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ISOLATION, IDENTIFICATION AND PRESERVATION OF *FUSARIUM* SPP. CAUSING DRY ROT OF POTATO TUBERS

ABSTRACT

Fungi of the genus *Fusarium* cause dry rot, a potato disease which develops during long-term storage of tubers. The disease-inducing capabilities differ among *Fusarium* spp., but may also vary within species universally considered main dry rot agents. Identification of *Fusarium* spp. present on diseased tubers in a surveyed area can help minimize crop losses and mycotoxin contamination by, for example, applying proper fungicides or planning crop rotation. Here, we present a procedure of obtaining single spore colonies of *Fusarium* spp. from potato tubers infected by dry rot, their identification using molecular methods and ways of preservation.

Key words: *β-tubulin*, ITS, single-spore culture, *Solanum tuberosum*, *tef-1α*

INTRODUCTION

Dry rot, caused by fungi of the *Fusarium* genus, is an important disease which develops during long-term storage of potato tubers. *Fusarium* spp. are abundant in the soil and the infection of tubers often starts at sites injured during harvest. The symptoms of the disease are hard, wrinkled skin and rotted cavities. *Fusarium* spp. recovered from diseased potato tubers vary depending on the geographic location, but might also change in time. Among the thirteen *Fusarium* spp. globally associated with dry rot (Cullen *et al.,* 2005), eight have been reported in Poland (Stefańczyk *et al.*, 2016). Their pathogenicity differs between and within species (Peters *et al.*, 2008a; Stefańczyk *et al.*, 2016). *Fusarium* spp. synthesize mycotoxins that contaminate the infected potato tubers and increase crop losses.

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Molecular methods, based on gene sequencing, were used to complement classical methods (Photo 1) and are currently used for identifying species in a daily basis.

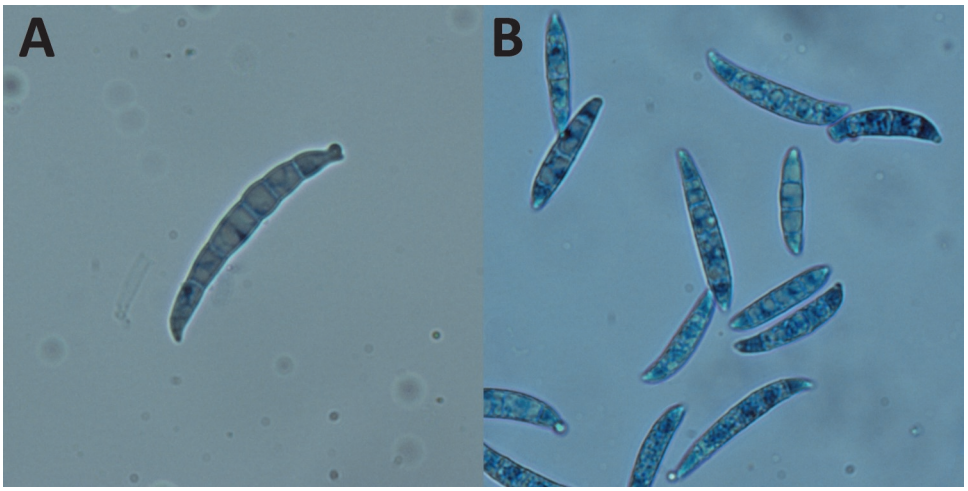

Photo 1. A microscopic observation of *F. cerealis* (A) and *F. sambucinum* (B) macroconidia stained with lactophenol blue. The species were identified using molecular methods.

The sequences of the widely used *tef-1α* (*translation elongation factor 1-α*; O'Donnell *et al.*, 1998) are available in most databases and are considered a good choice for *Fusarium* spp. identification; obtaining a PCR product of this gene might, however, require tedious optimization. Internal transcribed spacer (ITS; White *et al.*, 1990), referred to as the official fungal barcode, might sometimes fail to distinguish closely related species, because of the presence of ITS non-orthologous copies (O'Donnell and Cigelnik, 1997). A high level of sequence polymorphism is displayed by *bTub* marker gene (*β-tubulin*; O'Donnell and Cigelnik, 1997) which can be used as validation; however, not all *Fusarium* spp. have this sequence deposited in databases. Except for the *tef-1α*, the ITS and *bTub* marker genes employed for species identification at Młochów Research Center, other gene sequences, such as DNA-directed RNA polymerase II largest (RPB1) and the second largest subunit (RPB2), calmodulin (CaM) or histon H3 (HIS), might be used.

Identification of *Fusarium* spp. present on diseased tubers from a certain field area enables population structure monitoring. As the resistance to fungicides among isolates of different species might vary (Peters *et al.*, 2008b), survey data can help minimize crop losses by applying appropriate chemicals. The fungi of the *Fusarium* genus are capable of infecting a broad spectrum of hosts, thus data on the presence of a particular species can be used to plan crop rotation or the plants growing in the neighboring area. Having a collection of identified *Fusarium* spp., pathogenicity tests can be conducted to evaluate the resistance against dry rot in the chosen potato cultivars.

The *Fusarium* isolation and maintenance procedures described here are the modified methods by Zarzycka (2001). Changes apply to sucrose used instead of glucose in the potato-agar medium. Compared to the original procedure, the volume of agar (10 g instead of 20 g) and the incubation temperatures of the *Fusarium* cultures (16°C instead of 21°C) were also changed. The methods by Zarzycka (2001) are expanded in this manual by acquisition of single-spore cultures.

MATERIALS AND REAGENTS

1) Potato tubers with visible dry rot symptoms
2) Lab materials:
   a) round Petri dish
   b) sterile, sharp tool
   c) inoculation loop
   d) glass tubes
   e) cryovials
3) Sterile distilled water
4) Sterile paraffin oil
5) 70% ethanol (POCH, cat. No 396420420)
6) 0.1% mercuric chloride (Sigma-Aldrich, cat. No 429724)
7) Potato tubers for media preparation
8) Sucrose (Chempur, cat. No 427720906)
9) Agar (BTL, cat. No S-0001)
10) Rifamycin (Sigma-Aldrich, cat. No R8626)
11) Microtube pestles
12) Pipette tips
13) DNA isolation kit (Sigma-Aldrich, cat. No G2N70/G2N350)
14) PCR reagents
   a) PCR buffer (Thermo Scientific, cat. No B65)
   b) dNTPs (Sigma-Aldrich, cat. No DNTP100)
   c) synthesized primers (see Table 1)
   d) *Taq* Polymerase (Thermo Scientific, cat. No EP0701)
15) Agarose (GenoPlast Biochemicals, cat. No BMGPB826)
16) Ethidium bromide (Sigma-Aldrich, cat. No E1510)
17) PCR Clean-up kit (Sigma-Aldrich, cat. No NA1020)
18) Gel-extraction kit (Sigma-Aldrich, cat. No NA1111)

Note: Items from 1 to 12 are used in procedures of isolation and storage of fungal isolates. Items 13-18 are required for species identification.

EQUIPMENT

1) Sterile bench with UV light
2) Room with controlled temperature
3) Microscope (Carl Zeiss Microscopy, Jenaval)

4) Autoclave (HP Medizintechnik, Varioklav)
5) Freeze-dryer (Labconco, 7755511)
6) Pipettes
7) Thermomixer (Eppendorf, Thermomixer Compact)
8) Centrifuge (Eppendorf, 5417R)
9) Thermocycler (Bio-Rad, T100 Thermal Cycler)
10) Electrophoresis gel box with combs (Thermo Scientific, Owl EasyCast B2) connected to the power supply (Thermo Scientific, Owl EC300XL)
11) Gel imaging system (Vilber Lourmat, ECX-F26.MX)

RECEPIES

PSA medium ($g \times l^{-1}$)

1) 200 g peeled potato tubers
2) 20 g sucrose
3) 10 g agar

The peeled potato tubers are diced and boiled in tap water until not completely soft; the tuber fragments are discarded and the extract is mixed with 20 g of sucrose and 10 g of agar; the mixture is filled up with tap water to 1000 ml and autoclaved for 20 min.

If required, after autoclaving and cooling down to around 45°C, 3 ml of the stock rifamycin solution (1% stock in 70% ethanol) per 1000 ml medium are added to reach the final concentration of 30 $\mu g \times ml^{-1}$.

Agar medium ($g \times l^{-1}$):

1) 10 g of agar
2) Fill up with distilled water to 1000 ml and autoclaved for 20 min. A thin, approx. 2 mm, layer of the agar medium is spread on each Petri dish.

Liquid potato sucrose medium ($g \times l^{-1}$):

1) 200 g potato tuber extract
2) 20 g sucrose
3) Filled up with tap water to 1000 ml and autoclaved for 20 min.

PROCEDURE

Note: All steps are performed on UV light pre-treated sterile bench

Pure fungal cultures isolation

1) From the border of the healthy and diseased tissues, a 0.5 × 0.5 × 0.5 cm tuber fragment is cut out with a sterile tool and is disinfected in

70% ethanol for 15-20 s and de-aerated in 0.1% mercuric chloride for another 30 s afterwards. After washing three times in sterile distilled water, the tuber fragment is incubated at 16°C for around 2 weeks on a potato sucrose agar (PSA) medium with rifamycin.

Note: After the incubation period the fungus colony should have grown around the tuber, however, some species might require a longer incubation period.

2) A mycelium fragment from a side of the colony is then moved to a fresh PSA + rifamycin medium and incubated at 16°C for 1-2 weeks.
3) The third mycelium transfer to a fresh PSA medium (without rifamycin) is followed by incubation at 16°C for 1-2 weeks.
4) Conidia are gathered from a developed *Fusarium* colony by touching the fungus mycelium with an inoculation loop. By moving the inoculation loop in three to five parallel lines and an analogical set of lines in perpendicular orientation, the conidia are spread on the agar medium which is then incubated at 16°C for 24 h.
5) A Petri dish is turned upside down and single germinating spores located by microscopic observations are labelled with a marker pen and transferred further to a fresh PSA medium. Incubation is performed at 16°C for 2-3 weeks.

Note: For DNA isolation, a 0.5 cm fragment of a developed colony is moved to a liquid potato sucrose medium and incubated at 16°C for 1 week. Mature fungal colonies are transferred to 1.5 ml tubes and freeze-dried.

Storage of Fusarium isolates

1) For long-term storage, a *Fusarium* culture is passaged to PSA agar slopes with an inoculation loop. After incubation at 16°C for 1 week, the cultures are covered with sterile paraffin oil, sealed and stored at 4-7°C.

Note: Stored this way, the *Fusarium* isolates remain vital for at least 10 years. PSA agar slopes are prepared in the glass tubes angled during medium stiffening.

2) Alternatively, the fungal culture can be kept in liquid nitrogen. For this purpose, discs of ca. 0.7 cm diameter are cut out of a PSA medium coated with a developed *Fusarium* colony and moved to 1.5 ml cryovials. The procedure continues as described by Sobkowiak and Śliwka (2018).

Molecular identification of Fusarium species

1) Prior to isolation, the freeze dried fungal colonies are ground to powder using microtube pestles. The DNA isolated according to a protocol provided by DNA isolation kit manufacturers is further used in polymerase chain reaction (PCR) assays with markers amplifying genes commonly used for identifying the *Fusarium* species. The primer sequences, reagent concentrations and reaction conditions are shown in Table 1.

Table 1

The genes used for identifying *Fusarium* sp. in PCR assays, along with reaction conditions, primer sequences, product sizes and literature references.

Marker	Gene	PCR Mix	Primers	Ta	Product size [bp]	Reference
ITS	partial 18S, 28S and complete sequence of 5.8S ribosomal RNA genes	0.85 µM primers, 0.1 mM dNTPs	GGAAGTAAAAGTCGTAACAAG GTCCTCCGCTTATTGATATGC	55°C	568	White *et al*., 1990
TEF	app. 40% of *translation elongation factor 1-α* gene	0.5 µM primers, 0.2 mM dNTPs	ATGGGTAAGGARGACAAGAC GGARGTACCAGTSATCATGTT	60°C	713	O'Donnell *et al*., 1998
bTub	app. 60% of *β-tubulin* gene	0.2 µM primers, 0.25 mM dNTPs	AACATGCGTGAGATTGTAAGT GACCGGGGAAACGGAGACAGG	55°C	1015	O'Donnell and Cigelnik, 1997

2) The PCR products are examined in UV light on 1.5% agarose gels stained with ethidium bromide. If single bands are present, the products are purified with commercially available PCR clean-up kits. The products of expected sizes need to be extracted from the gel if additional bands are observed.
3) After the sequencing procedure, a consensus sequence, prepared using the nucleotide sequences obtained with forward and reverse primers, is queried against NCBI GenBank, the Fusarium-ID (Geiser *et al.*, 2004) and *Fusarium* MLST (O'Donnell *et al.*, 2012) databases using the default search parameters. The closest matching sequence from a database, demonstrated by a low *e* value, the greatest identity and coverage, represents the species of the query.

Note: Although the use of one identification marker might be sufficient, in some cases sequences of additional genes need to be obtained for correctness of the identification procedures. Sequence-based identification is performed in relation to the previously deposited data and the sequences which were incorrectly annotated will be propagated in the databases (Nilsson et al., 2014). It might also happen that rarely used genes find no matches in the queried databases, even though the species is prevalent and well described in literature. The polymorphism of some genes might also be too low to distinguish closely related species. Technical difficulties in PCR (additional products, products of unspecific sizes, no products) or illegible chromatograms which cannot be fixed by optimization, lead to the necessity of using a different gene. Phylogenetic analyses performed with multilocus sequence fragments exhibit a higher resolution than single genes and might sometimes help resolve the uncertain affinity of isolates.

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