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First record of *Rhizopus oryzae* from stored apple fruits in Saudi Arabia

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Abstract

During spring 2017, red delicious and Granny Smith apples with soft rot symptoms were collected from commercial markets in Saudi Arabia. The causal agent was isolated from infected fruits and its pathogenicity was confirmed by inoculation assays. To confirm the identity, total genomic DNA was extracted and multi-locus sequence data targeting two gene markers (ITS, ACT) was sequenced. Phylogenetic analysis confirmed the presence of two distinct clades; Saudi isolate was placed within a clade comprising *Rhizopus oryzae* and *R. delemar* reference isolates. Based on morphological characteristics, molecular identification and pathogenicity test, the fungus was identified as *Rhizopus oryzae*. This is the first report of Rhizopus soft rot caused by *R. oryzae* from stored apple fruits in Saudi Arabia.

Key words – Rhizopus soft rot – phylogeny – pathogenicity – morphological characters – sequence data

Introduction

Rhizopus soft rot caused by *Rhizopus oryzae* occurs worldwide and reduces the quantity and quality of harvested vegetables and ornamental crops during storage, transit, and marketing (Amadioha 1996, 2001, Agrios 2005). Rhizopus soft rot is one of the most important postharvest diseases of apple, banana, watermelon, sweet potato and other hosts in Korea (Kwon et al. 2000, 2010, 2011, 2012a, 2012b, 2014). *R. oryzae* is a complex of closely related, heterothallic species that are common, cosmopolitan saprotrophs in soil, dung, and rotting vegetation (Ellis 1985, Liou et al. 2001). Zheng et al. (2007) proposed that the name *R. oryzae* be replaced by *R. arrhizus*. Combined morphological and molecular based identification methods can clarify relationships among species of low morphological divergence such as *Rhizopus* species (Zheng et al. 2007, Abe et al. 2010, Hartanti et al. 2015). The main objective of the current research was to isolate and identify the soft rot fungus associated with recent outbreaks of soft rot on stored apple fruits in Saudi Arabia.

Materials & Methods

Fungal isolates

Twenty red delicious and Granny Smith apples were collected from commercial markets between March and April 2017 in Alquwayiyah, Saudi Arabia. Each apple was placed in a sterile

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plastic bag at room temperature for six days or until fungal growth covered the sample. Isolation was performed onto potato dextrose agar (PDA) as described previously (Kwon et al. 2011). Fungal mycelial tips produced on the diseased apple fruits were transferred to PDA.

Pathogenicity assay

Ten healthy apple fruits were artificially inoculated with the isolated fungus using the wound infection method. One hundred microliters of spore suspension (3×10^3 sporangiospores/mL) of the causal fungus was inoculated inside apple fruit and incubated at 28°C in a growth chamber with 100% relative humidity. After 4 days incubation, soft rot was observed on inoculated fruits, the symptoms being identical to those observed in the commercial markets. The same fungal pathogen was reisolated from the lesions to prove Koch's postulates. Morphological characteristics, such as colony colour, shape and size of sporangia, sporangiophores, and columellae, and rhizoid type were examined according to Zheng et al. (2007) by using a light microscope. Measurements of sporangiophore length and sporangiospore size were made in 20 replications ($n = 20$).

Multi-locus sequencing

Fungal DNA was extracted with Exgene Plant-Fungal SV Mini Kit (GeneAll Biotechnolgy Co., Seoul, Korea) following the manufacturer's instructions. The internal transcribed spacer (ITS) region of rDNA and the D1/D2 region of the large subunit (LSU) were amplified with primers ITS1/ITS4 (White et al. 1990) and parts of the actin (ACT) and sequenced. PCR conditions were followed according to methods described previously (Dolatabadi et al. 2014). PCR products were purified using a Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were directly used for a sequencing reaction at Macrogen Services (Daejeon, South Korea).

Phylogenetic analyses

Sequences generated from ITS gene regions and actin (ACT) gene were identified by BLAST analysis in GenBank database and sequences were analyzed with closely related taxa in *Rhizopus* from GenBank (Table 1). The sequences were automatically aligned in MAFFT v. 7 at the web server (http:// mafft.cbrc.jp/alignment/server; 2016). Maximum-likelihood (ML) analysis was performed in raxmlGUI v.0.9b2 (Silvestro & Michalak 2012). The RAxML software accommodated the general time reversible (GTR) model of nucleotide substitution with the additional options of modelling rate heterogeneity (Γ) and proportion invariable sites (I). Bootstrap support was obtained by running 1000 non-parametric bootstrap iterations. Rapid bootstrap analysis (Stamatakis et al. 2006) and searches for the best-scoring ML tree (RAxML option "-f a") were applied (Silvestro & Michalak 2012). Maximum likelihood bootstrap values (ML) equal or greater than 40% are given at each node. Phylograms were visualized with FigTree v1.4.0 program (Rambaut 2012) and reorganized in Microsoft Power Point (2007) and Photoshop CS3 Extended 10.0 (Adobe Systems, USA).

Results

Symptoms

The first symptom of soft rot on apple fruit was a water-soaked appearance to the affected tissue. The diseased parts later disintegrated into a mushy mass of disorganized cells that sloughed off. Rapid softening and disintegration of the diseased tissue followed. White mycelia formed on infection sites of apples and gradually covered the fruit with tufted whisker-like grey sporangiophores and sporangia. Pathogenicity tests gave typical symptoms in artificially inoculated fruits and appeared 4 days after inoculation (Fig. 1).

Morphological characteristics

Morphological characteristics of the fungi that were re-isolated from inoculated fruits were similar to those from naturally infected apple fruits. Colonies grown on PDA at 28ºC were initially

white and cottony, becoming heavily speckled with sporangia, and finally becoming brownish grey to blackish grey. They spread rapidly with stolons fixed at various points to the substrate by rhizoids. Both rhizoids and stolons were dark brown (Fig. 2). Characteristics of the fungus causing soft rot of apple caused by *Rhizopus oryzae* are compared with a Korean isolate from apple (Table 2).

Table 1 *Rhizopus* taxa used in the phylogenetic analysis and their corresponding GenBank numbers (Fig. 3).

Sr. No.	Rhizopus taxa	Isolate	Gene Sequence	
			(act) gene	ITS
1	*Rhizopus oryzae*	CBS 395.34	AB281522.1	AB181316.1
2	*Rhizopus oryzae*	CBS 112.07	AB281499.1	JN206323.1
3	*Rhizopus oryzae*	CBS:264.28	AB281506.1	KJ551404.1
4	*Rhizopus stolonifer*	CBS 609.82	AB512245.1	AB113023.1
5	*Rhizopus stolonifer* var. *lyococcos*	CBS 320.35	AB512234.1	JN206373.1
6	*Rhizopus delemar*	CBS 130965	KJ551436.1	KJ551398.1
7	*Rhizopus microsporus* var. *chinensis*	CBS 631.82	AB512246.1	DQ641310.1
8	*Rhizopus azygosporus*	CBS 357.93	AB512242.1	JN206343.1
9	*Rhizopus sexualis* var. *americanus*	CBS 340.62	AB512239.1	HM999967.1
10	*Rhizopus oryzae*	SQ.Saudi	MH156645	MH156644

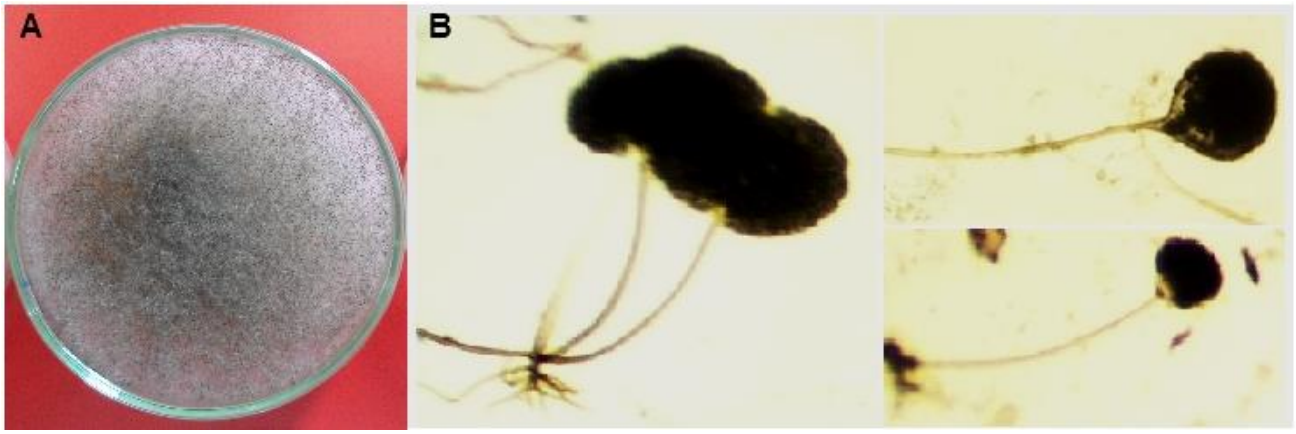

Fig. 1 – Morphological characteristics of *Rhizopus oryzae* isolated from soft rot lesions on apple. A potato dextrose agar (PDA) showing cottony growth with black spores after 7 days incubation. B sporangia, sporangiophores, columella, rhizoids (in lactophenol cotton blue, ×100).

Table 2 Comparison of mycological characteristics of soft rot fungus isolated from apple with previous descriptions of *Rhizopus oryzae* isolated from apple.

Characteristics		Isolates in the current research	*R. oryzae* (Kwon et al. 2011)
Colony	Colour	Brownish grey to blackish grey	Brownish grey to blackish grey
Sporangium	Shape	Globose	Globose
	Size	35~215 µm in diameter	40~200 µm in diameter
Sporangiospore	Shape	Sub-globose	Sub-globose or oval
	Size	4~12 µm in length	4~8 µm in length
Sporangiophore	Size	7~18 µm in diameter	8~20 µm in diameter
Columellum	Shape	Globose to sub-globose	Globose to sub-globose
	Size	90~120 µm in diameter	85~110 µm in diameter

Phylogenetic analysis

Two gene regions were chosen for the multilocus sequencing: the rDNA internal transcribed spacer (ITS) region, and the partial gene of actin (ACT), and concatenated sequences were used to generate the tree. A maximum-likelihood phylogenetic tree confirmed the correct concepts of the *Rhizopus* species and their separation in accordance with literature data. The present isolate was placed within a clade comprising *R. oryzae* reference isolates (Fig. 3). The multi-locus tree showed separation of two main clades, supported by a bootstrap value of 44% for *R. stolonifer* and *R. oryzae*. In *R. oryzae* separation of the two varieties was observed but this was only supported a by bootstrap value of 82% due to high sequence similarity between the varieties. Phylogenetic analysis proved monophyly for each species.

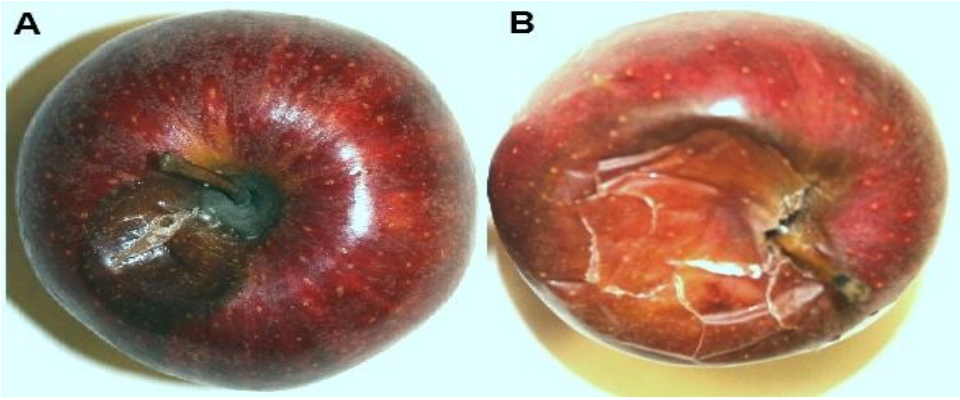

Fig. 2 – Symptoms of soft rot on apple caused by *Rhizopus oryzae*, A Soft rot symptoms on apple fruit sampled from commercial markets. B Symptom in an artificially inoculated fruit.

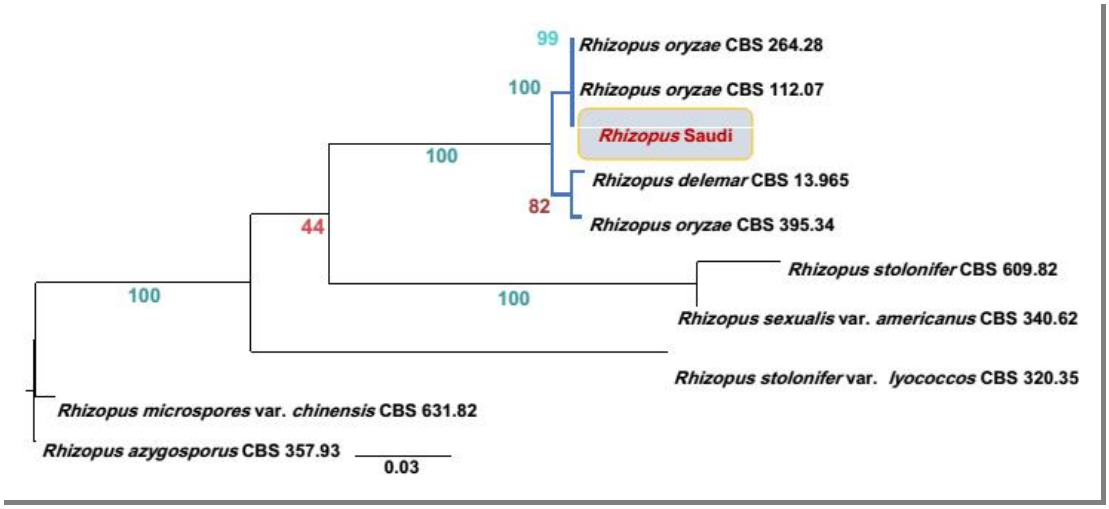

Fig. 3 – RAxML tree based on analysis of a combined dataset of ITS and ACT partial sequences gene. Bootstrap support values for maximum likelihood (ML) higher than 40%.

Discussion

Rhizopus oryzae is one of the most economically important members of Mucoromycotina (Gryganskyi et al. 2010). Although postharvest infection by *Rhizopus* spp. is more common than preharvest infection in the absence of injury or fruit cracking, *Rhizopus* strains were found, in some instances, to be associated with preharvest infection in several fruits (Kwon et al. 2000, Zhang et al. 2013). The measurements and taxonomic characters for isolates collected from apple coincided with those of *R. oryzae* (Lunn 1977, Kwon et al. 2011). Morphological characteristic of *Rhizopus* spp. based on size, shape and ornamentation of spores, which have been used in the past for species and variety distinction, remain inadequate for differentiation of these taxa (Gryganskyi et al. 2010,

Dolatabadi et al. 2014). Koch's postulates were completed by pathogenicity tests conducted on healthy fruits. The current isolate was placed within a clade comprising *R. oryzae* and *R. delemar* reference isolates. Multi-gene phylogenetic analyses support the existence of two cryptic species, *R. delemar* and *R. oryzae*. Results strongly support the hypothesis that genetic recombination is occurring within both *R. oryzae* and *R. delemar* (Gryganskyi et al. 2010). By using molecular phylogenetic analysis based on sequence of *Rhizopus* generated from 18S, internal transcribed spacer (ITS), and 28S ribosomal DNA (rDNA) regions, Abe et al. (2006) determined three major clusters, i.e., *R. microsporus* group, *R. stolonifera* group, and *R. oryzae*. The latest monograph of *Rhizopus* by Zheng et al. (2007) identified ten species, based on a combination of sporangial and zygosporic states morphology, maximum growth temperature, mating compatibility, and molecular systematics. However, based on multigene molecular phylogenetic analysis sequences generated from ITS rDNA, actin gene (ACT), the translation elongaton factor 1-α gene (EF), eight species were identified by Abe et al. (2010). Based on its morphological characteristics, pathological assay and molecular data, the current apple pathogen was identified as *R. oryzae*. To our knowledge, this is the first mycological and molecular identification of *R. oryzae* as a post-harvest causal of soft rot for apple fruits in Saudi Arabia.

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