Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. Novi Sad, № 137, 49—55, 2019

UDC 635.262:579.8
https://doi.org/10.2298/ZMSPN1937049I

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IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF *Fusarium proliferatum* ISOLATED FROM ELEPHANT GARLIC *Allium ampeloprasum* L.

ABSTRACT: Symptoms of cloves rot of *A. ampeloprasum* were noticed during 2018 in storage conditions. 16 isolates were obtained (AMP1-AMP16) and according to morphological and cultural characteristics they belong to *F. proliferatum* (sex. stage *Gibberella intermedia*), species of *Gibberella fujikuroi* complex. To confirm morphological identification, total genomic DNA was extracted from mycelium of the 16 isolates by amplification of TEF-1α gene, using polymerase chain reaction (PCR) that was performed with the primer pair EF1 and EF2. Results presented in this article clearly indicated that the new host of *Fusarium proliferatum* as the causal agent of cloves rot is „elephant garlic" *Allium ampeloprasum*. Pathogenicity test was confirmed on *Allium ampeloprasum* cloves. Pathogenicity assays revealed that all isolates caused symptoms on tested *Allium* spp., like naturally infected cloves.

KEYWORDS: *Allium ampeloprasum*, *Fusarium proliferatum*, cloves rot

INTRODUCTION

Allium ampeloprasum (Family Amaryllidaceae Subfamily-Allioideae) is a medicinal plant well known for its pharmaceutical potential with characteristic large mature cloves, commonly known as „elephant garlic" (Sharifi-Rad et al., 2016). It is native in range from southern Europe to western Asia, but it is naturalized and cultivated worldwide. In Serbia, wild form of *A. ampeloprasum* is kept in the collections of the Institute of Field and Vegetable Crops (curator

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Jelica Gvozdanović-Varga) and it is used in the garlic (*Allium sativum* L.) breeding research program. Symptoms of cloves rot of *A. ampeloprasum* were noticed during 2018 in storage conditions. Most of the diseased cloves did not show visible symptoms, but after cleaning and when they were peeled, deep lesions covered with fungal growth were observed (Figure 1a, 1b). According to Fuentes et al. (2013), the frequent occurrence of bulb rot during storage period has become a limiting factor to garlic production, emphasized *F. proliferatum* as a major postharvest issue. The first report of *F. proliferatum* as a causal agent of garlic rot came from Germany (Seefelder et al., 2002), and subsequently it was reported in North America (Dugan et al., 2003), Serbia (Stanković et al., 2007), Spain (Palmero et al., 2008) and India (Sankar and Prasad Babu, 2012). Based on pathogenicity tests, Stanković et al. (2007) claimed that *F. proliferatum* should be regarded as a potentially serious pathogen of garlic in Serbia.

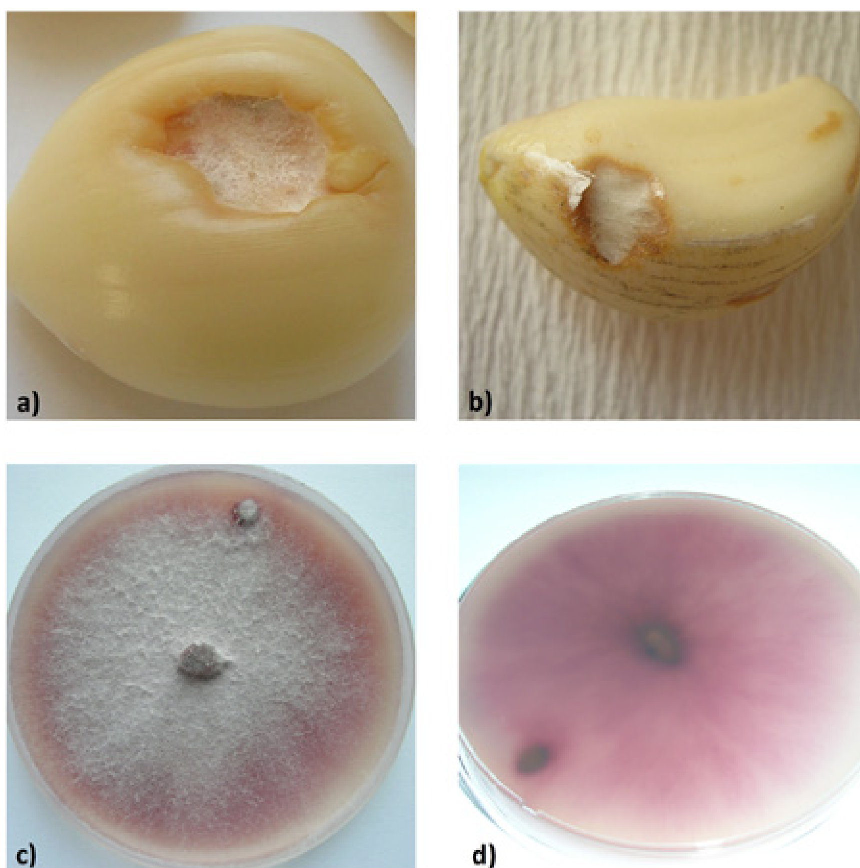

Figure 1. Fusarium proliferatum: a) and b) Symptoms on infected *Allium ampeloprasum* cloves – natural infection; c) Seven-day-old colony of isolate AMP11 grown on PDA – photographed from the top; d) Violet pigmentation released in PDA medium – bottom side of the agar plate.

MATERIAL AND METHODS

Isolation

In order to isolate the disease causing organism, cloves were separated, peeled off, surface disinfested in 1% NaOCl for 2 min, rinsed with sterilized distilled water, dried on a sterile filter paper and small parts of infected tissues were plated onto the medium surface. Potato dextrose agar (PDA) was used with the addition of 300 mg/l antibiotic (streptomycin sulfate). After 7 days at 25 °C, *Fusarium* colonies were examined and 16 isolates (AMP1-AMP16) were subcultured using a single spore technique. Growth rates are based on radial growth in a Petri dish. Plates were arranged in an incubator according to the experimental design and colony diameter measurements were taken at 3, 5 and 7 days after plating. Morphological and cultural characterization of isolates, cultured onto both PDA and Carnation leaf agar (CLA), was done according to Gerlach and Nirenberg (1982) and Leslie and Summerell (2006).

Pathogenicity test

Artificial inoculation of the five cloves of *Allium sativum* (autumn cv. Bosut, Ranko) and *Allium ampeloprasum* (cv. Biser), was done by using 7 day old mycelia from each isolate grown on PDA as an inoculum according to the method described by Palmero et al. (2012) and Dugan et al. (2007). Disinfection was done by dipping of cloves in 0.5% NaOCl for 60 seconds and rinsing in sterile water after which small pieces of inoculum were placed in a depth of 4 mm into the clove using a 1-mm diameter probe. Inoculated cloves were placed in aseptic sealed plastic boxes and incubated for three weeks at 25 °C, after which re-isolation of the fungi was done. For each isolate, another set of five cloves was inoculated with sterile PDA as negative control.

Molecular identification

In order to confirm morphological identification, sequencing of TEF-1α gene was performed using polymerase chain reaction (PCR) with the primer pair: EF1 and EF2 (O'Donnell et al., 1998). To obtain a DNA sequence, a total DNA of the 16 isolates and positive control FE-3 was extracted directly from the 7 day old mycelium (~ 100 mg wet weight), with a Dneasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Identification of isolate AMP11 was performed by sequencing the translation elongation factor TEF-1α gene. Purification and sequencing of the amplified fragments were performed in Company MACROGEN, Seoul, South Korea (http://dna.macrogen.com, Korea). Sequences were analysed in the program Finch TV Version 1.4.0., and filed in the GenBank database under the National Center for Biotechnology Information (NCBI).

Phylogenetic analysis

Phylogenetic tree on individual translation elongation factor TEF-1α gene sequences was constructed using the Maximum Likelihood (ML) phylogeny with 1,000 bootstrap repeats and pairwise deletion, implemented in MEGA 6 software (Tamura et al., 2013). Manual comparisons, corrections and alignments of the sequences were performed using CLUSTALW integrated into MEGA6 software. Kimura 2-parameter model, Gamma distributed (G) determined by Model test implemented in MEGA6 was used as the best fitting model of nucleotide substitution.

RESULTS AND DISCUSSION

Colonies of all investigated isolates were fast-growing, reaching 7–8.5 cm in diameter after 7 days, forming abundant, aerial white mycelium with violet to dark pigments released in PDA. According to Leslie and Summerell (2006), violet pigments are usually produced in the agar, but with overall pigmentation varying in intensity from nearly colorless to almost black. All isolates formed typical slightly curved macroconidia, with distinct foot cell, mostly three to five septate, measuring 32–55 µm x 3.4–4.2 µm. On CLA, one-celled, slightly pyriform microconidia were formed in long chains or coherent in the false heads. Chlamydospores were absent. According to morphological and cultural characteristics, all investigated isolates belong to *F. proliferatum* (sex. stage *Gibberella intermedia*), species of *Gibberella fujikuroi* complex, Liseola section. Results presented in this article are in compliance with species descriptions given by Gerlach and Nirenberg (1982) and Leslie and Summerell (2006).

Pathogenicity test was performed on *Allium sativum* (autumn cv. Bosut, Ranko) and *Allium ampeloprasum* (cv. Biser). Pathogenicity assays revealed that all tested isolates caused symptoms on tested *Allium* spp., like naturally infected cloves (Figure 2). Regarding the daily mycelial growth rate isolates AMP7, AMP11, and AMP13 were the fastest-growing and they caused rotting cloves after 7, 13 and 15 days, respectively. All isolates were re-isolated from symptomatic tissue thus fulfilling Koch's postulates. They were cryopreserved at -80 °C. No fungi recovered from control cloves.

As a result of molecular identification, the sequence of isolate AMP11 was deposited in the GenBank under Accession number MK061545. Genome sequence of TEF-1α gene is considered as highly significant information on species level for the entire *Fusarium* genus (Summerell et al., 2003; Geiser et al., 2004). This study based on analysis TEF gene sequences confirmed that strain originated from *A. ampeloprasum* had 100% homology to sequences of *F. proliferatum* strains obtained from NCBI database.

Figure 2. Pathogenicity test performed on *Allium ampeloprasum*:
Lesions on cloves covered with fungal growth of *F. proliferatum*

For better understanding of phylogenetic relationship of *F. proliferatum* isolate originated from elephant garlic, sequences were compared with sequences data set of *F. proliferatum* originated from garlic and *Fusarium* strains from different hosts. The sequence analysis of translation elongation factor EF-1α gene, grouped in the same cluster consisted of *F. proliferatum* isolates from garlic: strains F1119, F1131 from Italy, A6m1 from Spain, B3 from Serbia, but also with strains CBS 131570 from wheat from Iran, G16NH2-2-27S-1 from China and F90 from strawberry originated from Spain (Figure 3). Strain M14022 originated from grape in China was clustered separately.

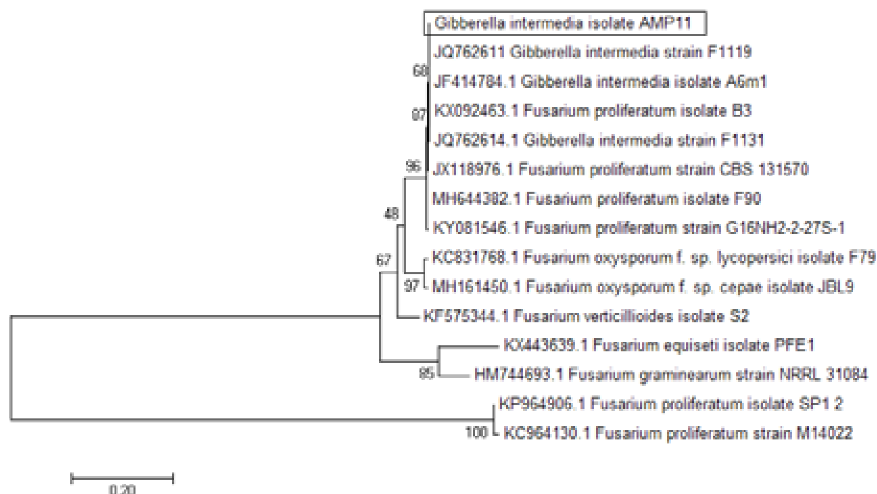

Figure 3. Maximum Likelihood phylogenetic tree of *F. proliferatum* isolates derived from a translation elongation factor EF-1α gene. Bar – estimated nucleotide substitutions per site is 0.2.

Results presented in this article clearly indicated that *Fusarium proliferatum* is the causal agent of cloves rots of *Allium ampeloprasum*. This species, although widespread as a pathogen of sorghum, maize, asparagus, onion, and garlic has not been described as a pathogen of *Allium ampeloprasum*.

CONCLUSION

Garlic bulbs are routinely stored at room temperature for several months or in refrigerated chambers with a high percent of moisture. The presence of *Fusarium proliferatum* cause rot of bulbs and cloves and it can be a threat to the collection of various Allium species preserved in storages. It can increase rot progression and severity with impact on reduction in germination. To our knowledge *Allium ampeloprasum* in the new host of *Fusarium proliferatum* as causal of cloves and bulbs rot.

ACKNOWLEDGMENTS

This research was supported by the Project TR31030 of Ministry of Education, Science and Technological Development, Republic of Serbia.

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ИДЕНТИФИКАЦИЈА И ФИЛОГЕНЕТСКА АНАЛИЗА *Fusarium proliferatum* ПРОУЗРОКОВАЧА ТРУЛЕЖИ ЛУКА *Allium ampeloprasum* L.

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РЕЗИМЕ: Симптоми трулежи ускладиштених ченова белог лука на врсти познатој као „elephant garlic" (*A. ampeloprasum*) запажени су током 2018. године. Прикупљен је велики број заражених узорака и након изолације одабрано је 16 изолата (AMP1-AMP16) за даља истраживања. На основу морфолошких и одгајивачких карактеристика је установљено да је проузроковач трулежи ченова гљива *F. proliferatum* (телеморф *Gibberella intermedia*), која припада комплексу *Gibberella fujikuroi*. Проучавањем патогености сви изолати су проузроковали симптоме трулежи на различитим врстама рода *Allium* spp. који су идентични природној инфекцији. У циљу потврде морфолошких одлика извршена је молекуларна идентификација методом ланчане реакције полимеразе (PCR) коришћењем пара прајмера EF1 и EF2 који амплификују TEF-1α ген, секвенционирање ДНК и филогенетска анализа добијених секвенци. Добијени резултати потврдили су да је *Fusarium proliferatum* проузроковач трулежи на врсти *A. ampeloprasum* као новом домаћину.

КЉУЧНЕ РЕЧИ: *Allium ampeloprasum*, *Fusarium proliferatum*, ченови белог лука