Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. Novi Sad, № 132, 9—17, 2017

UDC 635.25:631.55
https://doi.org/10.2298/ZMSPN1732009I

Maja V. IGNJATOV[1], Tatjana Č. POPOVIĆ[2], Dragana N. MILOŠEVIĆ[1], Zorica T. NIKOLIĆ[1], Gordana R. PETROVIĆ[1], Jelica M. GVOZDENOVIĆ-VARGA[1], Žarko J. IVANOVIĆ[2]*

[1]Institute of Field and Vegetable Crops,
Maksima Gorkog 30, Novi Sad 21000, Serbia
[2]Institute for Plant Protection and Environment,
Teodora Drajzera 9, Belgrade 11000, Serbia

IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF *Fusarium* sp. FIESC3 THE CAUSAL AGENT OF SEED ROT IN ONION (*Allium cepa* L.)

ABSTRACT: Onion (*Allium cepa* L.) is one of the most important vegetable crops in Serbia, where it is grown on an approximate surface of 20,000 ha. During the routine quality control analysis of onion seed in 2014, fungal infection was observed in an average of 28% of the seed. The objective of this paper was to isolate, determine, and identify *Fusarium* sp. based on the pathogen's morphological and molecular characteristics. Onion seed samples were collected from different localities in the region of Vojvodina. To obtain a DNA sequence-based identification, a total DNA of the 25 isolates was extracted directly from the mycelium (~ 100 mg wet weight), with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Following DNA extraction, the translation elongation factor 1-alpha region was amplified by PCR with the primer pair EF1 and EF2. An amplicon of 700 bp was amplified in 25 tested isolates. Identification of one isolate was performed by sequencing the translation elongation factor *EF-1α* gene, which was deposited in the NCBI GenBank database under accession number KP658211 (*Fusarium* sp. FIESC3).

KEYWORDS: FIESC3, *Fusarium* sp., onion, *EF-1α* gene, seed, sequencing

INTRODUCTION

Onion (*Allium cepa* L.) is one of the economically most important vegetable crop in Serbia. During the routine quality control analysis of onion seed in 2014 in Serbia, *Fusarium* fungal infection was observed on an average of 28% of the seed. Species of *Fusarium oxysporum*, *F. proliferatum*, and *F. equiseti* have been described as the most common seed-borne fungi which attack and

* Corresponding author. E-mail: maja.ignjatov@nsseme.com

can be transmitted by vegetable seeds. Seed health is a critical component for a successful crop production, while reduction in seed germination and growing energy occurres due to seed-borne diseases (Jasnić *et al*., 2005). Lević *et al*. (2009) indicate that *Fusarium* species periodically cause significant diseases, especially wilt of onion, garlic, and tomato, but there is a lack of information about *Fusarium* species associated with onion seed (*Allium cepa* L.) in Serbia. The significance of *Fusarium* basal rot of onion has been increasing in Serbia, not only in the production of green onion, but also in the production of onion sets and bulbs (Lević *et al*., 2009). The most frequent species isolated from onion sets and seedlings, as well as from the soil in Serbia are *Fusarium oxysporum* Schlecht. f. sp. *cepae* (Hanz) Snyd. & Hans, *F. oxysporum*, *F. moniliforme*, *F. solani*, *F. equiseti*, and *F. accuminatum* (Klokočar-Šmit *et al*., 1990; Lević 2008, Lević *et al*., 2009). The identification of *Fusarium* species traditionally relies on morphological and physiological characteristics, which is the most difficult step in the process of identification (Rahjoo *et al*., 2008), considering that *Fusarium* species can produce three types of asexual spores (Agrios, 1988). This is especially evident among very closely related *Fusarium* species, such as members of FIESC, which represents a complex of morphologically similar species (*F. equiseti*/*F. semitectum*/*F. incarnatum*) (O' Donnell *et al*., 2009, 2012). Recently, a multilocus sequence typing scheme analysis has been applied to members of FIESC and revealed that FIESC comprises 30 phylogenetically distinct species (O'Donnell *et al*., 2009, 2012). DNA sequence-based identification of some unknown isolates can be achieved using the translation-elongation factor *1-α TEF* gene region which has become the marker of choice as a single-locus identification tool in *Fusarium* (Geiser *et al*., 2004). Moreover, members of FIESC have been reported to produce type A and B trichothecene mycotoxins that cause toxicosis in humans and animals (O'Donnell *et al*., 2009).

The objective of this paper was isolation, morphological and molecular DNA sequence-based identification of *Fusarium* sp. isolates from onion seed samples collected from storage and warehouses at different localities in the region of Vojvodina, Serbia.

MATERIALS AND METHODS

Isolation and morphological characteristics

Blotter method was used for seed incubation during the routine seed health analysis (Mathur and Kongsdal, 2003). Onion seeds were immersed in NaOCl solution (1% available chlorine) for 3 min, washed in sterile water and drained. A set of three filter papers were immersed in sterile water, and placed completely wet in a Petri dish (ø9mm). Plating of 18 samples (400 seeds per sample) was done aseptically, a maximum of 10 seeds per plate were placed onto a blotter surface of each plate (Mathur and Kongsdal, 2003). Plates were incubated at 22 ºC for 7 days in alternating cycles of 12 hours light (NUV) and 12 hours darkness. After incubation, fungi which developed on each seed were examined under different magnifications of a stereomicroscope and identified.

Infected seeds were transferred to a potato dextrose agar (PDA), and incubated for seven days at 25 °C in alternating cycles of 12 hours light and 12 hours darkness, in order to induce sporulation and pigmentation in culture (Burgess *et al*., 1994). Light source consisted of three neon tubes measuring 40 W and a black light tube (Philips TLD 36W/08). For morphological identification, 25 isolates were single-spored and sub-cultured on both PDA and Carnation leaf agar (CLA) (Leslie and Summerell, 2006). Incubation lasted 7–10 days at 25 °C, in alternating cycles of 12 hours light and 12 hours darkness. Colony morphology was recorded from cultures grown on PDA and CLA.

Pathogenicity test

Pathogenicity test of 25 isolates was conducted using Knop agar slants, in controlled conditions in the laboratory (Tuite, 1969). A piece of mycelium (approximately 2-3 mm) of each isolate, grown on PDA for 7 days, was placed at the bottom of each test tube. Onion seeds were disinfested in 1% NaOCl for 2 to 3 min, rinsed with sterillized distilled water three times, and then dried on a sterile filter paper under aseptic conditions. Seeds were carefully placed and slightly pressed, approximately 2 cm above the inoculum. As a positive control, determined isolate designated as FE-3 (*Fusarium equiseti*) from our collection, was used. Onion seeds placed on a solid agar without mycelia were used as a negative control. Tubes were kept in the laboratory for two weeks, in a vertical position at room temperature (21−25 ºC) with day/night shift.

Sequencing and phylogenetic analysis

To obtain a DNA sequence, a total DNA of the 25 investigated 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 (forward primer: 5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF2 (reverse primer: 5'- GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') (Geiser *et al*., 2004). The amplification was performed in Eppendorf Mastercycler PCR device, using the modified program by Abdel-Satar *et al*. (2003): following 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 1µl of fungal DNA. Amplification fragments were determined using electrophoresis on 1% agarose gel containing ethidium bromide (0.5 µg/mL). The expected size of the amplified fragments was estimated by comparison with O'RangeRuler^{TM} 100 bp DNA Ladder (SM0623), ready-to-use (Fermentas, Lithuania). The agarose gel was visualised on UV transilluminator, and the images were captured with DOC PRINT system (Vilbert Lourmat, USA).

Identification of one isolate was performed by sequencing the translation elongation factor *EF-1α* gene. Purification and sequencing of the amplified fragments were done in the biotechnology company MACROGEN in Seoul, South Korea (http://dna.macrogen.com, Korea). Sequences were analyzed using the program FinchTV Version 1.4.0. Sequence of Serbian L1 isolate was compared with the previously reported isolates available in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/BLAST) and the *Fusarium* ID-database (Geiser *et al*., 2004), using the ClustalW program (Thompson *et al*., 1994) and MEGA5 software (Tamura *et al*., 2011). Manual corrections of aligned database, phylogenetic and molecular evolutionary analyses were conducted using MEGA 6 software package (Tamura *et al*., 2013). These gene sequences were assembled and edited using FINCHTV v.1.4.0 (http://www.geospiza.com). Multiple alignments and comparisons with reference strains for each of the genes were performed using CLUSTALW integrated into MEGA 6 software (Tamura *et al*., 2013). The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the analyzed taxa. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2011) and are presented as units of the number of base substitutions per site.

RESULTS

Morphological characteristics

Onion seeds placed on the blotter surface were covered with white, cottony mycelium, reddish to purple pigmentation observed under the seeds. The presence of macroconidia typical for *Fusarium* sp. on the infected onion seed was confirmed by microscopic examination on an average of 28% of the seed. The development of mycelium of 25 isolates on PDA occurred at medium mycelial growth rate and formed a colony 6 cm in diameter after seven days of incubation in darkness at 25 °C. A total of 25 isolates (L1-L25) formed whitish to pale salmon colonies, and the colour was uniform throughout the entire colony. The orange pigmentation developed on the reverse surface of the colony. On CLA, isolates formed hyaline, thin-walled, slightly curved, fusoid macroconidia, with 4–6 septae (23–40×3.5–6 μm). Microconidia and chlamydospores were not observed.

Pathogenicity test

Pathogenicity of onion seedlings under *in vitro* conditions, in a test tube on a Knop agar, was confirmed in 25 tested isolates. Five days after the inoculation, isolates caused a change in tissue colour and emergence of necrotic spots on the roots and shoot basis, spreading to the upper part of the shoot. The roots were completely infected by fungal mycelia after 10 days. After 14 days fungal mycelia of 25 isolates completely covered the seedlings, causing root

necrosis and seedling decay. No symptoms were observed on seedlings in the tubes which were used as negative control. Pathogen was reisolated and morphological identity was confirmed on PDA and CLA.

Sequencing and phylogenetic analysis

Primers EF1/ EF2 successfully detected the presence of *Fusarium* sp. in all tested isolates and amplified DNA fragments of predicted size. One clear band of 700 bp was visible in all tested isolates as well as in positive control. No amplicon was recorded in negative control. Identification of one isolate was performed by sequencing the translation elongation factor *EF-1α* gene, which was deposited in the NCBI GenBank database under accession number KP658211. BLASTn queries of GenBank and the *Fusarium* ID-database (Geiser *et al*., 2004), showed 100% identity to accessions GQ505648.1 (NRRL36323), and GQ505646.1 (NRRL36318) from an unnamed phylogenetic species within the *Fusarium incarnatum-equiseti* species complex designated FIESC3 (O'Donnell *et al*., 2009). TEF partial gene sequence of *F. proliferatum* was analysed to conduct a phylogenetic tree. Sequences generated in this study were added to the sequences of different *Fusarium* species selected from a BLAST search in NCBI GenBank for better understanding of their phylogenetic relationship (Geiser *et al*., 2004). A NJ tree constructed showed that the onion isolates were grouped together with *Fusarium equiseti* (KF754798, KP881270) and *Fusarium incarnatum* (JN092338) (Figure 1) strains from database:

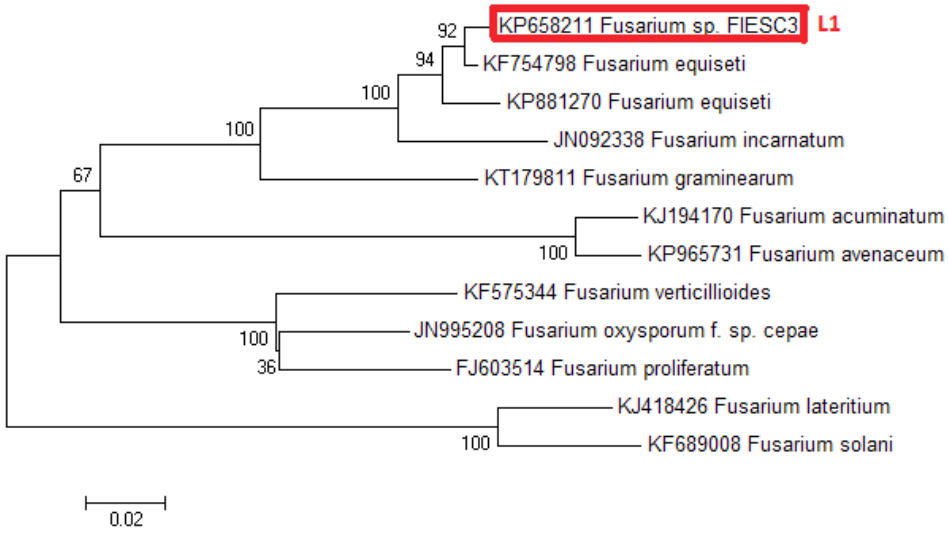

Figure 1. Phylogenetic tree based on Neighbour-Joining (NJ) analysis of TEF gene sequences for L1 isolate from onion and other Fusarium reference strains from NCBI database. Bar – estimated nucleotide substitutions per site is 0.02.

DISCUSSION

Fusarium species, members of FIESC, represent a complex of morphologically similar species (*F. equiseti*/*F. semitectum*/*F. inarnatum*) that comprises 30 phylogenetically distinct species (O'Donnell *et al.,* 2009, 2012). The correct identification of these species is therefore very important together with morphological identification. Using primers EF1 and EF2 to amplify translation elongation factor *1-alpha* gene region a specific band at 700 bp was obtained by PCR for 25 isolates in this study. This part of genome sequence is considered a highly significant information on species level for the entire *Fusarium* genus (Summerell *et al*., 2003; Geiser *et al*., 2004; Kristensen *et al*., 2005). Identification of one isolate was performed by sequencing the translation elongation factor *EF-1α* gene, which was deposited in the NCBI GenBank database under accession number KP658211. In our study, we recovered only one phylogenetic species, designated as FIESC 3. BLASTn queries of GenBank and the *Fusarium* ID-database (Geiser *et al*., 2004) showed 100% identity with accessions GQ505648.1 (NRRL36323), and GQ505646.1 (NRRL36318) from an unnamed phylogenetic species within the *Fusarium incarnatum-equiseti* species complex designated FIESC3 (O'Donnell *et al*., 2009). Castella and Cabanes (2014) used this tool for the analysis of phylogenetic diversity of *Fusarium incarnatum-equiseti* species (FIESC) complex of 51 strains isolated from Spanish wheat. Pathogens of genus *Fusarium* are well known as seed-borne as well as soil borne species able to produce various mycotoxins. Geographic area and climate are the most important factors that influence the occurrence of *Fusarium* and pattern of infestation by various *Fusarium* species (Castella and Cabanes, 2014). Our results showed that causal agent of seed rot in onion is a new species in Serbia designated as *Fusarium* sp. FIESC3. This is not unexpected considering that recent studies in Northern Europe have shown that the predicted climate changes towards 2050 are expected to change the *Fusarium* species composition in world (Parikka *et al*., 2012). Infection of seed results in reduced germination and *Fusarium* rot are hard to control being seed-borne, long persistant, capable of infection and spread in field and storage (Özer & Köycü, 2004; Klokočar-Šmit *et al*., 2008). Previously, Jasnić *et al*. (2005) showed that the decrease in seed germination and plant emergence of soybean is due to seed infection by *Fusarium* sp. in agroecological conditions of Serbia. Morphological identification is time consuming, because these species have small morphological differences and it is difficult to distinguish them from each other. On CLA, all tested isolates formed hyaline, thin-walled, slightly curved, fusoid macroconidia. Microconidia and chlamydospores were not observed. According to Nelson *et al*. (1983) and Burgess *et al*. (1994), morphology of macroconidia, microconidia, and chlamydospore can be assessed in cultures grown on CLA.

CONCLUSION

Knowledge of the composition of populations of *Fusarium* species transmitted by onion seed is of great importance for the establishment of appropriate measures for protection of seeds and seedlings. Based on the completion of Koch's postulates and sequence analysis, investigated isolates belong to *Fusarium* sp. FIESC3, a causal agent of pre-emergence dumping off, decay and rot of onion seed in Serbia. Due to its ability to reduce seed germination, the presence of this pathogen could significantly impact onion production in Serbia. These findings will provide the base to develop the effective disease management strategies.

ACKNOWLEDGMENTS

This research was supported by the Ministry of Science and Technological Development of the Republic of Serbia, project: TR31030 – *Development of new vegetable varieties and hybrids for outdoor growing and greenhouses.*

REFERENCES

Abdel-Satar MA, Khalil MS, Mohmed IN, Abd-Elsalam KA, Verreet JA (2003): Molecular phylogeny of Fusarium species by AFLP fingerprint. *Afr. J. Biotechnol.* 2: 51–56.

Agrios GN (1988): *Plant Pathology*, 3rd. ed. Academic Press, Inc. New York, pp 803.

Burgess LW, Summerell BA, Bullock S, Gott KP, Backhouse D (1994): *Laboratory Manual for Fusarium Research*. Third edition, Sydney.

Castella G, Cabanes FJ (2014): Phylogenetic diversity of Fusarium incarnatum-equiseti species complex isolated from Spanish wheat. *Antonie van Leeuwenhoek* 106: 309–317.

Geiser DM, Del Mar Jiménez-Gasco M, Kang S, Makalowska I, Narayanan N, Ward TJ, Zhang N, Kuldau GA, O'Donnell K (2004): *FUSARIUM*-ID v. 1.0: A DNA Sequence Database for Identifying *Fusarium*. *Eur. J. Plant. Pathol.* 110: 473–479.

Jasnić S, Vidić M, Bagi F, Đorđević V (2005): Pathogenicity of Fusarium species in soybean. *Matica Srpska J. Nat. Sci.* 109: 113–121.

Klokočar-Šmit Z, Jandrić-Ševar M, Kovačev D, Petrov M (1990): *Proučavanje fuzariozne truleži luka i efikasnosti hemijskog tretiranja pred uskladištenje*. Jugoslovenski simpozij, Intenzivno gajenje povrća i proizvodnja u zaštićenom prostoru. Ohrid: 133–140.

Klokočar-Šmit ZD, Lević J, Maširević S, Gvozdanović-Varga J, Vasić M, Aleksić S (2008): *Fusarium* rot of onion and possible use of bioproduct. *Matica Srpska J. Nat. Sci.* 114: 135–148.

Kristensen R, Torp M, Kosiak B, Holst-Jensen A (2005): Phylogeny and toxigenic potential is correlated in *Fusarium* species as reveald by partial translation elongation factor *1 alpha* gene sequences. *Mycol. Res.* 109: 173–186.

Leslie JF, Summerell BA (2006). *The Fusarium Laboratory Manual*, Blackwell: Iowa, pp. 388.

Lević J (2008): *Vrste roda Fusarium u oblasti poljoprivrede, veterinarske i humane medicine*. Beograd: Cicero, pp. 1226.

Lević J, Stanković S, Krnjaja V, Bočarov-Stančić A (2009): *Fusarium* species: The occurrence and the importance in agricultural of Serbia. *Matica Srpska J. Nat. Sci.* 116: 33–48.

Mathur SB, Kongsdal O (2003): *Common Laboratory Seed Health Testing Methods for Detecting Fungi*, ISTA, Switzerland.

Nelson PE, Tousson TA, Marasas WFO (1983): *Fusarium* species. An illustrated manual for identification. The Pennsylvania State University Press, University Park, Pennsylvania.

O'Donnell K, SuttonDA, Rinaldi MG, Gueidan C, Crous PW, Geiser DM (2009): Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-equiseti* and *F. chalmydosporum* species complexes within the United States. *J. Clin. Microbiol.* 47: 3851–3861.

O'Donnell K, Humber RA, Geiser DM, Kang S, Park B, Robert VA, Crous PW, Johnston PR, Aoki T, Rooney AP, Rehner SA (2012): Phylogenetic diversity of insecticolous fusaria inferred from multilocus DNA sequence data and their molecular identification via *FUSARIUM*-ID and *Fusarium* MLST. *Mycologia* 104: 427–445.

Özer N, Köycü D (2004): Seed-borne Fungal Diseases of Onion, and their control Fruit and Vegetable Diseases. *Disease Management of Fruits and Vegetables* 1: 281–306.

Parikka P, Hakala K, Tiilikkala K (2012): Expected shifts in *Fusarium* species composition on cereal grain in Northern Europe due to climatic change. *Food Addit. Contam.* 29: 1543–1555.

Rahjoo V, Zad J, Javan-Nikkhah M, Mirzadi Gohari A, Okhovvat SM, Bihamta MR (2008): Morphological and molecular identification of Fusarium isolated from maize ears. *Iran. J. Plant Pathol.* 90: 463–468.

Summerell BA, Salleh B, Leslie JF (2003): A utilitarian approach to *Fusarium* identification. *Plant dis.* 87: 117–128.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011): MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731–2739.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* 30: 2725–2729.

Thompson JD, Higgins DG, Gibson TJ (1994): CLUSTAL W.: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.

Tuite J (1969): *Plant pathological methods: fungi and bacteria*. Minneapolis: Burgess, pp. 239.

ИДЕНТИФИКАЦИЈА И ФИЛОГЕНЕТСКА АНАЛИЗА *Fusarium* sp. FIESC 3 ПРОУЗРОКОВАЧА ТРУЛЕЖИ СЕМЕНА ЦРНОГ ЛУКА (*Allium cepa* L.)

Маја В. ИГЊАТОВ[1], Татјана Ч. ПОПОВИЋ[2], Драгана Н. МИЛОШЕВИЋ[1], Зорица Т. НИКОЛИЋ[1], Гордана Р. ПЕТРОВИЋ[1], Јелица М. ГВОЗДЕНОВИЋ ВАРГА[1], Жарко Ј. ИВАНОВИЋ[2]

[1]Институт за ратарство и повртарство
Максима Горког 30, Нови Сад 21000, Србија
[2]Институт за заштиту биља и животну средину
Теодора Драјзера 9, Београд 11000, Србија

РЕЗИМЕ: Црни лук (*Allium cepa* L.) спада у најзначајније повртарске биљне врсте у Србији. Гаји се на површини од око 20.000 хектара. Током рутинске контроле квалитета семена црног лука у 2014. години, примећена је појава *Fusarium* sp. у високом проценту – од 28%. Циљ рада био је изолација и идентификација *Fusarium* sp. на основу морфолошких и молекуларних карактеристика патогена. Узорци семена лука сакупљени су с различитих локалитета и из великог броја складишта у Војводини. Након изолације патогена одабрано је и морфолошки окарактерисано 25 изолата *Fusarium* sp. Изолација ДНК извршена је директно из мицелија гљиве (~ 100 mg), коришћењем Dneasy Plant Mini Kit (Qiagen, Hilden, Germany). Амплификација ДНК циљаног гена (translation elongation factor EF-1α gene) обављена је помоћу PCR коришћењем пара прајмера EF1 и EF2. У свим проучаваним изолатима формирани су ампликони величине 700 bp. Идентификација једног одабраног изолата извршена је секвенцирањем транслационог фактора EF-1α гена, који је депонован у NCBI базу података под бројем KP658211 (*Fusarium* sp. FIESC 3).

КЉУЧНЕ РЕЧИ: FIESC3, *Fusarium* sp., црни лук, *EF-1α* ген, семе, секвенцирање