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Immuno-assessment of *Pseudomonas syringae* lipodepsipeptides (syringomycins and syringopeptins)

Monica Gallo[1], Vincenzo Fogliano[1], Alberto Ritieni[1], Lucia Peluso[1], Maria Luigia Greco[2], Rosa Lops[2] and Antonio Graniti[2]

[1]Dipartimento di Scienza degli alimenti, Università di Napoli "Federico II", Parco Gussone, 80055 Portici, Italy
[2]Dipartimento di Biologia e Patologia vegetale, Università, Via G. Amendola 165/A, 70126 Bari, Italy

Summary. Following a previous work on the immunological detection of syringopeptins (SPs), polyclonal antibodies with a high specificity for syringomycins (SRs) were raised in rabbits and purified. Assayed in a competitive ELISA, the most common forms of SR, i.e. SR-E and SR-G, were recognised with a detection limit of 0.1 mg per well, whereas other structurally related bacterial lipodepsipeptides (LDP), such as SPs, pseudomycins (PSs) and syringotoxins (STs) were not recognised. The immuno-assay (competitive ELISA) method developed in this work is about 100 times more sensitive than the current chromatographic (HPLC) method and requires no previous extraction of the toxin. The production of LDP in culture by strains of three pathovars of *Pseudomonas syringae* (pv. *aptata*, pv. *lachrymans* and pv. *syringae*) was found to range from 0.026 to 0.055 mg ml^{-1} for SRs and from 0.02 to 0.06 mg ml^{-1} for SPs. Both the concentration of LDP in aqueous extracts from zucchini cotyledons infected by *P. syringae* pv. *lachrymans* and the severity of symptoms were shown to increase progressively after infection. The immunologically estimated concentration of SRs in the infected cotyledons averaged 0.22 mg g^{-1} f wt after 12 hours, and 0.39 mg g^{-1} after 4 days. The corresponding values for SPs were 0.11 and 0.37 mg g^{-1}. In a recovery experiment, solutions of pure toxins (0.22 mg SR-E and 0.14 mg SP_{25A} g^{-1} f wt) were injected in healthy cotyledons. After 2 days, overestimation due to toxin complexing *in planta* was of 10% for (SR-E) and 40% for (SP_{25A}). Applying these percentages to the values estimated for infected cotyledons, the net concentrations were as follows: 12 h after inoculation: 0.20 (SRs) and 0.07 (SPs) mg g^{-1} f wt; four days after inoculation: 0.35 (SRs) and 0.22 (SPs) mg g^{-1} f wt. The values obtained with aqueous extracts from infected plants are relatively high if compared to the figures of the *in vitro* experiments. It is assumed that the high reactivity of ELISA to the immune-LDP-related compounds present in the water extracts from infected plants is due to the presence of high molecular weight LDP complexes having a cross-reactivity with antibodies substantially higher than that of free toxins.

Key words: *Pseudomonas syringae* pv. *lachrymans*, lipodepsipeptides, syringomycins, syringopeptins, zucchini.

Abbreviations used in the text: CFA, complete Freund adjuvant; DABA, 3,3'-diazobenzidine tetrachloride; KLH, keyhole limpet hemocyanin; LDP, lipodepsipeptides; PAGE, polyacrylamide gel electrophoresis; PS-A, pseudomycin A; SPs, syringopeptins; SP_{22A}, SP_{22B}, syringopeptins 22-A and 22-B; SP_{25A}, SP_{25B}, syringopeptins 25-A, 25-B; SRs, syringomycins; SR-E, syringomycin E; SR-G, syringomycin G; ST, syringotoxin.

Introduction

Many pathovars and strains of *Pseudomonas syringae*, as well as some other species of *Pseudomonas*, are known to produce *in vitro* biologically active (e.g. phytotoxic and antibiotic) lipodepsipeptides (LDP). According to the size of the cyclic peptide moiety, these metabolites may be grouped in classes, such as: (i) lipodepsinonapeptides, e.g. syringomicins (SRs), syringostatins (SSs), syringotoxins (STs) and pseudomycins (PSs); and (ii) the larger, more hydrophobic and phyto-

Corresponding author: A. Graniti
Fax: +39 080 5442906
E-mail: granitia@agr.uniba.it

toxic LPDS, e.g. syringopeptins (SPs), whose peptide moiety includes 22 (SP_{22A} and SP_{22B}) or 25 (SP_{25A} and SP_{25B}) amino acids (Ballio *et al*., 1991, 1994; Sorensen *et al*., 1996; Dalla Serra *et al*., 1999). These toxic metabolites are believed to be major virulence factors for plant pathogenic pseudomonads (Gross *et al*., 1997).

Extraction of bacterial LDP with organic solvents and subsequent HPLC purification give reliable results with culture filtrate, but they hardly work with infected plant tissue.

In a previous paper (Fogliano *et al*., 1999), polyclonal antibodies with a high specificity for SPs served to develop an immunological method (ELISA) for detecting these toxins in the aqueous extract from zucchini cotyledons infected with *Pseudomonas syringae* pv. *lachrymans*. Concentrations of approximately 0.1 mg SPs per g^{-1} f wt of infected plant tissue were assessed. With the present investigation a similar method was developed for the LDP of smaller molecular weight (lipodepsinonapeptides), exemplified by the syringomycins. Both immuno-assays were then used to check the production in culture of LDP by several strains of three pathovars of *P. syringae* (pv. *aptata,* pv. *syringae* and pv. *lachrymans*), as well as to assess the four toxins known to be produced by pv. *lachrymans* (SR-E, SR-G, SP_{25A} and SP_{25B}: Greco *et al*., 1998) both *in vitro* and *in planta*.

Apart from the relatively inexpensive and expeditious quantification of SPs and SRs in culture filtrates of bacterial strains they make possible, immuno-assays can be used for studies on the biosynthesis, biological activity and role in pathogenesis of these toxins.

Materials and methods

Bacterial strains and culture

Strain S91 of *P. syringae* pv. *lachrymans,* isolated from zucchini plants affected with "angular spot" in central Italy (Scortichini and Tropiano, 1991; Fogliano *et al*., 1999), was used both for routine production of LDP and for plant inoculation experiments. Time-course production in culture of SRs and SPs by this strain was monitored by growing the bacterium in 1-litre Roux flasks containing 200 ml IMM medium (Surico *et al*., 1988) at 25°C for 4 days and collecting samples every 12 or 24 hours.

In addition, the following pathovars and strains of *P. syringae* were used in the immunological assessment of LPD production: *P. syringae* pv. *aptata* strains NCPPB 871, 872 (from sugarbeet, USA) and 2664 (from sugarbeet, Italy); *P. syringae* pv. *syringae* strains B359 (from millet, USA), B382-4 (from maize, USA) and B426 (from walnut, USA); Y 27 and Y 37 (from bean, USA). All these strains were grown as above for 5 days.

Toxin preparation

Pure samples of SR-E, SR-G, SP_{25A} and SP_{25B} were obtained by HPLC fractionation of toxic preparations from culture filtrates of *P. syringae* pv. *lachrymans* (Ballio *et al*., 1991; Iacobellis *et al*., 1992; Fogliano *et al*., 1999). Reference samples of SP_{22A}, SP_{22B}, pseudomycin A (PS-A) and syringotoxin (ST) were kindly supplied by Professor A. Ballio, University of Rome, Italy.

Preparation of plant material

Batches of five hundred zucchini seeds (*Cucurbita pepo* cv. Striata d'Italia) were grown in the dark in a growth chamber at 25°C, RH near saturation, and inoculated at the cotyledon stage as described by Fogliano *et al*. (1999).

After inoculation, samples of infected cotyledons and the corresponding non-inoculated controls were collected every 12 or 24 hours for 4 days. Each sample of about 9 g f wt was mixed (1:1 w:v) with cold (4°C) water brought to pH 3 with 0.1M HCl, and ground in a Waring Blendor. After 1 h at 4°C, the homogenised plant tissue was filtered through cheesecloth and glass wool, and centrifuged at 7000 *g* for 15 min. The clear aqueous extract was frozen at -20°C and lyophilised.

Symptoms shown by the cotyledons were scored on a 0–4 scale, where 0 = no symptoms; 1 = small (2–4 mm diam.) water-soaked areas around the inoculation site; 2 = larger (up to 1 cm) water-soaked areas with tissue starting to collapse; 3 = necrotic areas surrounded by a halo of collapsed tissue, covering about half-lamina; 4 = inconsistent, necrotic tissues extended to cover from more than one half to the entire lamina of the cotyledon.

In the toxin recovery experiment, a total of 500 ml of a solution of either 2 mg SR-E ml^{-1} or 1.2 mg SP_{25A} ml^{-1} were injected into healthy cotyledons, corresponding to an average dose of 0.2 (SR-E) or 0.1 (SP_{25A}) mg g^{-1} f wt. Controls received an equivalent injection of sterile distilled water. After two

days, the cotyledons treated with toxins showed severe symptoms. They were weighed (5 g f wt treated with SR-E, and 5.5 g f wt treated with SP_{25A}), processed as above and assayed (ELISA) for toxin content.

Antigen preparation and antibody production

Samples of HPLC-purified SR-E were conjugated with a carrier protein, either bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) (Harlow and Lane, 1988), using diazobenzidine tetrachloride (DAB) as a cross linking agent, and either 3 mg BSA or 6 mg KLH as carrier proteins (Fogliano *et al.*, 1999). Coupling between SR-E and the carrier proteins was checked by SDS-PAGE (Laemmly, 1970).

Two 5-month-old New Zealand rabbits were immunised by injecting them with 0.5 mg of KLH–SR-E emulsified with CFA (1:4 v:v). The rabbits received a booster injection 4 weeks after the first injection. Blood was collected 20 days after the last injection by ear puncture. The serum was separated and stored at -20°C until use.

Immunological assay

Titre and characterisation of the antiserum were performed by indirect and competitive ELISA, following standard procedures (Harlow and Lane, 1988).

In the indirect assay, 0.1 ml samples of pure bacterial LDP (SR-E, SR-G, ST, PS-A and SP_{25A}), each tested at concentrations from 0.01 to 1 mg ml^{-1}, were added to 96-well plates and incubated at 4°C overnight. The available sites were blocked with horse serum (10% in PBS). After saturation, 0.1 ml aliquots of rabbit antiserum, diluted in PBS at rates ranging from 1:100 to 1:2000, were added at 37°C for 1 h. Peroxidase-conjugated antirabbit IgG secondary antibodies (Bio-Rad, Hercules, CA, USA) diluted in PBS 1:4000 (v:v) were used to develop suitable absorbance. Each LDP was tested in triplicate, and three independent sets of determinations were performed.

In competitive ELISA, wells were coated with 0.1 ml of a 0.1 mg ml^{-1} solution of BSA-SR-E. The plate was kept at 4°C overnight. After treatment with horse serum, the primary antibodies diluted 1:200 in PBS were added together with amounts of SR-E ranging from 0.01 to 1 mg per well, maintaining a total volume of 0.1 ml. The secondary antibodies were used at a dilution of 1:4000 in PBS. When detection of LDP was attempted in plant extracts, a 1 mg ml^{-1} solution of lyophilised aqueous extract from zucchini cotyledons infected by *P. syringae* pv. *syringae* was used as a coating agent in the competitive ELISA.

Results

Antibody characterisation

Unlike anti–SP_{25} polyclonal antibodies (Fogliano *et al.*, 1999), indirect ELISA with anti–SR-E antiserum gave low specific recognition (data not shown). This was probably due to the different molecular size of the two toxins (2400 Da for SP_{25}, 1254 Da for SR-E). As an antigen, the nonapeptide ring of SR-E, even though protein-bound, was close to the lower size limit for obtaining an efficient recognition in the assay. SR-E recognition was, however, satisfactory using competitive ELISA: the calibration curve was linear in a range between 0.01 and 1 mg ml^{-1} of free SR-E, with a detection limit of 0.1 mg per well. This limit is higher than that for SP_{25} (0.01 mg per well). The assay was highly reproducible: the inter-assay standard variation was below 10%, and that of the intra-assay was less than 5%. Data on specific recognition and cross-reactivity with related LDP are shown in Fig. 1. As expected, SR-E antibodies recognised SR-G, which differs from SR-E only in the length of the fatty acid chain (14 carbon atoms for SR-G, 12 for SR-E), but they did not recognise SP_{22} or SP_{25}. Interestingly, the other two nonapeptides assayed, PS-A and ST, gave no relevant cross-reaction with anti–SR-E antibodies.

The structural differences among SR-E, PS-A and ST are restricted to the 5 amino acids located between the first (serine with the fatty acid chain) and the last three (dehydrobutyric acid, hydroxy aspartic acid, and chloro-threonine), which remain unchanged. This means that the central part of the peptide ring plays a pivotal role in the antigen-antibodies interaction.

Production of LDP by various pathovars or strains of *P. syringae*

Six strains of *P. syringae* pv. *syringae* from various hosts and provenances, whose production of LDP in culture was only partially known, and three strains of *P. syringae* pv. *aptata* were tested by the

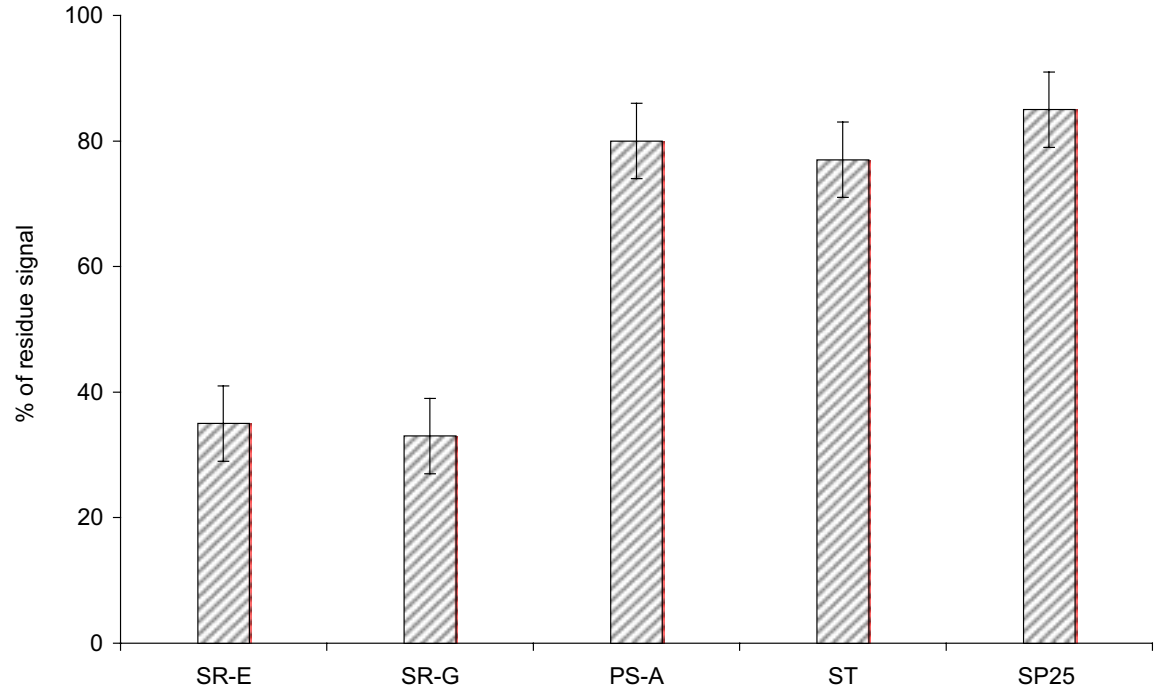

Fig. 1. Specificity of SR-antibodies. Competitive ELISA using anti–SR-E antiserum diluted to1:200 and 1 µg per well of various *Pseudomonas* LDP. Analysis of each sample was performed in triplicate.

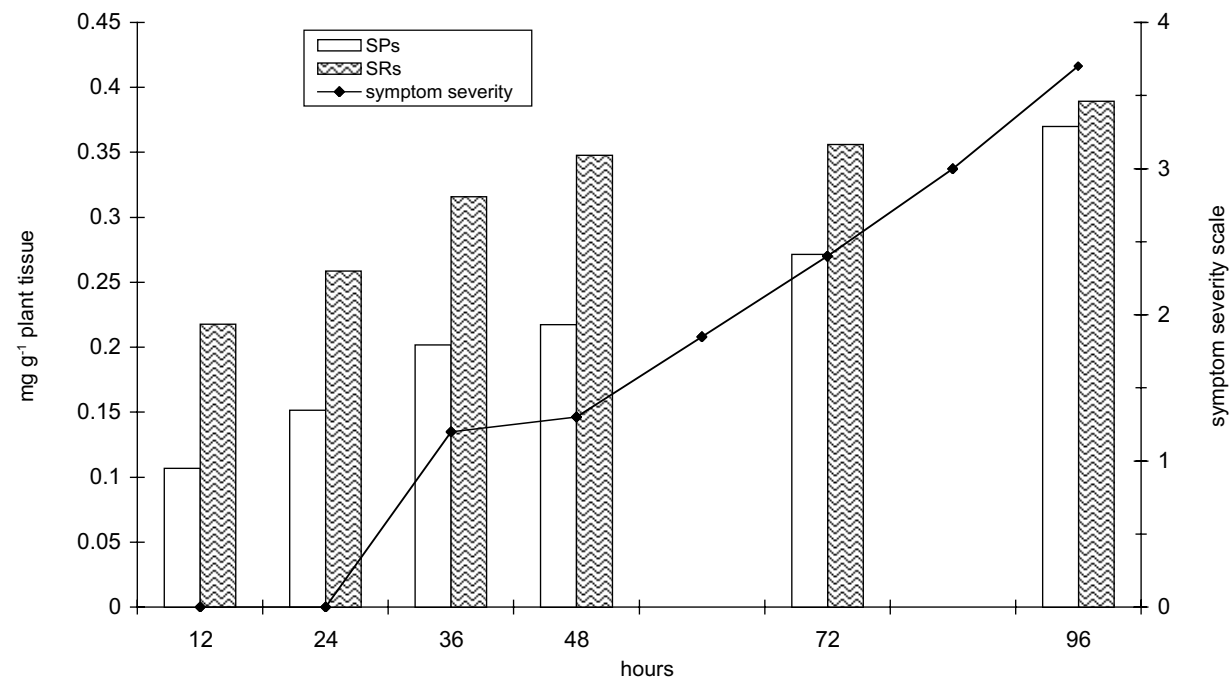

Fig. 2. Time course of the infection on zucchini cotyledons by *P. syringae* pv. *lachrymans* strain S91. The scale on the left indicates the concentration of LDP; that on the right refers to symptom severity (see Materials and methods). Values are the means of 3 replications.

immunological assays developed for SRs (this paper) and for SPs (Fogliano *et al*., 1999). Since the antibodies do not distinguish among different forms of SRs and SPs, the total amount of each class of LDP was determined (Table 1). The yield of SRs ranged from 26 to 55 mg l^{-1}, and that of SPs from 20 to 60 mg l^{-1} of culture filtrate. The best producer was the strain Y27 of *P. syringae* pv. *syringae*, isolated from bean by J.E. Crosse in Wisconsin, MI, USA, which is a highly virulent strain (G.L. Ercolani, personal communication). There was no apparent relation between the amount of the two classes of metabolites produced by each strain. The values were comparable to those reported in the literature, thus confirming the reliability of the data obtained by ELISA.

Detection of LDP in aqueous extracts from infected zucchini cotyledons

Both the anti–SR and the anti–SP antisera gave a strong reaction when aqueous extracts from cotyledons of zucchini infected by *P. syringae* pv. *lachrymans* were assayed. As previously reported for SPs (Fogliano *et al*., 1999), this reaction is specific and is due to the bacterial infection. No recognition occurred when extracts from non-inoculated controls were tested. The reaction was completely inhibited when either pure SR-E or SP_{25} was added to anti–SR or anti–SP antiserum.

In order to check whether development of symptoms was related to concentration of LDP in infected leaf tissue, and whether there was any difference between production *in vitro* and *in planta* of the two classes of toxins, aqueous extracts from samples of plant tissue collected at various stages of infection were assayed. The results shown in Fig. 2 indicate that the concentration of both classes of LDP in plant tissue increased with the severity of symptoms. Twelve hours after inoculation, when initial symptoms appeared, the immunologically determined concentration of SPs was 0.11 mg g^{-1} f wt; it increased progressively to 0.37 mg g^{-1} f wt by the end of the four-day experiment. The corresponding values for SRs were higher (particularly at the earlier recording date): 0.22 and 0.39 mg g^{-1} f wt.

Using the same procedure with healthy cotyledons injected with a known amount of pure toxins, 2 days after injection the immunoassay gave an estimated recovery of 0.22 mg SR-E and 0.14 mg SP_{25A} per g f wt. The expected concentration of the two toxins was 0.20 mg g^{-1} for SR-E and 0.10 mg g^{-1} for SP_{25A}, hence the overestimations compared to free toxins were 10% and 40% respectively.

Applying these percentages to the values estimated for infected cotyledons, the net concentrations were as follows: 12 h after inoculation: 0.20 (SRs) and 0.07 (SPs) mg g^{-1} f wt; four days after inoculation: 0.35 (SRs) and 0.22 (SPs) mg g^{-1} f wt.

Table 1. Concentration of syringopeptins (SPs) and syringomycins (SRs) in 5-day-old culture filtrates of nine strains of three pathovars of *Pseudomonas syringae*[a].

P. syringae pathovars and strains	SPs (mg l^{-1})	SRs (mg l^{-1})
P. syringae pv. *aptata*		
NCPPB 871	20.5 ± 0.7	44 ± 1.4
NCPPB 872	28 ± 0.8	50 ± 1.3
NCPPB 2664	46 ± 1.3	53 ± 1.7
P. syringae pv. *syringae*		
B359	46.5 ± 1.7	55 ± 2.1
B382-4	46 ± 1.1	50 ± 1.2
B426	55 ± 0.9	37 ± 1.0
Y27	60 ± 1.9	48 ± 1.1
Y37	25 ± 0.6	32 ± 0.7
P. syringae pv. *lachrymans*		
S91	33 ± 0.7	26 ± 0.4

[a] Data are mean values from three replicates of one experiment ± SD.

Discussion

The immuno-assay described in this study is a highly specific, simple and reliable method for the quantitative assessment of SRs in culture filtrates of plant-pathogenic pseudomonads. It is about 100 times more sensitive than the current chromatographic (HPLC) method and does not require previous extraction of the toxin with organic solvents.

With this method, and the SPs immuno-assay developed in a previous paper (Fogliano *et al.*, 1999), the ability of 9 strains of 3 *P. syringae* pathovars to produce LDP in culture was assessed. Under the conditions of the experiment, the maximum yield of these substances was about 0.05 (SRs) and 0.06 (SPs) mg ml^{-1} culture medium. All but three strains produced more SRs than SPs.

Although indications of the presence of SRs in infected plant tissues have been reported in the literature (Painter and Alconero, 1976; Grgurina *et al.*, 1997), reliable data on the production and accumulation of these toxins *in planta* are so far lacking.

In the infection of zucchini cotyledons by *P. syringae* pv. *lachrymans* strain S91, the maximum net concentration of SRs in plant tissue (0.35 mg g^{-1} f wt), as estimated by competitive ELISA 4 days after inoculation, was substantially (about 60%) higher than that of SPs (0.22 mg g^{-1}). Production of LDP *in planta* seems to have occurred fairly early. At the initial stages of the disease, 12 hours after inoculation, the net concentration of both toxins (0.20 and 0.07 mg g^{-1}, respectively) was well above that known to be active in various biological systems (Iacobelllis *et al.,* 1992; Di Giorgio *et al.,* 1996; Lavermicocca *et al.,* 1997). However, the values obtained with aqueous extracts from infected plants appear to be overestimated and are high when compared to the figures for the *in vitro* experiments reported above. This can be linked with previous observations (Iacobellis *et al.*, 1992a; Fogliano *et al.*, 1999) that high-molecular weight complexes with bacterial toxins occur in infected plant tissues and their aqueous extracts. It is therefore assumed that the high reactivity of ELISA to the immune-LDP-related compounds present in the water extracts from infected plants is due to the presence of LDP complexes having a cross-reactivity with antibodies which is substantially higher than that of free toxins. A further explanation may be that toxin production is activated at a higher level in plant tissues as compared to *in vitro* experiments.

The SRs and SPs immuno-assays, successfully applied in this study for assessing LDP *in vitro* and *in planta*, can be used to study the biosynthesis, biological activity and role in pathogenesis of bacterial LDP toxins. Apart from this, however, immuno-assays can also be used for diagnostic purposes.

Toxin-based identification procedures have been adopted for differentiating *P. syringae* pathovars and LDP-producing strains. Cultural methods and antimicrobial assays based on growth inhibition of bacteria, yeasts and hyphomycetes supposed to be most sensitive to SPs or to SRs, e.g. *Bacillus megaterium, Rhodotorula pilimanae,* and *Geotrichum candidum*, are used routinely. However, these tests are time-consuming, require a careful selection of the nutrient medium, and sometimes give false results. Recently, a PCR test developed for detecting the bacterial *syrB* gene involved in SRs and SPs secretion has been proposed to screen pseudomonads for their ability to produce LDP (Bultreys and Gheysen, 1999). However, the PCR test does not discriminate between SR- and SP-producing bacteria and does not indicate the amount of toxin produced. The immuno-assays developed for SRs for SPs can be applied for both purposes.

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