INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 7, No. 5, p. 153-164, 2015

RESEARCH PAPER OPEN ACCESS

Detection and identification of some botryosphaeriaceae species associated with grapevine in Taif Governorate at Saudi Arabia

Mohamed A. El-Awady[1,2], Mohamed M. Hassan[1,3*], Emad A. M. Gado[4,5], Abd El-Latif A. Sorour[1], Adel E. El-Tarras[1,2]

[1]*Scientific Research Deanship, Biotechnology Research Unit, Taif University, Kingdom of Saudi Arabia* [2]*Department of Genetics, Cairo University, Giza, Egypt*

[3] *Department of Genetics, Minufiya University, Sheben El-Kom, Egypt*

[4]*Department of Biology, Taif University, Kingdom of Saudi Arabia*

[5]*Department of Plant Pathology, Ain Shams University, Egypt*

Key words: Grapevine, *botryosphaeriaceous,* ITS region, phylogenetic analyses, *Guignardia bidwellii*

http://dx.doi.org/10.12692/ijb/7.5.153-162 Article published on November 30, 2015

Abstract

Grapevine is among the most famous fruit crops grown in Taif governorate in Saudi Arabia that characterized with its special quality and test. Grapevine disease caused by *botryosphaeriaceous* fungi include leaf spots, fruit rots, shoot dieback, bud necrosis, vascular discoloration of the wood, and perennial cankers among the most dangerous Grapevine disease. However, there are no studies about the status (occurrence or absence) of these important diseases in KSA generally or in Taif region has been reported. Therefore, the resent study was aimed to use the modern molecular genetics tools to report the status of the *Botryosphaeria spp* and its associated diseases in Taif. In this respect, the symptoms of four diseases caused by *Botryosphaeria spp* (Black rot, Macrophoma rot, Black dead arm and Botryosphaeria canker) were monitored in the grapevine farms in Taif. Among the four diseases, only the symptoms of Black rot were observed in some farms. Subsequently, the symbiotic samples of the Black rot were collected and their associated fungus spp were isolated in the lab. Ten isolates were obtained, characterized and identified at the morphological level. However, the fungus of the black rot disease, *G. bidwellii*, was not identified. Alternatively, the phylogenetic analyses of ribosomal DNA internal transcribed spacer (ITS) regions performed by conventional PCR. Sequencing analysis of the amplified fragment was obtained and aligned to the known sequences in the gene bank. A homologues percentage between the obtained sequence and the sequence of *G. bidwellii* was 99%. In conclusion, the occurrence of only one disease was reported and the presence of its associated fungus (*G. bidwellii*) was confirmed by PCR and sequencing techniques and bioinformatics tools, however we could not isolate and purify it.

* **Corresponding Author:** Mohamed M. Hassan ✉ khyate_99@yahoo.com

Introduction

Recently, *Botryosphaeria* spp. has been identified as important grapevine pathogens worldwide. Grapevine disease symptoms caused by botryosphaeriaceous fungi include leaf spots, fruit rots, shoot dieback, bud necrosis, vascular discoloration of the wood, and perennial cankers. Whereas grapevine fruit rot symptoms caused by species in the Botryosphaeriaceae have been known and studied over the years (Clayton, 1975; Kummuang *et al.*, 1996). In total, there are currently 21 different Botryosphaeriaceae species known to infect grapevines (Wilcox, 2003). Although species of the *Botryosphaeria spp.* have been reported to cause disease symptoms in grapevines in different production regions worldwide, but only in the last decade has the significance of these fungi as grapevine pathogens been recognized (Phillips, 2002; Taylor *et al.*, 2005; Van Niekerk *et al.,* 2004). In addition, recent studies have shown geographical differences in *Botryosphaeria spp* distribution, mainly attributed to climatic conditions (Úrbez-Torres *et al.*, 2008; Úrbez-Torres and Gubler, 2009; Pitt *et al.,* 2010; Úrbez-Torres, 2011).

Black rot is caused by *G. bidwellii* was the first grapevine disease attributed to a botryosphaeriaceous fungus. Although all young green vine tissues (young leaves, petioles, shoots, tendrils, pedicels, and peduncles) are susceptible to infection throughout the growing season, infection of the fruit is a major concern among growers. Leaves are highly susceptible to the disease when they emerge becoming more resistant as they fully expand (Wilcox, 2003). Infection on the leaves appear as small circular lesions of a light-brown color in the center which are bordered by dark brown bands. Pycnidia develop within a few days in the center of the lesions and appear as minuscule black spheres. Macrophoma rot is caused by *B. dothidea*, and its symptoms appear when berries reach full size and increase in prevalence as they ripen. At first, lesions appear as one or more dark circular spots with a tan color in the centre and may be associated with rachis blight. Lesions are flat or slightly sunken and can vary from 1 to 4 mm in diameter.

Trunk diseases of grapevine are caused by fungal pathogens that invade through pruning wounds located on the woody parts of the plant. The disease, formerly named black dead arm (BDA), was identified for the first time in a field in Tokaj, Hungary (Lehoczky, 1972), where it was observed on the basis of wood lesions only. Symptoms include a slow decline and dieback of the vine as a result of interruption of xylem conductivity or toxin production. Whoever, a recent survey however, provided evidence that foliar symptoms attributed to BDA in fact belonged to esca syndrome (Lecomte *et al.*, 2012). Grapevine cankers caused *B. dothidea*. The disease was characterized by the presence of wedged- or pie-shape perennial cankers in spurs, cordons and/or trunks, which associated with different grapevine symptoms worldwide (Úrbez-Torres *et al.*, 2006; Úrbez-Torres and Gubler, 2009).

Detection and accurate identification of plant pathogens is one of the most important strategies for controlling plant diseases to initiate preventive or curative measures. Special interest should be taken in the early detection of pathogens in seeds, mother plants and propagative plant material to avoid the introduction and further spreading of new pathogens in a growing area where it is not present yet. For that reason, the availability of fast, sensitive and accurate methods for detection and identification of fungal pathogens is increasingly necessary to improve disease control decision making.

Traditionally, the most prevalent techniques used to identify plant pathogens relied upon culture-based morphological approaches. These methods, however, are often time-consuming, laborious, and require extensive knowledge of classical taxonomy. Other limitations include the difficulty of some species to be cultured in vitro, and the inability to accurately quantify the pathogen (White *et al.,* 1990). This study aims to report the status (occurrence or absence) of *Botryosphaeria spp* that associated with four grape vine diseases (Black rot, Macrophoma rot, Black dead

arm and Botryosphaeria canker) in Taif Governorate, KSA.

Materials and methods

This work was carried out in the Fungal Genetics laboratory, Biotechnology and Genetic Engendering Unit, Scientific Research Center, Taif University, KSA.

Isolation and identification of some grapevine pathogens fungus

Sample from symptomatic grapevine tissue (roots, leaves, stems, flowers, fruits) of the desired diseases (Black rot, Macrophoma rot, Black dead arm and Botryosphaeria canker) were collected from Grapevine farms in four regions (Haweya, Hada, Shafa, Bani Saad) at Taif Governorate, KSA. After surface sterilization of the plant tissue (e.g. with 1% sodium hypochlorite or 50% hydrogenperoxide) small pieces were transferred to Petri dishes containing an Potato Dextrosa Agar (PDA) supplemented with antibiotics to prevent bacterial contaminants (usually streptomycin) and incubated at (25- 30°C) for pathogen development. All isolated colonies were stored on PDA slants for further identification.

Morphological identification of some pathogenic fungus isolates

The selected cultures were isolated at Fungal Genetics laboratory, Biotechnology and Genetic Engineering Unit, Scientific Research Center, Taif University, KSA. Different selected isolates were grouped and stored on PDA slants and were identified according to the key developed by (Rifai, 1969; Bissett, 1991).

DNA extraction, PCR, sequencing and multigene phylogenic analysis

Isolation of DNA from infected plant samples

Total DNA from the plant and the fungi can be isolated together from the infected plant tissue. That allows skipping the fungi culture step, although DNA from different fungal species or strains may be obtained. Genomic DNA Extraction from infected plant samples using the isolation kit (Jena Bioscience, Germany) for extracting the genomic DNA from plants and fungus tissues.

PCR amplification of the fungus ITS region of ribosomal RNA genes

Two specific primers were used for the amplification of the ribosomal DNA internal transcribed spacer (ITS) regions of the fungus using Polymerase Chain Reaction. Amplification of ITS1-5.8S-ITS2 was performed using the primers ITS1/ITS4 (White *et al.,* 1990). The sequence of the forward and reverse primers are, ITS-1 5'-TCTGTAGGTGAACCTGCGG-3'and ITS-4 5'-TCCTCCGCTTATTGATATGC-3', respectively. The reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µ1) containing 0.25% bromophenol blue, 40 % w/v sucrose in water and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis

Direct Sequencing of the specific PCR product and phylogenetic analysis

The desired PCR products were extracted, purified from the agarose gel and it was analyzed by direct Sequencing of the specific PCR product and phylogenetic analysis. The PCR product was sequenced using an automated DNA sequencer (3130 Genetic Analyzer, Applied Biosystems, Japan). The obtained sequences were used for BLAST searching against GenBank databases at using the BLAST tool of the NCBI web site. The nucleotide sequences of 5.8S-ITS region were determined using the sequencer (Gene analyzer 3121). The deduced sequence was aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 5.10. The forward and reverse sequences were checked and edited manually when needed. Then, a consensus sequence was generated from each alignment made. The sequencing

data were compared against the Gene Bank database (http://www.ncbi.nlm.nih.gov/BLAST/), where a nucleotide blast program was chosen to identify the homology between the PCR fragments and the sequences on the Gene Bank database.

Results

Screening of fungus diseases symptoms at the grape vine farms

Grapevine farms in four regions at Taif governor (Haweya, Hada, Shafa, Bani Saad) were surveyed to detected the symptoms of the desired diseases (Black rot, Macrophoma rot, Black dead arm and Botryosphaeria canker). No clear symptoms of the three grapevine diseases Macrophoma rot, Black dead arm and Botryosphaeria canker were detected. However, the typical symptoms of grapevine disease Black rot were detected (Fig. 1).

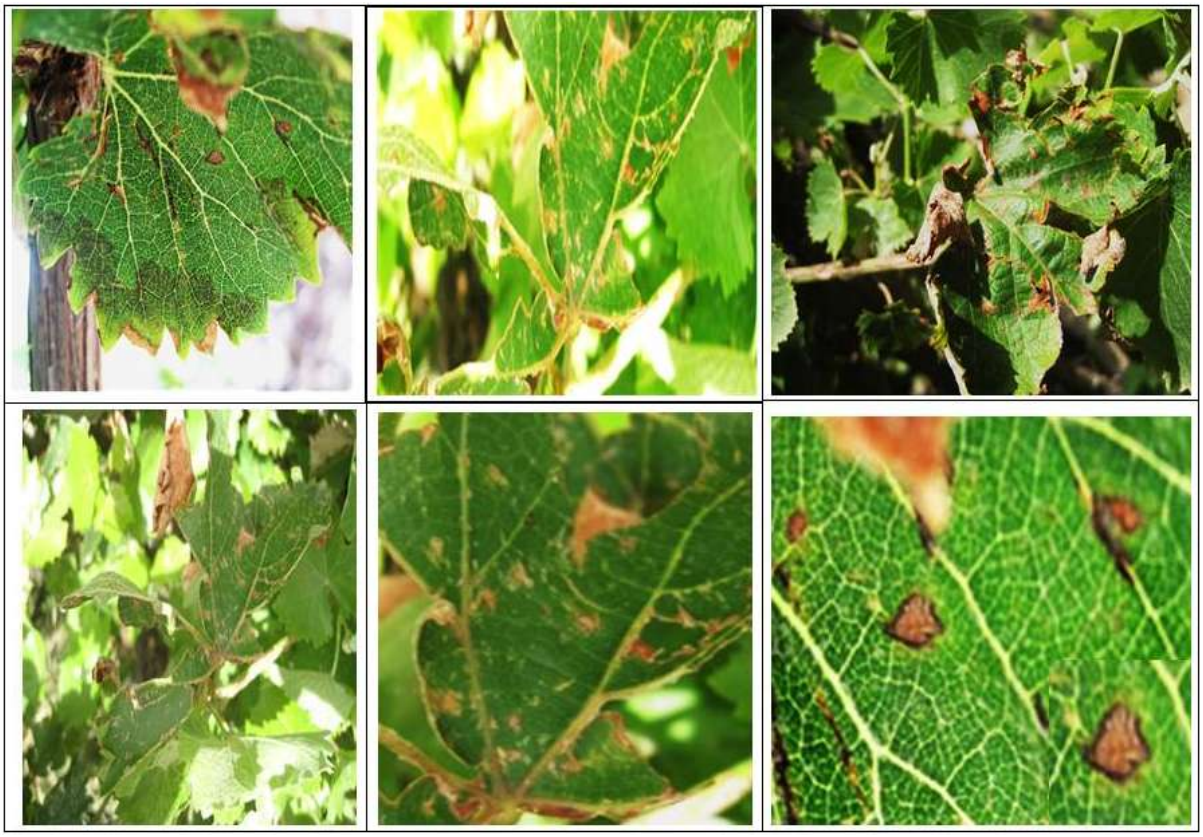

Fig. 1. Symptoms of the black rot detected in the leaves of grape vine plants grown in a grape farm in Bani Saad at Taif governorate.

The symptoms include the appearance of small circular lesions in the leaves. As infection progresses lesions expand and pycnidia develop in the center of the lesions. The infection of the fruit first appears light in color (Figure 2) and as fruit infection progresses, berries turn dark in color and eventually shrivel becoming raising- like body masses named “mummies” and covered with pycnidia.

Morphological Identification of some pathogenic fungus isolates

The growth patterns of different Grapevine fungus isolates after four days of incubation at 25°C showed significant differences in nature of culture growth and sporulation patterns (Data not show). The conidial wall patterns and shape were rough and subglobose and their color was red, white, green and black. The growth characters of reverse colony and mycelia colors patterns varied noticeably within and between the fungi isolates from colorless to yellow and watery white to white. The *Guignardia bidwellii* could not isolate as pure culture as *Fusarium*, *Pythium* and *Retroconis*, so we used the molecular tools to identify this fungus.

Fig. 2. Symptoms of the black rot detected in the steam and fruits of grape vine plants grown in a grape farm in Bani Saad at Taif governorate.

Identification of pathogenic fungus by PCR and sequencing analysis

As we could not isolate the fungus, *G. bidwellii*, the etiological agent of grape black rot, we tried to confirm the presence of it in the infected samples using the molecular genetics tools.

PCR amplification of the fungus ITS region of ribosomal RNA genes

Total DNA (plant and fungus) was isolated from infected tissues by two methods. The quality and quantity of the isolated DNA were proved using the spectrophotometer and electrophoresis in 1% agarose gel. Subsequently, the total genomic DNA were used as a template for PCR reaction to amplify the ITS region of the fungus rRNA gene. The reaction was carried out using specific primers for the ITS1-5.8S-ITS region of the genus *Guignardia* and it successfully amplify a proximately expected 600 bp. fragment that shown as a characteristic band when visualized in agarose gel (Figure 3).

Sequencing and phylogenic analysis of the PCR product

The amplified fragment was then extracted from the agarose gel and when submitted to sequencing, successfully showed a fragment of around 580 bp in length. The deduced sequenced was subjected to the alignment against GenBank databases.

The universal primers ITS-1 and ITS-4 were used to amplify the internal transcribed spacer regions of rDNA yielding products of approximately 580 bp as estimated by agarose gel electrophoresis (Fig. 3).

The amplified PCR products was eluded and sequenced by automated sequencer which encompass 5.8S rDNA gene. The sequences so obtained were subjected to BLAST search for its identity and confirmation, and subsequently submitted to National Centre for Biotechnology Information (NCBI) Gen Bank.

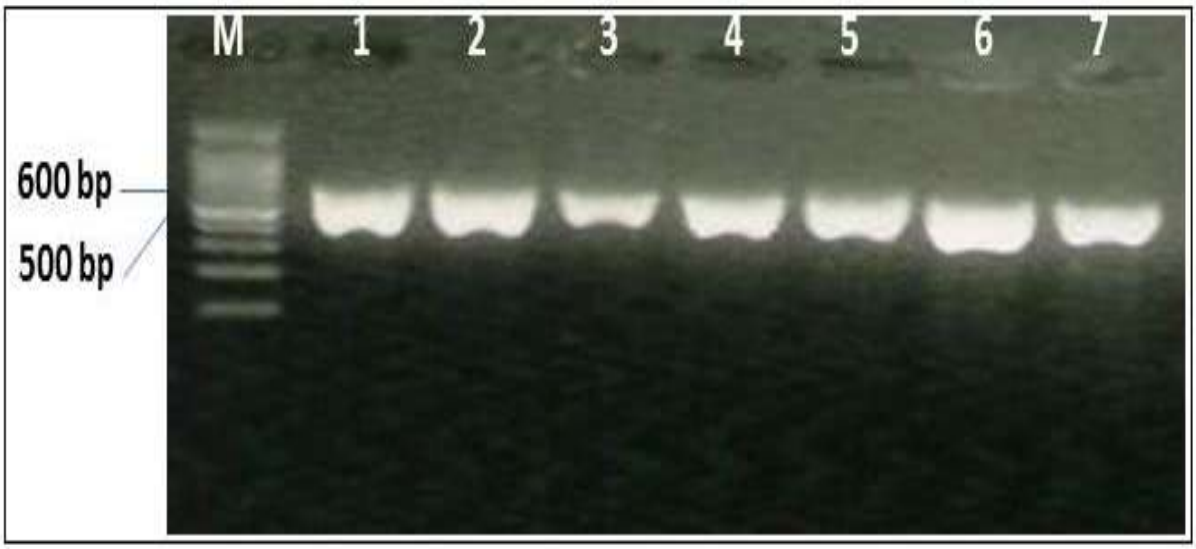

Fig. 3. PCR amplification of the fungus ITS1-5.8S-ITSof ribosomal RNA genes.

The highest homology percentage (99%) was recorded with the sequence of *G. bidwellii* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence and *G. bidwellii* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: NBRC 9903 with the accession no.AB095505.1 and AB095511.1, respectively.

The multiple nucleotide alignment of ITS regions was analyzed using BioEdit programme version 7.2.5. There was sub-stantial disparity in length of ITS sequences between *G. bidwellii* Tu-20 (580 bp) and *G.bidwellii* NBRC-9903 (581 bp) isolates. The isolates of *G. bidwellii* showed 98% homology in nucleotide sequence. Conversely *G. bidwellii* isolates showed nucleotide divergence of 2 % in ITS region. The isolates *G. bidwellii* NBRC-9466 (580 bp) and *G. bidwellii* NBRC-9903 displayed 100% homology in nucleotide sequence, (Fig. 4 and 5). To elucidate the genetic closeness of the *G. bidwellii* isolates a phylogenetic tree was constructed based on sequence analysis of ITS regions using the neighbour-joining method in MEGA 5.1 for windows version on sequences aligned. A random sequence was used as an out-group to demonstrate the situation of the root. Bootstrap analysis of ITS region with 1000 bootstrap replication demonstrated two main branches. The most isolates of *G. bidwellii* formed one group which supported with a bootstrap value of 99.9% except isolates *G. bidwellii* ATCC 200578 which grouped in sub-group of the first main branch. The other cluster consisted of other grapevine fungi, which separated into two subgroups supported by bootstrap value higher than 75%.

Discussion

Guignardia bidwellii is the etiological agent of grape black rot, a disease affecting *Vitis* and other Vitaceae that can cause heavy crop losses in vineyards. Its identification is mainly based on morphological characters and the symptoms on plants but, due to their variability, they may be difficult to interpret to reliably distinguish the pathogen at the species level. In the present study, we could detect the typical symptoms of the grape black rot (Fig. 1 and 2), however, we could not isolate the etiological agent, *G. bidwellii*. Therefore we used the molecular tools of PCR amplification followed by sequencing and alignment of the ITS region. As we could amplify the fragment corresponding to the fungus rRNA gene that showed 99% homology with two 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S gens of *G. bidwellii*, we could confirm the infection of the grapevine grown in Taif with black rot that caused by the fungus *G. bidwellii*. However, the identification could be a challenge when using BLAST analysis with ITS sequences because there can be minimal or no differences between some species or, in some cases, intraspecific variation can confuse the boundaries between species (e.g., *P.*

fragariae var. fragariae and *P. fragariae* var. rubi have identical ITS sequences). the occurrence of only one disease was reported and the presence of its associated fungus (*G. bidwellii*) was confirmed by PCR and sequencing techniques and we could not isolate and purify it.

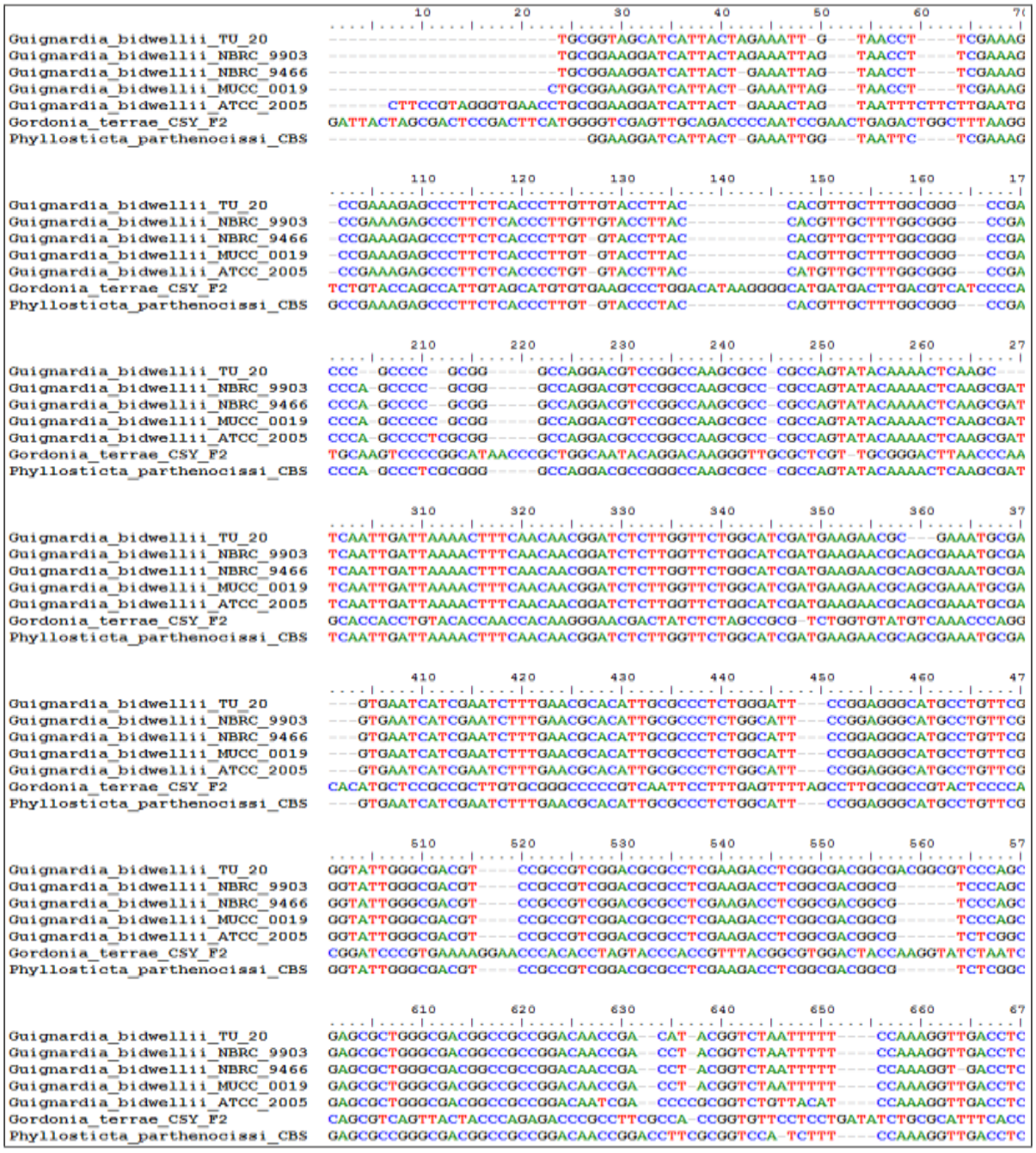

Fig. 4. Multiple nucleotide alignment of ITS region of some *Guignardia bidwellii* isolates using BioEdit programme version 7.2.5.

These results need to be confirmed in other farms and using other molecular tools. The nucleotide sequence alignment of ITS 1 and ITS 2 region revealed the presence of hotspots with nucleotide substitution at four positions supported the distribution of *G. bidwellii* NBRC 9903 into one subgroup. This authenticates the soundness of our perception that the bioefficacy of *G. bidwellii* Tu-20 was manifested at the molecular level which made them to distinguish from other *G. bidwellii* isolates. (Conversely *et al.*, 2002) reported that there was no relationship between the polymorphism showed by the some fungi

isolates and their hardness based on molecular marker. Similar kind of result was reported by (Shalini *et al.*, 2006; Shanmugam *et al.*, 2008). However the results of molecular markers employed in this present study was complementary and confirmatory in nature which substantiates our corroboration between bioefficacy of some fungi isolates and molecular characters.

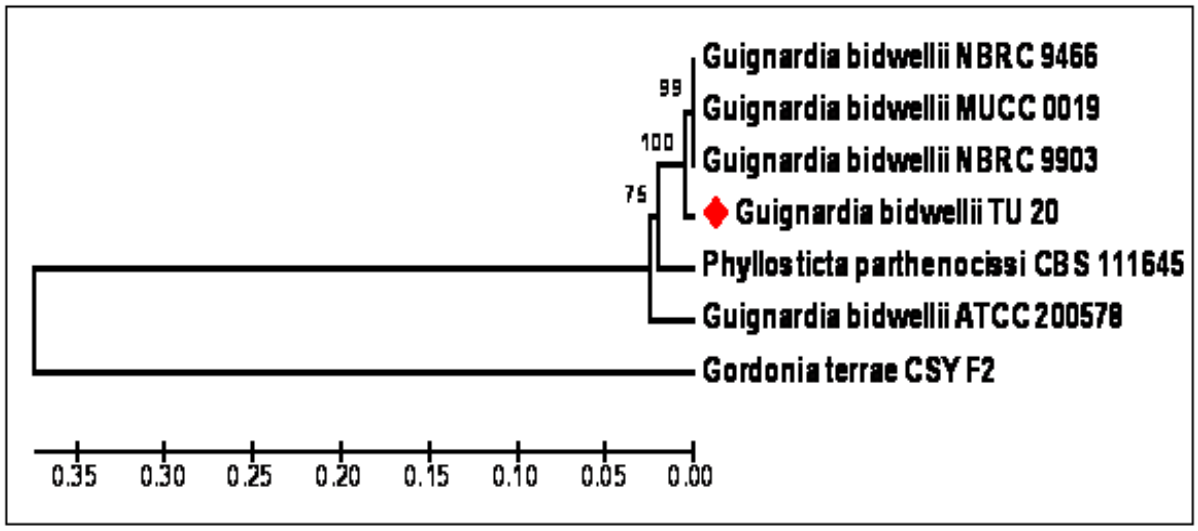

Fig. 5. Phylogenetic relationship of *Guignardia bidwellii* TU-20 and related genera based on ITS region sequences. The tree was constructed using neighbor joining algorithm with Kimura 2 parameter distances in MEGA 5.1 software. The bar indicates the Juke-Cantor evolutionary distance.

The authors also provided the first sequence data of the 5·8S rDNA internal transcribed spacer 1 (ITS) of this species. The b-tubulin-2 gene sequence has also been used extensively during phylogenetic studies because it is slightly more informative than analysis of the ITS region as shown in a study of ripe rot *Colletotrichum acutatum* (Whitelaw-Weckert *et al.*, 2007). Moreover, Phylogenetic analysis based on molecular sequence data is used for reconstructing fungal evolutionary relationships at intraspecific and interspecific levels (James *et al.*, 2006). Moreover, (Farr *et al.*, 2001) placed *G. uvicola* into the order Diaporthales, class Pyrenomycetes, phylum Ascomycota based on morphological and molecular analyses of the 28S rDNA. (Navarrete *et al.*, 2009) compared grapevine fungi isolates collected from wood tissues in Uruguay and their relationship to an isolate from Ohio described by (Farr *et al.*, 2001) on the basis of 28S rDNA.

Conclusion

among the four survived diseases (Black rot, Macrophoma rot, Black dead arm and Botryosphaeria canker) in some grapevine farms in Taif governorate, KSA, the occurrence of only Black rot was reported and the presence of its fungus (*Guignardia bidwellii*), it was confirmed by PCR and sequencing techniques and bioinformatics tools.

Acknowledgment

The authors would like to express their thanks and appreciation to Taif University, KSA, for financial support to carry out this work. This work was supported by Taif University, KSA under project No. 1-433-2130.

References

Bissett J. 1991. A revision of the genus *Trichoderma*. II. Infrageneric classification. Canadian Journal of Botany **69,** 2357-2372.
http://dx.doi.org/10.1139/b91-297

Clayton CN. 1975. Diseases of muscadine and bunch grapes in North Carolina and their control. North Carolina Agricultural Experiment Station Bulletin 451, 73.

Crowhurst RN, King FY, Hawthorne BT, Sanderson FR, Choi-Pheng Y. 1995. RAPD characterization of *Fusarium oxysporum* associated with wilt of angsana (*Pterocarpus indicus*) in Singapore. Mycological Research **99,** 14-18. http://dx.doi.org/10.1016/S0953-7562(09)80310-9

Farr DF, Castlebury LA, Rossman AY, Erincik O. 2001. Greeneria uvicola, cause of bitter rot of grapes, belongs in Diaporthales. Sydowia **53,** 185-199.

James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ. 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature **443,** 818-822.

Kummuang N, Diehl SV, Smith BJ, Graves CHJ. 1996. Muscadine grape berry rot in Mississippi: Disease epidemiology and crop protection. Plant Disease **80,** 244-247.

Lecomte P, Darrieutort G, Liminana JM, Comont G, Muruamndiaraz A, Legorburu FJ, Choueiri E, Jreijiri F, Elamil R, Fermaud M. 2012. New insights into Esca of grapevine: the development of foliar symptoms and their association with xylem discoloration. Plant Disease **96**, 924-934.

Lehoczky J. 1972. Deadarm disease of grapevines in Hungary. Acta Phytopathologica Academie Scientiarum Hungaricae **7**, 401-407.

Navarrete F, Abreo E, Bettucci L, Martı´nez S, Lupo S. 2009. First report of Greeneria uvicola as cause of grapevine dead-arm dieback in Uruguay. Australasian Plant Disease Notes **4**, 117-119.

Phillips AJL. 2002. Botryosphaeria species associated with diseases of grapevines in Portugal. Phytopathologia Mediterranea **41,** 3-18.

Pitt WM, Huang R, Steel CC, Savochia S. 2010. Identification, distribution and current taxonomy of Botryosphaeriaceae species associated with grapevine decline in New South Wales and South Australia. Australian Journal of Grape and Wine Research **16,** 258-271.

Rifai MA. 1969. A revision of the genus *Trichoderma*. Mycology **116,** 1-56. http://dx.doi.org/10.1007/s10482-013-9975-4

Shalini N, Lata KP, Kotasthane AS. 2006. Genetic relatedness among *Trichoderma* isolates inhibiting a pathogenic fungi *Rhizoctonia solani*. African Journal of Biotechnology **5**, 580-584.

Shanmugam V, Sharma V, Ananthapadmanaban. 2008. Genetic relatedness of *Trichoderma* isolates antagonistic against *Fusarium oxysporum* f. sp. *Dianthi* inflicting carnation wilt. Folia Microbiology **53,** 130-138.

Taylor A, Hardy GE, St J, Wood P, Burgess T. 2005. Identification and pathogenicity of Botryosphaeria species associated with grapevine decline in Western Australia. Australian. Plant Pathology **34,** 187-195.

Úrbez-Torres JR, Gubler WD. 2009. Pathogenicity of Botryosphaeriaceae species isolated from grapevine cankers in California. Plant Disease **93,** 584-592.

Úrbez-Torres JR, Leavitt GM, Guerrero JC, Guevara J, Gubler WD. 2008. Identification and pathogenicity of *Lasiodiplodia theobromae* and *Diplodia seriata*, the causal agents of bot canker disease of grapevines in Mexico. Plant Disease **92,** 519-529.

Úrbez-Torres JR, Leavitt GM, Voegel TM, Gubler WD. 2006. Identification and distribution of Botryosphaeria species associated with grapevines cankers in California. Plant Disease **90,** 1490-1503. http://dx.doi.org/org/10.1094/PD-90-1490

Úrbez-Torres JR. 2011. The status of Botryosphaeriaceae species infecting grapevines.

Phytopathologia Mediterranea **50,** S5-S45. doi http://dx.doi.org/10.14601/Phytopathol_Mediterr-9316

Van Niekerk JM, Crous PW, Groenewald JZ, Fourie PH, Halleen F. 2004. DNA phylogeny, morphology and pathogenicity of Botryosphaeria species on grapevines. Mycologia **96**,781-798.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in PCR Protocols: A Guide to Methods and Applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds., pp. 315-322, Academic Press, New York, NY, USA.

Whitelaw-Weckert MA, Curtin SJ, Huang R, Steel CC, Blanchard CL, Roffey PE. 2007. Phylogenetic relationships and pathogenicity of *Colletotrichum acutatum* isolates from grape in subtropical Australia. Plant Pathology **56,** 448-463. http://dx.doi.org/10.1111/j.1365-3059.2007.01569.x.

Wilcox WF. 2003. Black rot. Cornell Cooperative Extension, Disease identification sheet No. 102GFSG-D4, 3 p.