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Diversity of *Fusarium* spp. associated with dry rot of potato tubers in Poland

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Abstract *Fusarium* spp. belong to the division
Ascomycota and cause important plant diseases; these
fungi may contaminate food products with mycotoxins,
endangering human and animal health. Several *Fusarium*
spp. have been associated with potato dry rot. The most
frequent and devastating of these species are
F. sambucinum, *F. solani* and *F. oxysporum*, depending
on the geographic location and the season. Samples of
potato tubers with dry rot symptoms were collected, and
their putative fungal isolates were identified as *Fusarium*
species using partial nucleotide sequences of the internal
transcribed spacer, *translation elongation factor 1-α* and
β-tubulin genes. Among 149 isolates, 12 species were
identified. *F. oxysporum* was the most frequent (45 % of
the isolates), followed by *F. avenaceum* (12.1 %),
F. solani (10.7 %) and *F. sambucinum* (7.4 %).
Phylogenetic analyses confirmed the species identifica-
tions and revealed a high diversity of *F. solani* and a low
diversity of *F. oxysporum*. Potential producers of
zearalenone and trichothecenes were identified within
the obtained isolates using PCR markers. Isolates that
were pathogenic to potatoes in laboratory tests were found
in four species: *F. sambucinum*, *F. avenaceum*,
F. culmorum, and *F. graminearum*. The effects of in-
creased temperature and mixed inoculum on the pathoge-
nicities of chosen species were evaluated. This study adds
434 potato-derived *Fusarium* sequences to the NCBI
GenBank database and demonstrates that the list of
Fusarium species and mycotoxins present in potato tubers
may be richer than previously believed, regardless of
whether these species cause dry rot or live as saprophytes.

Keywords ITS · mycotoxin · pathogenicity · *Solanum
tuberosum* · *tef-1α* · *β-tubulin*

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Introduction

Potato (*Solanum tuberosum* L.) is a member of the
Solanaceae family and is a highly nutritious crop grown
worldwide. Potatoes are particularly valued in develop-
ing countries as a rich source of starch, vitamins C and
B6 and essential amino acids. In 2013, over 376 million
tons of potatoes were produced worldwide (FAOSTAT
2014) and stored for various lengths of time to provide a
continuous supply of this staple food to consumers and
industry. During long-term storage, potato tubers are
vulnerable to many diseases. Possible injuries to the
potato skin during harvesting can lead to, among other
problems, potato dry rot, a disease caused by species of
the genus *Fusarium* (Nelson et al. 1981). Dry rot, char-
acterized by variously coloured cavities and hard, wrin-
kled skin, causes yield losses of up to 60 % during

storage (Theron 1991), with average losses of 6 to 25 % (Chełkowski 1989).

Fusarium spp. belong to the division Ascomycota and occur in asexual and sexual (perfect) states. The discovery of perfect state can result in a species receiving a new genus and species name, which may cause confusion in the nomenclature. *Fusarium* species exhibiting such perfect stages belong to the genera *Calonectria* de Not., *Gibberella* Sacc., and *Nectria* Fries (Nelson et al. 1981), but these species are generally better known by their imperfect state names. Sexual states of many *Fusarium* have not yet been discovered and maybe never will, since some of them appear to have lost their ability for sexual reproduction (Nelson et al. 1981). A single-name system proposed by Geiser et al. (2013) unified the current usage of the name *Fusarium* and is used in this work.

The *Fusarium* spp. that colonize potato tubers vary depending on geographic location and time of the survey. *F. sambucinum* was the species most frequently isolated from potato tubers among eight species recovered in 1992–1993 in northeastern USA (Hanson et al. 1996), but also more recently in China (Du et al. 2012). *F. oxysporum* was reported to be the primary agent responsible for potato dry rot amidst 11 species in Michigan, USA (Gachango et al. 2012) and South Korea (Kim and Lee 1994). *F. avenaceum* dominated potato *Fusarium* population investigated between 1997 and 2000 in Scotland (Choiseul et al. 2007), while *F. solani* var. *coeruleum* dominated in four regions of Great Britain, including eastern Scotland, in 2000–2002 (Peters et al. 2008a). Globally, 13 *Fusarium* species are associated with potato dry rot (Cullen et al. 2005). However, this list can be extended to include *F. ciliatum*, *F. reticulatum*, *F. torulosum* and *F. verticillioides* (Kim and Lee 1994; Choiseul et al. 2007; Gachango et al. 2012) and most likely is still not complete. The pathogenicities of *Fusarium* spp. to potatoes differ considerably (Peters et al. 2008b) and the presence of less pathogenic species on dry rot-infected tubers might be opportunistic.

Dry rot in potato not only reduces the crop yield but can also contaminate the tubers with mycotoxins that cause cyto-, geno-, neuro- and hepatotoxic effects in animals and humans and thus threaten health when eaten directly (in *Fusarium*-infected plants) or indirectly (in the milk or meat of animals that have been fed with infected feed). The major types of mycotoxins produced by *Fusarium* spp. include beauvericin, moniliformin,

fumonisins, trichothecenes and zearalenone, with the latter two considered the most important due to their common occurrence. Trichothecenes are produced by *F. culmorum*, *F. graminearum*, *F. poae*, *F. cerealis*, *F. langsethiae*, *F. sporotrichioides* and *F. sambucinum*, among other species (Nicholson et al. 2004), and zearalenone is produced by *F. culmorum*, *F. graminearum*, *F. cerealis* and *F. equiseti* (Stępień 2013). The potential to produce mycotoxins might be established by confirmation of the presence of *Tri* cluster (trichothecenes) and *PKS* (polyketide synthases) genes (zearalenone; Baturo-Cieśniewska and Suchorzyńska 2011). However, PCR-based assays are only a weak indicator of potential to produce mycotoxins. The actual production of mycotoxins is a complex process involving many enzymes and diverse mutations of genes coding these enzymes or their regulators may affect the final mycotoxin profile of the fungal strain. Many of these mutations may be not detected by simple PCR markers. Moreover, the production of mycotoxins may change depending on environmental conditions (Mateo et al. 2002) and the susceptibility of a given potato cultivar (Xue et al. 2014).

The biological role of *Fusarium* spp. in potato tubers either as dry rot agents, secondary pathogens or saprophytes is difficult to estimate, because even within species considered as the dry rot agents, non-pathogenic isolates were found and isolates of different species cause lesions of different sizes, i.e. isolates of *F. sambucinum* have caused significantly larger lesions than the isolates of other tested species (Gachango et al. 2012). In potato dry rot samples, *Fusarium* isolates that are non-pathogenic to potato in the laboratory assessment are frequently identified (Peters et al. 2008a; Peters et al. 2008b). Therefore, it is interesting to know which species are present in potato tubers regardless of their role, so the risk of possible mycotoxin contamination can be estimated.

Knowledge of the present European *Fusarium* population that is attacking potatoes is limited. Only one survey investigated Central Europe (Latus-Ziętkiewicz et al. 1987). According to this study, in 1985–86, *F. sambucinum* Fuckel was the main pathogen causing dry rot in Poland. Other reports have focused on intraspecific studies of certain *Fusarium* species, e.g., *F. culmorum* (Baturo-Cieśniewska and Suchorzyńska 2011), *F. equiseti* (Stępień et al. 2012) and *F. sambucinum* (Baturo-Cieśniewska et al. 2014).

The objectives of the present study were to: (1) identify the *Fusarium* species isolated from dry rot-

infected potato tubers using the ITS (internal transcribed spacer), *tef-1α* (*translation elongation factor 1-α*) and *bTub* (*β-tubulin*) genes; (2) evaluate their pathogenicities; (3) investigate their intraspecific diversity; and (4) examine the toxigenic potentials of the obtained isolates.

Materials and methods

Sampling and isolation of pure fungal cultures

Potato tubers with dry rot symptoms were collected in 2012–2014 from central (Chwałowice, Młochów, Zazdrość), western (Naroczyce, Świebodzin) and northern locations in Poland (Zamarte). The exact dates of isolation and descriptions of the potato stocks from which the isolates were extracted are listed in Supplementary Table 1. Together, 14 potato cultivars were sampled grown from 13 independent seed stocks. Cultivar Denar was grown in two locations: Chwałowice and Młochów in 2012, cv. Jelly was grown in Młochów in 2010 and in Świebodzin 2013 and the remaining 12 cultivars were planted only once (Supplementary Table 1). Each cultivar sample consisted of 10 dry rot infected tubers that yielded from one to five *Fusarium* isolates, each one from a different tuber. 101 *Fusarium* spp. isolates were recovered from breeding lines. Different breeding lines were grown in Chwałowice, Młochów, Naroczyce and Zamarte, each one only at one location. Breeding lines were at the early stage of breeding and grown in the places of origin, providing information about *Fusarium* population associated with locations but not with the stock. Additionally, fungi were isolated from three imported potato tubers exhibiting disease symptoms that were bought in a local grocery store. We also included one isolate of unknown origin that was isolated in 1999 and 24 collected in 2011 from potatoes grown in Młochów. A total of 149 *Fusarium* isolates were analysed (Supplementary Table 1).

To obtain pure fungal cultures, 0.5 × 0.5 × 0.5 cm fragments of the tuber were taken from the borders of the healthy and diseased tissues and then disinfected in 70 % ethanol and 0.1 % mercuric chloride. The inoculum was rinsed in a sterile water and further incubated at 16 °C on potato sucrose agar (PSA) medium (Zarzycka 2001, modified). The conidia from a developed *Fusarium* colony were preselected via microscopic observations and spread on a thin layer of water agar

Table 1 List of genes used in this study with the concentrations and conditions of the corresponding PCR assays, product sizes and literature sources. All reaction mixtures contained 0.5–1.25 U *Taq* Polymerase, PCR buffer and 10–50 ng of template DNA

Marker	PCR mix	Primers 5' → 3'	Ta	Product size	Reference
ITS	0.85 µM primers, 0.1 mM dNTPs	GGAAGTAAAAGTCGTAACAAGG TCCTCCGCTTATTGATATGC	55 °C	568 bp	White et al. 1990
TEF	0.5 µM primers, 0.2 mM dNTPs	ATGGGTAAGGARGACAAGAC GGARGTACCAGTSATCATGTT	60 °C	713 bp	O'Donnell et al. 1998
bTub	0.2 µM primers, 0.25 mM dNTPs	AACATGCGTGAGATTGTAAGT GACCGGGGAAACGGAGACAGG	55 °C	1015 bp	O'Donnell and Cigelnik 1997
Tri5	0.2 µM primers, 0.1 mM dNTPs	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCTG	60 °C	545 bp	Nicholson et al. 2004
PKS4	0.2 µM primers, 0.1 mM dNTPs	CGTCTTCGAGAAGATGACAT TGTTCTGCAAGCACTCCGA	60 °C	~280 bp	Meng et al. 2010

medium using the inoculation loop. The next day, single germinating spores were moved to PSA medium.

PCR and sequencing

Single-spore cultures were moved to liquid potato sucrose medium. The mature fungal colonies were transferred to 1.5 mL Eppendorf tubes and freeze dried. The mycelia were ground to powder using a microtube pestle, and DNA was isolated using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The DNA of each isolated fungus was used for the PCR amplifications of the ITS, TEF and bTub markers for species identification (Table 1) and the amplification of markers of the ability to produce trichothecenes (Tri5) and zearalenone (PKS4). The ITS marker covers the partial 18S, 28S and complete sequence of 5.8S ribosomal RNA genes as well as full sequence of the internal transcribed spacers 1 and 2; TEF and bTub markers cover approximately 40 % and 60 % of corresponding genes sequences, respectively (including introns). The PCR reactions with the ITS (ITS5/ITS4), TEF (EF-1/EF-2) and bTub primers (T1/T222) were performed in 40 μL and assays with Tri5 (Tri5F/Tri5R), and the reactions with the PKS4 primers (PKS 4-2-F1/PKS 4-2-R1) were performed in 20 μL reactions under the conditions presented in Table 1. The quantities and qualities of the PCR products were examined on 1.5 % agarose gels stained with ethidium bromide. The products obtained with the ITS, TEF and bTub primers were purified with a GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. When more than one band was present, the product of the expected size was cut out from the gel under UV light and extracted with a GenElute Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). DNA sequencing was performed by an external company (Genomed S. A, Warsaw, Poland).

Phylogenetic analysis

The qualities of the retrieved sequences were evaluated with ChromasLite 2 software. The sequences obtained with the forward and reverse primers were used to prepare the consensus sequences using MultAlin online software (Corpet 1988). The sequences of ITS, *tef-1α* and bTub from the isolates examined in this study were queried against NCBI GenBank, the Fusarium-ID

(Geiser et al. 2004) and *Fusarium* MLST (O'Donnell et al. 2012) databases using the megablast algorithm, BLOSUM62 matrix and pairwise sequence alignment, respectively, for the species identification. The species from which the closest matching sequences (i.e., the lowest *e* value and the greatest identity and maximum coverage) originated were considered to be the identified species of the query.

A dataset for each isolate consisting of three gene regions was aligned using the ClustalW alignment method in MEGA6 software (Tamura et al. 2013) and trimmed manually to the same length when needed. The same software was used to analyse the phylogenetic relationships using the maximum parsimony (MP) approach with the "gaps ignore" option. The most parsimonious trees were evaluated by bootstrapping with 1000 replications. The trees were prepared with two separate input data sets. The first data set contained the combined ITS, TEF and bTub sequences of 73 *Fusarium* isolates with *Colletotrichum gloeosporioides* (MAFF237919) as outgroup. Only five *F. oxysporum* isolates of various origins were used in this data set because *F. oxysporum* was recovered most often and the large number of sequences of this species might have disturbed the tree readability. The second data set used for MP tree preparation consisted of multilocus sequences of 65 *F. oxysporum* isolates with the MF1 *F. sambucinum* isolate used as outgroup.

Pathogenicity tests

Single-spore fungal cultures were grown on PSA for three weeks at 16 °C. The spores were rinsed from the culture with sterile water and used to prepare the inocula. The concentration was adjusted to 2.5×10^6/mL using a haemocytometer.

Pathogenicity tests were performed on 119 *Fusarium* isolates: *F. oxysporum* ($N = 44$); *F. avenaceum* (17); *F. solani* (15); *F. sambucinum* (10); *F. cerealis* (7); *F. culmorum*, *F. graminearum* and *F. tabacinum* (5 isolates each); *F. redolens* (4); *F. venenatum* (3); single isolates of *F. equiseti* and *F. merismoides*; and two unidentified *Fusarium* isolates (M87A and M115). Additionally, *Ilyonectria* sp. and *Colletotrichum* sp. isolates from dry rot-infected tubers were used in tests to evaluate their potential role in the development of disease. Each test was performed with two replications. In each replication, five tubers from four potato cultivars (cvs.) were inoculated with fungal isolates.

Table 2 List of the species identified and grouped according to their origins. The section 'other' contains three locations, i.e., Naroczyce, Świebodzin and Zazdrość, and three isolates from imported potato tubers bought in a grocery store (imported from unidentified countries)

Species	Place of origin				Sum	% of all
	Młochów	Chwałowice	Zamarte	Other		
F. avenaceum	4		11	3	18	12.1
F. cerealis	2		1	4	7	4.7
F. culmorum	8				8	5.4
F. equiseti			1		1	0.7
F. graminearum	3		1	1	5	3.4
F. merismoides				1	1	0.7
F. oxysporum	47	15	4	1	67	45
F. redolens	2	2	1		5	3.4
F. sambucinum	9			2	11	7.4
F. solani	7	3	5	1	16	10.7
F. tabacinum			4		4	2.7
F. venenatum		1	2		3	2
Unidentified *Fusarium*	3				3	2
Sum	85	21	30	13		
	146 isolates of **12** *Fusarium* species +3 unidentified					

Limited information on the cultivars' resistance to dry rot was available. The Hinga (starch, late) and Kuba (starch, mid-early) cultivars have been described as moderately resistant to dry rot, with a grade of 6 on a 1–9 scale, in which 9 indicates the greatest resistance (Boguszewska et al. 2011). The resistances of the cvs. Bartek (table, mid-early) and Gawin (table, mid-early) were unknown. The apical ends of the tubers were wounded with a specially prepared tool tipped with a metal cylinder to cause a wound of 10 mm in depth and 5 mm in diameter. Fifty microlitres of inoculum was pipetted into the wound and incubated for four weeks at 16 °C in a plastic box covered with glass to maintain the humidity. To test whether the temperature affected the pathogenic capacity, a subgroup of 23 fungal isolates were tested with four replications in which two were incubated at 16 °C and two were inoculated at 20 °C. The same setup was also used to test how co-infection influenced pathogenicity. For these tests, equal amounts of spore suspensions (2.5×10^6/mL) of *F. oxysporum* isolate Z157 were combined with other *Fusarium* species in the following three mixes: Mix 1 contained Z155B (*F. avenaceum*), Mix 2 contained Z174A (*F. graminearum*), and Mix 3 contained Z162 (*F. solani*). After the incubation period, the tubers were cut in half along their longer axes and two perpendicular diameters of the lesions were measured. The lesion size was calculated for each tuber as the mean of the two diameters. Statistical analyses were performed using Statistica 10 software (Statsoft Inc., 2011). Analyses of variance were performed to test the

Table 3 *Fusarium* spp. identified in potato dry rot samples and grouped according to the results of the PCR assays with the Tri5 and PKS4 markers to indicate the potential abilities to produce trichothecenes and zearalenone, respectively

Product presence		*Fusarium* spp.
Tri5	PKS4	
−	−	*F. avenaceum*, *F. merismoides*, *F. oxysporum*, *F. redolens*, *F. solani*, *F. tabacinum*, M87A, M115 and M93
+	−	*F. sambucinum* and *F. venenatum*
+	+	*F. cerealis*, *F. culmorum*, *F. equiseti* and *F. graminearum*

effects of the species, cultivar and their interaction as well as isolate, temperature and their interaction on pathogenicity. Post-hoc Tukey tests were applied to prepare homogenous groups of *Fusarium* spp. according to their pathogenicities. Together, each isolate or isolate mixture was tested on five tubers of four potato cvs. in two replicates.

Accession numbers

The sequences of the ITS, *tef-1α* and *bTub* genes of the strains used in this study were submitted to NCBI GenBank, and their accession numbers are listed in Supplementary Table 1. The alignments and trees are deposited in TreeBASE (http://www.treebase.org/): accession 17,844.

Results

Identification of the *Fusarium* species and mycotoxin production potential

In 2012–2014, 352 potato tubers with dry rot symptoms were collected from Chwałowice (66 tubers), Młochów (139), Naroczyce (50), Świebodzin (5), Zamarte (86), Zazdrość (3) and a local store (3). From the diseased tubers, 121 *Fusarium* spp. isolates were recovered and identified. An additional 25 isolates collected in 1999 (an MF1 isolate of unknown origin) and in 2011 (24 isolates collected in Młochów) were included in the analyses. Despite the use of three genes for species identification, three isolates remained unidentified (ITS acc. Nos.: M87A – KP295503, M115 – KP265344 and M93 – KP265346) because no relevant identified reference sequences were available in neither database browsed. Though the TEF sequences of M87A and M115 isolates shared around 92 % similarity with an *F. virguliformae* isolate, these findings found no confirmation in either ITS or bTUB sequences. In total, 149 fungal isolates were collected (Table 2, Supplementary Table 1). Additionally, single isolates of fungal species belonging to *Colletotrichum*, *Rhizoctonia*, *Ilyonectria* and *Alternaria* genera were also recovered (data not shown). The most abundant *Fusarium* species, which was present at all of the surveyed locations, was *F. oxysporum*, with 67 isolates that comprised 45 % of all of the species identified. *F. oxysporum* was most frequently recovered in Młochów (47 of 85 isolates) and in Chwałowice (15 of 21), where it accounted for

55 and 71 % of the *Fusarium* populations, respectively. The dominant species in Zamarte was *F. avenaceum*, with 11 of 30 (37 %) isolates belonging to this species. The least frequent were *F. merismoides* and *F. equiseti*, of which only single isolates were found. The most diversified *Fusarium* population was in Zamarte, where nine *Fusarium* spp. were found, whereas only four species were identified in Chwałowice. In total, 149 ITS, 141 TEF and 144 bTub sequences were deposited in NCBI GenBank.

The isolates are listed in Table 3 according to their potential abilities to produce trichothecenes and zearalenone. PCR assays were applied to divide the tested *Fusarium* species into the following three groups: 1) species unable to produce the tested mycotoxins (both markers absent), 2) potential producers of trichothecenes only, and 3) producers of trichothecenes and zearalenone (both markers present; Table 3). No intraspecific variations in the presence of the Tri5 and PKS4 markers were detected. No species that produced only zearalenone were observed.

Phylogeny

To illustrate the relationships among the 10 *Fusarium* spp. from potato (73 isolates including five *F. oxysporum* isolates), an MP tree was constructed (Fig. 1), with a consistency index (CI) of 0.71 and a retention index (RI) of 0.95. The phylogenetic reconstruction revealed six main clades. The trichothecene and zearalenone producers comprised clade one (*F. cerealis*, *F. culmorum* and *F. graminearum*; branch support value of 99 %) and shared a common ancestor with the trichothecene-only producers *F. sambucinum* and *F. venenatum*. An independent branch was formed by a single *F. equiseti* isolate that was also capable of synthesizing both of the examined mycotoxins. A separate branch was formed by the *F. avenaceum* isolates (99 % bootstrap value). Another clade was formed by the *F. redolens* and *F. oxysporum* species. The *F. solani* isolates with two unidentified isolates (M87A and M115) were clustered together with a 99 % bootstrap value. An independent branch was formed by the unidentified isolate M93.

The intraspecific diversity amongst the *F. oxysporum* isolates was investigated in a separate analysis that resulted in the MP tree shown in Fig. 2 (CI = 0.99, RI = 0.98). The intraspecific variation among the 65 tested isolates appeared to be low. No clear groups
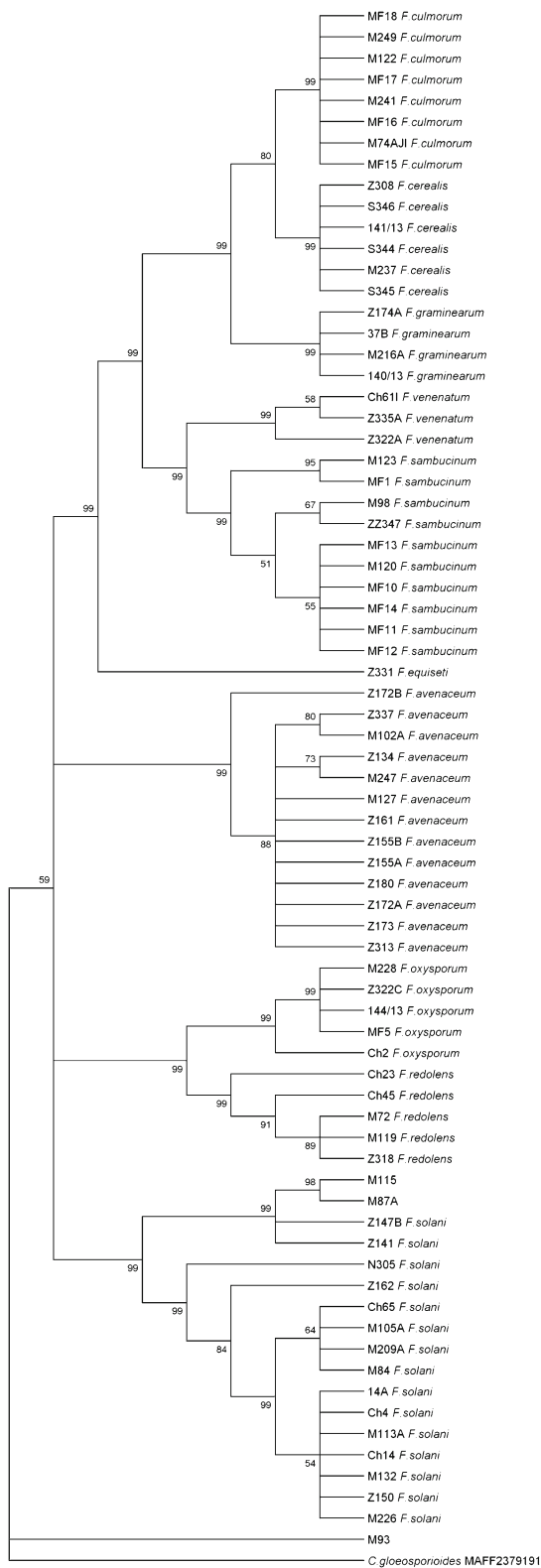
◀ **Fig. 1** Maximum parsimony tree obtained using the combined ITS, TEF and bTub sequences of 73 isolates belonging to 10 *Fusarium* species. The tree consisting of 1315 sites (391 parsimony-informative) was tested by bootstrapping (1000 replicates) with a cut-off value of 50 %. *C. gloeosporioides* was used as outgroup. TreeBASE accession number: 17,844

distinguishing isolates with different origins were observed. Isolate 144/13 of unknown origin exhibited no divergence and was grouped with the Polish isolates.

Pathogenicity of *Fusarium* spp.

An analysis of variance revealed significant influences of species, potato cultivar and the interaction of species × cultivar on the size of the lesion (Supplementary Table 2). Among the tested fungal species, *F. sambucinum* was the most pathogenic (Supplementary Table 3) and produced average lesions of 17.0 mm on the cv. Bartek, 19.7 mm on cv. Gawin, 29.8 mm on cv. Hinga and 19.7 mm on cv. Kuba (10 isolates; Fig. 3). Lesion sizes less than 13 mm did not exceed the size of the mechanical wound; thus this size was taken as the threshold between pathogenicity and non-pathogenicity. Based on this assumption, pathogenic isolates were found for *F. sambucinum* (9/10 isolates), *F. avenaceum* (2/17), *F. culmorum* (1/5) and *F. graminearum* (2/5; Supplementary Table 4). The average lesion size caused by *F. avenaceum* was 10.9 mm, whereas the two pathogenic isolates developed considerably larger lesions of 17.5 and 16.4 mm. One *F. culmorum* (lesion size: 13.1 mm) and two *F. graminearum* (lesions of 15.1 and 14.1 mm) isolates were significantly more pathogenic than the other isolates that belonged to these species. In contrast, the majority of the *F. sambucinum* isolates were pathogenic to the potato and caused an average lesion of 21.6 mm. Only one *F. sambucinum* isolate caused an average lesion size that was considerably lower, at 12.1 mm (Supplementary Table 4).

Resistance of potato cultivars

The lesion sizes on four different potato cultivars, calculated as averages from 37 isolates from four of the most pathogenic *Fusarium* species, are shown in Fig. 4. Cv. Gawin was the most resistant cultivar, with an average lesion size of 12.2 mm. Cvs. Kuba and Bartek exhibited statistically similar resistance to the *Fusarium* spp. tested, with average lesion sizes of 13.1 and 13.7 mm, respectively. The most susceptible cv., Hinga, exhibited an average lesion size of 17.0 mm.
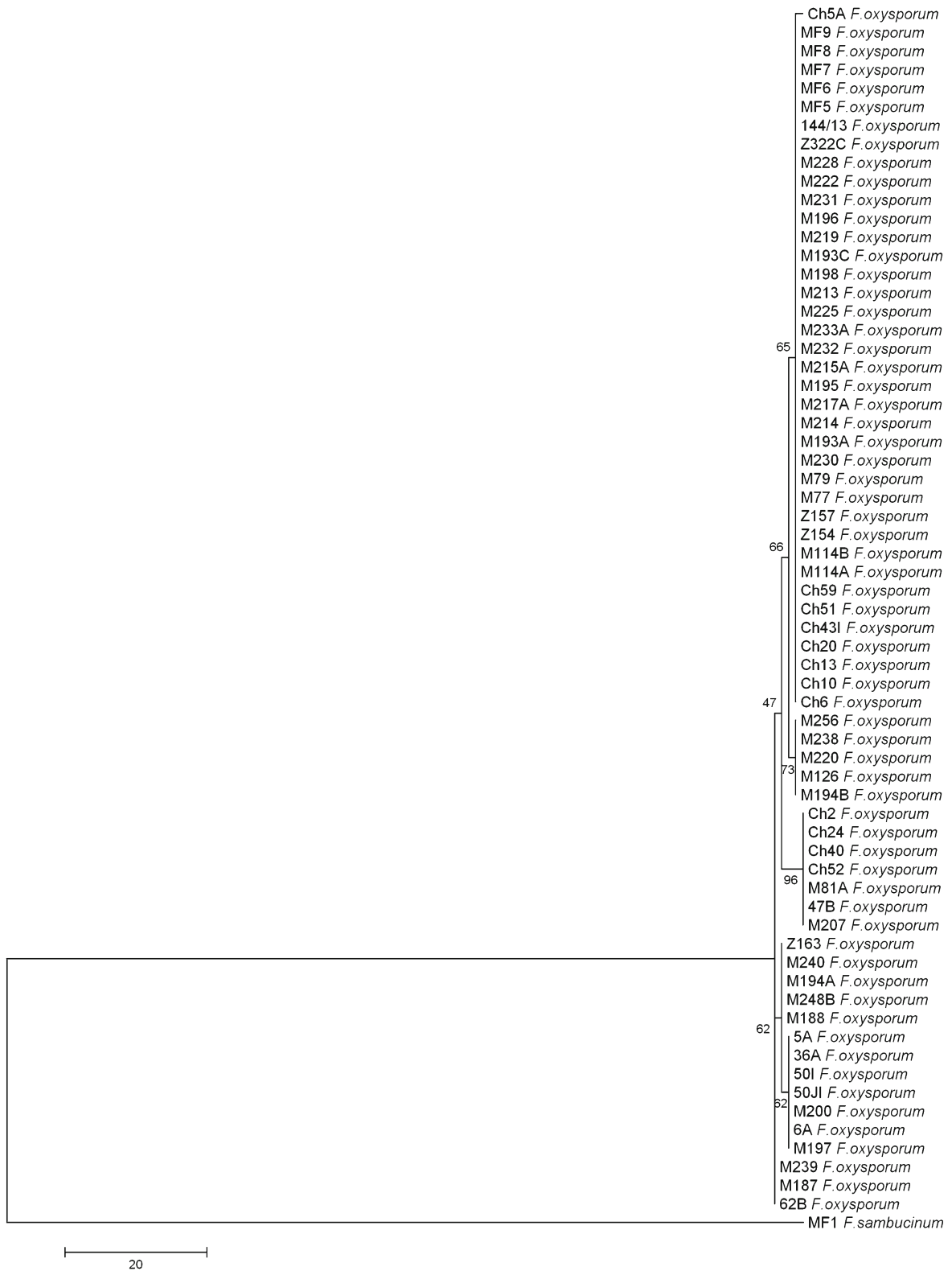
Ch5A *F.oxysporum*
MF9 *F.oxysporum*
MF8 *F.oxysporum*
MF7 *F.oxysporum*
MF6 *F.oxysporum*
MF5 *F.oxysporum*
144/13 *F.oxysporum*
Z322C *F.oxysporum*
M228 *F.oxysporum*
M222 *F.oxysporum*
M231 *F.oxysporum*
M196 *F.oxysporum*
M219 *F.oxysporum*
M193C *F.oxysporum*
M198 *F.oxysporum*
M213 *F.oxysporum*
M225 *F.oxysporum*
M233A *F.oxysporum*
65 M232 *F.oxysporum*
M215A *F.oxysporum*
M195 *F.oxysporum*
M217A *F.oxysporum*
M214 *F.oxysporum*
M193A *F.oxysporum*
M230 *F.oxysporum*
M79 *F.oxysporum*
M77 *F.oxysporum*
Z157 *F.oxysporum*
Z154 *F.oxysporum*
66 M114B *F.oxysporum*
M114A *F.oxysporum*
Ch59 *F.oxysporum*
Ch51 *F.oxysporum*
Ch43I *F.oxysporum*
Ch20 *F.oxysporum*
Ch13 *F.oxysporum*
Ch10 *F.oxysporum*
47 Ch6 *F.oxysporum*
M256 *F.oxysporum*
M238 *F.oxysporum*
M220 *F.oxysporum*
73 M126 *F.oxysporum*
M194B *F.oxysporum*
Ch2 *F.oxysporum*
Ch24 *F.oxysporum*
Ch40 *F.oxysporum*
Ch52 *F.oxysporum*
96 M81A *F.oxysporum*
47B *F.oxysporum*
M207 *F.oxysporum*
Z163 *F.oxysporum*
M240 *F.oxysporum*
M194A *F.oxysporum*
M248B *F.oxysporum*
M188 *F.oxysporum*
62 5A *F.oxysporum*
36A *F.oxysporum*
50I *F.oxysporum*
50JI *F.oxysporum*
62 M200 *F.oxysporum*
6A *F.oxysporum*
M197 *F.oxysporum*
M239 *F.oxysporum*
M187 *F.oxysporum*
62B *F.oxysporum*
MF1 *F.sambucinum*

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Fig. 2 Maximum parsimony tree created for the combined ITS, TEF and bTub sequences of 65 isolates of *F. oxysporum*. The tree consists of 1445 sites, of which 10 were parsimony informative. Bootstrap support values >50 % are shown at the nodes. The names of the isolates encode their places of origin: Ch – Chwałowice; M, MF or numbers only – Młochów; Z – Zamarte; and imported isolate 144/13. TreeBASE accession number: 17,844

Influence of temperature on pathogenicity

The pathogenicities of 23 isolates of 5 *Fusarium* species, 1 isolate of *Ilyonectria* sp. and three mixtures of isolates of *Fusarium* spp. were tested at 20 °C and 16 °C. The average lesion sizes after incubation at both temperatures were compared, and the results are shown in Fig. 5. The analysis of variance revealed that temperature, isolate and the temperature × isolate interaction exerted significant effects on the lesion size ($p = 0.005$; Supplementary Table 5). Post-hoc Tukey tests revealed significant differences between the tested temperatures and indicated that 16 °C (mean lesion size: 10.8 mm) was a more suitable incubation temperature than 20 °C for disease progression (mean lesion size: 10.1 mm). However, three of the isolates were more pathogenic at 20 °C, i.e., Z173, Z155B (*F. avenaceum*), and Z162 (*F. graminearum*), as were the isolates in mixes 1 (*F. oxysporum* + *F. avenaceum*) and 2 (*F. oxysporum* + *F. graminearum*).

Discussion

ITS sequences are frequently used to identify fungal species, and vast numbers of these sequences are already available in databases. However, as reported by O'Donnell and Cigelnik (1997), the existence of non-orthologous copies of these sequence in some clades may lead to erroneous phylogenies. Errors that resulted in incorrect annotations of some ITS sequences may have been propagated over time in the databases (Nilsson et al. 2014). Hence, in addition to ITS, we sequenced fragments of two genes, i.e., the single-copy *translation elongation factor 1-α* (TEF) gene, which typically displays a high level of sequence polymorphism (O'Donnell et al. 1998), and *β-tubulin* (O'Donnell and Cigelnik 1997). Multilocus sequence fragments consisting of these three genes (i.e., ITS, *tef-1α*, *bTub*) were analysed to resolve the relationships among the species and to provide information regarding the intraspecific variation. Among the 12 recovered species, eight (*F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. sambucinum* and *F. solani*) were in the group that has been globally associated with dry rot in potatoes (Cullen et al. 2005), and two (*F. merismoides* and *F. redolens*) had previously been reported to be pathogenic to potatoes (Førsund 1980; Salami and Popoola 2007). No reports on the pathogenicities of
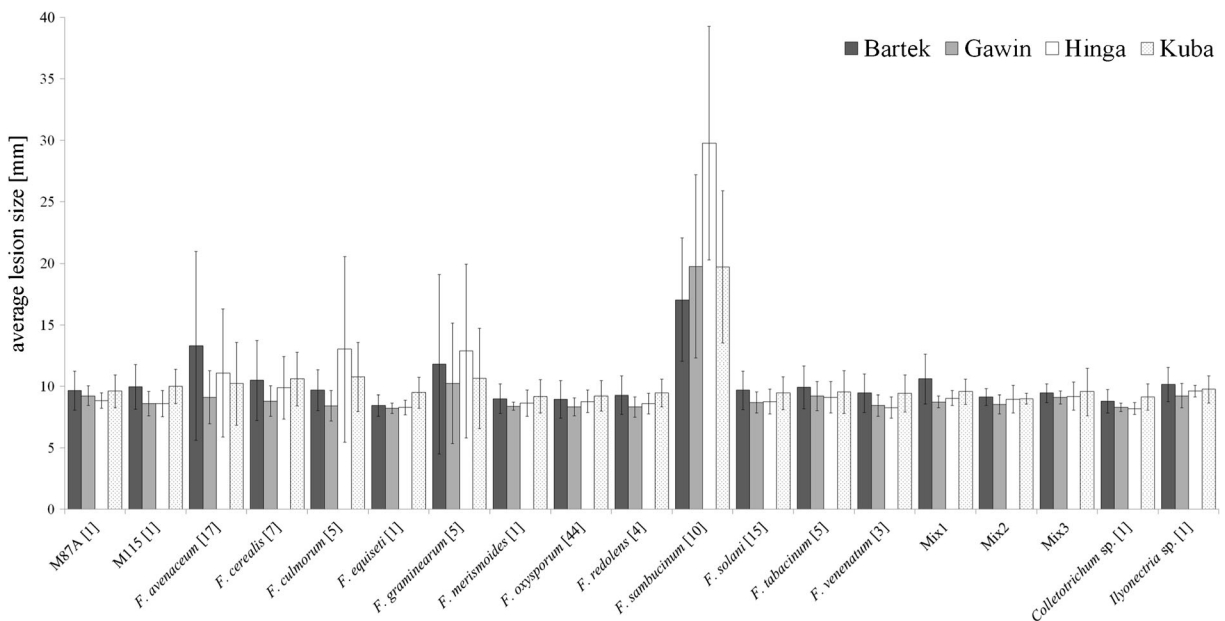

Fig. 3 Pathogenicity test results shown as the average lesion sizes caused by the tested fungal species on four potato cultivars. The numbers of isolates representing a given species are shown in brackets. The standard deviations are indicated by the error bars
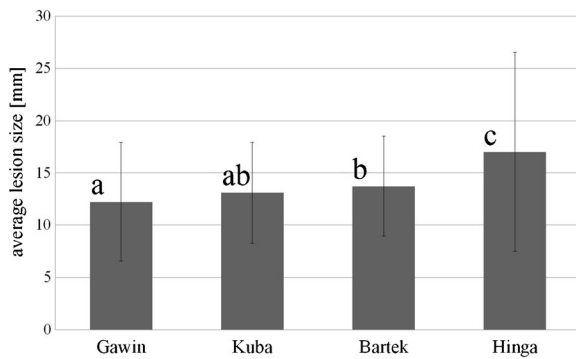
Fig. 4 The average lesion sizes calculated for all 37 *F. avenaceum*, *F. culmorum*, *F. graminearum* and *F. sambucinum* isolates in four potato cultivars used in pathogenicity tests at 16 °C. Values marked with the same letters were not significantly different from each other according to Tukey's post-hoc tests. The standard deviations are shown as the error bars

F. venenatum or *F. tabacinum* have been published; however, the latter was isolated in large numbers from potato roots (Scholte et al. 1985).

Analyses were performed with 73 isolates of 10 *Fusarium* species (Fig. 1). The resulting phylogenetic relationships remained in agreement with previous studies (Du et al. 2012; Kristensen et al. 2005; Zhang et al. 2006). The isolates of the same species were grouped together in the resulting MP tree, supporting their correct identification. *Fusarium culmorum*, *F. cerealis* and

F. graminearum formed monophyletic groups with strong support of 99 % bootstrap values. The species that belong to this clade are potentially trichothecene and zearalenone producers and formed a sister clade to a clade comprising *F. sambucinum* and *F. venenatum*; these latter species produced only trichothecenes (each species was supported by a 99 % bootstrap value). A distinct branch was formed by *F. equiseti*, which was a trichothecene and zearalenone producer. These results agree with those of previous studies (Kristensen et al. 2005) suggesting that the ancestor of the three clades described above gained the abilities to synthesize trichothecenes and zearalenone. The zearalenone-producing capacity was then lost in the clade formed by *F. sambucinum* and *F. venenatum*. A separate branch was formed by *F. avenaceum* (99 % bootstrap value). Among the isolates from that species, some sub-lineages were formed; however, these sub-lineages were not distinguished according to their geographic origins. Five isolates of different origins were chosen to represent the *F. oxysporum* species. Only one, Ch2, formed a separate branch, and the remaining four clustered together. Similarly, the isolates Ch23 and Ch45 formed separate branches in the *F. redolens* clade. Two unidentified isolates, i.e., M115 and M87A, grouped within the *F. solani* clade, and they most likely belonged to the *F. solani* species complex (FSSC). Multilocus sequence
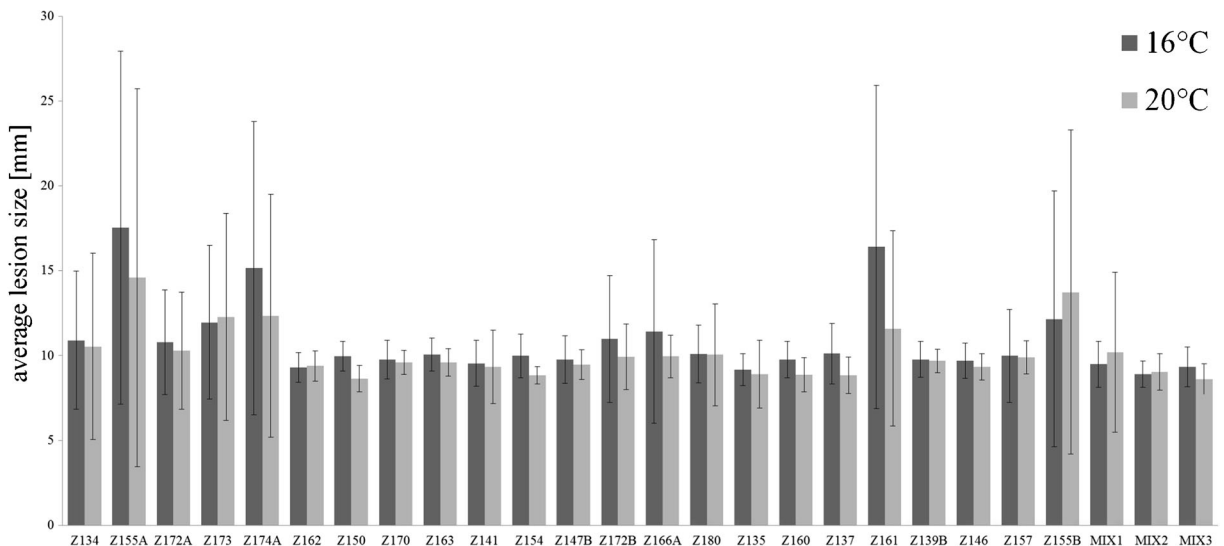

Fig. 5 Average lesion sizes of the isolates incubated at two temperatures. Standard deviations are shown as error bars. *F. avenaceum*: Z134, Z155A, Z172A, Z173, Z172B, Z166A, Z180, Z161, and Z155B; *F. graminearum*: Z174A; *F. solani*: Z162, Z150, Z141, Z147B, and Z135; *F. tabacinum*: Z170, Z160, Z137, and Z139B; *F. oxysporum*: Z163, Z154, and Z157; *Ilyonectria* sp.: Z146.; Mix 1: Z157 + Z155B (*F. oxysporum* + *F. avenaceum*); Mix 2: Z157 + Z174A (*F. oxysporum* + *F. graminearum*); and Mix 3: Z157 + Z162 (*F. oxysporum* + *F. solani*)

analyses (Zhang et al. 2006) have revealed the existence of over 45 species belonging to the FSSC that had previously been identified as *F. solani* due to these species' morphological similarities. A high level of diversity of the *F. solani* isolates was also detected in our study, supporting the idea that these isolates are members of the FSSC rather than a single species. The M115 and M87A isolates shared similar levels of pathogenicity as the *F. solani* isolates, as shown in our tests. In an additional analysis (data not shown), the *tef-1α* sequences of *F. solani* isolates from this study were analysed along with sequences deposited in the NCBI GenBank database under *F. solani* and *F. solani* var. *coeruleum* (syn. *F. coeruleum*) species names. The results revealed a strong resemblance between the first two groups of sequences, whereas *F. coeruleum* differed notably. Hence, we conclude that the isolates collected in our study were *F. solani*. The unidentified isolate M93 appeared to be distinct from the other species included in this study, and the divergence appears to have occurred early (Fig. 1). Its TEF and ITS sequences shared a high level of similarity with a CBS 119875 strain described as *Fusarium* of unknown species. Further morphological analyses should be performed to identify this isolate to the species level.

Fusarium species can be passively dispersed via the atmosphere; however, because of their abundance in the soil, they are often referred to as soil borne, and *F. oxysporum* has been reported to be the most common *Fusarium* in the soil (Nelson et al. 1981). In the sampled potatoes with dry rot symptoms, 45 % of all of the isolates were identified as *F. oxysporum*; however, none of the tested isolates of this species were pathogenic to the potato tubers in our study, or they were at least unable to cause dry rot under the tested conditions. Manici and Cerato (1994) showed that of the 8 *F. oxysporum* isolates tested, all were pathogenic to potato tubers when incubated at 20 °C. Hence, we tested the pathogenicities of three isolates of *F. oxysporum* and some other species following exposure to two different temperatures (16 °C and 20 °C). An analysis of variance showed that the temperature exerted a significant influence on the lesion size, with the average lesions formed at 16 °C larger than those formed at 20 °C; however, none of the *F. oxysporum* isolates displayed enhanced pathogenicity with increased temperature. Incubation at 20 °C was more favourable for two of the *F. avenaceum* and one of the *F. solani* isolates and for *F. avenaceum* and *F. graminearum* when mixed with *F. oxysporum*.

The effect of temperature on disease development differed among the species and among the isolates of the same species. These findings might explain why our results contradict those of Manici and Cerato (1994).

The frequent presence of *F. oxysporum* in sampled dry rot lesions might be explained by another hypothesis that this species infects the tuber either together with some other, more pathogenic species or after the tissue has already been attacked by other pathogens. According to the literature, fungal species that simultaneously infect the same host can interact with each other. For example, Velluti et al. (2000) reported decreases in the colony forming units of *F. verticillioides* and *F. proliferatum* when they co-infected maize kernels together with *F. graminearum*. Such interactions have also been observed in another experiment (Tajrin 2013), in which eight oat genotypes were inoculated with *F. graminearum* and *F. langsethiae* together and separately. Stronger *F. graminearum* growth, as measured by the amount of fungal DNA, was reported on six of the oat genotypes with which *F. graminearum* was co-infected along with *F. langsethiae* compared to inoculation with *F. graminearum* alone. These results may be explained by either possible competition between species attacking the same host, leading to the increased growth of one or the other species, or a synergistic interaction between species (Tajrin 2013). Xu et al. (2007) observed a negative effect of co-infection in an experiment in which wheat ears were inoculated separately or with combinations of two or three isolates of *F. avenaceum*, *F. culmorum*, *F. graminearum* and *F. poae*. The co-inoculations led to decreases in fungal biomass compared the single-isolate inoculations, and the single inoculations also exhibited 1000-fold greater nivalenol production. In our experiment, we used three mixtures of *F. oxysporum* with *F. avenaceum*, *F. graminearum* or *F. solani* to inoculate potato tubers. These co-infections did not enhance the pathogenicities of any of the isolates compared to the single inoculations. Interestingly, the use of isolate Z174A of *F. graminearum* in Mix 2 even decreased its pathogenicity. Concentrations of mycotoxins should be measured to determine whether this isolate shifted its energy allocation to produce more toxins at the expense of pathogenicity. Low pathogenicities of the abundant *F. oxysporum* isolates might indicate that we did not isolate a dry rot causal agent but, rather, a contaminant from soil because this species is very abundant and is adapted to long-term survival in the soil (Nelson et al. 1981). Another possibility is that the *F. oxysporum*

isolates were secondary colonizers in the dry rot-infected potato tubers because *F. oxysporum* has also been reported to be a vigorous saprophyte (Nelson et al. 1981).

Twelve *Fusarium* species were identified in rotten potato samples in this study and may have caused contamination with mycotoxins other than the expected mycotoxins based on the typical dry rot-causing *Fusarium* species. *F. equiseti* and *F. oxysporum* were isolated; therefore, potential risks of contamination with beauvericin, moniliformin and fumonisins B_1 (Stępień 2013) along with zearalenone and trichothecenes exist in potato tubers. Depending on a population structure resulting from a survey, a range of mycotoxins might be anticipated and further research for their presence carried out. Even in this relatively small sample of 149 isolates, we found three samples that were unidentifiable based on the existing nucleotide sequence databases, which indicates that the knowledge of *Fusarium* spp. in the potato is limited and requires further study. The large group of *F. oxysporum* isolates recovered that were not obviously pathogenic in the laboratory tests might still be pathogenic in the field and in storage. These isolates might be secondary pathogens or might require specific environmental factors that were not reproduced in the laboratory to cause infection. Less abundant but definitely pathogenic were *F. sambucinum*, *F. avenaceum*, *F. culmorum* and *F. graminearum*, and these results are in accordance with those of earlier reports (Peters et al. 2008b; Du et al. 2012). In our study, statistical analysis performed on the four of pathogenic *Fusarium* spp. divided the isolates according to their pathogenicity (Supplementary Table 4). High number (90.6 %) of the recovered isolates caused lesions of size below the threshold which may be puzzling. The range of a lesion size between 1.3 and 351.4 mm^2 caused by the *F. cerealis* isolates in the study of Gachango et al. (2012) indicates that among the 17 tested isolates some would be considered non-pathogenic in our tests, since the lesion would not significantly exceed the size of a mechanical wound. Large ranges in a disease development capabilities of different *Fusarium* spp. were also reported in other studies (Choiseul et al. 2007; Peters et al. 2008a; Peters et al. 2008b).

The main agents that cause dry rot in potato tubers differ depending on location, but might also change over time, particularly under conditions of climate change. The data from our survey can serve for monitoring *Fusarium* spp. in potato. Knowing which *Fusarium* spp. are present in the potato tubers with dry rot could help minimizing crop losses and mycotoxin contamination by fungicide application, since the resistance to fungicides might differ in frequency among isolates of different species (Peters et al. 2008a). Moreover, the acquired data may be helpful for planning a crop rotation, but also a crop in the vicinity of the area surveyed. Some of the *Fusarium* spp. we have isolated from potato tubers are known to be important cereal pathogens and growing such cereal host species before or next to potatoes should be avoided to limit the risk of dry rot of potato tubers.

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