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Short Notes

Phytotoxins produced by *Lasiodiplodia laeliocattleyae* involved in Botryosphaeria dieback of grapevines in Brazil

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Abstract. Botryosphaeria dieback (BD) is an important trunk disease affecting grapevines. Several *Lasiodiplodia* species have been shown to be involved in BD affecting the perennial organs of grapevine, mainly causing cankers. (*R*)-(-)-mellein and tyrosol, two well-known fungal phytotoxins, were isolated from the organic extract of culture filtrate of *Lasiodiplodia laeliocattleyae* (syn. *egyptiacae*), which had been isolated from grapevines affected by BD in Brazil. This increases knowledge of the secondary metabolites produced by *Lasiodiplodia* species, confirming that (*R*)-(-)-mellein is a toxin typically produced by Botryosphaericeae species.

Keywords. Grapevine wood disease, phytotoxins, (*R*)-(-)-mellein, tyrosol.

INTRODUCTION

Botryosphaeria dieback (BD) is a grapevine trunk disease that causes serious problems for grape production, including emerging wine-producing countries such as Brazil. The economic impacts of grapevine trunk diseases result from significant yield reductions from diseased vines and increased production costs for application of control measures. Many efforts have been made to find new and effective management practices for the disease (Mondello *et al.*, 2018). BD also affects many fruit tree crops, including mango, olive, walnut and almond (de Oliveira Costa *et al.*, 2010; Olmo *et al.*, 2016; Rodríguez-Gálvez *et al.*, 2017).

BD of grapevine is caused by several Botryosphaeriaceae species, associated with decline symptoms including dieback, wood canker and spur dieback (Úrbez-Torres, 2011; Billones-Baijens and Savocchia 2018; Gramaje *et al.*, 2018; Mondello *et al.*, 2018).

Like other trunk disease pathogens, Botryosphaeriaceous species can produce toxic metabolites belonging to different compound classes including aromatic compounds, isocoumarins, jasmonates, naphthalenones, polyketides, and phenols (Martos *et al.*, 2008; Masi *et al.*, 2018). In particular, *Lasiodiplodia* species were investigated for production of phytotoxic metabolites and other substances (including jasmonic acid, mellein, lasiodiplodin, theobroxide, butyrolactones, botryosphaeran, botryorodines, lasiodiplodan) are produced *in vitro* by different isolates of *L. theobromae* and other *Lasiodiplodia* spp. such as *L. mediterranea*. These toxic metabolites have also been purified from *in vitro* cultures of strains isolated from host pants other than grapevine, and tested for their toxic activity (Aldridge *et al.* 1971, Husan *et al.* 1993, Matsuura *et al.*, 1998, He *et al.*, 2004, Miranda *et al.*, 2008, Kitoaka *et al.*, 2009, Andolfi *et al.*, 2014).

In a recent study, phytotoxic metabolites produced in liquid cultures by six species of *Lasiodiplodia* isolated in Brazil, and causing Botryosphaeria dieback of grapevine, were chemically identified. As determined by LC-MS, *L. brasiliense, L. crassispora., L. iraniensis, L. pseudotheobromae* produced jasmonic acid, while *L. brasiliense* synthesized jasmonic acid and (3*R*,4*S*)-4-hydroxymellein. *Lasiodiplodia euphorbiaceicola* and *L. hormozganensis* produced some low molecular weight lipophilic toxins, that were isolated and identified (Cimmino *et al.*, 2017). In particular, from culture filtrate of *L. euphorbiaceicola*, (*R*)-(-)-mellein, (3*R*,4*R*)-(-)- and (3*R*,4*S*)-(-)-4-hydroxymellein, and tyrosol were isolated, and identified. Tyrosol and *p*-hydroxybenzoic acid were also isolated from culture filtrates of *L. hormozganensis* (Cimmino *et al.*, 2017).

Knowledge of the non-specific phytotoxins produced by Botryosphaeriaceous species is increasing (Masi *et al.*, 2018), and it is increasingly important to determine the full spectrum of these metabolites to understand their roles in disease and symptom development (Meh *et al.*, 2013). This note reports the isolation of phytotoxins produced *in vitro* from *L. laeliocattleyae* (syn. *egyptiacae*) (Jayawardena *et al.*, 2018), a pathogen of grapevine (Correia *et al.*, 2016) and agent of mango dieback and fruit rot (Rosado *et al.*, 2016). To our knowledge this is the first report of phytotoxic metabolites isolated from *L. laeliocattleyae*.

MATERIALS AND METHODS

Fungal isolates and culture conditions

The strain of *L. laeliocattleyae* (CMM0206) used in this study was obtained from the collection of Universidade Federal Rural de Pernambuco, Recife, Brazil. It was inoculated and grown in stationary culture, as reported for other strains of *Lasiodiplodia* (Cimmino *et al.*, 2017), in modified Difco Czapek Dox medium (Benton), with 0.5% yeast and 0.5% malt extract (Difco). The cultures were grown for 21 d at 25 °C in the dark. The mycelium was removed and the liquid cultures were lyophilized prior to the extraction procedure.

Extraction of low molecular weight phytotoxic metabolites

The lyophilized residues of the culture filtrates (2.85 L) were dissolved in 300 mL of water and extracted with EtOAc (3 × 300 mL) at the same pH as the original culture (pH 8). The organic extracts were then combined, dried (Na_2SO_4), filtered, and evaporated under low pressure. The organic residue (264.0 mg) was purified by silica gel column chromatography using $CHCl_3$-*i*-PrOH (95:5, v/v), and six fractions of homogenous groups were collected. The residue (21.9 mg) of fraction #1 was purified by preparative TLC on silica gel using $CHCl_3$ as eluent. This yielded a white solid, which was identified as *R*(-)- mellein (**1**, Figure 1, 13.7 mg). The residue of fraction #3 was purified on preparative TLC on silica gel, using $CHCl_3$-i-PrOH (97:3, v/v) as eluent, yielding a white solid, which was identified as tyrosol (**2**, Figure 1, 12.4 mg).

Chemical analyses and characterization

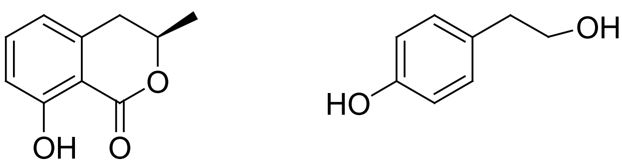
Optical rotations were measured in MeOH on a Jasco P-1010 digital polarimeter (Jasco). ^{1}H NMR spectra were recorded at 400 or 500 MHz in $CDCl_3$ on Bruker and Varian instruments, with MeOH as an internal standard. ESI MS and LC/MS analyses were performed using the LC/MS TOF system AGILENT (Agilent Technologies) 6230B, HPLC 1260 Infinity. Analytical and preparative TLC was carried out on silica gel plates (Kieselgel 60, F254, 0.25 mm and 0.5 mm) (Merck). TLC spots were visualized by exposure to UV radiation, or by spraying first with 10% H_2SO_4 in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110°C for 10 min. Column chromatography was performed using silica gel (Kieselgel 60, 0.063-0.200 mm) (Merck). Standard sample of (*R*)-(-)-mellein was obtained from the culture filtrates of *Sardiniella urbana* (Cimmino *et al.*, 2018), and of tyrosol from *Lasiodiplodia euphorbiaceicola* (Cimmino *et al.*, 2017).

Phytotoxicity bioassay

The phytotoxic activity of chromatographic organic extract fractions was assayed on lemon fruit, using previously reported protocol (Andolfi *et al.*, 2014b; Cimmino *et al.*, 2017).

RESULTS

The culture filtrates of *L. laeliocattleyae* were exhaustively extracted with ethyl acetate. The organic extract and the resulting aqueous phases were assayed for phytotoxic activity, and both showed some phytotoxicity in the bioassay conditions. The phytotoxicity results were essentially the same as previously outlined (Cimmino et al, 2017). The organic extract was purified by column chromatography. When assayed on lemon fruit, the residue of fractions #1 and #3 produced intense necrotic spots. These were further purified by preparative TLC, obtaining two pure metabolites. The purified compounds were identified as (*R*)-(-)-mellein and tyrosol (**1** and **2**, Figure 1) by comparison with standard samples (Cimmino *et al.*, 2017; 2018). The identity of metabolite **1** was confirmed comparing the $[a]^{25}_{D}$, ^{1}H NMR and ESIMS(+) data with those reported in previous studies (Djoukeng *et al.*, 2009; Evidente *et al.*, 2010). Metabolite **2** was identified by comparing its ^{1}H NMR and ESIMS data with those reported in literature (Kimura *et al.*, 1973; Evidente *et al.*, 2010).



1, (*R*)-(-)-Mellein **2,** Tyrosol

Figure 1. Structures of *R*(-)-mellein **1** and tyrosol **2** isolated from *Lasiodiplodia laeliocattleyae* H141a.

DISCUSSION

Melleins are metabolites produced by many fungi in different genera which are involved in numerous plant diseases. These compounds give different phytotoxic, zootoxic and moderate antifungal effects. (*R*)-(-)-mellein (**1**), produced by different Botryosphaeriaceae, produces toxic effects on grapevine leaves and grapevine calli (Vankatasubbaiah *et al.*, 1991; Djoukeng *et al.*, 2009,; Evidente *et al.*, 2010). Furthermore, this compound was detected in infected wood samples and in green shoots of grapevines affected with Botryosphaeria dieback (Abou-Mansour *et al.*, 2015). *R*-(-)-mellein was produced *in vitro* by several different species of Botryosphaeriaceae such us *Diplodia mutila, Neofusicoccum parvum, Neofusiccoum australe, Neofusicoccum luteum* isolated from grapevine in different grape-growing areas in the world, posing questions about its involvement in the virulence of Botryosphaeria dieback pathogens (Reveglia *et al.*, 2018).

Melleins are isocoumarins, and together with jasmonic acid, its esters, dihydrofuranones and closely related compounds, these are specifically related to Botryosphaeriaceous pathogens. Eutypine and analogues are only produced by *Eutypa* species, the cause of Eutypa dieback (Masi *et al.*, 2018).

Tyrosol (**2**), is a ubiquitous phytotoxic secondary metabolite that has been isolated from *N. parvum* (Evidente *et al.*, 2010) and *N. australe*, both of which are well-known Botryosphaeria dieback agents (Andolfi *et al.*, 2012). Furthermore, metabolite **2** was produced by some *Lasiodiplodia* species such as *L. euphorbiaceicola* and *L. hormozganensis* (Cimmino *et al.*, 2017). More recently, it was also isolated from *Diplodia seriata*, *N. luteum* and *D. mutila* associated with grapevine wood infections (Reveglia *et al.*, 2018). The phytotoxic activity of tyrosol was shown on tomato cuttings (Evidente *et al.*, 2010), but other results point to activity of tyrosol as an active quorum sensing compound in *Candida albicans* (Chen *et al.*, 2004), controlling of this organism. Tyrosol was also shown to have a synergistic inhibitory effect on radish and grain sorghum when tested with vanillic acid (Einhelling *et al.*, 1978,;Yu *et al.*, 1994; Evidente *et al.*, 2010).

This first report on phytotoxic metabolites produced by *L. laeliocattleyae*, one of the pathogens associated with cankers and diebacks on grapevine in Brazil, adds further information on the complex interactions between virulence factors produced by the internationally common pathogens involved in Botryosphaeria dieback of grapevine and the diseases they cause.

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LITERATURE CITED

Aldridge D.C., Galt S., Giles, D., Turner W.B., 1971. Metabolites of *Lasiodiplodia theobromae. Journal Chemical. Society C: Organic* 1623–1627.

Abou-Mansour E., Débieux M., Ramírez-Suero M., Bénard-Gellon M., Magnin-Robert A.,… Larignon P., 2015. Phytotoxic metabolites from *Neofusicoccum parvum*, a pathogen of Botryosphaeria dieback of grapevine. *Phytochemistry* 115: 207–215.

Andolfi A., Maddau L., Cimmino A., Linaldeddu B.T.,Franceschini A.,… Evidente A., 2012. Cyclobotryoxide, a phytotoxic metabolite produced by the plurivorous pathogen *Neofusicoccum australe*. *Journal of Natural Products* 75: 1785–1791.

Andolfi, A., Maddau L., Cimmino A., Linaldeddu B.T., Basso S.,… Evidente A, 2014. Lasiojasmonates A-C, three jasmonic acid esters produced by Lasiodiplodia sp., a grapevine pathogen. *Phytochemistry* 103: 145–153.

Billones-Baaijens R., Savocchia S., 2018. A review of Botryosphaeriaceae species associated with grapevine trunk diseases in Australia and New Zealand. *Australasian Plant Pathology* Doi.org/10.1007/s13313–018-0585-5.

Chen H., Fujita M., Feng Q., Clardy J. G., Fink R., 2004. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proceedings of the National Academy of Sciences*, 101: 5048–5052.

Cimmino A., Cinelli T., Masi M., Reveglia P., da Silva M.A.,… Evidente A., 2017. Phytotoxic lipophilic metabolites produced by grapevine strains of *Lasiodiplodia* species in Brazil. *Journal of Agricultural and Food Chemistry* 65: 1102–1107.

Cimmino A., Maddau L., Masi M., Linaldeddu B.T., Evidente A., 2018. Secondary metabolites produced by *Sardiniella urbana*, a new emerging pathogen on European hackberry. Natural product research. doi.org/10.1080/14786419.2018.1477154.

Correia K. C., Silva M. A., de Morais Jr M. A., Armengol, J., Phillips A.J., Michereff S.J. 2016. Phylogeny, distribution and pathogenicity of *Lasiodiplodia* species associated with dieback of table grape in the main Brazilian exporting region. *Plant Pathology*, 65: 92–103.

de Oliveira Costa V. S., Michereff S. J., Martins R. B., Gava C. A. T., Mizubuti, E. S. G., Câmara M.P. S. 2010. Species of Botryosphaeriaceae associated on mango in Brazil. *European Journal of Plant Pathology,* 127: 509–519.

Djoukeng, J.D., Polli S., Larignon P., Abou-Mansour E., 2009. Identification of phytotoxins from *Botryosphaeria obtusa*, a pathogen of black dead arm disease of grapevine. *European Journal of Plant Pathology* 124:303–308.

Einhelling, F.A., Rasmussen J.A, 1978. Synergistic inhibitory effects of vanillic and p-hydroxybenzoic acids on radish and grain sorghum. *Journal of Chemical Ecology* 4: 425–436.

Evidente A., Punzo B., Andolfi A., Cimmino A., Melck D., Luque J., 2010. Lipophilic phytotoxins produced by *Neofusicoccum parvum*, a grapevine canker agent. *Phytopathologia Mediterranea* 49: 74–79 (and references therein cited).

Gramaje, D., Úrbez-Torres J.R, Sosnowski M.R., 2018. Managing grapevine trunk diseases with respect to etiology and epidemiology: current strategies and future prospects. *Plant Disease* 102, 12–39.

He G., Matsuura H., Yoshihara T., 2004. Isolation of an α-methylene-γ-butyrolactone derivative, a toxin from the plant pathogen *Lasiodiplodia theobromae*. *Phytochemistry* 65: 2803–2807.

Husain A., Ahmad A., Agrawal K.P., 1993. (-)-Jasmonic acid, a phytotoxic substance from *Botryodiplodia theobromae*: characterization by NMR spectroscopic methods. *Journal of Natural Products* 56: 2008–2011.

Jayawardena R. S., Purahong W., Zhang W., Wubet T., Li X., Liu M., ... & Yan J., 2018. Biodiversity of fungi on *Vitis vinifera* L. revealed by traditional and high-resolution culture-independent approaches. *Fungal Diversity* 90: 1-84.

Kitaoka N., Nabeta K., Matsuura H., 2009. Isolation and structural elucidation of a new cyclohexenone compound from *Lasiodiplodia theobromae*. *Biosciences, Biotechnology & Biochemistry* 73: 1890–1892.

Kimura Y., Tamura S., 1973. Isolation of l-β-phenyllactic acid and tyrosol as plant-growth regulators from *Gloeosporium laeticolor*. *Agricultural and Bioogical Chemistry* 37: 2925–2925.

Martos S., Andolfi A., Luque J., Mugnai L., Surico G., Evidente A., 2008. Production of phytotoxic metabolites by five species of Botryosphaeriaceae causing decline on grapevines, with special interest in the species *Neofusicoccum luteum* and *N. parvum*. *European Journal of Plant Pathology* 121: 451–461.

Masi M., Cimmino A., Reveglia P., Mugnai L., Surico G., Evidente A., 2018. Advances on fungal phytotoxins and their role in grapevine trunk diseases. *Journal of Agricultural and Food Chemistry*, 66: 5948–5958 (and references therein cited).

Matsuura H., Obara N., Chisaka N., Ichihara A., Yoshihara T., 1998. Novel cyclohexene compound from *Lasiodiplodia theobromae* IFO 31059. *Bioscience, Biotechnology & Biochemistry* 62: 2460–2462.

Mehl, J. W., Slippers B., Roux J., Wingfield M. J., 2013. 14 Cankers and other diseases caused by the Botryosphaeriaceae. *Infectious Forest Diseases,* 298.

Miranda C.C.B.O., Dekker R.F.H., Serpeloni, J. M., Fonseca E.A.I, Cólus I.M.S., Barbosa A.M. 2008. Anticlastogenic activity exhibited by botryosphaeran, a new exopolysaccharide produced by *Botryosphaeria rhodina* MAMB-05. *International Journal of Biology and Macromolecules* 42: 172–177.

Mondello V., Songy S., Battiston E., Pinto C., Coppin C.,Trotel-Aziz P.,…Fontaine F., 2018. Grapevine Trunk Diseases: A Review of Fifteen Years of Trials for Their Control with Chemicals and Biocontrol Agents. *Plant Disease* 102: 1189–1217.

Olmo D., Armengol J., León M., Gramaje D., 2016. Characterization and pathogenicity of Botryosphaeriaceae species isolated from almond trees on the island of Mallorca (Spain). *Plant Disease*, 100: 2483–2491.

Reis P., Magnin-Robert M., Nascimento T., Spagnolo A., Abou-Mansour E., Fontaine F., 2016. Reproducing Botryosphaeria dieback foliar symptoms in simple model system. *Plant Disease* 100: 1071–1079.

Reveglia P., Savocchia S., Billones-Baaijens R., Masi M., Cimmino A., Evidente A., 2018. Phytotoxic metabolites by nine species of Botryosphaeriaceae involved in grapevine dieback in Australia and identification of those produced by *Diplodia mutila*, *Diplodia seriata*, *Neofusicoccum australe* and *Neofusicoccum luteum*. *Natural Product Research*. in press.

Rodríguez-Gálvez E., Guerrero P., Barradas C., Crous, P. W., Alves A., 2017. Phylogeny and pathogenicity of *Lasiodiplodia* species associated with dieback of mango in Peru. *Fungal Biology* 121: 452–465.

Rosado A., Machado, W.C., Freire A. R., F. D. C. O., Pereira O.L. 2016. Phylogeny, identification, and pathogenicity of *Lasiodiplodia* associated with postharvest stem-end rot of coconut in Brazil. *Plant Disease* 100: 561–568.

Úrbez-Torres J.R. 2011. The status of Botryosphaeriaceae species infecting grapevine. *Phytopathologia Mediterrerranea* 50: 5–45.

Venkatasubbaiah P., Sutton T.B., Chilton W.S., 1991. Effect of phytotoxins produced by *Botryosphaeria obtusa*, the cause of black rot of apple fruit and frogeye leaf spot. *Phytopathology* 81: 243–247.

Yu J.Q., Matsui Y, 1994. Phytotoxic substances in root exudates of cucumber (*Cucumis sativus* L.). *Journal of Chemical Ecology* 20: 21–31.