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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *Fusarium tricinctum* AND *Fusarium acuminatum* AS CAUSAL AGENTS OF GARLIC BULBS ROT IN SERBIA

ABSTRACT: Garlic (*Allium sativum* L.) is considered to be one of the oldest crops in the world. During 2016, infected garlic bulbs occurred in storages on several localities of the Province of Vojvodina. Symptomatic cloves showed typical rot symptoms such as softened and spongy areas covered with white fungal growth with deep lesions formed on the cloves which became dry over time. A total of 36 isolates of *Fusarium* species were obtained from diseased cloves of garlic. Colony morphology and microscopic properties of isolated *Fusarium* species were recorded from the cultures grown on PDA and CLA, respectively. Identification of two chosen isolates was performed by sequencing the EF-1α gene. The TEF sequence of isolate JBL12 showed 100% similarity with several *F. tricinctum* sequences and sequence of JBL539 showed 99% identity with several *F. acuminatum* sequences and they were deposited in the NCBI GenBank. Based on the results of the morphological and molecular identification, isolates JBL12 and JBL539 were identified as *F. tricinctum* and *F. acuminatum*, respectively, as new causal agents of garlic bulbs rot in Serbia. Specific primers were designed for the PCR identification of the *F. tricinctum*.

KEYWORDS: garlic (*Allium sativum*), bulb and clove rot, *Fusarium* spp., EF-1α gene

INTRODUCTION

Garlic (*Allium sativum* L.), a bulbous vegetable, is considered to be one of the oldest horticultural crops in the world (Moyers, 1996). This garlic species is grown worldwide, particularly in mild climate regions, with the total world annual production being 24 million tons on average. The top producers are China, Egypt, India, Korea and USA. Garlic is cultivated in Serbia, covering more

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than 7,000 ha, mostly concentrated in the northern part of Serbia, Vojvodina Province.

Fungal pathogens such as *Fusarium* spp. can cause significant economic losses at the postharvest stage of garlic (Kim *et al*. 2003; Palmero *et al*. 2013). The significance of *Fusarium* rot of garlic has been increasing in Serbia and according to Lević *et al*. (2009) *F. proliferatum*, *F. oxysporum*, *F. solani* and *F. verticillioides* species were previosly identified and isolated from infected cloves of garlic in Serbia. Stanković *et al*. (2007) reported that *F. acuminatum* and *F. equiseti* were isolated from onion, whereas *F. proliferatum*, *F. oxysporum* and *F. solani* were species detected on both onion and garlic in Serbia.

During 2016, infected garlic bulbs occurred in storage and warehouses in several localities of the Province of Vojvodina. Disased cloves showed typical rot symptoms such as softened and spongy areas covered with white or reddish fungal growth with deep lesions formed on the cloves which became dry and small over time. Symptomatic bulbs were subjected to phytopathological analysis in order to identify the causative agent of the disease.

MATERIAL AND METHODS

Morphological characterization

To isolate the causative organism, cloves were separated from the bulbs, the margins of the lesions were cut into small pieces, surface-sterilized with 1% NaOCl for 2–3 min, and washed three times with sterile distilled water and plated onto a Potato Dextrose Agar (PDA) medium amended with 300 mg/l streptomycin sulphate (Gerlach and Nirenberg, 1982). Plates were incubated at 26 °C in the dark. Seven days later, *Fusarium* colonies were recognized morphologically and chosen isolates were subcultured in PDA using a single spore technique. A total of 36 isolates of *Fusarium* species were obtained. Colony morphology and microscopic properties of isolated *Fusarium* species were recorded from the cultures grown on PDA and CLA, respectively.

DNA extraction and molecular species identification

To obtain a DNA sequence, a total DNA of the 36 isolates and one positive control FE-3 was extracted directly from the 7 days old mycelium (~ 100 mg wet weight), with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following DNA extraction, the translation elongation factor 1-*alpha* gene region was amplified by PCR with the primer pair EF1 and EF2 (Geiser *et al*. 2004). The amplification was performed on the Eppendorf Mastercycler PCR device, using the modified program by Abdel-Satar *et al*. (2003): 35 repeated cycles: 94 °C 1 min, 53 °C 1 min, 72 °C 2 min. The PCR mixture with a total volume of 25 µl consisted of 2x Eppendorf Master Mix (Taq DNA polymerase 1.25 U, 30mM Tris-HCl, 50mM KCl, 1.5mM

MgCl2; 0.1% Igepal-CA630; 0.2 mM dNTP); 0.6 µM of each primer and 1 µl of fungal DNA. Amplification fragments were determined using electrophoresis on 1.5% agarose gel containing ethidium bromide (0.5 µg/mL). The expected size of the amplified fragments was estimated by comparison with ready-to-use O´RangeRulerTM 100 bp DNA Ladder (SM0623, Fermentas). The agarose gel was visualised in UV transilluminator and the images were captured with DOC PRINT system (Vilbert Lourmat, USA).

Identification of isolates was performed by sequencing the translation elongation factor EF-1*αlpha* gene. Purification and sequencing of the amplified fragments was done in Company MACROGEN, Seoul, South Korea (http://dna.macrogen.com). Sequences were analyzed in the program FinchTV Version 1.4.0. Sequence of isolates JBL12 and JBL539 was compared with the previously reported isolates available in the NCBI GenBank and the *Fusarium* ID-database (Geiser *et al*. 2004), using the ClustalW program (Thompson *et al*. 1994) and MEGA5 software (Tamura *et al*. 2011).

PCR assay specificity

On the basis of the EF-1*αlpha* gene fragment sequence, two primer sets were designed for specific identification of *F. tricinctum* using NCBI tool for finding specific primers based on specific sequence of isolate JBL12 (accession no. KX611146). PCR were performed in 25-µl reaction (Table 1), with all investigated isolates of *Fusarium* spp. (JBL1-JBL36). The PCR temperature profile comprised an initial denaturation step at 94 °C for 2 min, 35 cycles at 94 °C 1 min, 60 °C 1 min, 72 °C 2 min and a final extension at 72 °C for 10 min. Amplicons were electrophoresed in 1.5% agarose gel (Invitrogen) with ethidium bromide.

Table 1. Designed new primer sets for specific identification of *Fusarium tricinctum* and PCR conditions

Primer name	5'....'3	PCR components	Final concentration	25 µl reaction
BL12-3FUSTRF BL12-3FUSTRR	TTCGCTCCCTCACTCGAAAC TGAAGGAACCCTTTCCGAGC	2x MMix	1x	12.5 µl
		10 µM Primer F	1 µM	2.5 µl
BL12-5FUSTRF BL12-5FUSTRR	AGTGCGGTGGTATCGACAAG GTTTCGAGTGAGGGAGCGAA	10 µM Primer R	1 µM	2.5 µl
		DNA	~1,000 ng	1.0 µl
		Nuc.-free water		6.5 µl

RESULTS

Morphological characterization

The fungal isolate (JBL539) formed a fast-growing (7 cm in 6 days), abundant, pale ochraceous, whitish-pink and partly carmine aerial mycelium.

This isolate also produced dark to blood – red pigmentation in agar which later turned amber with a dark tan colour at the edge of PDA, which is typical of *F. acuminatum* (Gerlach and Nirenberg, 1982). On CLA macroconidia were abundant, slender, equilaterally curved with elongated apical cell and pedicellate basal cell, mostly three to five septate (rarely 0–1 septate) and measuring 32–44 µm x 3.5–4.7 µm. The fungus formed globose to subglobose chlamydospores, mostly in pairs, chains or clusters. Microconidia were not observed. Based on the colony morphology and the description of fungal structures, the isolated fungus was identified as *F. acuminatum* (Ell. & Kellerm) (Gerlach and Nirenberg, 1982).

The isolate (JBL12), when grown on PDA, rapidly produced abundant, dense, white, aerial mycelium that became pink with age and formed red pigments in the medium. On CLA, macroconidia were abundant, relatively slender, curved to lunate and three to five septate. Microconidia were napiform, oval or pyriform, zero to one septate and commonly clustered in false heads, without chlamydospores. On the basis of fungal morphology, the fungus was identified as *F. tricinctum* (Corda) Saccardo (Gerlach and Nirenberg, 1982).

DNA extraction and molecular species identification

To confirm the morphological identification, total genomic DNA was extracted from the mycelium of the isolates JBL12 and JBL539 with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and the EF-1*alpha* region was successfully detected in all tested samples amplified by PCR with the primer pair EF1 and EF2 and obtaining fragments of predicted size (700bp). The PCR product derived from the isolates JBL12 and JBL539 was directly sequenced in both directions using the EF1/EF2 primer pair as in PCR and deposited in GenBank (Accession No. KX611146, KX752419). The sequences were compared to those in GenBank. TEF sequence of isolate JBL12 showed 100% similarity with several *F. tricinctum* sequences (e.g., HM068307, EU744838, and EU744837) while the EF sequence of JBL539 showed 99% identity with several *F. acuminatum* sequences (e.g., EF531698, KP868658, KJ194170).

PCR assay specificity

These new designed primers (BL12-3FUSTRF/BL12-3FUSTRR and BL12--5FUSTRF/BL12-5FUSTRR) successfully identified *F. tricinctum* and its separates from other different *Fusarium* species. One clear band of 223bp and 112bp, respectively, were visible in tested sample designated as JBL12 (*F. tricinctum*).

DISCUSSION

Garlic bulbs are quite perishable because their high moisture content makes them vulnerable to microbial decay as well as physiological deterioration (Eckert

and Ogawa, 1988). Also *Fusarium* sp. are one of the most important pathogens in the growing season and during the storage. The control of *Fusarium* rot of garlic is difficult due to the epiphytic survival and spreading of the pathogen during storage. During 2016, infected garlic bulbs occurred in storages and warehouses on several localities of the Province of Vojvodina. Symptoms appeared during the storage, as spongy, softened, cloves covered with white, light pink or reddish mycelium. Over time the cloves became dry and small.

Based on the results of the molecular identification, macromorphological and micro-morphological characteristics of isolates JBL12 and JBL539 were identified as *F. tricinctum* and *F. acuminatum*, respectively, as new diseases causal agents of garlic bulbs rot in Serbia. Stanković *et al*. (2007) reported that *F. acuminatum* and *F. equiseti* were isolated from onion, whereas *F. proliferatum*, *F. oxysporum* and *F. solani* were species detected on both onion and garlic in Serbia. Recently, *F. tricinctum* has been described as a new pathogen of garlic in Serbia (Ignjatov *et al*. 2017) which, unlike *F. acuminatum* (JBL539), has citriform microconidia and falcate, strongly curved macroconidia, with a well--marked foot cell.

Fusarium species caused similar symptoms on stored garlic and it was difficult to distingush them based on the symptoms and morphological characteristics. Polymerase chain reaction (PCR) with primers designated as EF1 and EF2 were created as choice of a single locus identification tool in *Fusarium* genus (Geiser *et al*. 2004). The presence of a 700 bp amplicon in all investigated isolates was confirmed by comparing the amplified DNA fragments with the marker and positive control. The translation elongation factor 1-*alpha* (TEF) gene which encodes an essential part of the protein machinery is highly informative at the species level in *Fusarium* (Geiser *et al*. 2004). Primers EF1 and EF2 were first developed in the fungi to investigate lineages within the *F. oxysporum* complex and these primers amplify an ~700 bp region of TEF in all known fusaria (O'Donnell *et al*. 1998).

Nucleotide sequence differences were found when sequence of the EF-1*αlpha* region of the *F. acuminatum* was compared to those of *F. tricintum*, separating those two species and diverse clusters. Our report on these pathogens provides a basis for epidemiological studies and supports other efforts towards the development of effective disease management strategies for this pathosystem.

The disease tends to occur more frequently in garlic and it is more often a problem in storage than in the field. *Fusarium* species infecting garlic affect the health safety of agricultural workers, especially those associated with processing and store houses, as well as the consumers. Presence of *Fusarium* cloves infection decreases physiological properties of garlic, especially seed health and germination potential.

Using tool for finding specific primers based on specific sequence of *F. tricinctum* (JBL12/NCBI Acc. KX611146), a PCR-based assay was developed for the specific detection of *F. tricinctum*, which has been validated using 36 strains of the *Fusarium* sp. from different garlic varietes and geographical origins. Two primers sets were designed for specific PCR identification: BL12--3FUSTRF/BL12-3FUSTRR and BL12-5FUSTRF/BL12-5FUSTRR. One clear

band of 223 bp and 112 bp was visible in tested isolate JBL12 and no amplicon was recorded in negative control and other strains.

This finding will provide the basis to develop the effective disease management strategies and specific identification of *F. tricinctum* as causal agent of garlic bulb rot in Serbia.

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МОРФОЛОШКА И МОЛЕКУЛАРНА ИДЕНТИФИКАЦИЈА *Fusarium tricinctum* И *Fusarium acuminatum* ПРОУЗРОКОВАЧА ТРУЛЕЖИ БЕЛОГ ЛУКА У СРБИЈИ

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РЕЗИМЕ: Бели лук (*Allium sativum* L.) сматра се једном од најстаријих биљних врста на свету. Током 2016. године, запажена је појава трулежи белог лука у складиштима и магацинима на неколико локалитета у Војводини. Симптоми су се испољавали у виду лезија, трулежи белог лука као и појавом мицелије на инфицираним ченовима. Циљ рада био је изолација и идентификација *Fusarium* spp. на основу морфолошких и молекуларних карактеристика патогена. Изолацијом је добијено 36 изолата *Fusarium* spp. Детекција и идентификација одабраних изолата потврђена је методом PCR коришћењем прајмера EF1 и EF2 који амплификују производе величине 700bp. У свим проучаваним изолатима формирани су ампликони величине 700bp. Изолација ДНК два одабрана изолата извршена је директно из мицелије гљиве (~100 mg), коришћењем DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Идентификација изолата JBL539 и JBL12 извршена је секвенцирањем EF-1α гена, који су депоновани у NCBI базу података под бројем KX611146 (*F. tricinctum*) и KX752419 (*F. acuminatum*). Креирани су специфични прајмери за PCR идентификацију врсте *F. tricinctum*.

КЉУЧНЕ РЕЧИ: бели лук (*Allium sativum*), трулеж луковица и ченова, EF-1 alpha ген