cosmopolitans and heterogeneous. Some *X. maltophilia* strains contain a respiratory nitrate reductase. It might enable cells to use nitrate as an electron sink under semi-aerobic conditions (WOODARD *et al.*, 1990). Nevertheless, the earlier strains of *X. maltophilia* were strictly aerobic (HUGH and RYSCHENKOW, 1961) in contrast to *E. carotovora* (BRADBURY, 1986).

Fifteen distinct somatic **serotypes** were encounted among 26 strains of *X. maltophilia* (HUGH and RYSCHENKOW, 1961), while for *E. carotovora* 36 serogroups have been differentiated (DE BOER and Mc NAUGHTON, 1987). Since different serogroups have often been attributed to different LPS-structures, especially the O-chains, it can be assumed that different O-chain structures occur in both of the heterogeneous species *E. carotovora* and *X.*

maltophilia. Therefore, the here reported finding of identical O-chain structures in both species may be **accidentally** and not reproducible for the whole spectrum of different strains occurring in both species.

4.2 Preliminary structure of the LPS-O-chain of *Erwinia carotovora ssp. atroseptica*

Interestingly, the LPS-O-chain of *Eca* strain 2967 was completely different from that of *Ecc* strain 436. It is yet unknown, whether all *Eca* strains have a similar structure, and what is the exact structure of the higher branched sugar. Its similarity to caryophyllose from *Pseudomonas caryophilli* (ADINOLFI *et al.*, 1995 a and b) appears interestingly. This pathogen causes bacterial wilt of carnation with similar symptoms than blackleg of potato plants, that is a sticky, brown, basal stem rot (LELLIOTT and STEAD, 1987). Future studies have to reveal to which extent different LPS-O-chains occur in the *Erwinia carotovora* subspecies *carotovora* and *atroseptica*.

4.3 Virulence of the Japanese "rough" *Eca*-strain in comparison to other "smooth" *Erwinia carotovora* strains

Our results clearly showed a strong difference between the two *Eca* strains. The German strain (with intact O-chain) was **highly virulent**, whereas the Japanese strain (without O-chain) was **very weakly** virulent on potato discs in laboratory experiments. Also, in the green house experiments potato plants developed typical **blackleg symptoms** by the German strain one week after inoculation, whereas the Japanese strain did not cause any symptoms one month after inoculation. Similarly, thick leaves of Chinese cabbage showed an extended soft-rotting by strain *Eca* 2967, but only small soft-rotted lesions by *Eca* 2962. Thus, the Japanese strain was **weakly virulent** on **Chinese cabbage** in spite that this strain was originally isolated from Chinese cabbage, whereas the German strain showed strong softening symptoms one day after inoculation.

The pathogenicity of *Eca* and *Ecc* strains for Chinese cabbage has been observed by several other workers. CHIU *et al.* (1965) reported that *Erwinia phytophthora* (*E. atroseptica*) infected Chinese cabbage and formed a decayed area of 40 mm diameter within 24 h at 28 °C and relative humidity of 100%. Also, BRADBURY (1986) reported that *Ecc* infects Chinese cabbage (*B. chinensis*) so that larger fleshy organs become softened to a pulp very quickly, and

that *Eca* infects the same plant and is able to cause stalk rot of cabbage. KELLER and KNÖSEL (1980) reported that the pathogen caused about 30% losses of Chinese cabbage yield.

A different pattern of symptoms was provoked on **leaves** of tomato, potato, tobacco and Chinese cabbage (Figs. 4, 5, 6 and 7), where either no symptoms appeared (on tobacco and Chinese cabbage), or HR-like necrotic leaf spots occurred shortly after inoculation (on leaves of tomato and potato) (Figs. 4, 5 and 6). These symptoms should be regarded as a resistant reaction because they were only caused by high bacterial concentrations (10^8–10^9 cfu/ml). MÜLLER *et al.* (1998) investigated the role of whole LPS as well as its subunits O-chain and core region on **induced resistance** in compatible and incompatible systems and found that lipid A alone can cause this effect, while the O-chain or the core region did not induce resistance. Therefore, the higher capability of the rough *Eca* strain (GSPB 2962) to cause necrotic spots on tomato leaves may be due to the fact that the LPS of this strain was not masked by long O-chains.

Also, other laboratories reported a lowered virulence of *Erwinia carotovora* strains which had defects in the LPS-O-chain. TOTH *et al.* (1999) found that from 40 spontaneous bacteriophage-resistant mutants of *Eca* one mutant, A5/22, showed significantly reduced virulence *in planta* (blackleg symptoms) and a weaker ability to cause potato soft rot compared with the wild-type strain. On the other hand, this mutant also showed reduced synthesis of the exoenzymes pectate lyase (Pel) and cellulase (Cel) in the supernatant. However, enzyme activities were not compared with bacterial numbers, as was done in our studies. PAGE showed that the mutant strain A5/22 had a defect in the LPS-O-chain compared to the wild-type strain. SCHOONEJANS *et al*. (1987) used bacteriophages ΦEC2 and Mu to isolate lipopolysaccharide (LPS)-defective mutants of *Ech* that also exhibited reduced virulence when inoculated into *Saintpaulia ionantha* plants. Also, PIRHONEN *et al*. (1991) screened for LPS-defective mutants of *Ecc* after transposon mutagenesis. These authors described three LPS-defective mutants which exhibited a galactose-sensitive phenotype and were impaired in virulence.

Also, for other phytopathogenic bacteria several data indicate that LPS, and especially the O-chain, contribute to bacterial virulence (NEWMANN *et al.,* 1995), whereas the role of exopolysaccharides, for instance xanthan, in pathogenesis has not been completely unravelled. In the case of *Xanthomonas campestris pv. campestris* the O-chain polysaccharide (OPS) of the LPS was regarded as a factor of pathogenicity (STEINMANN, 1996). Similarly, the results obtained by LAUX (1998) imply that LPS from a smooth(s)-strain and a **less virulent**

rough(r)-mutant of *Pseudomonas syringae pv. phaseolicola* react differently with pectins of a sensitive bush bean. Therefore, the low virulence of the rough(r)-*Eca* strain 2962 might be explained by its incomplete O-specific chain. However, before reaching this conclusion the question had to be answered whether strain *Eca* 2962 was also impaired in other factors of virulence, especially the release of extracellular enzymes.

Most strains of *Erwinia carotovora* produce several extracellular enzymes that degrade plant cell walls, such as pectate lyase, pectin lyase, polygalacturonase, pectin methyl esterase, cellulase and protease (PEROMBELON and KELMAN, 1980; BARRAS *et al.,* 1994; SALMOND *et al.,* 1994; PEROMBELON and SALMOND, 1995). These different enzymes enable the bacterium to cause tissue maceration and necrosis (COLLMER and KEEN, 1986; KOTOUJANSKY, 1987). Most of these enzymes have been shown to be important in virulence, since mutations affecting their synthesis led to reduction or loss of virulence (KOTOUJANSKY, 1987). Thus, mutations that affect synthesis or secretion of extracellular enzymes from *Erwinia spp.* (*Eca, Ecc* and *Ech*) led to a reduction or **loss of virulence in *planta*** (MURATA *et al.,* 1990; REEVES *et al.,* 1993). Therefore, we determined the pectinase activity of the two *Eca*-strains 2962 and 2967. According to our results as shown in Tables 2 and 3 it might be concluded that the Japanese strain (GSPB 2962) possesses a lower pectinase activity than the German strain (GSPB 2967) when determined in the supernatant. However, when the pectinase activity was calculated per bacterial cell *in vitro* both strains showed the same pectinase activity. We concluded from these results that the weak virulence of the Japanese strain can not be attributed to a lower pectinase activity.

On the other hand, the slow growth of the Japanese strain in the liquid pectin medium might be explained by the low pectinase activity of this strain. This possibility was excluded by our finding that in King's medium B (without pectin) the Japanese strain also grew much slower than the smooth and virulent strain 2967, the difference being even higher than in liquid medium. Obviously, the Japanese strain (2962) possessed a **lower growth vigour** than the virulent *Eca* strain. In most other cases of phytopathogenic bacteria the rough variants grow even faster or as fast as the smooth wild strains, for instance in case of *Ralstonia solanacearum* (HUSSAIN and KELMAN, 1958) or *P. syringae pv. phaseolicola* (GERWE *et al.*, 1987). Therefore, it appears not very probable that the low growth vigour of strain 2962 was related to the loss of the LPS-O-chain.

However, dependencies between growth, nutrient composition and LPS production have been reported for *E. carotovora.* The results obtained by FUKUOKA *et al.* (1989) show that the combination of two types of carbon sources enhanced LPS productivity, cell growth, and

furthermore, productivity per unit cell. When *E. carotovora* was cultivated on a basal medium or the medium containing a **single carbon source**, the LPS yield was poor.

Since the reason for the low growth vigour of the Japanese strain is completely unknown it is still impossible to answer the question whether the loss of the LPS-O-chain, or the low growth vigour, or both deficiencies are responsible for the low virulence of the Japanese *Eca* strain (GSPB 2962).

5 SUMMARY

1- Electrophoresis of the LPS of the Japanese *Eca* strain (2962), originally isolated from Chinese cabbage, revealed an absence of the O-chain, that is a "rough" LPS. In contrast, the German strains of *Eca* (2967) and *Ecc* (436) all contained "smooth" LPS with an O-chain, indicated by the typical ladder-like pattern after electrophoresis.

2- The repeating unit of the O-chain of *Ecc* 436 was elucidated as a tetrasaccharide of the following structure:

```
→3)-ß-L-Rhap-(1→4)-α-L-Rhap-(1→3)-α-D-Fucp-(1→
                          3
                          ↑
                          1
                      α-D-Glcp
```

3- The repeating unit of the O-chain of *Eca* 2967 was identified as a pentasaccharide containing three galactose residues, one rhamnose and a new higher branched sugar (similar to caryophyllose).

4- The Japanese *Eca*-strain (2962) was very weakly virulent, as indicated by a weak soft rotting of potato slices and Chinese cabbage leaves and the inability to cause blackleg symptoms of potato plants, in contrast to the highly virulent *Eca*-strain 2967.

5- When inoculated into young leaves at high concentrations (10^8 to 10^9 cfu/ml), the Japanese *Eca* strain (2962) caused a little bit stronger necrotic leaf spots and chlorosis on tomato compared to those by the German *Eca* strain (2967), whereas similar symptoms were caused by both strains on potato leaves, and no symptoms were detectable on leaves of tobacco and Chinese cabbage.

6- The Japanese strain *Eca* (2962) produced lower **pectinolytic activity** in the supernatant of *in vitro* cultures than the German *Eca* strain (2967). However, when the pectinolytic activities were calculated per bacterial cell, the enzyme activities did not differ significantly between the two strains.

7- However, the German *Eca* strain (2967) showed a much **higher growth vigour** in liquid **pectin** medium as well as in **King's** medium B than the Japanese strain 2962.

8- Similarly, the German *Eca*-strain grew faster and produced deeper cavities on the CVPM medium than the Japanese strain.

9- The results obtained clearly showed that the Japanese strain had a slower growth vigour on any artificial medium, as well as a lower disease causing capability on different plants.

Whether this defect was caused by the confirmed loss of the O-specific LPS-chain is unknown but does not appear very probable.

CHAPTER IV

Detection of *Erwinia carotovora subsp. atroseptica* and *Erwinia carotovora subsp. carotovora* by Polymerase Chain Reaction (PCR)

1 INTRODUCTION

Under temperate conditions, soft rot of potato crops is primarily caused by two of the five subspecies recognized within *Erwinia carotovora*. While *E. carotovora ssp. carotovora* is widespread and has a wide host range besides potato, *E. carotovora ssp. atroseptica* is more specifically associated with potato and is prevalent under cold to temperate climates. Both subspecies are also responsible for severe losses in the field (HELIAS *et al.,* 1998). Often, the bacteria occur as latent contamination on potato tubers, and the actual occurrence of soft rot depends on environmental factors (PEROMBELON and KELMAN, 1980).

Different methods to assess microbial contamination of potato tubers have been the subject of various studies. **Classical methods** based on biochemical and biological tests of the isolated bacteria on selective media have proved to be useful tools for identification and enumeration of soft rot *Erwinia spp.* (STEWART, 1962; CUPPLES and KELMAN, 1974; BDLIYA, 1995) but are **laborious and time consuming** (SMID *et al*., 1995). Therefore, serological and more recently molecular methods, such as the polymerase chain reaction (PCR), have been developed to detect and characterize *Erwinia carotovora.* However**,** not all of these methods can be used routinely for analyses of potato tubers, or allow specific detection of each *Erwinia spp.* (HELIAS *et al*., 1998). **Serological** methods are one way of detecting erwinias on potato tubers (De BOER and McNAUGHTON, 1987), but the serological diversity of strains in some countries **do not** allow **reliable** detection by these methods (KANKILA, 1989).

Most recently, the polymerase chain reaction (**PCR**) was proposed for the detection of tuber contamination by *Erwinia spp.* (ANONYMOUS, 1992). In the PCR a target DNA fragment is amplified enzymatically. Thus, PCR combines a high degree of specificity with a high degree of sensitivity. PCR technology has been successfully developed as a tool for specific and sensitive detection of micro-organisms in clinical (HARTSKEER *et al*., 1989) and environmental samples (BEI *et al*., 1991; ATLAS *et al*., 1992).

When detecting plant pathogenic microorganisms in infested potato tubers by means of PCR, there is always the **problem** of high amounts of plant substances, such as starch and phenolic compounds, being coextracted, interferring with or even **inhibiting** the enzyme **Taq-polymerase** necessary for amplifying the target DNA in the PCR reaction (NIEPOLD, 1994;

VAN DER WOLF *et al*., 1996; HYMAN *et al*., 1997). Recently, several DNA primers shown to be specific for *Eca* or *Ecc* regardless of serogroup, have been generated and used in PCR-based assays (DE BOER and WARD 1995; FRECHON *et al*., 1995; SMID *et al*., 1995).

The purpose of **this study** was to test the feasiblity, reliability and sensitivity of the PCR for assessing the latent contamination of potato tubers by erwinias. For this aim **two primer sets** were compared to assess and evaluate the best and most efficient method for detecting *Erwinia spp.*.

2 MATERIALS AND METHODS

2.1 Solutions and buffers for agarose gel electrophoresis

10 x-Electrophoresis buffer (tris-boric acid-EDTA (TBE), 1000 ml) (SAMBROOK *et al.*, 1989)

Tris	0.89 M
Boric acid	0.89 M
Na_2EDTA X 2 H_2O	0.02 M

107.8 g Tris, 55.0 g boric acid and 7.44 g Na_2EDTA were dissolved in 1000 ml distilled water and stored at room temperature (stock solution). For electrophoresis, the solution was diluted 1:10 with distilled water.

5 x- Loading buffer (10 ml)

Glycerol	50%
TBE	5 x half strength of stock solution
Bromophenol blue (BPB)	0.3 %

30 mg BPB were dissolved in 4.25 ml of 10 x-TBE and 5.75 ml of 87% glycerol were added. The solution was stored at room temperature.

2.2 Amplification by PCR

The PCR procedure was carried out according to the methods described by NIEPOLD (1994) DE BOER and WARD (1995). **The bacterial strains** (see 2.4) were grown on King's medium B agar plates and incubated at 27 °C for 24 h. Bacterial suspensions were washed off the agar plates, adjusted to an OD_{660} of 0.06 (ca. 1.5 X 10^7 cfu/ml) and ten-fold dilutions were prepared in sterile distilled water. Detection of *Erwinia carotovora* in each dilution was carried out by serial dilution and plating on King's medium B as well as by PCR.

For **PCR**, the bacterial suspension was boiled at 100 °C for 5 min and stored at -20 °C until use. To reduce the risk of contamination, all materials used were sterilised. Sterile pipette tips and microvials from "biozym Diagnostic GmbH" (Hameln, Germany) were used throughout. The *Erwinia carotovor*a **primers** Y1/Y2 (NIEPOLD, 1994) and Eca1f/Eca2r (DE BOER and WARD, 1995) were generated by NIPAS, Göttingen.

PCR **amplification** of DNA was achieved by using two specific **primers** as follows: primer Y1 (5` GGACGCCGAGCTGTGGGGT3`) together with primer Y2 (5`CAGGAAGATGT CGTTATCGCGAGT3`) (NIEPOLD, 1994) and primers Eca1f (5`CGGCATCATA AAAACACG3`) together with Eca2r (5`GCACACTTCATCCAGCGA3`) (WARD & DE BOER, 1995). A negative control was enclosed by running the PCR reaction with sterile water and the positive control was used as bacterial DNA template. The enzyme *Taq* polymerase (5 U/µl) was added directly to the reaction mix. Each PCR reaction containing a final volume of 50 µl was prepared as described in Table 1 and covered with 50 µl sterile mineral oil (Sigma, Deisenheim).

The PCR was performed by heating in a thermal cycle (Hybaid-Omni Gene, Heidelberg). PCR ampilification by two primers was achieved by three stages. The first stage was the initial cycle of denaturation at 95 °C for 1 min. The second stage was performed by running fourty cycles of a denaturating temperature at 94 °C for 1 min, annealing temperature of 65 °C for 1 min and an extension phase of 72 °C for 45 sec. The same temperature was used for both primer pairs. The third stage, an 8 min extension period, was added after the final cycle.

2.3 Analysis of the PCR product using agarose gel electrophoresis

Gel electrophoretic analysis was performed by using 1.5% agarose NEEO Ultra-Quality gels (Roth, Karlsruhe) in 1 x TBE separation buffer, pH 8.0. The gel electrophoresis apparatus used was a horizontal gel electrophoresis GNA-100 and GNA-200 (Pharmacia, Freiburg). Agarose was weighed, suspended in buffer and melted in a microwave until a transparent solution was achieved. After cooling until about 45–50 °C, the agarose was poured into a plastic mold and allowed to solidify at room temperature. Air bubbles were removed with a pipette tip direct after pouring. After the gel was completely polymerized, it was tranferred to a gel tank containing the electrophoresis running buffer (1 x TBE, pH 8.0) and the same buffer was added just enough to cover the top of the gel to a depth of 1-2 mm (DARLING and BRICKELL, 1994).

The **samples** were prepared as follows: Two µl of the loading buffer was pipetted onto a para film strip equal to the number of the performed PCR reactions and 8 µl from each of the PCR reaction were pipetted out by mixing with the loading buffer on the parafilm strip. The whole volume was transferred into the slots of the agarose gel. The lid of the electrophoresis tank was closed and the electrical leads connected to the power pack (H. Hölzer, Dorfen). A voltage of 5 V/cm was set, and the sample migration from the anode to the cathode was visualised by the migration of the 5 x Loading Dye solution along the gel, that was allowed to run for 30-60 min

at room temperature. After DNA fragments were separated (in the electric field), the gel was removed carefully and put in 0.5 µg/ml ethidium bromide (Fluka, New-Ulm) solution for 10 min. The gel was removed again and submersed in distilled water for 5 min.

The gel was photographed with a Polariod MP4 camera provided with a red filter at 590 nm, using a black and white coaterless film polapan 667 (Polariod, St. Albans, Hertfordshirre, UK) under UV apparatus Intas 254 nm designed by Polariod (Intas, Göttingen). The gel was also visualised under UV transilluminator (Bio-Rad Gel Doc 1000, Munich), and saved as data by Multi Analyst program. The expected size of the PCR product was 430 bp for primers Y1 &Y2 and 690 bp for primers Eca1f and Eca2r, respectively.

Table 1: Reaction mix for PCR reagents used for DNA amplification

Reagents	Concentration per reaction
Dist. water (sterile) to final volume	50 µl
10 x PCR buffer	1% (v/v)
DNTP (Fermentas, St. Leon-Rot)	0.2 mM
Each primer solution (Y1 & Y2)	1 µM
Each primer solution (ECAIf & ECA2r)	1 µM
Tween 20	10% (v/v)
Taq-DNA polymerase	1.0 U
Bacterial template	3 µl

2.4 Determination of the detection limit of the PCR method

The bacterial strains were grown on King's medium B plates at 27 °C for 24 h. For this experiment the bacterial strains were adjusted to an OD_{660} of 0.06 (ca.10^8 cfu/ml) in sterile distilled water and then boiled at 100 °C for 5 min. From each dilution 3 µl were added to the PCR reaction mixture and analysed following the procedure described in 2.2.

2.5 Determination of *Eca* and *Ecc* with specific primers

Different **bacterial strains** of *Eca* and *Ecc*, five *Eca* (GSPB nos. 2962, 9201, 2967,1405 and 2231) and four *Ecc* (GSPB nos. 426, 429, 436 and 1405) were grown as described in 2.2. Each

bacterial strain was adjusted to an OD_{660} of 0.06 (ca.10^8 cfu/ml) in sterile distilled water and then boiled at 100 °C for 5 min and analysed following the procedure as described in 2.2.

2.6 Effect of potato peel extract on the detection limit of *Eca* by PCR

Four methods of DNA extraction from potato peel extract artificially contaminated by *Erwinia carotovora ssp. atroseptica* or *carotovora* were compared as follows:

2.6.1 DNA isolation by using the method of LI and DE BOER (1995)

Potato tubers were peeled (ca. 1 g peel) with a knife hand peeler and crushed in a sterile mortar with 4 ml TE buffer pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and 0.1 ml of bacterial suspension (ca. 1.8 X 10^8 cfu/ml) was inoculated into the homogenate and serially diluted. Aliquots (100 µl) from the plant homogenate were centrifuged at 8.000 g for 10 min at 4 °C in Eppendorf tubes. The PCR protocol steps for DNA purification were applied as follows:

A - The pellet was frozen at –20 °C for 1 h, thawed at room temperature for 10 min, mixed with 100 µl cold acetone (–20 °C) and incubated for 10 min, suspended in 500 µl of TE buffer, followed by addition of 50 µl of 250 mM EDTA, pH 8.0, 50 µl of 14% SDS, and 10 µl proteinase K (Merck, Darmstadt) and incubated for 1 h at 55 °C.

B – An equal volume of 7.5 M ammonium acetate was added to separate most the cell debris from the DNA by pelleting the precipitate in a centrifuge at 14.000 g for 20 min at 4 °C.

C – The clear supernatant (0.8 ml) was transferred to a new tube, and 0.8 volume isopropanol (– 20 °C) were added and incubated for 30 min at – 20 °C.

D - The DNA was pelleted by centrifugation at 14.000 g for 20 min at 4 °C. The pellet was washed with 70% ethanol and precipitated by a second centrifugation at 14.000 g for 20 min at 4 °C.

E – After decanting the supernatant, the DNA pellet was dried in the tube at room temperature for 0.5 h, and finally the DNA was resuspended in 50 µl TE buffer (pH 8.0) and incubated at 4 °C overnight. All samples were stored at –20 °C. For the PCR procedure 2 µl of each DNA sample were used.

2.6.2 DNA isolation by using the method of MÖLLER *et al*. (1992)

1- Peel 0.5 g potato tuber with a knife hand peeler and grind the peel with a sterile mortar in liquid nitrogen.

2- Mix the homogenate with 2 ml TES buffer (0.1 mM Tris pH 8.0, 10 mM EDTA, 2% SDS), and inoculate 0.1 ml from a bacterial suspension (ca. 10^8 cfu/ml) to the homogenate, which is then serially diluted.

3- 500 µl are taken from the homogenate and 5 µl proteinase K (Merck, Darmstadt) are added to each E-cup tube and incubated at 55- 60 °C for 1 h.

4- Add 140 µl 5 M NaCl and 65 µl 10% CTAB into each Eppendorf tube and incubate at 65 °C for 10 min.

5- Add 1 volume (700 µl) of chloroform/isoamylalcohol = 24:1 (v/v), mix gently, place on ice for 30 min, centrifuge at 12.000 g for 10 min at 4 °C.

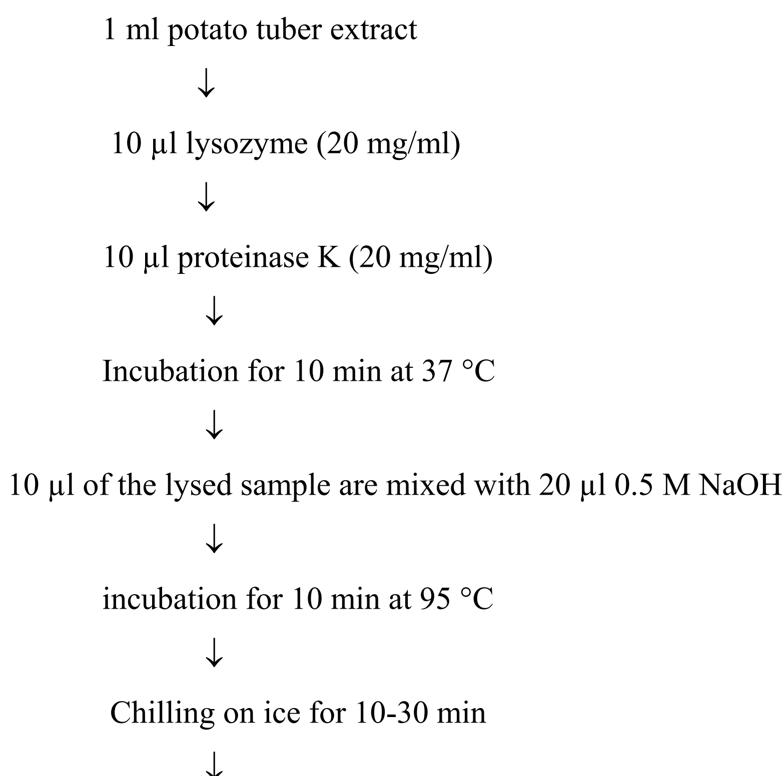
6- Transfer the supernatant to a fresh tube, add 225 µl 5 M NH_4 acetate, mix gently, place on ice for 30 min, centrifuge at 12.000 g for 5 min at 4 °C.

7- Transfer the supernatant to a fresh tube, add 0.55 vol (510 µl) to precipitate DNA, centrifuge immediately for 5 min at 12.000 g for 5 min at 4 °C.

8- Decant the supernatant and wash the pellet twice with cold 70% ethanol, dry the pellet at room temperature and dissolve the pellet in 50 µl TE buffer pH 8.0.

2.6.3 DNA isolation by using the NaOH method (NIEPOLD, 1999a)

Potato tubers were peeled with a knife hand peeler and crushed in a sterile mortar with 4 ml sterile water, and 0.1 ml from a bacterial suspension (ca. 10^8 cfu/ml) were inoculated into the homogenate and serially diluted. DNA was extracted a shown on the following flow chart:



Transfer of 5 µl of the lysate into a new tube

Add 20-45 µl Tris/HCl/BSA (3%) buffer (pH 7.5) to the 5 µl lysate sample for extraction of a low starch potato variety, mix and incubate at room temperature for 5 min (1:5-10 dilution), 45 µl for extraction of a high starch potato variety, mix and incubate at room temperature for 5 min (1:5-10 dilution).

5 µl are used for performing the PCR

2.6.4 DNA isolation by using the method of the German Plant Protection Service, Hannover (PASTRIK and RAINEY, 1999)

1- Potato tubers are peeled with a knife hand peeler and crushed in a sterile mortar with 4 ml sterile water, and 0.1 ml of bacterial suspension (ca. 10^8 cfu/ml) is inoculated into the homogenate and serially diluted. The plant homogenate aliquots (100 µl) are centrifuged at 8.000 g for 10 min at 4 °C in Eppendorf tubes.

2- After decanting, the pellet is resuspended in 220 µl Lysis-buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0) in a 1.5 ml Eppendorf tube.

3- Tubes are kept for 10 min at 95 °C in a heating block and the samples put on ice for 5 min.

4- Add 80 µl of lysozyme stock solution (50 mg/ml lysozyme in 10 mM Tris-HCL, pH 8.0) and incubate at 37 °C for 30 min.

5- 220 µl of solution A (Invitogen BV, Netherland) are added, mixed well and incubated at 65 °C for 30 min.

6- 100 µl of solution B (Invitogen BV, Netherland) are added and mixed by vortexing until the precipitate moves freely in the tube and the sample is uniformly viscous.

7- 500 µl chloroform are added and vortexed until viscosity decreased and the mix is homogeneous.

8- The samples are centrifugated at 15.000 g for 20 min at 4 °C to separate the phases forming an interphase, and the upper phase is transferred into a fresh microcentrifuge tube.

9- 1 ml ethanol 100% (-20 °C) is added, vortexed briefly and incubated on ice for 10 min.

10- The pellet is obtained by centrifugation at 15.000 g for 20 min at 4 °C.

11- The pellet is washed with 500 µl ethanol 80% (-20 °C), mixed by inverting the tube, and again centrifuged at 15.000 g for 20 min at 4 °C.

12- The pellet is dried by decanting the supernatant and remaining the tubes openly at room temperature for 0.5 h. Finally the pellet is resuspended in 100 µl sterile H_2O, and 2 µl of the DNA extract are used in the PCR.

3 RESULTS

3.1 Detection and identification of *Erwinia spp.* using the polymerase reaction

The first step in this study was to evaluate the specificity of the two *Erwinia spp.* primer pairs Y1&Y2 and Eca1f & Eca2r. For comparison 9 bacterial strains, 5 from *Eca* and and 4 from *Ecc*, were tested. For extracting the DNA, the bacteria were boiled in water (see 2.3). The results obtained show that all bacterial strains from *Eca* and *Ecc* yielded a distinct band of 434 bp in size with primers Y1 and Y2 **(Fig. 1 a)** while only *Eca* strains showed a signal product of 690 bp using the primers Eca1f & Eca2r **(Fig.1 b)**. It can be concluded from these results that the specificity of primers Y1 and Y2 was broader because they could detect *Eca* and *Ecc* equally well, whereas primers Eca1f & Eca2r detected only *Eca.*

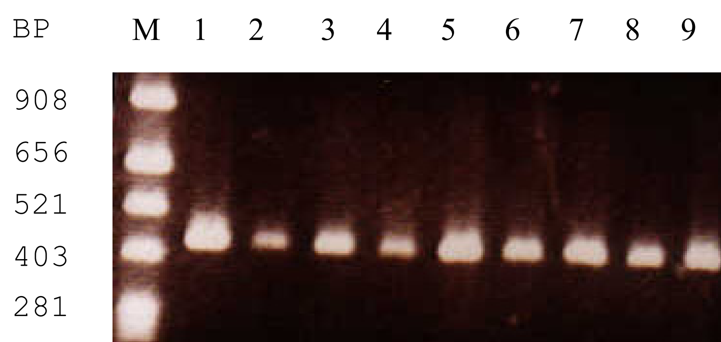


Fig. 1a: Reaction of *Eca* and *Ecc* strains using primers Y1 & Y2. The PCR product after 40 cycles was separated by agarose gel electrophoresis. Lanes: (M) marker pBR/ *AIu* 20, (1) *Eca* GSPB 2231, (2) *Eca* GSPB 1401, (3) *Eca* GSPB 2967, (4) *Eca* GSPB 2962, (5) *Eca* GSPB 9201, lanes: (6) *Ecc* GSPB 436, (7) *Ecc* GSPB 426, (8) *Ecc* GSPB 1405, and (9) *Ecc* GSPB 429.

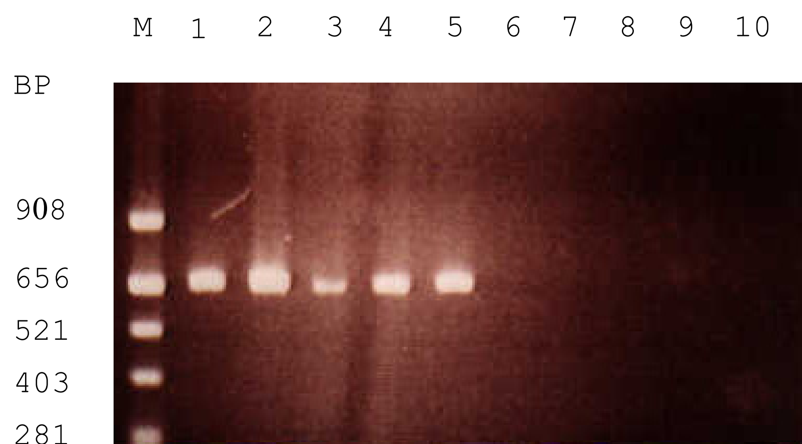


Fig. 1b: Reaction of *Eca* and *Ecc* strains using primers Eca1f & Eca2r. The PCR product after 40 cycles was separated by agarose gel electrophoresis. Lanes: (M) marker pBR/ *AIu* 20, (1) *Eca* GSPB 2231, (2) *Eca* GSPB 1401, (3) *Eca* GSPB 2967, (4) *Eca* GSPB 2962, (5) *Eca* GSPB 9201, lanes: (6) *Ecc* GSPB 436, (7) *Ecc* GSPB 426, (8) *Ecc* GSPB 1405, *Ecc* GSPB 427 and (9) *Ecc* GSPB 429.

3.2 The detection limit of the PCR method using primers Y1 and Y2

For determining the detection limit of *Erwinia carotovora ssp. atroseptica* from bacterial cultures, it was not necessary to extract DNA from the bacteria. The method was performed by boiling the bacterial samples (100 µl) from each dilution to 100 °C for 5 min. The sensitivity of PCR technique was determined by assaying 10-fold serial dilutions of a bacterial suspension from *Eca* (GSPB no. 2967) as well as by determining the bacterial concentrations by plating serial dilutions on King's medium B. The results in **Fig. 2** clearly show that according to the microbiological determination (plating) of bacterial concentration the detection limit of the PCR method using the *Eca* primers Y1 and Y2 was 1.5 X 10^3 cfu/ml.

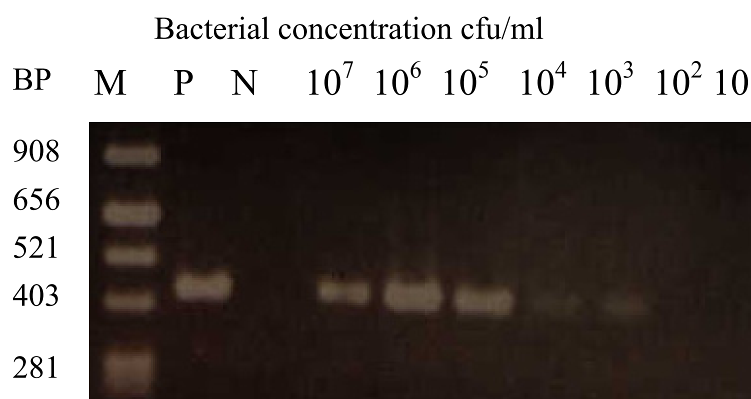


Fig. 2: Determination of the detection limit of the PCR using primers Y1 and Y2. The test was performed with a pure culture of *Eca* GSPB 2967. Ten–fold dilutions were prepared in sterile distilled water and boiled at 100 °C for 5 min. The PCR product after 40 cycles was separated on agarose by electrophoresis. M = Marker, P = positive control, N = negative control.

3.3 The detection limit of the PCR method using primers Eca1f and Eca2r

In a standard PCR protocol performed with primers Eca1f and Eca2r and using a pure culture of *Eca* as DNA template a 690 bp product was obtained **(Fig. 3)**. The results show that the detection limit of the PCR using the primers Eca1f and Eca2r was 1.5 X 10^3 cfu/ml.

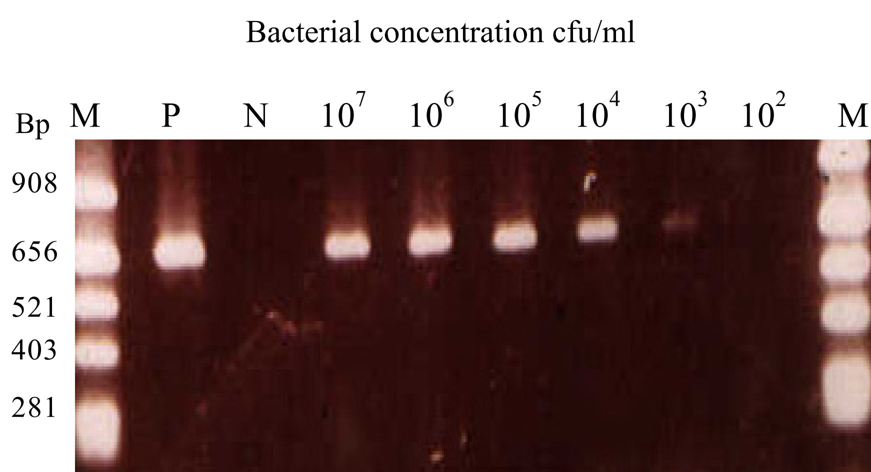


Fig. 3: Determination of the detection limit of the PCR using primers Eca1f and Eca2r. The test was performed with a pure culture of *Eca* GSPB 2967. Ten–fold dilutions were prepared in sterile distilled water and boiled at 100 °C for 5 min. The PCR product after 40 cycles was separated on agarose by electrophoresis. M = Marker, P = positive control, N = negative control.

3.4 Detection of *Eca* in artificially contaminated potato peel extract by PCR with primers Y1 and Y2

Isolation of DNA from artificially contaminated potato peel extract was achieved by different protocols (see subchapter 2.5) for detecting soft rot bacteria from peel extracts. PCR amplification with primers Y1 and Y2 yielded a product of 434 bp only by the method of LI an DE BOER (1995) which, thus, allowed to detect *Erwinia carotovora* in the peel extract. By the other protocols a DNA amplification was not successful. The results as shown in **Fig. 4** revealed bands only after the third sample dilution, whereas from peel extracts containing high bacterial concentrations a positive signal was not detectable. This discrepancy can be attributed to substances from the potato peel extract that inhibited the PCR reaction. The detection limit was **10^2 cfu/ml**.

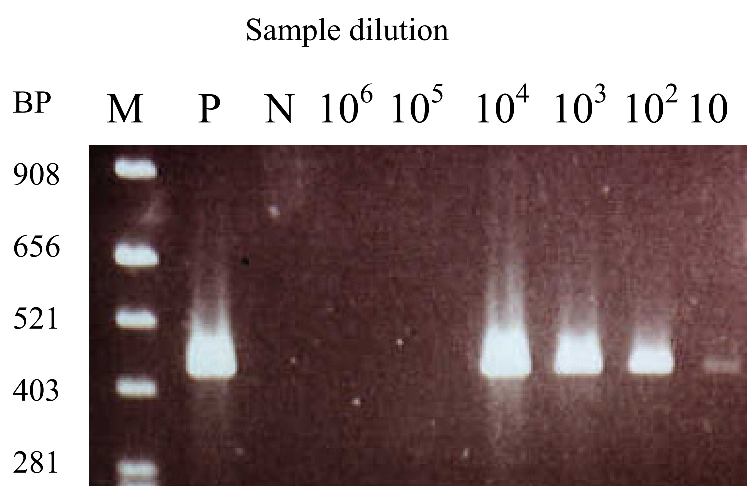


Fig. 4: Determination of *Eca* in artificially inoculated potato peel extract by PCR using *Eca* primers Y1 and Y2. The test was carried out with ten-fold dilutions of the inoculated tuber peels. The PCR product amplified after 40 cycles was separated on agarose gel by electrophoresis. M = Marker, P = positive control, N = negative control.

3. 5 Detection of *Eca* in artificially contaminated potato peel extract by PCR with primers Eca1f and Eca2r

Fig. 5 shows a distinct signal after the second sample dilution, representation a sensitivity of the PCR of **10^2 cells/ml**.

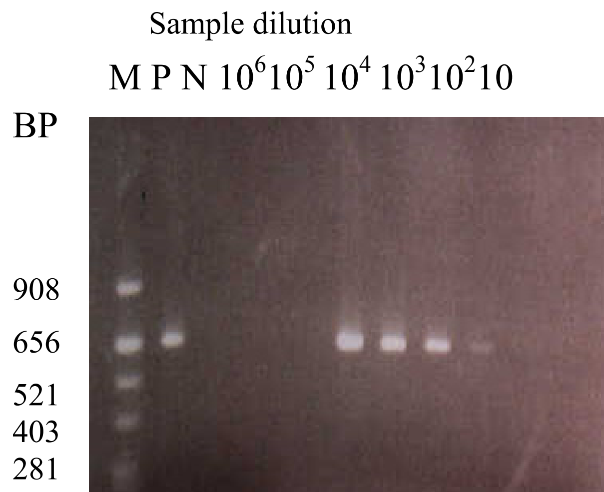


Fig. 5: Determination of *Eca* in artificially inoculated potato peel extracts by PCR with the *Eca* primers Eca1f and Eca2r. The test was carried out with ten-fold dilutions of the inoculated potato peel extracts. The PCR product amplified after 40 cycles was separated on agarose gel by electrophoresis. M = Marker, P = positive control, N = negative control.

4 DISCUSSION

The polymerase reaction (PCR) which allows a target DNA fragment to be amplified enzymatically has been applied successfully for detecting **latent contamination** by *Erwinia spp.*. The method is based on the use of *Eca*-specific DNA primers to amplify a targeted *Eca* DNA fragment to faciliate detection of both living and dead cells (HENSEN and FRENCH, 1993). Classical techniques of bacterial isolation and characterization are time consuming and relatively insensitive because of the high level of saprophytes occurring in potato samples (LELLIOTT and STEAD, 1987). Therefore, several alternative methods have been developed for detecting *Erwinia carotovora* in potato tubers. These involve semi-selective media (STEWART, 1962; CUPPLES and KELMAN, 1974; BYDLIA, 1995 and AHMED *et al.,* 2000), serological methods, such as DAS-ELISA (PEROMBELON and HYMAN, 1995; FRAAIJE *et al.,* 1997) and IFC (VAN VUURDE, 1990; PEROMBELON *et al.,* 1998), and the detection of known DNA sequences following amplification by the PCR using pathogen specific oligonucleotide primers (NIEPOLD, 1994; DE BOER and WARD, 1995; SMID *et al.*, 1995; HYMAN *et al.,* 1997; HELIAS *et al*., 1998).

In this study two sets of primers and different **extraction procedures** were compared to improve the practice of seed certification by a simple, fast, sensitive and specific method for determining the level of *E. carotovora*-contamination of seed tubers in order to overcome the problem of inhibition of the polymerase chain reaction by potato tuber derived compounds.

4.1 Specificity of the two *Ec* primer pairs

The specificity and reliability of the *Eca* primers to amplify a fragment of *Erwinia carotovora* were verified by testing different bacterial strains. In the first screening step, two primer sets were tested for their ability to differentiate the two *Erwinia* subspecies. For comparison 5 *Eca* and 4 *Ecc* strains were tested, which can infect potatoes. The results showed that the primers Y1 and Y2 amplified DNA from all *Eca* and *Ecc* strains tested and confirmed previous reports of HELIAS *et al*. (1998) who found that PCR performed on 140 *Erwinia* strains yielded an amplification product of 434 bp in 109 strains from *Eca* and *Ecc* only, whereas none of the surveyed *E. carotovora subsp. betavasculorum* strains showed an amplificate. Also, BDYLIA (1995) obtained similar results when different strains of *Eca, Ecc* and *Ech* were tested by these primers. In his studies, only *Eca* and *Ecc* gave a product of 434 bp, while *Ech* strains did not show amplificates.

In contrast, the primers ECAIf/ECA2r were suited to specifically detect the blackleg pathogen *Eca*. We confirmed by our experiments that the 690 bp fragment from *Eca* strains was not amplified when using DNA from *Ecc* strains. The same results were obtained by DE BOER and WARD (1995) who amplified a 690 bp fragment from *Eca* strains but not from strains of other *Erwinia carotovora* subspecies isolated from various hosts.

4.2 Determination of the detection limit in pure bacterial cultures of *Erwinia carotovora*

Our results obtained in this study revealed that **it is not necessary** to extract and purify bacterial DNA from pure cultures. The specific DNA amplificates were detected from all *Eca* and *Ecc* strains tested. The PCR performed on boiled bacterial suspensions yielded a 434 bp fragment by the *Eca* and *Ecc*-specific primers Y1 and Y2, whereas a 690 bp fragment was obtained by the ECA1f/ ECA2r primers, specific for *Eca.*

The detection limit determined in these studies was similar for the two primers **(10^3 cfu/ml)**, as confirmed by plating aliquots of serial dilutions onto King's medium B. Boiling of bacterial suspensions proved to be a useful step prior to PCR assays because of its easiness. Previous publications (BDLIYA, 1995; HYMAN *et al.,* 1997) reported detection limits of bacterial suspensions after extracting the DNA, which were similar to our results with *Erwinia spp.*. Thus, BDLIYA (1995) determined a detection limit of 10^4–10^5 cfu/ml which was still within the range **reported earlier** (ANONYMOUS, 1992; LÜDTKE, 1994). Our results confirmed those obtained by MARTINS (2000) who reported a detection limit of 4 X 10^2 cfu/ml from bacterial suspensions of *Ralstonia solanacearum,* while NIEPOLD (1994) could detect only 10^4 cfu/ml of *Eca.*

It is concluded that **boiling** of bacteria pure cultures is a **simple** and cheap method requiring very little equipment. Only 5 min are needed to boil the bacterial suspensions in contrast to 4 h or more required for most of the DNA extraction procedures.

4.3 Comparison of four DNA extraction methods

This study describes a PCR assay for specifically detecting *Eca* in potato tubers. As peel extract contains **PCR inhibitory substances** (VAN DER WOLF *et al.,* 1996), it was necessary to extract DNA beforehand, taking the opportunity to concentrate it and, thus, to increase sensitivity of the assay. In the past few years, PCR assays based on three different sets of DNA primers have been developed for detecting contamination by *Erwinia carotovora.*

The method of LI and DE BOER (1995) involving maceration in Tris-EDTA buffer, washing with cold acetone, purification with proteinase and ammonium aceteate precipitation of cell debris, and DNA precipitation with isopropanol allowed to obtain high yielding bacterial DNA from potato peel extracts. The procedure used by LI and DE BOER to prepare DNA for PCR amplification, avoids the use of organic solvents for separating DNA from cell debris. This method was relatively easy to use on a large number of samples. Successful amplification of DNA extracted from peel extracts indicated a wide applicability of the PCR test. This DNA purification method proved to be advantageous since the extract did not contain plant derived compounds which inhibited amplification of DNA in peel extract in comparison to other protocols described in 2.3 which were not successful in this study. The centrifugation of the macerated peel extract showed to be a useful step in the DNA purification procedure.

In the here reported PCR experiments, inhibitory substances occurred in undiluted samples and 1:10 diluted homogenates of potato peel extract (Fig. 4 and Fig. 5). The inhibiting effect of potato tissue, probably by phenolic compounds, appeared to be strong. Thus, ELPHINSTONE *et al.* (1996) reported that the PCR product could not be amplified when macerates of potato cv. Desire´ were used. FRECHON *et al.* (1998) reported that evaluation in five different laboratories using similar DNA extraction procedures confirmed the sensitivity of this method, which ranged from 10 to 10^3 cfu/ml of *Eca* depending on the *Eca* strain, potato cultivars/stocks and the speed and ease of procedure. The sensitivity of PCR when detecting bacteria directly in crude peel extracts, was ca. 10^6 cfu/ml (VAN DER WOLF *et al.,* 1996). This low sensitivity was attributed to the presence of inhibitory compounds in peel extracts.

4.4 Determination of the detection limit of *Ec* in potato peel extracts

The detection limit by PCR obtained in this study, when determined by plating aliquots of serial dilutions on the semi-selective medium, was **10^2 cfu/ml peel extract**. PCR detection avoids the difficulty of different serogroups within *Ec* by using *Eca*-specific primers (DE BOER and WARD, 1995; FRECHON *et al.,* 1998; SMID *et al.,* 1995) detecting all serogroups of *Ec.* However, the sensitivity in plant material has been limited, since concentrations of 1 X 10^5 – 1 X 10^8 cells/ml peel extract were needed to obtain positive PCR signals (FRAIIJE *et al.,* 1996; VAN DER WOLF *et al.,* 1996; HELIAS *et al.,* 1998). These detection thresholds can be greatly improved by either enrichment or immunomagnetic separation of the bacteria before PCR detection, which allows a detection of 10^4 cells /ml peel extract (FRAIIJE *et al.,* 1996) down to 2 X 10^3 cells/ml peel extract (VAN DER WOLF *et al.,* 1996). The lower sensitivity of PCR for peel extract was due to the presence of inhibitory compounds in the potato peel extract prepared. Since we applied the DNA extraction method of LI and DE BOER (1995) excluding

the inhibitory compounds, the sensitivity could be improved to detect **10^2 cfu/ml** peel extract. By comparing the detection limit from contaminated potato peel extract to that from bacterial pure cultures it appeared that the detection limit from peel extract was a little bit lower (10^2 cfu/ml) than that from the pure culture (1.5 X 10^3 cfu/ml). This difference may be due to the extraction methods applied. The bacterial pure culture were only boiled for 5 min, whereas the potato peel homogenate was extracted by acetone and EDTA (see IV, 2.5.1). By the latter method the DNA is concentrated which increases the sensitivity of the test.

4.5 Comparison of microbiological and molecular genetic methods for detection of *Erwinia carotovora* on or in potatoes

The PCR assay is the most specific method for detecting of *E. carotovora.* It also has a satisfactory sensitivity level and can be completed within one day. By comparing the PCR method with the semi-selective medium used in this study it can be concluded that the detection limit obtained was a slightly lower by the microbiological method (2 X 10^1 cfu/ml) (Chapter I, see 3.3) than by the PCR (10^2 cfu/ml).

However, the PCR method requires more specialized facilities than the microbiological method, especially if carry-over of contaminations from previous amplification is to be avoided. These include separate containment facilities such as biosafety cabinets, dedicated laboratory equipment, use of ultra-pure water, pipette tips with filters. Furthermore, costs tend to be high, since not only expensive equipment and reagents are needed but this method requires more labor time than the microbiological method. In addition, the latter method allows to quantify the degree of contamination. The disadvantage of the semi-selective medium is the difficulty to differentiate between *Eca* and *Ecc*.

In conclusion, the microbiological method proved to be very sensitive, and thus should be recommended as a standard procedure for detection of *Erwinia carotovora* strains. In contrast, the PCR technique can be a useful additional method and is very valuable for differentiating *Eca* from *Ecc* isolates.

5 SUMMARY

1 – The aim of this study was to compare two specific *E. carotovora* primer sets to determine the method best suited for detecting latent contamination of potato tubers.

2 – The specificity of primers Y1 and Y2 was broader by detecting *Eca* and *Ecc*, whereas primers Eca1f and Eca2r detected only *Eca*.

3 – The results revealed that the detection limit of pure cultures of *Eca* was **1.5 X10^3** cfu/ml for both primer pairs Y1 & Y2 and Eca1f & Eca2r when the bacterial suspensions were boiled for 5 min without any DNA-extraction.

4 – The detection limit from potato peel extract contaminated by *Eca* was **10^2** cfu/ml by both primer sets (Y1/ Y2 and Eca1f /Eca2r) when the DNA-extraction method of LI and DE BOER was applied.

5- Considering the costs and requirements for a PCR detection the use of the semi-selective agar medium appears to be more advantageous for detecting and quantifying latent contamination of potato tubers by *Eca* and *Ecc*.

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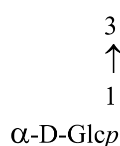
SUMMARY

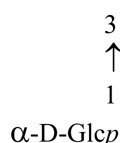
For a **sensitive detection** of latent contamination of potato tubers by soft rot causing erwinias *[Erwinia carotovora subsp. atroseptica* (*Eca*), and *Erwinia carotovora subsp. carotovora* (*Ecc*)*]*, the semi-selective double layer medium of Bdliya (1995) was improved resulting in a **detection limit** as low as **2 X 10^1 cfu/ml**. **Field experiments** proved that seed potato lots assessed as moderately or strongly contaminated by *Ecc* gave lower yields and less quality than potato lots assessed as weakly contaminated. By applying **PCR** and the primer setsY1/ Y2 or Eca1f /Eca2r the detection limit of *Eca* in potato peel extract was **10^2** cfu/ml. Field experiments with seed potatoes treated with **Degaclean** revealed a reduced germination rate of the treated potatoes and increased percentage of tubers with soft rot, so that **yields** after treatment of seed potatoes were **reduced**. The repeating unit of the LPS-O-chain of *Ecc* 436 was elucidated as a tetrasaccharide of the following structure:



The repeating unit of the O-chain of *Eca* 2967 was identified as a pentasaccharide containing three galactose molecules, one rhamnose and a new higher branched sugar (similar to caryophyllose). The *Eca*-strain 2962 with a rough LPS was very weakly virulent.

ZUSAMMENFASSUNG

Für einen **empfindlichen Nachweis** der latenten Kontamination von Kartoffelknollen mit Weichfäule erregenden Erwinien *[Erwinia carotovora subsp. atroseptica* (*Eca*) und *Erwinia carotovora subsp. carotovora* (*Ecc*)*]* wurde das semi-selektive Zweischichten-Medium von Bdliya (1995) so verbessert, dass die Nachweisgrenze nur **2 X 10^1 cfu/ml** betrug. **Feldversuche** ergaben, dass Saatkartoffel-Partien, die als mittel oder stark mit *Ecc* kontaminiert eingestuft worden waren, nach der Ernte niedrigere Erträge von schlechterer Qualität ergaben als schwach kontaminierte Partien. Bei Anwendung der **PCR** mit den Primer-Paaren Y1/Y2 oder Eca1f /Eca2r betrug die Nachweisgrenze von *Eca* in Kartoffelschalen-Extrakten **10^2 cfu/ml**. Feldversuche mit Saatkartoffeln, die mit **Degaclean** behandelt worden waren, ergaben eine verminderte Keimungsrate der behandelten Kartoffeln und einen erhöhten Anteil von Kartoffeln mit Weichfäule, so dass die **Ernteerträge** deutlich **reduziert** waren. Die Wiederholungseinheit der **LPS-O-Kette** von ***Ecc*** 436 wurde als ein **Tetrasaccharid** mit folgender Strukturformel aufgeklärt:



Die Wiederholungseinheit der LPS-O-Kette von ***Eca*** 2967 wurde als ein **Pentasaccharid** identifiziert, das drei Galaktose-Moleküle, ein Rhamnose-Molekül und einen neuen stärker verzweigten Zucker (ähnlich der Caryophyllose) enthielt. Der *Eca*-Stamm 2962 mit **rauher** LPS war nur **sehr schwach virulent**.