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NEW OR UNUSUAL DISEASE REPORTS - 9TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

First report of *Neofusicoccum mangiferae* associated with grapevine dieback in China

Asha J. DISSANAYAKE[1,2,3,*], Wei ZHANG[1,2,*], Xinghong LI[2], Ying ZHOU[2], Thilini CHETHANA[1,2,3], Ekachai CHUKEATIROTE[3], Kevin D. HYDE[3], Jiye YAN[2], Guozhen ZHANG[1] and Wensheng ZHAO[1]

[1] Department of Plant Pathology, China Agricultural University, Beijing 100097, China
[2] Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China
[3] Institute of Excellence in Fungal Research, and School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand

Summary. The Botryosphaeriaceae represents an important, cosmopolitan family of pathogens infecting woody plants. Grapevines (*Vitis vinifera*) suspected of being affected by Botryosphaeria dieback were collected from different Provinces of China and several Botryosphaeriaceae species were identified. This research was conducted to further study the species of Botryosphaeriaceae associated with grapevines in China and to estimate the prevalence and severity of the disease. Symptoms were characterized by partial or total death of grapevine cordons, with brown U-shaped necrotic sectors and brownish-black spots in cross-sections of affected trunks and arms. A fungus isolated from diseased vines in Henan and Anhui Provinces was identified as *Neofusicoccum mangiferae* on the basis of its morphological and cultural characteristics as well as an analysis of combined sequence data from the internal transcribed spacer (ITS) cluster of the ribosomal DNA together with partial sequences of the β-tubulin (BT) and translation elongation factor (EF1-α) genes. Pathogenicity was assessed by inoculating the fungus onto detached green grapevine shoots under controlled laboratory conditions and mature wood of standing vines cv. Summer Black in a greenhouse. This is the first record of *N. mangiferae* associated with grapevine dieback in the world.

Key words: Botryosphaeria dieback, canker, pathogenicity, *Vitis vinifera.*

Introduction

Grapevine trunk diseases are some of the main factors limiting vineyard longevity and productivity (Úrbez-Torres, 2011). Several species in the family Botryosphaeriaceae are important vascular pathogens causing severe and economically significant decline and dieback symptoms on grapevines. Presently 24 Botryosphaeriaceae species are known from grapevines worldwide (Úrbez-Torres, 2011; Linaldeddu *et al*., 2014). Species in the genera *Botryosphaeria, Diplodia, Lasiodiplodia* and *Neofusicoccum* (Crous *et al*., 2006), have been reported causing various decline symptoms in grapevine (van Niekerk *et al*., 2006; Luque *et al*., 2009; Úrbez-Torres, 2011). These symptoms include cankers, bud and wood necrosis, spur and shoot dieback, vascular discoloration of the wood and perennial cankers with retarded growth of the vine (van Niekerk *et al*., 2006; Úrbez-Torres *et al*., 2006, 2008; Úrbez-Torres, 2011).

Botryosphaeria dothidea, Diplodia seriata, Lasiodiplodia theobromae and *Neofusicoccum parvum* were recently reported to be associated with Botryosphaeria dieback of grapevine in China (Yan *et al*., 2013). 'Cluster and fruit dropping' was the most prominent symptom recorded in Chinese vineyards, which is different from other known dieback symptoms such as vine canker, bud necrosis, vascular discoloration and whole plant death (Yan *et al*., 2013). The research reported here focused on the identification and characterization of *Neofusicoccum mangiferae* (Syd. & P. Syd.) Crous Slippers & A.J.L. Phillips as one of the

Corresponding authors: W. Zhao, G. Zhang
E-mail: mppzhaws@cau.edu.cn, zhanggzh@cau.edu.cn
* These authors contributed equally to this work.

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Botryosphaeriaceae species associated with grapevine decline in China.

Materials and methods

Fungal isolation and morphological characterization

Grapevines with symptoms resembling Botryosphaeria dieback (*sensu* Úrbez-Torres, 2011) were collected from Henan and Anhui Provinces in China. Small pieces (<5 mm^2) were cut from the margins of healthy and infected tissues, disinfected in 75% ethanol for 1 min, rinsed three times in sterile distilled water, dried and plated on potato dextrose agar (PDA). The plates were incubated at 28°C for at least 5 days in a 12-12 h light-dark regime until fungi developed from the symptomatic tissue. Hyphal tips of colonies resembling Botryosphaeriaceous fungi were transferred to fresh PDA plates and incubated until they produced conidiomata and conidia. Fungi were initially identified based on their morphological characteristics. Conidia of these isolates were spread over the surface of water agar (WA) plates and, after incubation at 28°C overnight, single germinating conidia were transferred to fresh PDA plates to obtain pure cultures. Spore dimensions (40 conidia) were recorded with a Nikon DS-RiI digital camera connected to a Nikon, NIS-Elements F3.0 microscope.

DNA amplification and phylogenetic studies

Genomic DNA was extracted from 50–100 mg of mycelium following the method described by Doyle and Doyle (1987). Primer pairs ITS1 and ITS5 (White *et al*., 1990) were used to amplify the internal transcribed spacers (ITS) of the ribosomal DNA. Primers EF1–728F and EF1–986R (Carbone and Kohn, 1999) and Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify and sequence part of the translation elongation factor 1-alpha (EF1-α) gene and part of the β-tubulin gene respectively. PCR products were separated on 1% agarose electrophoresis gels, stained with ethidium bromide and visualized under UV light. Sequencing was carried out by Sunbiotech Company, Beijing, China. Sequences were aligned with those retrieved from GenBank (Table 1) using MAFFT v. 6.0 (Katoh and Toh, 2010) and adjusted manually when necessary. The nucleotide substitution models were determined individually for each gene region using MrModelTest v. 2.3 (Nylander, 2004). Bayesian analyses employing a Markov Chain Monte Carlo (MCMC) (Larget and Simon, 1999) method were performed with MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). Four MCMC chains were run simultaneously, starting from random trees, and ending after 10^6 generations with trees saved every 100^{th} generation. Maximum parsimony analysis (MP) was performed with PAUP* v.4.0b10 (Swofford, 2003) using the heuristic search option with 1000 random stepwise addition. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications. Sequences generated in this study were deposited in GenBank (Table 1).

Pathogenicity tests

Two *N. mangiferae* isolates were used in the pathogenicity tests, namely AH-I-002 and ZZ1-1-

Table 1. Isolates used in this study. *Neofusicoccum mangiferae* isolates obtained in this study are in bold.

Neofusicoccum **Species**	**Isolate Number**	**Origin**	**Host**	**Collector**	**GenBank Accession Numbers**		
					ITS	**EF1-α**	**β-tubulin**
N. eucalypticola	CBS 115679	South Africa	*Eucalyptus grandis*	H. Smith	AY615141	AY615133	AY615125
N. eucalyptorum	CMW 10126	South Africa	*Eucalyptus grandis*	H. Smith	AF283687	AY236892	AY236921
N. macroclavatum	CBS 118223	Australia	*Eucalyptus globulus*	T.I. Burgess	DQ093196	DQ093217	DQ093206
N. mangiferae	CBS 118532	Australia	*Mangifera indica*	G.I. Johnson	AY615186	DQ093220	AY615173
N. mangiferae	**AH-I-002**	**Anhui, China**	***Vitis vinifera***	**X. H. Li**	**KJ146837**	**KJ146835**	**KJ146833**
N. mangiferae	**ZZ1-1-2-05s**	**Henan, China**	***Vitis vinifera***	**X. H. Li**	**KJ146838**	**KJ146836**	**KJ146834**

2-05s. Nine detached, 30 cm long, green grapevine shoots and three standing grapevines of cv. 'Summer Black' were inoculated with each isolate. A mycelial plug (3–5 mm^2) taken from the margin of an actively growing colony on PDA was placed in a shallow wound (~3 mm) made with a scalpel on the middle of each shoot and wrapped with Parafilm (BEMIS, USA). Nine shoots and three standing vines were inoculated with sterile PDA plugs as controls. Inoculated detached shoots were placed in pots filled with soil, watered with sterile distilled water and maintained in an incubator at 28°C with 12–12 h light-dark regime. Standing vines were maintained in a greenhouse at 28°C. Lengths of the bark cankers were measured after 5 to 12 days for detached shoots and after 2 to 4 weeks for standing vines. The significance of differences in lesion lengths between the treatments was determined by one-way ANOVA and means were compared using Duncan's multiple range test at the 5% significance level. SPSS software version 17 (SPSS Inc., Chicago, IL) was used for the statistical tests.

Results and discussion

The symptoms of diseased grapevine samples from Henan Province appeared in partial or total death of affected cordons, U-shaped necrotic sectors. The bark tissue of the diseased cordons was collapsed at the sites of infection, becoming dark-brown. The cross-sections of affected trunks that showed brownish-black spots were collected from Anhui Province. After colonization by the fungus, the cross sections of the wood became brown spots

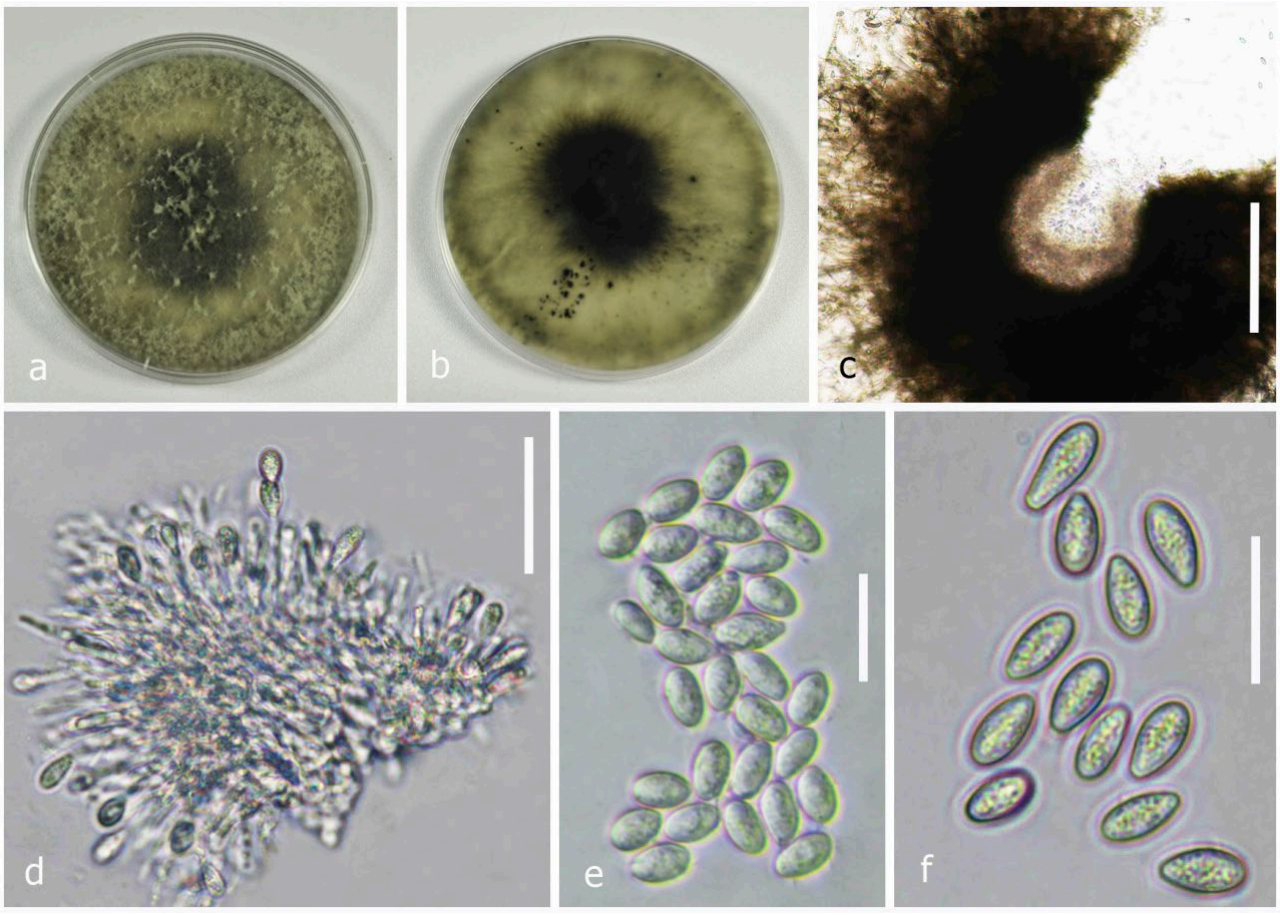

Figure 1. *Neofusicoccum mangiferae*. a. Upper view of a 14-days old culture; b. Reverse view of a 14-days old culture; c. Cross section of a conidioma; d. Conidiogenous cells and young conidia. e, f. Conidia. Bar = 200 μm (c), 40 μm (d), 20 μm (e, f).

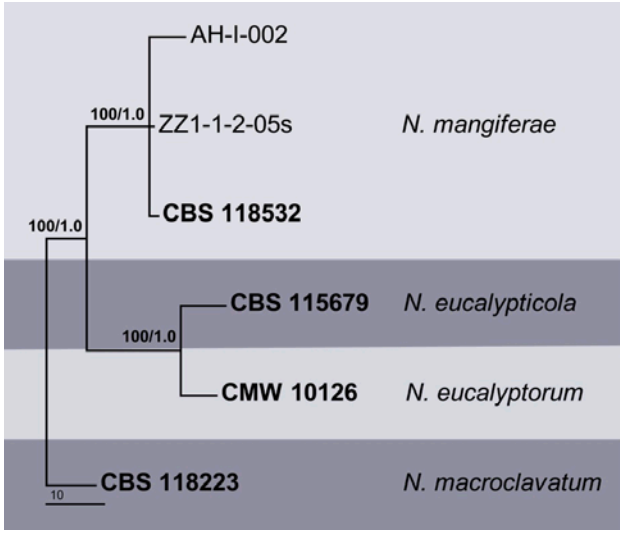

Figure 2. One of the 1000 equally most parsimonious trees obtained from the phylogenetic analysis of the combined dataset of ITS, EF1-α and β-tubulin sequences alignment. The scale bar shows 10 changes. Bootstrap support values for maximum parsimony (MP) greater than 90% and Bayesian posterior probabilities above 0.95 are given above the nodes. Ex-type and ex-epitype isolate numbers are in bold. The tree is rooted to *Neofusicoccum macroclavatum* (CBS 118223).

and this was observed downwards and upwards in a narrow streak inside the trunk.

Two representative isolates were used in this study (Table 1) each from Henan and Anhui Provinces. Colonies of *N. mangiferae* isolates on PDA were initially grey-white and became dark grey-black after 14 days (Figure 1). Colonies produced hyaline, ellipsoidal, aseptate conidia with rounded apices measuring 12–16 × 5–8 µm (n=40) after 35 days incubation at 28°C. Conidial morphology and cultural features of the isolates were in close agreement with the morphological description of *N. mangiferae* (Crous *et al*., 2006; Phillips *et al*. 2013).

PCR amplifications of the ITS, EF1-α and β-tubulin gave products of approximately 0.5, 0.3, and 0.4 kbp, respectively. The combined data set of ITS, EF1-α and β-tubulin consisted of six taxa, which comprised the two *N. mangiferae* isolates obtained in this study and four additional isolates including the outgroup (*N. macroclavatum*). The combined dataset comprised 1226 characters after the uneven ends were truncated. Of these characters, 1146 were constant, 42 were parsimony uninformative and 38 were parsimony informative. A heuristic search produced 1000 most parsimonious trees of 84 steps (CI = 0.952, RI = 0.925 and RC = 0.881) and the first is shown in Figure 2. Maximum-parsimony and Bayesian inference produced trees with nearly identical topologies (Bayesian tree not shown). Isolates obtained in this study clustered together in a well-supported clade (bootstrap value = 100%; posterior probability = 1.0) with *N. mangiferae* CBS 118532, which was used as representative for the species by Slippers *et al*. (2005) and Phillips *et al*. (2013), thus confirming the identification of the studied isolates.

Inoculation with both isolates resulted in necrosis of the shoots (Figure 3). Differences in lesion lengths among two isolates of *N. mangiferae* on green shoots were significant at the 5% level (Figure 4), but on standing vines, no significant differences could be de-

Figure 3. a. Detached grapevine shoots artificially infected with *Neofusicoccum mangiferae* (after 7 days); b. Cross section of inoculated shoots (after 7 days); c, d. Pathogenicity test on standing grapevines. (c) Control. (d) Cane death 30 days after inoculation.

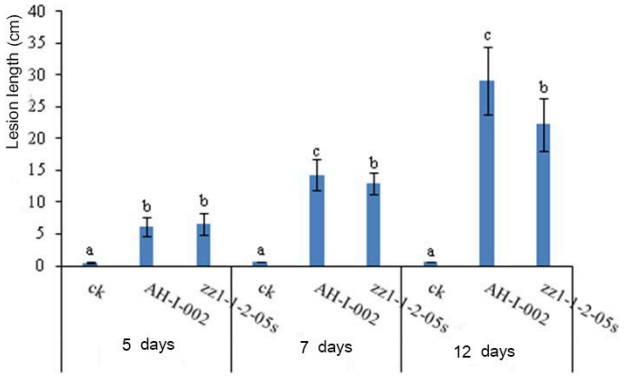

Figure 4. Necrosis length caused by *Neofusicoccum mangiferae* isolates (AH-I-002 and ZZ1-1-2-05S) inoculated on green detached grapevine shoots (N=9) after 5, 7 and 12 days. ck: non-inoculated control. Error bars indicate standard deviation of the mean. Significant differences (P<0.05) between means are indicated with different letters according to Duncan's multiple range test.

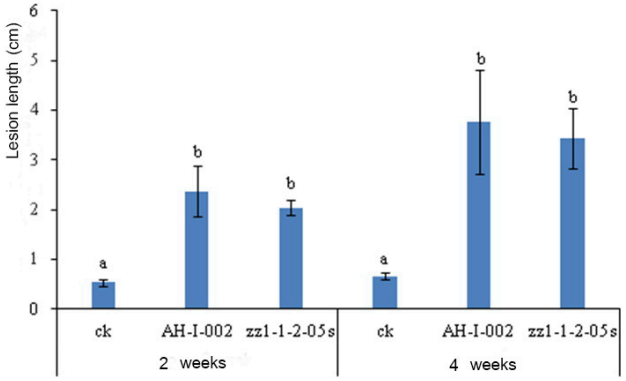

Figure 5. Necrosis length caused by *Neofusicoccum mangiferae* isolates (AH-I-002 and ZZ1-1-2-05S) inoculated on shoots of standing grapevines (N=3) after 2 and 4 weeks. ck: non-inoculated control. Error bars indicate standard deviation of the mean. Significant differences (P<0.05) between means are indicated with different letters according to Duncan's multiple range test.

tected (Figure 5). The longest bark lesions observed in both detached shoots and standing vines were caused by isolate AH-I-002 (Figures 4, 5). Both *N. mangiferae* isolates were successfully reisolated from the margin of symptomatic tissues, thus fulfilling Koch's postulates and confirming the pathogenicity of this species on grapevine. To our knowledge, this is the first report of *N. mangiferae* associated with grapevine dieback in the world. Further studies are currently in progress to study the distribution of this pathogen in Chinese vineyards and its role in grapevine dieback.

Acknowledgments

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Literature cited

Carbone I. and L.M. Kohn, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91, 553–556.

Crous P.W., B. Slippers, M.J. Wingfield, J. Reeder, W.F.O. Marasas, A.J.L. Phillips, A. Alves, T. Burgess, P. Barber and J.Z. Groenewald, 2006. Phylogenetic lineages in the Botryosphaeriaceae. *Studies in Mycology* 55, 235–253.

Doyle J.J. and J.L. Doyle, 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19, 11–15.

Glass N.L. and G.C. Donaldson, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied Environmental Microbiology* 61, 1323.

Katoh K. and H. Toh, 2010. Parallelization of the MAFFT multiple sequence alignment program. *Bioinformatics* 26, 1899–1900.

Larget B. and D. Simon, 1999. Markov chain Monte chain Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* 16, 750–759.

Linaldeddu B.T., A. Deidda, B. Scanu, A. Franceschini, S. Serra, A. Berraf-Tebbal, M. Zouaoui Boutiti, M.L. Ben Jamâa and A.J.L. Phillips, 2014. Diversity of Botryosphaeriaceae species associated with grapevine and other woody hosts in Italy, Algeria and Tunisia, with descriptions of *Lasiodiplodia exigua* and *Lasiodiplodia mediterranea* sp. nov. *Fungal Diversity*, DOI: 10.1007/s13225-014-0301-x.

Luque, J., S. Martos, A. Aroca, R. Raposo and F. Garcia-Figueres, 2009. Symptoms and fungi associated with declining mature grapevine plants in Northeast Spain. *Journal of Plant Pathology* 91, 381–390.

Nylander J.A.A., 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden.

Phillips A.J.L., A. Alves, J. Abdollahzadeh, B. Slippers, M.J. Wingfield, J.Z. Groenewald and P.W. Crous, 2013. The Botryosphaeriaceae: genera and species known from culture. *Studies in Mycology* 76, 51–167.

Ronquist F. and J.P. Huelsenbeck, 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574.

Slippers B., G.I. Johnson, P.W. Crous, T.A. Coutinho, B.D. Wingfield and M.J. Wingfield, 2005. Phylogenetic and morphological re-evaluation of the Botryosphaeria species causing diseases of *Mangifera indica*. *Mycologia* 97, 99–110.

Swofford D.L., 2003. PAUP*. Phylogenetic analysis using parsimony (and other methods). Version 4. Sinaur Associates, Sunderland, MASS. USA.

Úrbez-Torres J.R., 2011. The status of Botryosphaeriaceae species infecting grapevines. *Phytopathologia Mediterranea* 50, 5–45.

Úrbez-Torres J.R., G.M. Leavitt, J.C. Guerrero, J. Guevara and W.D. Gubler, 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 J.R., G.M. Leavitt, T.M. Voegel and W.D. Gubler, 2006. Identification and distribution of *Botryosphaeria* spp. associated with grapevine cankers in California. *Plant Disease* 90, 1490–1503.

Van Niekerk J.M., P. Fourie, F. Halleen and P.W. Crous, 2006. *Botryosphaeria* spp. as grapevine trunk disease pathogens. *Phytopathologia Mediterranea* 45, S43–S54.

White T.J., T. Bruns, S. Lee and J. Taylor 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies. In: PCR protocols: A guide to Methods and Applications (M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, ed.). Academic Press, San Diego, CA, USA, 315–322.

Yan J.Y., Y. Xie, W. Zhang, Y Wang, J.K. Liu, K.D. Hyde, R.C. Seem, G.Z. Zhang, Z.Y. Wang, S.W. Yao, X.J. Bai, A.J. Dissanayake, Y.L. Peng and X.H. Li, 2013. Species of Botryosphaeriaceae involved in grapevine dieback in China. *Fungal Diversity* 61, 221–236.

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