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Original scientific paper

Characterization of sirodesmins isolated from the phytopathogenic fungus *Leptosphaeria maculans*

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Abstract: The pathogenicity of phytopathogenic fungi is associated with phytotoxins, especially with their chemical nature and quantity. Sirodesmins are phytotoxins from the epipolythiodioxopiperazines group, produced by the fungus *Leptosphaeria maculans*, which are a cause of blackleg and stem canker in oilseed rape (*Brassica napus* L.). The aim of this work was to obtain a detailed chemical profile of sirodesmins in five fungal isolates (four from Vojvodina, Serbia, and one from the Centre for Agricultural Research, Rothamsted, UK). Sirodesmins showing different phytotoxicity on treated cotyledons of cv. Quinta were separated and detected by thin layer chromatography in all analysed isolates (L.m, C-3, St-5 and S-11) except K-113, which neither contained sirodesmin congeners nor did it exhibit activity. By use of high performance liquid chromatography coupled with tandem mass spectrometer, it was possible to identify total of 10 sirodesmins, together with their precursor – phomamide. It was found that the dominant epipolythiodioxopiperazines of the investigated *L. maculans* isolates were sirodesmin PL, sirodesmin C, and their de-acetylated derivatives.

Keywords: epipolythiodioxopiperazine; thin layer chromatography; liquid chromatography; mass spectrometry; phytotoxicity.

INTRODUCTION

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.[1] In some cases, toxins cause death of plant cells to release nut-

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rients or disrupt plant metabolism in favour of the pathogenic fungi.[2] Sphingolipids originating from fumonisin B_1, produced by *Fusarium* species (*Fusarium verticillioides*), cause cell death by depletion of extracellular ATP.[3] Particular races of the pathogenic fungus *Cochliobolus carbonum* produce HC-toxin that does not directly destroy the cell, but inhibits the enzyme histone deacetylase by disrupting the regulatory gene in the plant cell.[4] Many selective toxins play a role in the virulence of pathogenic fungi.[2,5] The role of non-selective toxin in virulence is complex and the production of toxins is not always correlated with virulence.[6] 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.[7,8] The diketopiperazine ring originates from cyclic dipeptides and sulphur bridges are responsible for all the known toxic effects of these molecules.[9] Gardner[8] 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.[10,11] In addition, these compounds have antibacterial and antiviral properties.[12]

The aim of this study was to perform crude separation of fungal toxins using TLC, to evaluate phytotoxic effects of the separated components on oilseed rape (*Brassica napus* L.), and to identify individual toxins using liquid chromatography with a tandem mass-spectrometric detector (LC–MS–MS).

EXPERIMENTAL

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, 123 isolates of fungi were ob-

tained. All isolates were analysed at the morphological and molecular level. Based on morphological (colony appearance, shape, size and colour of the pycnidia and picnospores[13,14]) and molecular characteristics (Polymerase Chain Reaction – Restriction Fragment Lenght Polymorphism, PCR–RFLP[15]), it was determined that 115 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.

Extraction and isolation of phytotoxins

Four isolates of *L. maculans* were used for the extraction and isolation of the phyto toxins. 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,[17,18] but using a two-stage thin layer chromatography (TLC) fractionation. 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 μL of chloroform at room temperature. The chloroform solution was applied on a TLC plate (silica gel G, 20 cm×20 cm×0.25 mm, Macherey–Nagel) using a glass capillary and the plate was developed using ethyl acetate:chloroform (1:1) as the solvent. After drying, the plate was examined under UV light (254 nm) (Fig. 1). Based on literature data,[17,19] these spots were tentatively 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 phytotoxins (for chemical and phytotoxic activity analysis). The final chloroform solution of the dry extract was applied on a preparative plate (silica gel G, thickness 2 mm, Macherey–Nagel), and the plate was developed by the aforementioned procedure. Three spots were detected under UV light, designated 1 (Rf = 0.15), 2 (Rf = 0.44) and 3 (Rf = 0.60). The spots were removed from plates and individually extracted in absolute ethanol (20 mL) at room temperature with shaking for 2 h. The spot 2 extract was evaporated under a N_2 stream and further separated using on additional TLC plate by the aforementioned procedure, yielding fractions 2a (Rf = 0.49), 2b (Rf = 0.40), 2c (Rf = 0.32) and 2d (Rf = 0.22). Spots were removed and extracted as described above. All the obtained extracts were purified on a Sephadex column (SPE Bakerbond Sephadex G-25) previously conditioned with 10 mL of ethanol. The ethanol filtrate was evaporated under a N_2-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 obtained 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 Zor-

bax Eclipse XDB-C18 rapid resolution column 50 mm×4.6 mm, 1.8 µm (Agilent Technologies), held at 40 °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 effluent 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 °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 (MS^1 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 (MS^2 experiment), using $[M+H]^+$ 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).

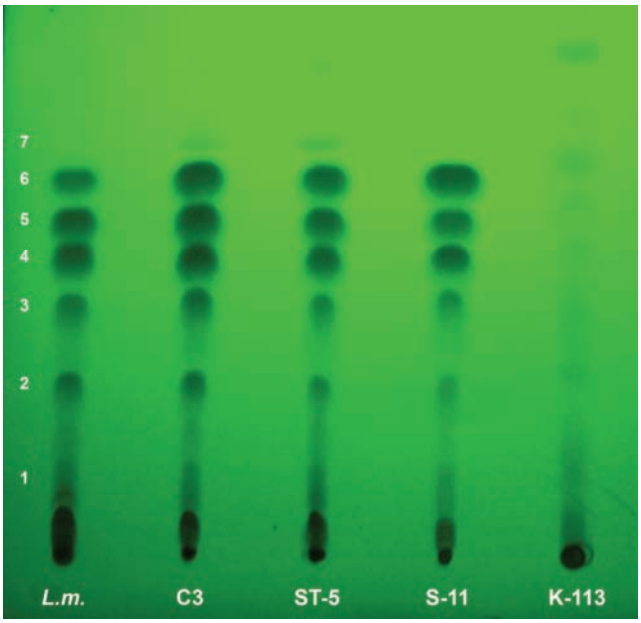


Fig. 1. Thin layer chromatogram of the ethyl acetate fraction from the culture filtrates of four *L. maculans* isolates (*L.m.* St-5, C-3 and S-11) and one *L. biglobosa* isolate (K-113). The ethyl acetate fractions were separated on silica gel with chloroform:ethyl acetate (1:1) as solvent. The compounds were examined under UV light (254 nm). Fractions 1–7 (*Rf* values: 0.11, 0.17, 0.27, 0.34, 0.42, 0.51 and 0.60). These findings are in accordance with previously published results.[10-14]

Phytotoxicity test

Fractions 1, 2a, 2b, 2c, 2d and 3 from the preparative plate were extracted in ethanol and diluted with water to final ethanol concentration of 0.5 % by volume. Different toxin fractions were applied as small 5 µL droplets over a puncture wound on cotyledons of the oilseed rape cv. Quinta. Plants were kept at 22±2 °C and 70/80 % relative humidity in a 12 h photoperiod. Symptoms were rated after 2 days using the scale of Badawy and Hoppe:[19] – (no symptoms),

+ (slight lesions), ++ (moderate lesions), +++ (severe lesions). The crude extract of isolate K-113 was used as a negative control.

RESULTS AND DISCUSSION

Identification of phytotoxins

In the LC–MS chromatograms of the investigated samples, a number of peaks were detected (Figs. 2–5) with molecular weights, isotopic profiles and fragmentation patterns in agreement with those of sirodesmins and other secondary biomolecules (Fig. 6), already detected in *Leptosphaeria* species. For convenience, all peaks were given a designation in form P*xxxy*, where *xxx* is monoisotopic molecular weight (M_{mi}) and *y* is an additional letter added if several isobaric peaks were detected.

The peak with the retention time t_r = 1.57 min, designated P444, belongs to a compound with a monoisotopic molecular weight of 444 Da, corresponding to either sirodesmin J (deacetylsirodesmin A) or its 1-epimer (deacetylsirodesmin PL). In MS^1 spectrum (Table I), weak signals of adduct ions were detected at *m/z* 445 $[M+H]^+$, 467 $[M+Na]^+$ and 483 $[M+K]^+$, as well as a fragment ion at *m/z* 381 $[M+H–S_2]^+$ as the base peak. The isotopic peak profile, A (100 %), A+1 (22.9 %) and A+2 (12.3 %), is in good agreement with the theoretical profile for $C_{18}H_{24}N_2O_7S_2$ (100 %, 22.9 %, 12.8 %). The dominant peak in the MS^2 spectrum is the fragment at *m/z* 381 $[M+H–S_2]^+$. The observed neutral loss of sulphur from a polysulphide bridge is consistent with the behaviour of epipolythiodioxopiperazines (ETPs).[20] Both possible isomers have already been detected in *L. maculans* cultures.[19,21–24] The *Rf* value of spot 4 in the thin layer chromatogram (Fig. 1) is identical to that of deacetylsirodesmin PL (synthesized from sirodesmin PL) reported by Badawy and Hoppe.[19] However, it should be noted that, without obtaining the *Rf* value of deacetylsirodesmin A, identification with absolute certainty is not possible, especially since (unlike in the Badawy–Hoppe experiment) the extracts of *L. maculans* described herein contained significant amounts of sirodesmin A in addition to sirodesmin PL.

Peak P454, eluting at 1.42 min, corresponds to sirodesmin H (monosulphide analogue of sirodesmin PL), already identified in *L. maculans* cultures.[22–24] In addition to the adduct ions at *m/z* 455 $[M+H]^+$, 477 $[M+Na]^+$ and 493 $[M+K]^+$, the MS^1 spectrum also contains fragment ions at *m/z* 437 $[M+H–H_2O]^+$ and 393 $[M+H–H_2O–CO]^+$. The isotopic peaks profile: A (100 %), A+1 (22.6 %), A+2 (8.4 %) is consistent with the theoretical values for $C_{20}H_{26}N_2O_8S$ (100, 24.4 and 8.9 %). In the MS^2 spectrum, a number of fragments were detected, corresponding to loss of alcoholic OH (as H_2O), acetyl (as ketene, C_2H_2O) and an unidentified group at *m/z*=78: 437 $[M+H–H_2O]^+$, 413 $[M+H–C_2H_2O]^+$, 393 $[M+H–CO_2]^+$, 377 $[M+H–78]^+$, 351 $[M+H–CO_2–C_2H_2O]^+$, 315 $[M+H–CO_2–78]^+$, *etc*.

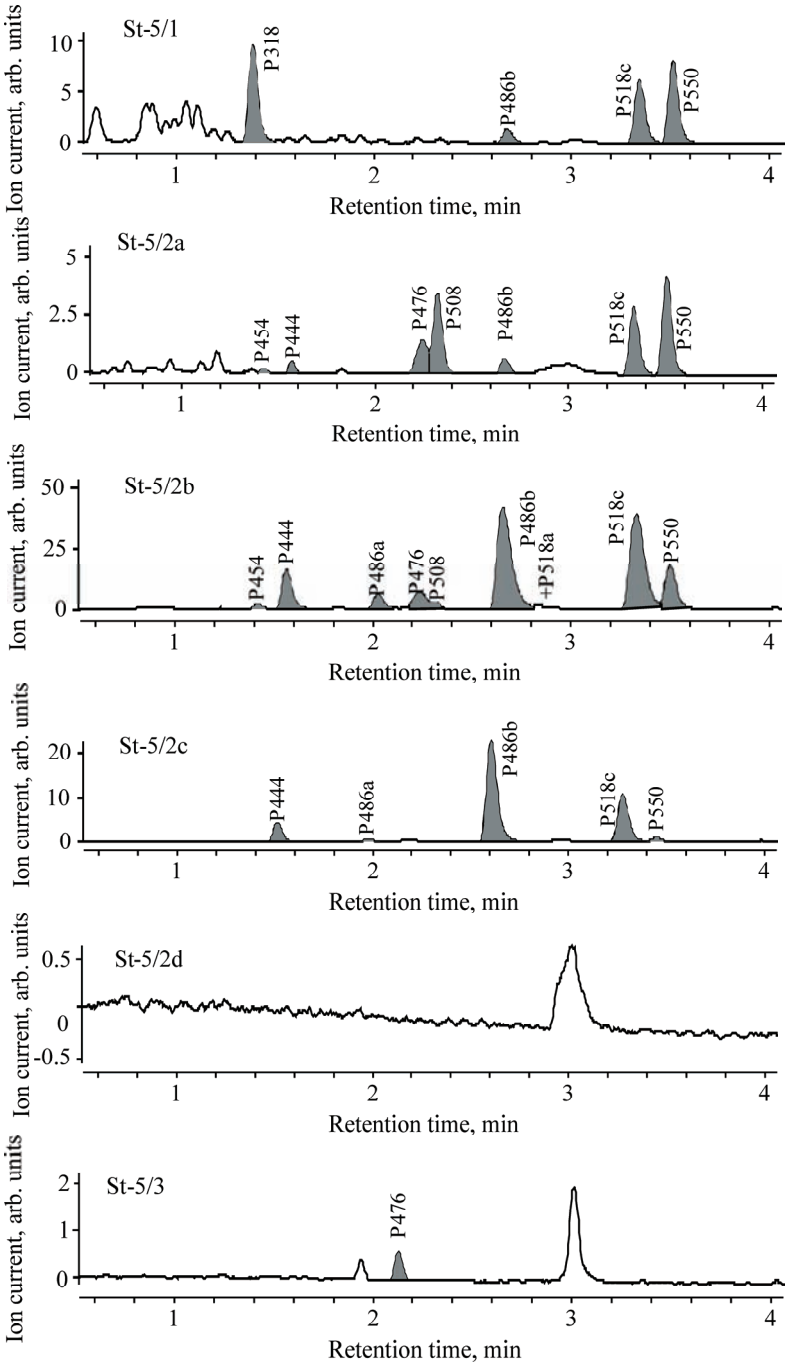


Fig. 2. LC–MS base peak chromatograms of the fractions of the St-5 isolate. The chromatographic conditions are given in the text.

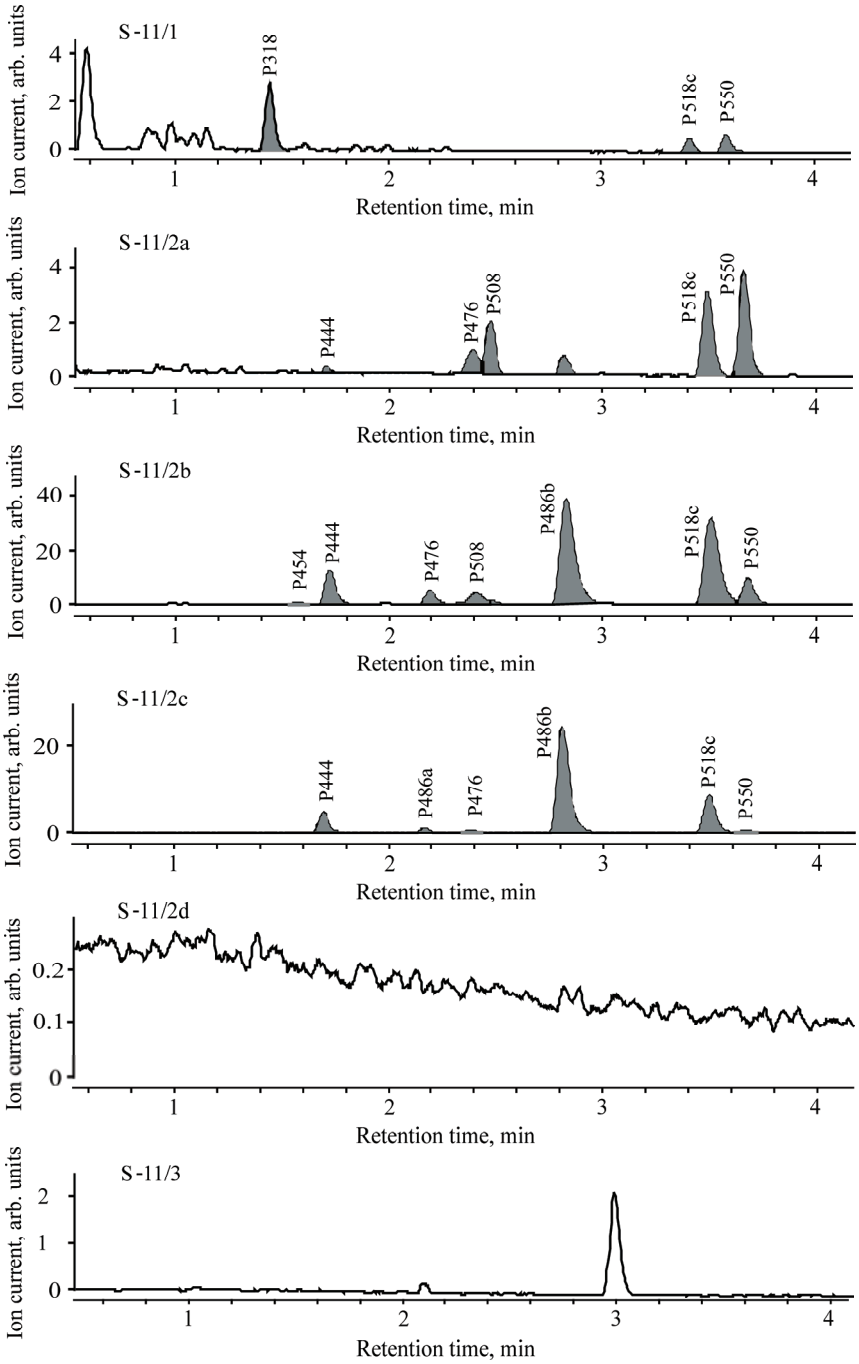


Fig. 3. LC–MS base peak chromatograms of the fractions of the St-11 isolate. The chromatographic conditions are given in the text.

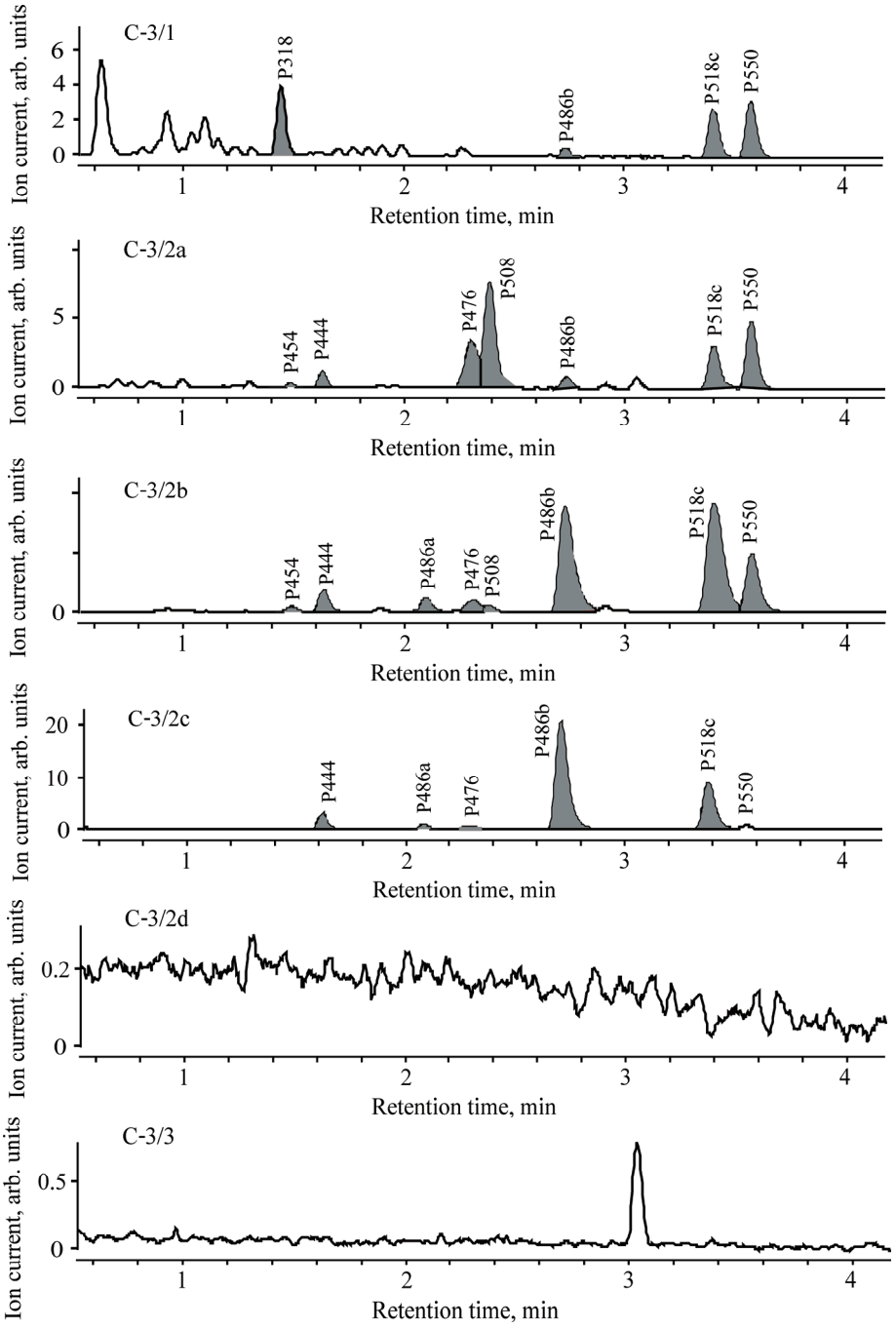


Fig. 4. LC–MS base peak chromatograms of the fractions of the C3 isolate. The chromatographic conditions are given in the text.

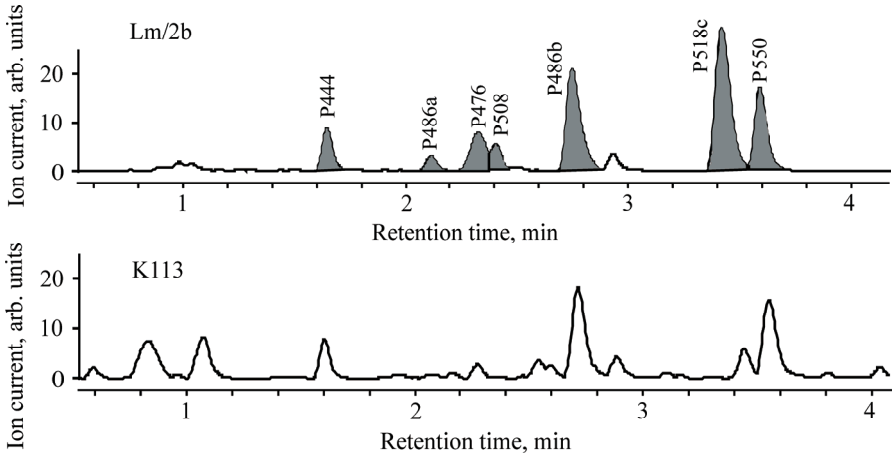
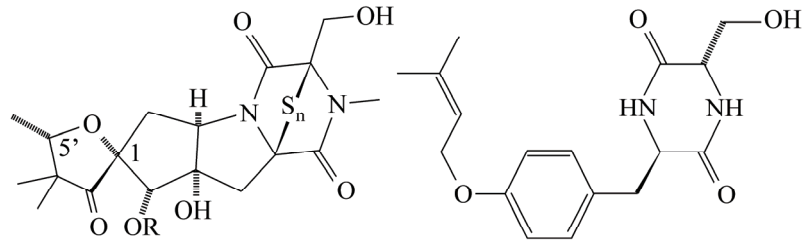


Fig. 5. Comparison of the LC–MS base peak chromatograms of L.m./2b and K-113. While several peaks with similar t_R were detected in both samples, they do not represent same compounds (as they differ completely in their mass spectra).



n=1, R=Ac sirodesmin H
n=2, R=H sirodesmin J, 1-*epi*-sirodesmin J
n=2, R=Ac sirodesmin A, sirodesmin PL
n=3, R=H de-*O*-acetylsirodesmin C/F
n=3, R=Ac sirodesmin C/F
n=4, R=H de-*O*-acetylsirodesmin B/E/K
n=4, R=Ac sirodesmin B/E/K

phomamide

Fig. 6. Structures of the detected epipolythiodioxopiperazines.

The MS^1 spectrum of peak P476, eluting at 2.22 min, contains (in addition to the low-abundance of adducts with H^+, Na^+ and K^+) an intense peak of a fragment at *m/z* 381 $[M+H–S_3]^+$. The molecular weight and the loss of an S_3-unit point out to deacetylsirodesmin C or F. Badawy and Hoppe already reported deacetylated sirodesmin C as a component of *L. maculans* extract, with an *Rf* value close to that of the spot designated 3 in Fig. 1.[19] However, due to lack of information on its epimer behaviour, compound P476 can only be tentatively identified as deacetylsirodesmin C (which stems from the more abundant epimer, sirodesmin PL). The experimental isotopic peak profile is in good agreement with the theoretical profile for $C_{18}H_{24}N_2O_7S_3$: 100, 25.0 and 17.2 % *vs.* 100, 23.7 and

17.4 % for A, A+1 and A+2, respectively. The MS^2 spectrum features fragment *m*/*z* 381 $[M+H–S_3]^+$ as a base peak, accompanied by *m*/*z* 353 $[M+H–S_3–CO]^+$ and 325 $[M+H–S_3–2CO]^+$. The same fragment ions were detected in the MS^2 of peak P444, differing only in the number of sulphur atoms in the bridge, which supports the assumed structure.

TABLE I. Positive ionization MS^1, MS^2 and pseudo-MS^3 spectra of the detected compounds (V_c – collision voltage)

Peak	Id	Order	V_c / V	Adduct and product ions, *m*/*z* (abundance)
P318	Phomamide	MS^1	–	659 (6), 357 (8), 341 (16), 319 (43), 251 (100)
P444	Deacetylsirodes-	MS^1	–	483 (7), 467 (5), 445 (9), 381 (100)
	min J or	MS^2	0	445 (30), 381 (100)
	1-*epi*-deacetyl-		10	381 (100), 363 (10)
	sirodesmin J		20	381 (100), 363 (22), 158 (6)
			30	381 (100), 353 (47), 325 (41), 229 (53), 205 (95), 193 (42), 140 (47)
P454	Sirodesmin H	MS^1	–	493 (22), 477 (14), 455 (100), 437 (33),
		MS^2		393 (21)
			0	455 (100), 437 (40), 413 (10), 393 (29), 377 (10)
			10	437 (9), 413 (41), 393 (100), 377 (25), 351 (17), 315 (32), 279 (18)
			20	413 (14), 393 (100), 351 (71), 315 (55), 279 (40), 245 (8), 217 (31)
			30	393 (13), 351 (100), 323 (9), 245 (12), 217 (78)
P476	Deacetylsirodes-	MS^1	–	515 (8), 477 (13), 381 (100)
	min C or F	MS^2	0	477 (43), 381 (100)
			10	381 (100)
			20	381 (100)
			30	381 (100), 253 (33), 325 (18), 205 (16), 140 (15)
P486a	Sirodesmin A	MS^1	–	525 (14), 509 (13), 487 (100), 423 (50)
		MS^2	0	487 (100), 423 (79)
			10	423 (100)
			20	423 (100), 229 (15), 140 (10)
			30	423 (100), 395 (19), 367 (34), 246 (26), 229 (83), 219 (13)
P486b	Sirodesmin PL	MS^1	–	525 (6), 487 (14), 423 (100)
		MS^2	0	487 (28), 423 (100), 409 (5)
			10	423 (100)
			20	423 (100), 327 (15), 346 (21), 229 (63)
			30	423 (19), 246 (47), 229 (100), 217 (19), 140 (19)
P508	Deacetylsirodes-	MS^1	–	547 (6), 381 (100)
	min B, D, E or K			

TABLE I. Continued

Peak	Id	Order	V_c / V	Adduct and product ions, *m/z* (abundance)
P518a	Sirodesmin F	MS^1	–	557 (19), 541 (24), 519 (100)
		MS^2	0	423 (100)
			10	423 (100)
			20	423 (100), 229 (8)
			30	423 (100), 395 (29), 367 (14), 246 (21), 229 (69), 219 (19), 140 (22)
P518a	Sirodesmin F	MS^{3a}	0	423 (100)
			10	423 (100), 327 (19), 229 (33)
			20	423 (17). 229 (100), 219 (7)
			30	229 (100), 219 (9), 201 (7), 173 (7)
P518b	Unknown	MS^1	–	557 (33), 541 (70), 519 (100), 501 (45)
		MS^2	0	501 (16), 441 (20), 423 (100), 345 (8)
			10	423 (100), 345 (41)
			20	423 (78), 345 (100), 246 (46)
			30	423 (33), 345 (100), 246 (32), 229 (14), 217 (44), 205 (44)
		MS^3	0	423 (100)
			10	423 (100)
			20	423 (100), 395 (25), 367 (41), 305 (15), 229 (22), 223 (12), 219 (25)
			30	367 (45), 305 (53), 229 (65), 219 (100), 201 (40) 191 (43), 188 (57)
P518c	Sirodesmin C	MS^1	–	557 (4), 423 (100)
		MS^2	0	423 (100)
			10	423 (100)
			20	423 (100), 229 (5)
			30	423 (100), 395 (15), 367 (28), 246 (28), 229 (36), 140 (10)
		MS^3	0	423 (100)
			10	423 (100), 327 (14), 229 (30)
			20	423 (25), 229 (100)
			30	229 (100)
P550	Sirodesmin B	MS^1	–	589 (4), 423 (100)

[a]Pseudo-MS^3 spectrum of ion 423 obtained by in-source fragmentation

Two peaks of compounds with a monoisotopic molecular weight of 486 were detected: P486a (eluting at 2.03 min) and P486b (at 2.66 min). The two corresponding compounds, sirodesmin A and sirodesmin PL (also designated G), are epimers and, thus, it was not possible to distinguish them solely using their mass spectra. From their relative amounts and knowing that sirodesmin PL is the dominant phytotoxin of *L. maculans*,[6,8,20,22–25] it could be assumed that peak P486a corresponds to sirodesmin A, while P486b represents the PL isomer. The *Rf* value of spot 6 (0.51) in the thin layer chromatogram is very similar to that of the sirodesmin PL reference standard (0.50) reported by Badawy and Hoppe.[19]

The MS^1 spectrum of both peaks are similar, containing H^+, Na^+ and K^+-adduct ions, as well as an intense fragment peak at *m*/*z* 423 $[M+H–S_2]^+$, which is in agreement with previous results.[6] The isotopic peak profile for A, A+1 and A+2 ions is 100, 25.0 and 14.0 % for P486a and 100, 23.6 and 14.2 % for P486b, which supports the assumed formula $C_{20}H_{26}N_2O_8S_2$ (theoretical profile: 100, 25.2 and 13.5 %). The most abundant fragments in the MS^2 spectra of both peaks were: 423 $[M+H–S_3]^+$, 395 $[M+H–S_3–CO]^+$, 367 $[M+H–S_3–2CO]^+$, 246 and 229.

Peak at 2.32 min, designated P508, exhibited minute ions at *m*/*z* 531 $[M+Na]^+$ and 547 $[M+K]^+$ and 381 $[M+H–S_4]^+$, as the base peak, in the MS^1 spectrum. Based on its molecular weight, as well as the observed loss of an S_4-unit, it could be assumed that the compound is a tetrasulphide homologue of deacetylsirodesmin A or PL, *i.e.*, deacetylsirodesmin B, D, E or K. For a more precise identification, its isolation and acquisition of its NMR spectrum would be necessary. None of possible isomers is indexed in the Dictionary of Natural Products (up to 2007),[26] but sirodesmin K has already been found in *L. maculans*.[24] It should be noted that Badawy and Hoppe succeeded in synthesising deacetylsirodesmin B (by sulphurization of previously deacetylated sirodesmin PL). While no spot at a corresponding *Rf* value (0.13) was observable in their unmodified *L. maculans* extract,[19] well-defined spots at *Rf* = 0.17 are present in extracts described herein. It remains unclear whether it corresponds to sirodesmin B or one of the other isomers.

Three peaks corresponding to compounds with M_{mi} = 518 were detected: the weak P518a (t_r = 2.71 min) and P518b (2.92 min) and the abundant P518c (3.34 min). The MS^1 spectra of all three compounds feature H^+, Na^+ and K^+ adducts, as well as intense fragment *m*/*z* 423 $[M+H–S_3]^+$. Two isomeric trisulphide sirodesmins with M_{mi} = 518 are known, *i.e.*, sirodesmin C and F.[26] The theoretical isotopic profile for the A, A+1 and A+2 ions of these compounds, with empirical formula $C_{20}H_{26}N_2O_8S_3$, is 100, 26.0 and 18.1 %, which is in good agreement with the experimental data: 100, 26.5 and 17.2 % for P518a, 100, 26.3 and 16.3 % for P518b and 100, 26.6 and 18.8 % for P518c. Since sirodesmins C and F are 1-epimers, similarity of their MS^2 spectra is to be expected. Indeed, the MS^2 fragmentation patterns of P518a and P518c show remarkable similarity, with *m*/*z* 423 $[M+H–S_3]^+$ as the base peak, accompanied by *m*/*z* 395 $[M+H–S_3–CO]^+$, 367 $[M+H–S_3–2CO]^+$, 246, 229 and 140. It is likely that P518c represents sirodesmin C, which is known to be one of the main sirodesmins in *L. maculans*.[19] In that case, P518a is probably sirodesmin F, which was reported in *Sirodesmium diversum*, but not in *L. maculans* (probably due to co-elution with some of major ETPs – sirodesmin PL in the present experiments).[26] The *Rf* value of spot 5 in the TLC chromatogram is identical to that of sirodesmin C, as determined by Badawy and Hoppe.[19] The MS^2 spectrum of the third peak, P518b, differs significantly from those of the other two – while base peak is *m*/*z* 423, several fragment ions that are absent in the other two peaks are observable, including an intense peak at *m*/*z*

345 $[M+H–S_3–78]^+$, as well as 501 $[M+H–H_2O]^+$ and 441 $[M+H–78]^+$. It is possible that P518b represents an isomer of sirodesmin C and F with the acetyl attached at a different position (at C2a-OH or hydroxymethyl).

The peak at 3.51 min, designated P550, contains a weak signal at *m*/*z* 589 $[M+K]^+$ and an intense one at *m*/*z* 423 $[M+H–S_4]^+$ in MS^1 spectrum. The molecular weight and the loss of a tetrasulphide unit indicate to a tetrasulphide homologue of sirodesmin A (or one of its isomers), *i.e.*, sirodesmin B, D, E or K. Both sirodesmins B and K have already been reported in *L. maculans* cultures, while sirodesmin E was found in *S. diversum*. [19,22–24,26]

Peak P318, eluting at 1.39 min, was detected only in fractions designated as 1. The MS^1 spectrum features a fragment at *m/z* 251 as the base peak, as well as adduct ions *m*/*z* 319 $[M+H]^+$, 341 $[M+Na]^+$, 357 $[M+K]^+$ and 659 $[2M+Na]^+$. This peak corresponds to dioxopiperazine phomamide, a biosynthetic precursor of sirodesmins, which was already detected in *L. maculans* cultures[22–24]. The fragment *m*/*z* 251 corresponds to the loss of *O*-bound prenyl as C_5H_5. The isotopic peaks profile: A (100 %), A+1 (18.0 %), A+2 (2.8 %) is in agreement with the assumed formula $C_{17}H_{22}N_2O_4$ (theoretical: 100, 20.1 and 2.7 %).

Phomalide, phomaligols, polanrazins, leptomaculins and maculansins, previously identified in *L. maculans*, were not detected in the investigated samples. However, it is well known that the nature of the synthesized metabolites is strongly dependent on both growing medium and on *L. maculans* group and subgroup.[20–25]

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 (preparative TLC spot) designated 2b. 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.

Due to differences in the response factors, which are to be expected when using ESI–MS, the peak areas (given in Table II) 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.[19] These results are also supported by the TLC plates (Fig. 1).

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 (Fig. 5.). This indicates that sirodesmins could be employed as markers for the differentiation of two species – *L. maculans* and *L. biglobosa*.

TABLE II. 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										
	P318	P444	P454	P476	P486a	P486b	P508	P518a	P518b	P518c	P550
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	3	1

Phytotoxicity of the sirodesmin fractions

Fractions 2b and 2c produced the most severe lesions (+++) on cotyledons for all four isolates (C-3, St-5, S-11 and L.m). Fraction 2a showed moderate phytotoxicity (++) except for isolate C-3, which was rated as slightly phytotoxic (+). Slight phytotoxicity was also observed for fraction 1 (all isolates). Fractions 2d and 3 of all isolates of *L. maculans* and K-113 (*L. biglobosa*) did not produce any lesions on cotyledons. The whitish spots on these cotyledons were the consequence of epidermis damage by the needle. The phytotoxicity activity of the fraction (Fig. 7) is in agreement with the results of LC–MS analysis, when the

presence of phytotoxic sirodesmins in fractions 1, 2a, 2b and 2c was clearly confirmed, which is also in accordance with the results of Badawy and Hope.[10,19]

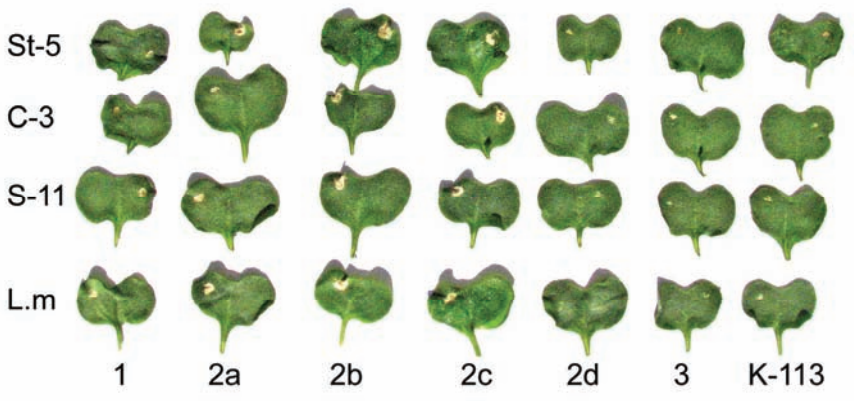


Fig. 7. Phytotoxic effects of the fractionated *Leptosphaeria* isolates on *Brassica napus* cv. Quinta cotyledons.

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.[7,8] It was demonstrated that ETPs non-selectively form mixed disulphide bonds with various cysteine-containing proteins, such as NF-κB (thus accounting for immunosuppressive effects of ETPs) and alcohol dehydrogenase. In addition, they can catalyze the formation of intramolecular disulphide bonds between physically close Cys residues within some proteins, including creatine kinase. However, the exact consequences of ETPs binding to proteins are still unknown. Other theories explain the toxicity of ETPs through redox cycling. A polysulphide bridge is easily reduced in cells; the spontaneous auto-oxidation back to disulphide could generate various reactive oxygen species (ROS), including H_2O_2 and $O_2^{\bullet -}$, that are known to cause adverse effects on cell constituents. However, the concentrations of ETPs that exhibit toxic effects would cause negligible oxidative stress; moreover, some effects (including apoptosis) were proved not to be ROS-related.[8]

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.[19]

CONCLUSIONS

The results obtained by thin-layer chromatography and high-performance liquid chromatography with mass spectrometric detection demonstrated the pre-

sence of sirodesmins in all the examined fungal culture isolates from Serbia (C-3, St-5, S-11) 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. The isolated sirodesmins exhibited phytotoxicity on oilseed rape (*Brassica napus* L.) and may act as virulency factors, contributing to development of *Leptosphaeria maculans*-caused disease. However, no sirodesmins were detected in *L. biglobosa*, suggesting these compounds as markers for the differentiation of the two species. These results are the first indication of the presence of two *Leptosphaeria* species in Serbia – *L. maculans* and *L. biglobosa*.

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И З В О Д

КАРАКТЕРИЗАЦИЈА СИРОДЕЗМИНА ИЗОЛОВАНИХ ИЗ ФИТОПАТОГЕНЕ ГЉИВЕ *Leptosphaeria maculans*

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Патогеност фитопатогених гљива повезана је са фитотоксинима, а нарочито са њиховом хемијском природом и количином. Сиродезмини су фитотоксини из групе епиполитиодиоксопиперазина, које производи гљива *Leptosphaeria maculans*, узрочник суве трулежи корена и рака стабла уљане репице. Циљ овог рада била је детаљна хемијска карактеризација сиродезмина у пет изолата гљива (четири из Војводине и један из Велике Британије, Центар за пољопривредна истраживања, Rothamsted). Код свих испитиваних изолата (*L. maculans*, C–3, St–3, S–11), осим K-113 (који није садржао сиродезмине нити показивао активност) танкослојном хроматографијом су раздвојени и детектовани сиродезмини који су показали различиту фитотоксичност на третираним котиледонима сорте Quinta. Применом течне хроматографије високе ефикасности, куповане са тандемским масеним спектрометром, било је могуће идентификовати укупно 10 сиродезмина, као и њихов прекурсор – фомамид. Утврђено је да су доминантни епиполитиодиоксопиперазини испитиваних изолата *L. maculans* сиродезмин PL, сиродезмин C и њихови деацетиловани деривати.

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