Mycologia Iranica 9(1): 51–57, 2022 **Original Article** DOI: 10.22092/MI.2022.360409.1238

Macrophomina vaccinii a new species for funga of Iran

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Abstract: In summer 2020, bean plants (*Phaseolus vulgaris* L.) with symptoms of wilting, chlorosis, drying and the formation of numerous microsclerotia in the stem were detected from Ghachsaran fields, Kohgiluyeh & Boyer-Ahmad provinces of Iran. Infected samples of stem were taken to the laboratory, cut into small sections, sterilized, washed in sterile water, dried on sterile paper towels, and cultured on Potato Dextrose Agar (PDA) amended with 50 µg of kanamycin to prevent bacterial contamination. Isolates were purified by hyphal tip technique. Four isolates from infected stems tissue were recovered. Fungal isolates were identified based on morphological characteristics and molecular data of *tef1*-α gene. According to the morphological and phylogenetic analysis, the isolates were identified as *Macrophomina vaccinii*. This is the first report of *M. vaccinii*, in Iran.

Keywords: Common bean, Iran, morphology, phylogeny, *tef1*-α,

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.), one of the most important crops in terms of both its economic and nutritional value, is cultivated in different parts of Iran (Osdaghi & Lak 2015).

Charcoal rot, which is caused by *Macrophomina* spp. species, is a devastating disease that occurs from the seedling stage to maturity stage, and is responsible for low yields in crops of great economic importance such as beans, soybean, sugarcane, cotton and under the dry and hot conditions of different regions throughout the world (Gupta et al. 2012).

The fungus belongs to the family *Botryosphaeriaceae*, causes diseases such as root rot, charcoal rot, leaf blight, seedling blight, pre- and post-emergence damping-off, and stem and pod rot on over 750 plant species (Farr & Rossman 2020) and produced either microsclerotia (the primary source of inoculum) or pycnidia in the roots and stems of the host, which enable it to survive in crop debris and the soil for prolonged periods (Cohen et al. 2016) and characterized by brown, septate mycelium with abundant production of black microsclerotia, pycnidial conidiomata, with aseptate, hyaline conidia with apical mucoid appendages, sometimes becoming dark and septate with age (Phillips et al. 2013).

Mostly, the identification of *Macrophomina* species by morphological analysis is considered impossible, mostly due to the overlap of the conidia morphology between the species (Machado et al. 2018). In recent years, different parts of the *Macrophomina* genome are used in molecular phylogeny-based taxonomy such as sequences of internal transcribed spacer (ITS) of rDNA, β-tubulin (βT), calmodulin (CAL) and translation elongation factor 1- alpha (TEF1-α) and β-tubulin (Sarr et al. 2014; Machado et al. 2018). Until now, five species are accommodated within *Macrophomina*, viz. *M. phaseolina*, *M. tecta* Vaghefi, B. Poudel & R.G. Shivas, *M. euphorbiicola* A.R. Machado, D.J. Soares va O.L. Pereira, *M. pseudophaseolina* Crous, Sarr va Ndiaye, and *M. vaccinii* Zhang ter & L. Zhao. (Sarr et al. 2014; Machado et al. 2018; Poudel et al. 2021).

Most isolates of *Macrophomina* have been attributed to the generalist phytopathogen *M. phaseolina*. *M. phaseolina* is the most common species and is the type of the genus that first was isolated from *Phaseolus vulgaris* in 1947 from Italy (Dhingra & Sinclair 1978).

M. pseudophaseolina was described by Sarr et al. (2014), reported on peanut, cowpea and okra in Senegal (Sarr et al. 2014), and then in peanut, cotton, and castor bean in Brazil (Machado et al. 2018). *M. euphorbiicola* was described by Machado et al. (2018), isolated on bellyache bush and castor bean in Brazil. *M. tecta* was described by Poudel et al. (2021), and isolated from *Vigna radiata* and *Sorghum bicolor* with charcoal rot symptoms in New South Wales and Queensland. *M. vaccinii* was described by Zhao et al. (2019), and indicated that could cause stem blight of blueberry.

Submitted 10 Feb 2022, accepted for publication 30 June 2022
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Previously, *M. phaseolina* has been detected in numerous host plants in Iran such as bean (Ershad 2022). To date, there is no available information related to other species as the agent of charcoal rot on bean in Iran. This study aimed to recognize and characterize the *Macrophomina* isolates recovered from bean plants in Iran based on morphological and molecular features.

MATERIALS AND METHODS

Fungal isolation and morphological characterization

In summer 2020, charcoal rot was observed in bean plants growing in Ghachsaran, Kohgiluyeh & Boyer-Ahmad provinces in southern Iran. Disease symptoms included general wilting, chlorosis, drying and the formation of numerous microsclerotia in the stem, crown and root of the plants.

Plant tissue surface with charcoal rot symptoms sterilized for one min. in 1% (v/v) sodium hypochlorite, rinsed three times in sterile distilled water, and examined under a dissecting microscope. Single microsclerotia were transferred to kanamycin-amended (50 µg/mL, Sigma-Aldrich, Australia) water agar plates. Pure fungal cultures were established by transferring hyphal tips of the growing fungi onto potato dextrose agar using a sterile needle under aseptic conditions.

Infected samples of the stem were taken to the laboratory in polyethylene bags and kept in the refrigerator until diagnostic laboratory isolations. Infected tissues were cut into small sections, 3-5 mm, with a sterile calpel from dead plants or the boundary of the necrotic lesions, sterilized in 70% ethanol for 30s and then, % (v/v) sodium hypochlorite for one min, washed in sterile water, dried on sterile paper towels and cultured on Potato Dextrose Agar (PDA) medium (PDA; 12 g/L potato extract, 11 g/L dextrose, 1.2% agar) amended with 50 µg of kanamycin to prevent bacterial contamination. The plates were incubated at 30°C and observed daily growth (Pickel et al. 2020). Isolates were purified by hyphal tip technique (Dasgupta, 1988).

Macrophomina species were identified using morphological characteristics i.e. conidiomata, conidia and conidiogenous cells, microsclerotia morphology, colony pattern and growth rate at different temperatures (Crous et al. 2006; Sarr et al. 2014). In order to obtain conidia and conidiophores, mycelial plugs of each isolate grew on PDA medium and transferred on 2% water-agar (WA) medium containing needles or twigs of *Pinus* sp. and maintained at 25°C under a 12-hour light/dark period for 8 weeks to induce sporulation (Crous et al. 2006; Sarr et al. 2014).

For morphological assay of the species, ninety conidia and microsclerotia of each isolate were measured using Olympus light microscope CX31, equipped with a Dino-eye microscope eye-piece camera in conjunction with Dino Capture version 2.0 software.

DNA extraction, amplification, and phylogenetic analysis

Isolates were grown in Potato Dextrose Broth (PDB) at 30°C for one week on a rotary shaker; the mycelium was harvested in a sterile 2 mL microcentrifuge tube, and freeze-dried. Total DNA was extracted with the CTAB technique as described by Murray and Thompson (1980). DNA quantification and qualification was measured by spectrophotometer and agarose gel, and the genomic DNA was stored at −20 °C for further analysis.

The pair of primers EF1-728F (Carbone & Kohn, 1999) EF2R (Jacobs et al. 2004) were used to amplify the translation elongation factor 1-α (*tef1*-α) gene. PCR amplifications were performed in volumes of 20 µl comprising 0.05 µM of each forward and reverse primer (CinnaGen), 0.4 µM dNTPs (MBI Fermentas), 1× Dream Taq buffer (MBI Fermentas), and 0.5 U Dream Taq DNA polymerase (MBI Fermentas). Thermal cycling was performed by using an initial denaturation step for 5 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 94°C, elongation step for 1 min at 72°C, and a final elongation step for 7 min at 72°C and lastly, the optimized annealing temperature for these primers were 1 min at 55°C (Machado et al. 2018).

The amplicons were analyzed on a 1% agarose electrophoresis gels, stained with ethidium bromide, and viewed under UV light to check the amplification size and purity (GelDoc, Bio-Rad Laboratories). PCR products were purified from agarose gels using a PCR purification kit (Fermentas) and DNA sequencing was performed at each of the forward and reverse directions by Macrogen Company (South Korea).

The nucleotide sequences of *tef1*-α sequence gene were blasted using Megablast to identify their closest neighbors, edited by BioEdit v. 7.2.5 (Hall 2012) and aligned using MAFFT v.7 (Katoh & Standley 2013). Manual adjustment of sequence alignments was performed to accommodate insertions/deletions. BLASTn search (which is available at https:// blast.ncbi.nlm.nih.gov/) was conducted to compare newly obtained sequences against the NCBI database. All sequences used in this research are listed in Table 1. Phylogenetic analyses were performed in MEGA5 (Tamura et al. 2011) using the Maximum likelihood method (Saitou & Nei 1987).

Cophinforma eucalypti were used as an outgroup in the analysis. New nucleotide sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov), and the accession numbers of sequences were OP672295 to OP672298 (Table 1).

T**able 1.** Details and GenBank accession numbers of *Macrophomina* isolates included in this study.

Species name	Isolates	Source	Accession number *tef1-α*
Macrophomina phaseolina	CMM4222	Zhao et al. 2019	KU058918
M. phaseolina	CMM4131	Zhao et al. 2019	KU058914
M. pseudophaseolina	CMM4032	Machado et al. 2018	KU058905
M. pseudophaseolina	CMM4029	Machado et al. 2018	KF553903
M. euphorbiicola	CMM4145	Zhao et al. 2019	KU058907
M. vaccinii	CGMCC3.19509	Zhao et al. 2019	MK687432
M. vaccinii	CGMCC3.19503	Zhao et al. 2019	MK687426
M. vaccinii	CGMCC3.19510	Zhao et al. 2019	MK687433
M. tecta	BRIP70713	Poudel et al. 2021	MW592270
M. tecta	BRIP70717	Poudel et al. 2021	MW592277
M. vaccinii	Macro-V1	This study	**OP672295**
M. vaccinii	Macro-V2	This study	**OP672296**
M. vaccinii	Macro-V3	This study	**OP672297**
M. vaccinii	Macro-V4	This study	**OP672298**
Cophinforma eucalypti	MFLUCC110425	Machado et al. 2018	JX646865

RESULTS AND DISCUSSION

In this work, four morphologically similar isolates were recovered from infected tissues in bean fields from Kohgiluyeh & Boyer-Ahmad provinces of Iran (Table 1). A large number of microsclerotia, dark brown to black, were observed on the infected stems of symptomatic plants (Fig. 1f). Overall, four isolates belonged to *M. vaccinii* species based on the morphological characteristics, obtained from symptomatic plants. None of the four isolates produced sexual stage on different medium. These isolates produced white colonies with fluffy aerial mycelium which gradually turned pale olivaceous to olivaceous-black (Fig. 1a-e) along with sclerotial masses that were hard, smooth, dark brown to black, ellipsoid to obovoid, 60-90 µm in diameter when produced in the laboratory (Fig. 1g-h).

Isolates produced pycnidia on WA medium overlaid with needles or twigs of *Pinus* sp., were black, globose, mostly solitary, up to 350 µm in diameter with a central ostiole. The conidia were hyaline, aseptate, smooth, straight to curved, ellipsoid to ovoid with apical sheath, 19-30 × 7.6-10.6 µm in size (Fig. 1i). The optimum temperature for colony growth was 27°C on PDA.

Isolates Macro-V1 to Maco-V4 were nominated as *Macrophomina vaccinii* Y. Zhang ter & L. Zhao, in morphological analyses because of the numerous morphological characteristics corresponding to the original description of *M. vaccinii* by Zhao et al. (2019). PCR amplification and sequencing were successful for four isolates. The obtained sequences of *Macrophomina* isolates were submitted to GenBank under the following accession numbers: OP672295 to OP672298 for *tef1*-α region (Table 1).

Phylogeny analyses of *tef1*-α sequences revealed that four isolates Macro-V1 to Maco-V4 are a monophyletic group with *M. vaccinii* in a well-supported clade (posterior probability = 98). phylogenetic analysis indicated an excellent distinction between *M. vaccinii* among the other *Macrophomina* species (Fig. 2). As a result, the overall topologies of our phylogenetic trees were consistent with previous studies (Poudel et al. 2021; Zhao et al. 2019).

In general, the distinction of *Macrophomina* species based only on morphological features is considered impossible, mainly due to the overlap of the conidia features between the species (Machado et al. 2019). Previous studies showed that only the ITS-rDNA region sequence data is not sufficient to distinguish the *Macrophomina* species (Machado et al. 2018) and the distinction of *Macrophomina* species was done based on multilocus phylogenetic analysis of sequences ITS- rDNA, actin, β-tubulin, calmodulin, TEF1-α (Sarr et al. 2014). Then, Hyde et al. (2014) reported that the *tef1*-α gene can present an adequate phylogenetic signal for species discrimination, and also Machado et al. (2018) suggested that the *tef1*-α gene can be used as a primary marker for *Macrophomina* species distinction in population screening studies. Consequently, *M. vaccinii* in the

present study was verified using *tef1*-α gene and led to a reliable and accurate identification and these results are consistent with the finding of Machado et al. (2018) and Hyde et al. (2014)

The source of primary introduction of *M. vaccinii* to bean fields is not known. This *Macrophomina* species may have been in the fields for a long time. Environmental parameters such as seasonal temperature fluctuations and global warming are factors affecting the incidence of *Macrophomina* species. Disease symptoms are more severe under dry and warm growing conditions (Melo et al. 2021). Drought stress may happen in any developmental phase of the common bean and increase crop predisposition to infection by *Macrophomina* species due to negative effects on the plant. Drought stress reduces stomatal conductance, transpiration rate, and water potential (Mayek-PÉrez et al. 2002).

The climate in southern Iran, with hot and dry summers and winters short and cool, is suitable for the activation of *Macrophomina* and causes a major reduction in sesame yield (Edraki and Banihashemi 2010). Therefore, intensive sampling of bean and other plants in many regions of Iran would help to detect other *Macrophomina* species, and then management strategies are essential to prevent the spread of this species.

So far, only *Macrophomina phaseolina* species had been reported on bean plants in Iran (Ershad 2022). It is worth mentioning that *M. vaccinii* can be another agent of charcoal rot on bean plants based on morphological and molecular diagnostics. According to our best knowledge, *M. vaccinii* is reported for the first time in Iran.

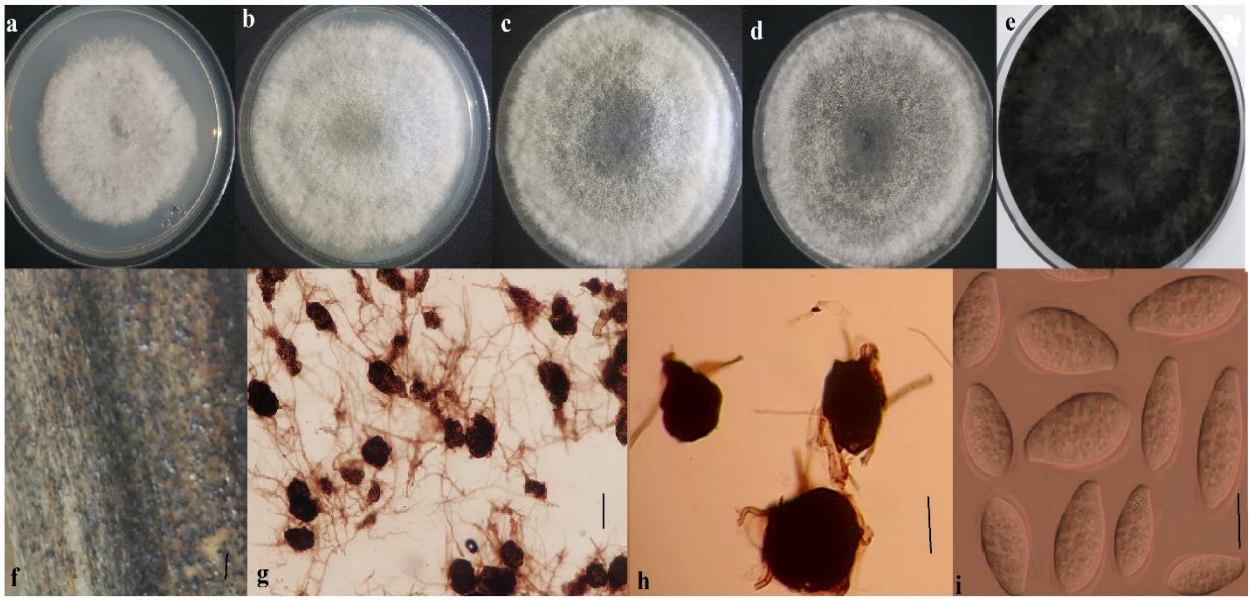

Fig. 4. Characteristics of *Macrophomina vaccinii* isolated from *Phaseolus vulgaris*; (a-d) White colonies with fluffy aerial mycelium, (e) Dark olive to black colony on PDA medium, (f) Microsclerotia on infected stem, (g-h) The formation of black microsclerotia on PDA, (i) Conidia extruded from the pycnidia. Scale bars (f) = 100µm, (g)= 20µm, (h, i) = 40µm.

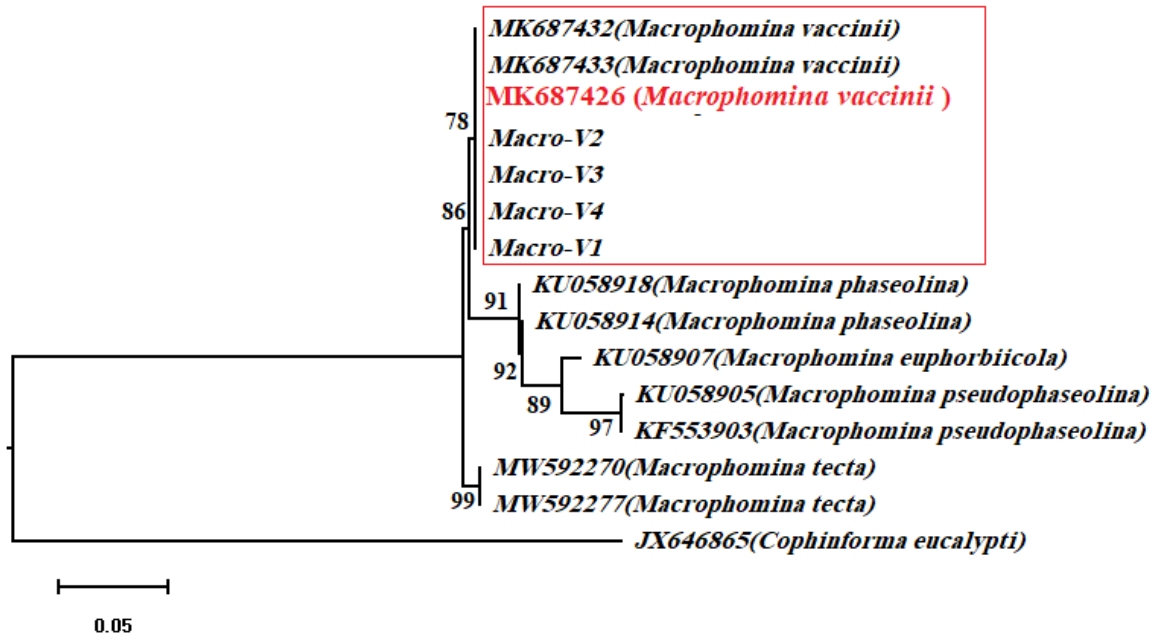

Fig. 2. Maximum likelihood phylogenetic tree inferred from *tef1*-α gene sequences of *Macrophomina* species. Bootstrap values are labeled on the branches. *Cophinforma eucalypti* isolate JX646845 is included as an outgroup. *Macrophomina vaccinii* isolates obtained in this study are indicated in the red box.

ACKNOWLEDGEMENTS

The author gratefully acknowledges Yasouj University (Yasouj, Iran) for financial support.

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Macrophomina vaccinii، گونه جدید برای قارچ‌های ایران

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گروه گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه یاسوج، یاسوج، ایران.

چکیده: در بهار ۱۴۰۰، گیاهان لوبیا با علائم پژمردگی، کلروز، خشکیدگی و حاوی تعداد زیادی سختینه روی ریشه، طوقه و ساقه از منطقه گچساران در استان کهگیلویه و بویراحمد جمع‌آوری شدند. ساقه گیاهان آلوده به قطعات کوچک‌تر بریده، ضدعفونی و روی محیط کشت سیب‌زمینی-دکستروز –آگار (PDA) کشت داده شدند. خالص سازی جدایه‌ها به روش نوک ریسه انجام شد. در مجموع چهار جدایه از بافت‌های آلوده جداسازی شد. بر اساس خصوصیات ریخت‌شناختی و داده‌های مولکولی به دست آمده از توالی‌یابی بخشی از ژن *tef1-α*، جدایه‌های به‌دست آمده متعلق به *Macrophomina vaccinii* بودند. این اولین گزارش از گونه *M. vaccinii* در ایران است.

کلمات کلیدی: لوبیا، ایران، مورفولوژیکی، فیلوژنی، *tef1-α*

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تاریخ دریافت: ۱۴۰۰/۱۱/۲۱ تاریخ پذیرش: ۱۴۰۱/۴/۹