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PATHOGENICITY AND POTENTIAL CAPACITY FOR PRODUCING MYCOTOXINS BY *FUSARIUM SAMBUCINUM* AND *FUSARIUM SOLANI* ISOLATES DERIVED FROM POTATO TUBERS

ABSTRACT

Studies of potential abilities of *F. sambucinum* to produce trichothecenes was conducted on isolates previously confirmed as belonging to this species by PCR. In all cases, A positive result for the presence of *Tri*5 gene, coding the ability to synthesize these mycotoxins. There was no potential to synthesize trichothecenes by *F. solani*.

Further analysis concerned the potential ability of *F. sambucinum* to produce group B trichothecenes (DON and NIV). No isolate gave the expected amplification product (282 bp for deoxynivalenol and 312 bp for nivalenol), which would indicate the potential for producing these mycotoxins. Studies have shown the ability to produce trichothecenes of group A.

Analysis of the potential ability for the synthesis of enniatins by *F. sambucinum* showed that 91% of isolates gave of 332 bp amplification product, which proves them as potencial producers of these mycotoxins.

There were significant differences in the pathogenicity of *F. sambucinu*m and *F. solani* represented by the size of decay caused by these species. The rotten tissue area caused by *F. sambucinum* was about 10 times bigger than after inoculation by *F. solani*. Furthermore, isolates within the same species (*F. sambucinum*) showed diverse pathogenicity. It should be noted, however, that the concentration of mycotoxins does not depend on the size of rotten tissue of potato tubers. Isolate, which caused the most severe disease symptoms, produced low concentrations of mycotoxins.

Key words: F. sambucinum, *F. solani*, mycotoxins, pathogenicity, PCR, potato

INTRODUCTION

Of many species isolated from potato tubers with dry rot symptoms, mostly *F. sambucinum (F. sulphureum), F. solani (F. coeruleum), F. oxysporum, F. avena-*

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ceum, F. culmorum, F. equiseti are listed. In different regions of the world the share of respective *Fusarium* species and their pathogenicity towards potato varies; thus it is difficult to define finally which is the main disease agent. At the same time tuber rotting caused only by A single pathogen is extremely rare. Tubers rot most often as A result of mixed infections. In Poland *F. sambucinum* (*F. sulphureum*) is considered to be main agent of that disease (Stachewicz *et al.* 1978; Latus-Ziętkiewicz 1993, Kurzawińska 1994). The species is also the main agent of disease in North America (Wharton *et al.* 2006).

Fungi of *Fusarium* genus do not only decrease the yield and deteriorate the potato quality but are also capable of producing secondary metabolites toxic for people and animals: mycotoxins [Sveeney and Dobson 1999]. Lenc *et al.* (2008) showed that *F. sambucinum* has A potential capacity for producing trichothecenes of A group, mainly *monoacetoxiscirpenol* (MAS) and *diacetoxyscirpenol* (DAS). Ellner [2002] reports on the content of DAS in potato tubers artificially infected by *F. sambucinum* can reach even 200 μg per tuber. The pathogen can also produce *beauvericin* and fusarine (Leslie & Summerell 2006) as well as eniatine (Altomare *et al.* 1995)

Due to the threat posed by potato tuber infection with *Fusarium* genus fungi, the aim of the present research was to determine the potential capacity for producing mycotoxins of the trichothecenes and eniatines groups by *F. sambucinum* and *F. solani* isolates derived from various arable environments in the country. To confirm the capabilities, the selected isolates of the pathogens researched were applied to inoculate tubers and their contents of mycotoxins were defined.

MATERIAL AND METHODS

The research of the potential capacity for producing trichothecenes and eniatines by *F. sambucinum* was performed using 71 and *F. solani* – using 77 single-spore isolates obtained from dry-rotting potato tubers obtained from different regions of Poland (the following provinces: mazowieckie, kujawsko-pomorskie, warmińsko-mazurskie, wielkopolskie, lubelskie and zachodniopomorskie).

The isolation of fungi from dry-rotting tubers was made with the method described by Kurzawińska (1994) and the identification was made based on the mycological keys.

The single-spore cultures of *F. sambucinum* and *F. solani* were reproduced in Erlenmeyer flasks on liquid PD medium (glucose-potato). The Kolby were shaken (150 rotations/m) by 5-6 days at the room temperature with the rotary shaker.

DNA was isolated with A modified method by Doyle and Doyle (1990). The DNA concentration in the solution was measured spectrophotometrically and then all the samples were made to reach the concentration of 100 $\mu g \times \cdot l^{-1}$.

The species classification of the isolates was verified and their potential mycotoxigenicity was determined with the polymerase chain reaction (PCR) using specific SCAR starters (Table 1). To do so, using *Taq* PCR Core Kit supplied by Qiagen, (the US), A mixture was made which included (the amounts given per reaction): H_2O miliQ – 3.9 µl, solution Q – 2.5 µl, buffer 10x – 1.25 µl, $MgCl_2$ – 0.5 µl, dNTP – 0.25 µl, starter I – 0.75 µl, starter II – 0.75 µl, Polimeraza Taq – 0.1 µl, DNA – 2.5 µl.

Table 1

Primers used in PCR assays

Test	Primer	Sequence (5' – 3')	Source
Fusarium sambucinum	FSF1	5'-ACATACCTTTATGTTGCCTCG-3'	Mishra *et al*., 2003
	FSR1	GGAGTGTCAGACGACAGCT	
Tri5	HA*Tri*/F	CAGATGGAGAACTGGATGGT	Edwards *et al*.,2001
	HA*Tri*/R	GCACAAGTGCCACGTGAC	
*Tri13*DON	*Tri*13F	5'-CATCATGAGACTACTTGTAGTTTGG-3'	Chandler *et al*.,2003
	*Tri*13DONR	5'-GCTAGATCGATTGTTGCATTGAG-3'	
*Tri13*NIV	*Tri*13NIVF	5'-CCAAATCCGAAAACCGCA-3'	
	*Tri*13R	5'-TTGAAAGCTCCAATGTCGTG-3'	
Eniatines	esysam 1	5'-TGATTCTCAACTCCGTCGTTCA-3'	Kulik *et al*., 2007
	esysam 2	5'-CACAGCCTTCATGTTCTTGGG-3'	

The mixture with adequately selected starters was placed in the thermocycler Biometra Uno II and the reaction was performed which involved 30 cycles PCR according to the specific thermal profile parameters (Tables 2 and 3).

Table 2

Parameters of thermal profile

PCR cycle steps	Temperature [^oC]	Time [min]
Initial denaturation	94	2
Denaturation	94	1
Annealing	by Table 3	1
Elongation	72	2
Final extension	72	5
Cooling	4	-

Table 3

Annealing conditions in particular PCR assays

Test	Temperature [°C]
F. sambucinum	60.5
*Tri*5	57.7
*Tri*13DON	61.0
*Tri*13NIV	61.4
Eniatines	53.7

The products obtained as A result of the PCR reaction were separated on 1.4% agar gel in TBE buffer in 110V electric field. The images were recorded with the electronic gel documentation system supplied by Vilber Lourmat and BioCapt software.

To investigate the pathogenicity, there were used 60 single-spore isolates of *F. sambucinum* obtained from dry-rotting potato tubers, derived from various regions of Poland. Potatoes were inoculated with pathogens following the method described by Latus-Ziętkiewicz (1993). The evaluation was made after 30 days of incubation. The diameter of the rot was measured, then the tubers were cut and its depth was measured. The evaluation criterion was made up by the sum mean of the measurements five tubers, minus the mean of the sum of the diameter and depth of the place formed after the tuber inoculation in the control combination.

To verify the content of mycotoxins, tubers inoculated with isolates F_{SA}-05-37, F_{SA}-05-55 and F_{SA}-07-09 which showed A similar pathogenicity were exposed to chemical analyses. The research was performed at the Institute of Experimental Biology, the Department of Physiology and Toxicology of the Kazimierz Wielki's University in Bydgoszcz applying the HPLC-MS/MS method.

RESULTS

To verify the species classification to of *F. sambucinum* isolates researched, the PCR reaction was performed with FSF1 and FSR1 starters. The test showed the presence of the expected product of amplification 315 pz long with 64 isolates researched (Fig. 1). The repeated reaction for the isolates marked with numbers *28, 29, 31, 32, 33, 56, 63* giving A negative or doubtful effect confirmed A lack of product conditioning the classification as *F. sambucinum*.

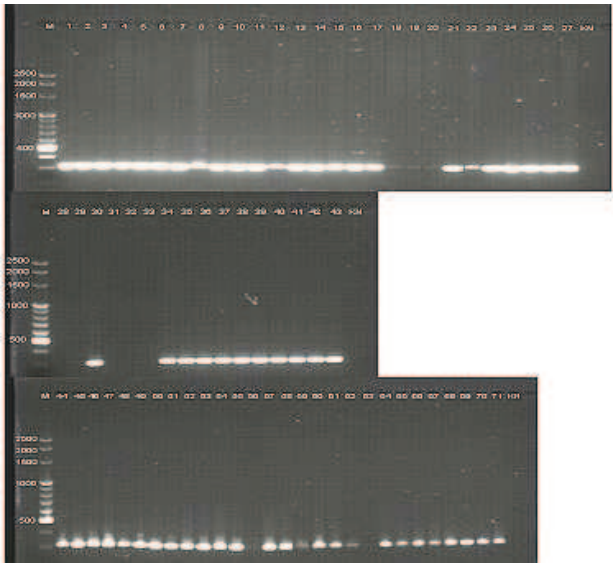

Fig. 1. Confirming the species classification of *F. sambucinum* with the PCR method

The research of the potential capacity for producing trichothecenes was made with the HA*Tri*/F and HA*Tri*/R starters with 64 previously positively verified isolates. In all the cases there was reported A positive result identifying the presence of gene *Tri*5, conditioning the capacity for synthesis of mycotoxins representing the trichothecenes group (Fig. 2).

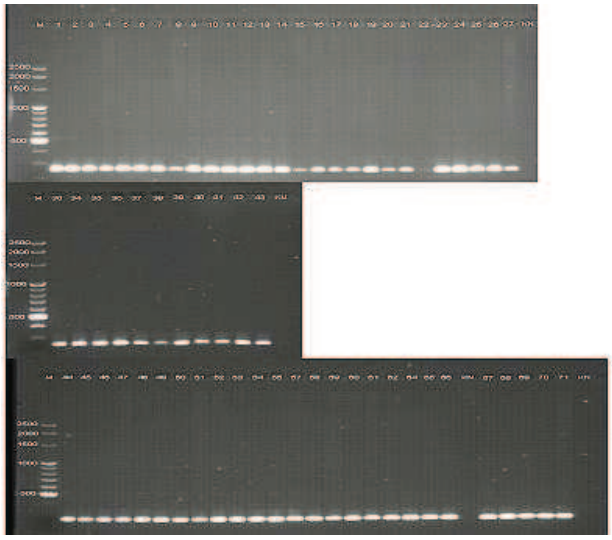

Fig. 2. Molecular analysis of potential capacity for producing trichothecenes in *F. Sambucinum*

Further analyses concerned the kind of the trichothecenes produced. The PCR test was made with starters *Tri*13F and *Tri*13DONR as well as *Tri*13NIVF and *Tri*13R to identify the presence of the gene conditioning the possibility of the synthesis of trichothecenes of B group (DON and NIV). In none of the cases was there recorded the expected product of amplification 282 pz long for *deoxynivalenol* and 312 pz for *nivalenol***.**

The analysis of the potential capacity of 64 isolates of *F. sambucinum* for the synthesis of eniatines showed that in 58 cases there was obtained the product 332 pz long, which points clearly to that property (Fig. 3). Repeated testing of the isolates no *12, 16, 19, 20, 22* and *30* coincided with the earlier reports.

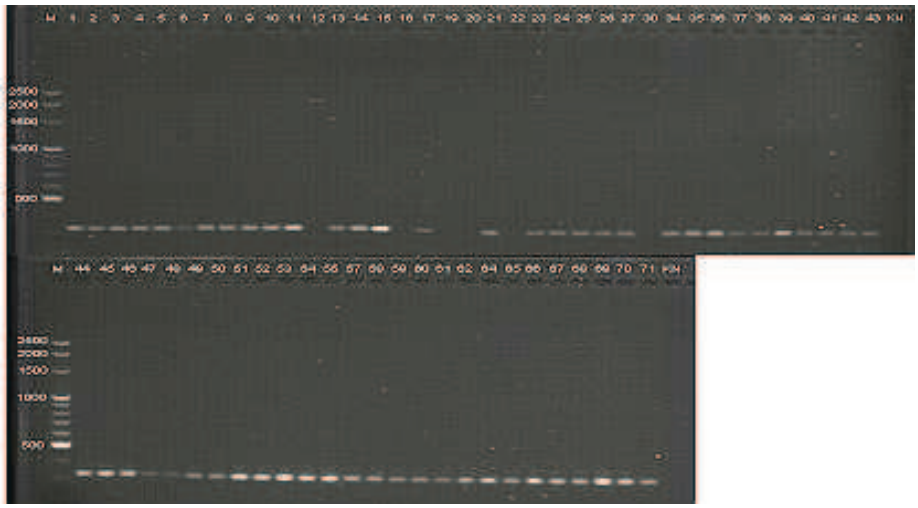

Fig. 3. Molecular analysis of potential capacity for producing eniatines in *F. sambucinum*

The molecular analysis of the potential capacity for producing trichothecenes by *F. solani* isolated from dry-rotting tubers did not identify the presence of the fragment of gene *Tri*5, 260 pz long, which points clearly that none of the isolates was capable for the synthesis of trichothecenes.

The study of the pathogenicity of *F. sambucinum* and *F. solani* demonstrated considerable differences in the extent of the rot caused by respective fungal species.

It was also observed that the isolates of the same species show A varied pathogenicity. Statistical calculations demonstrated that all the isolates of *F. sambucinum* studied caused significantly more extensive disease symptoms of dry rot than *F. solani*. The mean size of the rot caused by *F. sambucinum* isolates was 23.3 mm, while by *F. solani* 3.1mm. There was also found a high variation in the pathogenicity of *F. sambucinum* (Tables 4 and 5).

Table. 4

Average size of the tuber rot caused by *Fusarium sambucinum*

Isolate code		Size [mm]	Isolate code		Size [mm]	Isolate code		Size [mm]
1	F_{SA}-05-54	23.20	21	F_{SA}-05-22	20.07	41	F_{SA}-07-28	16.00
2	F_{SA}-05-26	20.40	22	F_{SA}-05-55	28.73	42	F_{SA}-07-27	22.33
3	F_{SA}-05-51	21.67	23	F_{SA}-05-07	26.27	43	F_{SA}-07-40	28.40
4	F_{SA}-05-52	20.60	24	F_{SA}-05-34	28.27	44	F_{SA}-07-29	21.33
5	F_{SA}-05-53	24.87	25	F_{SA}-05-01	40.27	45	F_{SA}-07-39	27.13
6	F_{SA}-05-57	29.93	26	F_{SA}-05-30	19.13	46	F_{SA}-07-18	23.73
7	F_{SA}-05-25	27.73	27	F_{SA}-06-13	17.07	47	F_{SA}-07-32	19.40
8	F_{SA}-05-28	15.00	28	F_{SA}-06-03	30.27	48	F_{SA}-07-21	24.60
9	F_{SA}-05-36	30.53	29	F_{SA}-06-23	31.80	49	F_{SA}-07-26	10.60
10	F_{SA}-05-37	25.00	30	F_{SA}-06-02	19.87	50	F_{SA}-07-41	23.47
11	F_{SA}-05-38	25.87	31	F_{SA}-06-30	10.33	51	F_{SA}-07-34	15.93
12	F_{SA}-05-39	24.80	32	F_{SA}-06-26	38.40	52	F_{SA}-07-23	27.13
13	F_{SA}-05-35	18.47	33	F_{SA}-06-31	18.33	53	F_{SA}-07-10	19.47
14	F_{SA}-05-62	35.93	34	F_{SA}-06-37	25.87	54	F_{SA}-07-37	13.20
15	F_{SA}-05-42	23.60	35	F_{SA}-07-30	29.73	55	F_{SA}-07-20	18.07
16	F_{SA}-05-43	33.27	36	F_{SA}-07-31	26.67	56	F_{SA}-07-09	32.27
17	F_{SA}-05-40	20.47	37	F_{SA}-07-36	15.47	57	F_{SA}-07-16	32.40
18	F_{SA}-05-33	9.53	38	F_{SA}-07-33	15.00	58	F_{SA}-07-19	28.80
19	F_{SA}-05-45	15.53	39	F_{SA}-07-04	18.53	59	F_{SA}-K-01	15.73
20	F_{SA}-05-31	23.13	40	F_{SA}-07-38	19.27	60	F_{SA}-B-01	27.60

Considering the growing system the isolates were obtained from and the extent of the rot, one can state that the greatest size of the rot was caused by the isolates obtained from tubers originated from organic farming. Similar results were reported from isolates derived from integrated farming. The smallest extent of the rot was reported for the isolates obtained from tubers derived from traditional farming they differed from the isolates from organic farming by 3.83 mm and by 2.45 mm from the integrated farming isolates. The χ^2 test, however, showed that the statistic recorded is lower than the critical value and so no significant differences in the size of rots were recorded (Table 6).

Table 5

Percentage of tubers and pathogenicity of *F. sambucinum*

Number of tubers	Percentage	The size of rot [mm]
1	1.6	0.0 – 9.9
20	33.4	10.0 – 19.9
30	50.0	20.0 – 29.9
9	15.0	> 30.0
The average size of rot		23.33

Table 6

Mean size of the putrid tissue caused by the isolates from tubers grown in various farming systems

Farming system	Rot size [mm]
Organic	26.27
Integrated	24.89
Traditional	22.44

The concentration of mycotoxins does not always depend on the size of the putrid tissue. The chemical analyses demonstrated that in tubers of similar putrid tissue size the content of mycotoxins ca vary. Isolate F_{SA}-05-37 responsible for the rot size of 25mm produced much more *monoacetoxiscirpenol* (MAS) and *diacetoxyscirpenol* (DAS) than isolates F_{SA}-05-55 and F_{SA}-07-09 which caused slightly greater disease symptoms (Table 7).

Table 7

Concentration of mycotoxins in dry-rotting cultivars

Isolate	Rot size [mm]	MAS	DAS
F_{SA}-05-37	25.0	970.0	574.0
F_{SA}-05-55	28.7	79.4	20.4
F_{SA}-07-09	26.3	Not detected	Not detected

DISCUSSION

Tuber dry rot caused by *Fusarium* spp. is an important potato disease not only from the economic perspective but also because of the threat it poses for the human and animal health.

The main disease agents are *Fusarium* fungi capable of producing mycotoxins. Hohn and Desjardins (1992) identified in *F. sambucinum* gene *Tri5* the coding enzyme – the synthesis of trichodiene, the first indirect product in metabolic tracks of the synthesis of trichothecenes, and Edwards *et al.* (2001) confirmed, using PCR, the capacity for producing trichothecenes by *F. sambucinum, F. sporotrichioides, F. graminearum, F. crookwellense, F. culmorum* and *F. poae.*

The identification of *Fusarium* genus fungi based on the morphological characters is very troublesome and doe no ensure the right classification. Identifying the *F. sambucinum* species classification with the PCR method showed highly applicable. The present research showed that 6 of 71 isolates determined based on the available mycological keys did not fall within that species. Further research with those six isolates, determined only with traditional methods, could lead to mistaken results and conclusions.

The research performed with the PCR method demonstrated that all the *F. sambucinum* isolates included gene *Tri*5 and thus they were potentially capable of producing trichothecenes. In the case of *F. solani*, there was observed no amplification product of that gene, which points to A lack of capacity for producing those mycotoxins.

The next research stage concerned the possibility of *F. sambucinum* producing trichothecenes B group: *nivalenol* (NIV) and *deoxynivalenol* (DON). The tests made showed that the isolates showed no capacity for the biosynthesis of those mycotoxins. The result was confirmed by chemical analyses of potato inoculated by *F. sambucinum*. There was found high concentration of A group trichothecenes, especially *monoacetoxiscirpenol* (MAS). In none of the samples was there identified *nivalenol*, d*eoxynivalenol* and its derivatives (3-acetylodeoksyniwalenol, 15-acetylodeoksyniwalenol) and toxin T-2 and toxin HT-2. As far as the present author is aware, so far in Poland there have been made no attempts at defining the potential capacity for producing toxins of *Fusarium* genus fungi isolated from potato with the molecular method.

The toxigenicity of those fungi with the use of thin layer chromatography (HPTLC) was investigated by Latus-Ziętkiewicz (1993), who found that all the isolates of *F. sambucinum* produced A group trichothecenes, especially *diacetoxyscirpenol* (DAS), however, they did not produce group B trichothecenes. She showed, similarly as in this research, that *F. solani* does not produce trichothecenes.

Investigations of the production of mycotoxins by *Fusarium* genus fungus are not clear and trigger some doubts. El-Banna *et al.* (1984) report on *F. solani*

var. *coeruleum* and *F. sambucinum* species being capable of producing trichothecenes both representing A group (HT-2), and B group (DON, ADON, NIV). They also found that *F. solani* var. *coeruleum* can produce *deoxynivalenol* (DON) on potatoes and in liquid medium. They also observed that the concentration toxins in the tubers infested, stored at the same temperatures, varied.

Desjardins and Plattner (1989) claim that *F. sambucinum* can produce A group trichothecenes, mainly DAS, at lower amounts – MAS, T-2 toxin, neosolaniol. JELEŃ *et al.* (1995) in all the tubers inoculated with various isolates of *F. sambucinum* identified group A trichothecenes (DAS, 15-MAS, 4-MAS), however, their content varied depending on the isolate**.** Earlier Ripperger *et al.* (1975) and Steyn *et al.* [1978] showed that *F. solani* and *F. sambucinum* produce only A type *trichotecenes* (DAS), however, they do not produce B type toxins.

Interestingly, not all the isolates of the species considered potentially mycotoxigenic must produce them. It depends on genetic properties of A given strain [Ward *et al.* 2002] and environmental conditions of infestation, including temperature, humidity and the content of nutrients in the substrate (Mateo *et al.* 2002).

Potatoes infected by *F. sambucinum* pose A potential threat for the health of animals fed with them (Desjardins and Plattner 1989). The contaminated feed consumed can cause poisoning referred to as *fusariotoxicoses***.** It is commonly known poorer quality tubers, also with rot symptoms, are fed to farm animals, mostly pigs, especially sensitive to the effect of trichothecenes. There was observed their unfavourable effect on the growth and development of piglets as well as A negative effect on the immune and circulatory systems. In the animal tissues fed with contaminated feed there occurs an accumulation of toxins which can then reach the consumer in A form of contaminated meat (Rotter *et al.* 1992; Rafai *et al.* 1995).

CONCLUSIONS

1. Researching the biology and the properties of fungi, it is necessary to verify the identification with molecular methods. With A considerable similarity of fungi, using traditional methods only can lead to mistaken results and conclusions. In the present research, as part of this paper, it was shown that 2 out of 28 isolates, determined based on the available mycological keys, did not belong to *F. sambucinum* species.
2. *Fusarium sambucinum* has A potential capacity for the production of trichothecenes. All the isolates investigated with the PCR method were identified with gene *Tri5*, conditioning their synthesis. Chemical analyses of the inoculated tubers showed high concentration of *monoacetoxiscirpenol* (MAS) and the presence of *diacetoxyscirpenol*

(DAS). The trichothecenes synthesis property was not identified in *F. solani*.

3. The *F. sambucinum* isolates studied did not demonstrate A potential capacity for the synthesis of group B trichothecenes (DON and NIV). In none of the cases was there reported an expected product of amplification 282 pz long for *deoxynivalenol* and 312 pz long for *nivalenol***.**
4. Potatoes even with slight symptoms with dry rot of tubers should not be used to animal feed as potatoes can be identified with fusarium toxins of trichothecenes group. The content of those toxins does not always depend on the extent of disease symptoms.

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