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IDENTIFICATION OF LEPTOSPHAERIA MACULANS AND LEPTOSPHAERIA BIGLOBOSA ISOLATES BASED ON PATHOGENICITY AND PRODUCTION OF SIRODESMIN PL

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Abstract

The aim of this study was to identify isolates within the pathogenic species of *Leptosphaeria maculans* and *Leptosphaeria biglobosa*, based on the pathogenicity and phytotoxin production. The fungus cultures were isolated from rapeseed plants showing the symptoms of stem canker. They were collected from nine locations in rapeseed growing region of northern Serbia. In the study were used 119 local isolates from Serbia and two reference isolates from the Center for Agricultural Research, Rothamsted, Great Britain, (L. m. for *Leptosphaeria maculans*, and L. b. for *Leptosphaeria biglobosa*). The pathogenicity of the isolates was examined on the Westar, Glacier and Quinta rapeseed cultivars. Eight isolates caused small necrotic spots on cotyledons in all tested cultivars, while 111 caused large necrotic spots in which the formation of pycnidia was observed. Four local isolates (K-113, C-3, St-5 and S-11) and reference isolate L.m. were used for the extraction and isolation of phytotoxins, based on their morphological and pathogenic characteristics. Isolates C-3, St-5, S-11 and L. m. did not produce a yellow brown pigment in the Czapek medium but produced sirodesmin PL while the K-113 isolate produced a yellow brown pigment and did not produce sirodesmin PL.

Keywords: *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, rapeseed, phytotoxin.

Introduction

Leptosphaeria maculans (Desm.) Ces and De Not (anamorph: *Phoma lingam* (Tode ex Fr.) Desm.) cause dry (black leg) on oil rapeseed worldwide. Disease is economically very important in Europe, Australia and Canada (West *et al.*, 2001). The appearance of the disease to a greater or lesser intensity depends on climatic factors, cultural practices and varieties resistance (Howlett, 2004; Aubertot *et al.*, 2006; The population of *L. maculans* can be divided into at least two subgroups, which are termed as virulent or avirulent (McGee & Petrie 1978 Koch *et al.*, 1989 Badawy & Hoppe 1989), Tox⁺ or Tox⁰ (Balesdent *et al.*, 1992), A or B (Johnson and Lewis 1994). Aggressive isolates (group A or Tox⁺) induce grey-green, sporulating lesions on leaves and destructive stem canker while non-aggressive isolates (group B or Tox⁰) cause leaf spot and superficial stem lesions (Koch *et al.*, 1989; Badawy *et al.*, 1991; Williams, 1992; Thürwächter *et al.*, 1999). Brun *et al.*, (1997) and Williams and Fitt (1999) indicated that group A is highly virulent, while group B is slightly virulent. Koch *et al.*, (1991), Mengistu *et al.*, (1991) & Kutcher *et al.*, (1993) have studied the virulence of different isolates of *L. maculans* on three varieties of oil rapeseed (Westar, Quinta and Glacier) and classified them into four pathogenic groups. Group B was then isolated and identified as a new species called *Leptosphaeria biglobosa* (Shoemaker and Brun, 2001). A number of plant pathogens produce secondary metabolites (toxins) in order to obtain nutrients from plant cells. In some fungi, the toxins have potential toxicity or carcinogenic properties that could endanger the health of humans, animals and plants. (Bohnert *et al.*, 2010) In some cases, toxins cause death of plant cells to release nutrients or disrupt plant metabolism in favour of the pathogenic fungi. (Howlett, 2006). Many selective toxins play a role in the virulence of pathogenic fungi (Howlett, 2006; Wolpert, 2002) The role of non-selective toxin

in virulence is complex and the production of toxins is not always correlated with virulence (Elliott *et al.*, 2007). Sirodesmin PL, the product of the pathogenic fungus *Leptosphaeria maculans*, belongs to the class of epipolythiodioxopiperazines (ETPs), and is characterized by the presence of disulphide bridges (Gardiner *et al.*, 2004; Gardiner *et al.*, 2005). The diketopiperazine ring originates from cyclic dipeptides and sulphur bridges are responsible for all the known toxic effects of these molecules (Mullbacher *et al.*, 1986). Gardner *et al.*, (2005) stated that disulphide bridges are a key structural element for the creation of a variety of reactive forms of oxygen and for connection with the cysteine residues of proteins. It is assumed that the toxicity of ETPs could be explained by these reactions. Sirodesmin PL is a non-selective toxin that causes chlorosis and necrosis, inhibits root growth and leads to the extinction of plant cells (Badawy *et al.*, 1989; Fox and Howlet, 2008). In addition, these compounds have antibacterial and antiviral properties. (Rouxel *et al.*, 1988). The aim of this study was to identify isolates within the pathogenic species of *Leptosphaeria maculans* and *Leptosphaeria biglobosa*, based on the pathogenicity test and to perform crude separation of fungal toxins using preparative TLC on silica-gel plates, and to identify individual toxins using HPLC coupled with a tandem mass-spectrometric detector (LC-MS-MS).

Materials and Methods

Isolation of fungi and obtaining monospore culture

Infected plants of oilseed rape were collected during 2009/10 in the region of Vojvodina, Serbia. Diseased plant organs (root, basal and upper stem, leaf, flower, pod and seed) with clearly defined symptoms of the disease were used for the isolation of the fungi. Diseased tissue fragments were soaked in a 3 % solution of sodium hypochlorite for 5–10 min and then washed with sterile water and naturally dried under controlled conditions. After drying, the fragments of diseased tissue were applied to the culture medium of potato dextrose agar (PDA) (Difco, Detroit, USA) that had previously been poured into petri plates. To prevent bacterial growth in the medium, 50 mg of streptomycin sulphate (Galenika, Belgrade, Serbia) was added per litre. The inoculated petri plates were incubated at 25±1°C. After 5 to 10 days, the formation of pycnidia and pycnidiospores was observed under a stereo microscope. Pure cultures were obtained by the following procedure: pycnidiospores, which were released from pycnidia serving as a single droplet originating from the culture media, were transferred with the tip of a spear needle into plastic tubes to which 2 mL of sterile water had previously been added. The prepared suspension of conidia was applied onto the aqueous agar medium, which had previously been poured into petri plates. After 48 h, germination of the conidia was observed under a stereo microscope. The germinated conidia, together with fragments of the substrate, were transferred onto PDA medium in petri plates and placed in an incubator at 25°C in order to develop monospore fungal isolates. In this way, 119 isolates of fungi were obtained. All isolates were analysed at the morphological and molecular level. Based on morphological (colony appearance, shape, size and colour of the pycnidia and pycnidiospores (Punithalingam and Holliday, 1972; Fitt *et al.*, 2006) and molecular characteristics (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism, PCR-RFLP Balesdent *et al.*, 1998) it was determined that 111 isolates belonged to *L. maculans* and 8 to *L. biglobosa* (data not shown). Using the method of random selection, four isolates (three of *L. maculans*, St-5, C-3 and S-11, and one of *L. biglobosa*, K-113) were taken for further research.

Phytopathogenic test

The pathogenicity of the isolates (total of 119 isolates from Serbia and two isolates from the Centre for Agricultural Research, Rothamsted, UK designated L.m. - *Leptosphaeria maculans* and L.b - *Leptosphaeria biglobosa*) has been examined by the method of Koch *et al.*, (1991), Chen and Fernando (2006). Seeds were disinfected by dipping in 3% solution of sodium-

hipohlorite (NaOCl) for 3 to 5 min. and then washed with tap water and dried at room temperature under controlled conditions. The success of disinfection was tested on PDA nutrient medium. Disinfected seeds of varieties Westar, Quinta and Glacier were applied in two Petri dishes, and not disinfected seeds of listed varieties were used as controls. In each box was placed 5 seeds. Thus prepared Petri dishes were placed in a thermostat at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the dark. After 15 days was carried out visual and microscopic examination of the seeds and substrate, after which the seeds were placed in a germination chamber. Germinated seeds were sown in plastic containers with a diameter of cells 4×5 cm, which were previously filled with sterile substrate. Each cell was seeded by one germinated seed. After germination (6-7 days), when the cotyledons separated, inoculation was performed as follows: sterile needle was used to injure one cotyledon, while the other cotyledon was not injured. Injured part was inflicted by micropipette with $5 \mu\text{l}$ (10^6) suspension of pycnospores. After inoculation, plants were transferred to controlled conditions at a temperature of 20°C , 95% RH and 12 h photoperiod. After 48 h the plants were placed in greenhouse conditions. During follow-up of symptoms true leaves were removed several times. Symptoms were rated after 15 days. using the scale – (no symptoms), + (slight lesions), ++ (moderate lesions), +++ (severe lesions). As a negative control, a variety of glacier was used where distilled water was applied to cotyledons.

Extraction and isolation of phytotoxins

Four isolates of *L. maculans* were used for the extraction and isolation of the phytotoxins. Three isolates (C-3, St-5 and S-11) originated from Vojvodina and the fourth, designated L.m. (*L. maculans*) and serving as the reference isolate, was received from the Centre for Agricultural Research, Rothamsted, UK. In addition, an *L. biglobosa* culture (designated K113) was prepared as a known negative. All five isolates were sown in Czapek liquid medium, 16 which had previously been poured into tubes. The tubes were placed in a climate chamber at 20°C with 12 h photo period. After 30 days, the cultures were filtered to separate the fungus mycelium from the liquid medium. Culture extracts were prepared and purified according to previously published procedures, (Koch *et al.*, 1989, Pedras and Bisesenthal, 2001) using a thin layer chromatography (TLC) fractionation, with double elution. The culture filtrate was first extracted with ethyl acetate (6 mL of ethyl acetate per 5 mL of filtrate). The organic extract was dried with anhydrous sodium sulphate. After removal of sodium sulphate by filtration through a qualitative filter paper, the samples were evaporated under a stream of nitrogen. The residue was redissolved in $100 \mu\text{L}$ of chloroform at room temperature. The chloroform solution was applied on an analytical TLC plate (Macherey-Nagel, Silicalgel G-25 UV₂₅₄, glass plates, 20×20 cm \times 0.25 mm) using a glass capillary and the plate was developed using ethyl-acetate : chloroform (1:1) as the eluent. After drying, the plate was examined under UV light at 254 nm (Mitrović *et al.*, 2012). According to the literature data, (Koch *et al.*, 1989, Badawy and Hoppe, 1989) spots that can be visually observed under the UV light at 254 nm, are unambiguously identified as phytotoxins. Based on the preliminary results, a larger-scale experiment (with a greater amount of media) was set up with the purpose of preparative isolation of the observed phytotoxins (for the analysis of their chemical properties and phytotoxicity.). The final chloroform solution of the dry extract was applied to a preparative (prep.) TLC plate: (Macherey-Nagel, Silicagel G-200 UV254, glass plates, 20×20 cm \times 2 mm), and the plate was developed in the binary eluent system, ethyl-acetate : chloroform (1:1). Three spots were identified under UV light at 254 nm, and designated as 1 (with retention time, $R_f = 0.15$), 2 (with retention time, $R_f = 0.44$) and 3 (with retention time, $R_f = 0.60$). The spots were removed from the TLC plate by scratching the silica gel layer, and individually extracted in absolute ethanol (20 mL) at room temperature with vigorously shaking for 2 h. The extract of TLC spot, designated as 2, was evaporated under a nitrogen stream, and then again applied on a preparative TLC plate and eluted,

according to the above mentioned procedure, yielding fractions 2a ($R_f = 0.49$), 2b ($R_f = 0.40$), 2c ($R_f = 0.32$) and 2d ($R_f = 0.22$). Spots were removed and extracted according to the same procedure as described earlier. All subsequent extracts were purified on a Sephadex column (SPE Bakerbond Sephadex G-25) previously conditioned with 10 mL of ethanol. The collected ethanolic filtrate was evaporated under a nitrogen-stream, and the dry residue was used in the identification of phytotoxins using the LC-MS-MS method.

LC-MS-MS characterization of phytotoxins

Chemical composition of isolated fractions was determined by reversed-phase high-performance liquid chromatography (Agilent Technologies Series 1200 Rapid Resolution liquid chromatograph) coupled with tandem mass spectrometric detection (Agilent Technologies Series 6410A Triple-Quad mass spectrometer with an electrospray ion source). 1 μL of undiluted sample was injected into the system. The components were separated using a Zorbax Eclipse XDB-C18 rapid resolution column 50 mm \times 4.6 mm, 1.8 μm (Agilent Technologies), held at 40 $^\circ\text{C}$. The samples were eluted using the gradient mode: 0 min 30 % B, 7–10 min 100 % B (phase A being 0.1 % aqueous formic acid and phase B – 0.1 % formic acid in acetonitrile) with a post time of 2.5 min. The mobile phase flow was 1 mL min^{-1} . The eluent was forwarded into the electrospray ion source (ESI) without flow splitting. The ESI parameters were as follows: nebulizer pressure 40 psi, drying gas temperature 350 $^\circ\text{C}$, drying gas flow 9 L min^{-1} , capillary voltage 4000 V and fragmentor voltage 100 V. All samples were analyzed in the MS2Scan mode (MS1 experiment), using positive polarity, in the m/z range 150–900. Afterwards, the representative sample (St-5/2b) was analyzed in the Product Ion Scan mode (MS2 experiment), using $[\text{M}+\text{H}]^+$ of suspected sirodesmins peaks as precursor ions, and a collision voltage of 0–30 V (in 10 V increments). All the acquired data were processed using MassHunter Workstation-Qualitative Analysis software, ver. B.03.01 (Agilent Technologies).

Results and Discussion

Isolates of L.m, St-5 and S-11 were caused by chlorotic spot (+ slight phytotoxicity) after 5 days, while no change in cotyledones was observed in the K-113 isolates compared to the control variant. After 10 days, isolates L.m, St-5 and S-11 showed moderate phytotoxicity (++) except for K-113. After 15 days, isolates of L.m, St-5, S-11 caused the most severe lesions (+++) on cotyledons while K-113 was assessed as slight virulent (+) compared to the control variant (Figure 1). Expressed aggressiveness of the isolates in the conducted studies indicates that L.m, St-5 S-11 belong to the species *L.maculans* while K-113 *L.boglobosa* (Fitt et al 2006, West et al. 2001, Brun et al. (1997)



Figure 1. Pathogenicity of isolates on varieties Westar, Glacier and Quinta

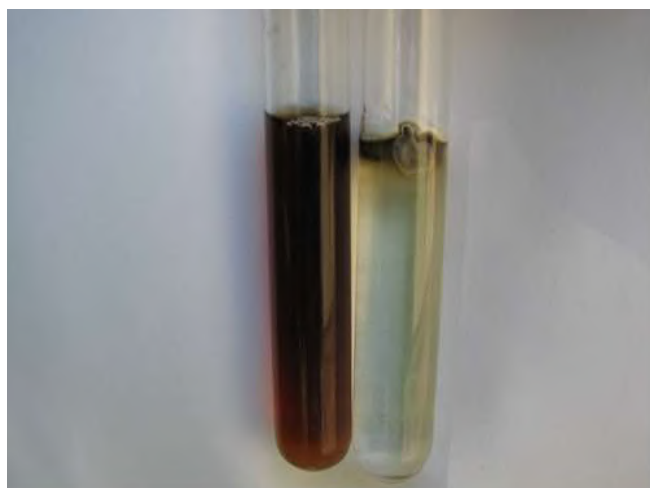


Figure 2: Production of pigment in Liquid Czapek medium

Isolates L.m., St-5, C-3, S-11 do not produce yellow pigment in Czapek liquid medium, compared to K-113 (Figure 2) The absence of yellow pigments in the examined isolates (L.m., St-5, C-3, S-11) classified them as a group of virulent (aggressive) isolates relative to K-113 which form pigments, which is in line with allegations (Williams & Fitt, 1999).

Chemical profile of *L. maculans* extracts

Since reference standards for sirodesmins were not available, the absolute concentrations of the detected compounds in the samples could not be determined. However, it was possible to compare the differences in content of each compound throughout the fractions. It was observed that the bulk of the identified sirodesmins were contained within the fraction (according to the spot from preparative TLC) designated 2b (Mitrović *et al.*, 2012). For the majority of compounds, only a small percentage was present in fractions 2a and 2c, with the exception of compounds with the strongest signals (sirodesmin PL, sirodesmin C and P444), that diffused into 2c spot to a greater extent. Another exception is P318 (phomamide), which occurs exclusively in fraction 1. Fractions 2d and 3 were practically devoid of sirodesmins (Figure 3, Table 2).

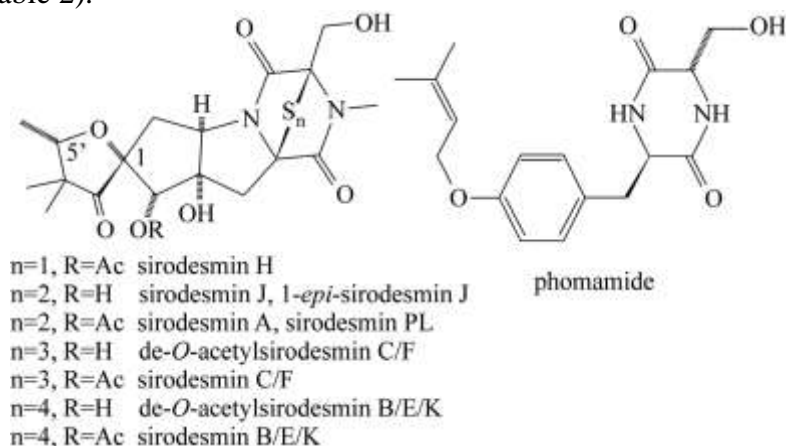


Figure 3: Structures of the detected epipolythiodioxopiperazines.

Table 1. Relative abundances of sirodesmin congeners, given as peak areas calculated from extracted ion chromatograms. For each compound, all abundant adduct and fragment ions were taken into account

Extract, fraction	Peak areas, arb. units										
	P31 8	P44 4	P45 4	P47 6	P486 a	P486 b	P50 8	P518 a	P518 b	P518 c	P55 0
St-5/1	324	0	46	8	0	45	12	7	1	160	181
St-5/2a	0	16	16	53	0	24	82	5	1	73	89
St-5/2b	0	485	79	290	338	2086	56	119	34	1423	461
St-5/2c	0	121	10	30	38	855	2	9	2	286	22
St-5/2d	0	0	0	0	0	0	0	0	0	0	0
St-5/3	0	0	0	0	0	3	0	0	0	2	0
C-3/1	106	0	17	5	0	15	6	1	0	56	56
C-3/2a	0	35	22	124	4	30	182	10	0	80	104
C-3/2b	0	232	87	164	261	1706	39	138	35	1307	524
C-3/2c	0	80	12	18	41	802	2	10	2	238	18
C-3/2d	0	0	0	0	0	0	0	0	0	0	0
C-3/3	0	0	0	0	0	3	0	0	0	4	1
S-11/1	103	0	6	0	0	5	3	0	0	16	18
S-11/2a	0	12	15	33	2	26	41	1	6	75	79
S-11/2b	0	370	35	159	263	1739	23	76	18	1014	232
S-11/2c	0	137	6	22	50	966	2	9	2	232	12
S-11/2d	0	0	0	0	0	1	0	0	0	2	1
S-11/3	0	0	0	0	0	0	0	0	0	1	0
L.m./2a	0	443	4	136	131	383	0	32	1	90	5
L.m./2b	0	252	31	353	185	924	129	227	19	971	476
L.m./2c	0 24	1	109	13	53	156	30	1	165	200	
L.m./2d	0	44	0	121	2	19	121	7	1	45	38
L.m./3	0	141	0	103	10	218	23	8	1	150	29
K113	0	0	0	0	0	0	0	0	2		

Due to differences in the response factors, which are to be expected when using ESI-MS, the peak areas (given in Table 1) can only be treated as a rough approximation of relative abundances within a sample. However, in our opinion, it is safe to assume the protonation constants of the sirodesmin congeners are sufficiently comparable to be able to state that the dominant ETP components of the investigated *L. maculans* cultures were sirodesmins PL and C, and their deacetylated derivatives, which is in agreement with previous results (Badawy and Hoppe, 1989b), and also supported by our results obtained by analyzing TLC plates (Mitrović *et al.*, 2012).

While it is known that a polysulphide bridge in the diketopiperazine ring is responsible for the observed toxic effects (while the nature of side groups does not affect toxicity), and the reduction thereof leads to complete loss of activity, the exact mechanism of toxicity is still a matter of debate (Gardiner *et al.*, 2004; Gardiner *et al.*, 2005).

Regardless of the mechanism (or a combination thereof) that is responsible for the phytotoxic effects of ETPs, activity could be expected for all detected polysulphide-bridge containing

sirodesmins, although it is likely that only the dominant components contribute significantly, as indicated by Badawy and Hoppe, (1989b).

Referring to our previously published results (Mitrović *et al.*, 2012), we came to the conclusion that there was no significant difference between investigated *L. maculans* extracts. No sirodesmins were found in the reference extract K113 prepared from an *L. biglobosa* culture (Mitrović *et al.*, 2012). This indicates that sirodesmins could be employed as markers for the differentiation of two species – *L. maculans* and *L. biglobosa*.

Conclusions

The results obtained by the phytopathogenic test, developed in the liquid Czapek medium, show that the investigated isolates of St-5, S-11 and C-3 originate from Serbia, cause severe symptoms in cotyledones in all varieties (Westar Glacier, Quinta) and do not form yellow-brown pigments while K-113 isolate forms a yellow-brown pigment and causes slight virulence. Isolates C-3, St-5, S-11 in thin-layer chromatography and high-performance liquid chromatography with mass spectrometric detection demonstrated the presence of sirodesmins except for K-113. It was found that the dominant epipolythiodioxopiperazines in the investigated *L. maculans* isolates were sirodesmin PL, sirodesmin C, and their deacetylated derivatives. Based on the conducted studies, the isolates C-3, St-5, S-11 belong to the species *Leptosphaeria maculans* while the K-113 type of *Leptosphaeria biglobosa*.

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