Macrophomina vaccinii sp. nov. causing blueberry stem blight in China

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Abstract
Blueberries (Vaccinium spp.) have been widely cultivated in China because of their nutritional benefits and economic value. Blueberry stem blight has become one of the most severe diseases influencing blueberry productivity and quality in China. In this study, eight fungal isolates were obtained from twenty stem blight lesions of blueberry collected in Nanping, Fujian province, China. Asexual stage was observed after inducing sporulation, the morphology of which agrees with Macrophomina in the black, smooth, hard sclerotia and ellipsoid to obovoid, smooth hyaline conidia with apical sheath. Furthermore, DNA sequences of concatenated ITS, tef1-α, TUB, and ACT loci indicated that these isolates belong to a novel fungal species. The distinguishing morphological characteristics, such as the wider conidia and larger conidiomata pycnidial, also support its new status. Thus a novel fungus, Macrophomina vaccinii, was described in this study. Pathogenicity tests indicated that M. vaccinii could cause stem blight of blueberry.

Keywords
Vaccinium, stem blight, Botryosphaericeae, taxonomy, pathogenicity

Introduction
Blueberries (Vaccinium spp.) are popular fruits because of their health benefits health, such as enhancing brain memory and preventing heart disease (Shi and Liu 2009, Popović et al. 2018). Blueberries have been commercially cultivated worldwide, particularly in the USA, Canada and a few European countries (Evans and Ballen 2014). Blueberry cultivation in China started in 1981, and the planted area has reached 31,210 hectares with total production of 114,905 t in 2017 (Li et al. 2018). Blueberries have
been widely cultivated mainly in Guizhou, Shandong and Liaoning province (Xu et al. 2015, Li et al. 2018). Stem blight disease was one of the most prevalent diseases in blueberry cultivation areas in China, which has seriously affected the growth of blueberry plants, fruit quality and productivity (Yu et al. 2012, 2013a, b, Xu et al. 2015, Xu 2016).

A number of fungal species have been reported causing stem blight, dieback or stem canker of blueberries. For instance, *Botryosphaeria dothidea*, *Lasiodiplodia theobromae*, *Neofusicoccum ribis*, and *N. parvum* caused stem blight of highbush or rabbiteyes blueberries in USA (Milholland 1972, Creswell and Milholland 1988, Smith 2004, Wright and Harmon 2009, 2010, Koike et al. 2014). *Macrophomina phaseolina* (Tassi) Goid caused stem blight of highbush blueberries in Serbia (Popović et al. 2018). *Neofusicoccum parvum* caused stem blight and dieback of highbush blueberries in Mexico (Boyzo-Marin et al. 2016).

*Diaporthe ambigua*, *D. australaficana*, *D. neotheicola*, *D. passiflorae*, *Pestalotiopsis clavispora*, *P. neglecta*, and *Truncatella angustata* caused stem canker and dieback of highbush blueberries in Chile (Espinoza et al. 2008, Elfar et al. 2013), and *Godronia cassandrae* caused stem dieback of highbush blueberry in Norway (Stromeng and Stensvand 2011).

The genus *Macrophomina* was introduced based on *M. phaseolina*, and assigned in the Botryosphaeriaceae (Botryosphaeriales) (Crous et al. 2006, Phillips et al. 2013). Thus far, three species are accommodated within *Macrophomina*, viz. *M. phaseolina*, *M. pseudophaseolina* Crous, Sarr & Ndiaye, and *M. euphorbiicola* A.R. Machado, D.J. Soares & O.L. Pereira (Phillips et al. 2013, Sarr et al. 2014, Machado et al. 2019). *Macrophomina phaseolina* is a soil- or seed-borne polyphagous pathogen, causing charcoal rot disease on about 500 plant species of more than 100 families throughout the world (Su et al. 2001, Babu et al. 2007, Sarr et al. 2014). In Serbia, *M. phaseolina* was reported as a causal agent causing foliage death and brown discoloration of internal vascular stem tissues of highbush blueberry in 2015 (Popović et al. 2018). So far, *M. pseudophaseolina* has been reported causing charcoal rot disease on six plant species, viz. *Abelmoschus esculentus, Arachis hypogaea, Hibiscus sabdariffa, Vigna unguiculata, Gossypium hirsutum, Ricinus communis*, and associated with seed decay of *Jatropha curcas* (Sarr et al. 2014, Machado et al. 2019). *Macrophomia euphorbiicola* has only been reported as the causal agent of the charcoal rot on *Ricinus communis* and *Jatropha gossypifolia* (Machado et al. 2019).

In the course of an ongoing survey of biodiversity of fungi causing stem blight of blueberries in China, a new taxon with general characteristics of *Macrophomina* was collected. The aim of this study was to identify the new isolates based on morphological characteristics and multigene phylogenetic analysis, and determine their pathogenicity on the blueberry.

**Materials and methods**

**Sample collection, fungal isolation and morphological studies**

This study was conducted at the Blueberry Production Garden in the suburb area of Nanping, Fujian province, China. Twenty diseased or dead stems (about 30 cm in length) were collected from blueberry branches in February, 2018. Wood segments (0.5 × 0.5
× 0.2 cm) cut from the diseased lesion boundary or dead tissue were surface sterilized (Pavlic et al. 2004) and incubated on malt extract agar (MEA, 2%) for fungal strains. Petri-dishes were incubated in the dark at 28 °C until fungal colonies were observed. Pure cultures were obtained by hyphal tips from the margin of the suspected Macrophomina colonies, which were subcultured on fresh MEA and maintained at 28 °C.

To induce sporulation of conidia, isolates were cultivated on synthetic nutrient-poor agar (SNA) with autoclaved pine needles placed onto the medium, and incubated at 25 °C under near-UV light (mainly 340 nm) (Dou et al. 2017b). Pycnidia produced on the pine needles were morphologically described and characterized following the protocol of Dou et al. (2017a, b). Measurements of conidia, conidiogenous cells and microconidia were made from water mounts. Measurements and digital photographs were made using a Nikon Coolpix 995 digital camera connected to a trinocular Leitz Orthoplan microscope and processed with Adobe Photoshop Elements 10 software. Fungal isolates and specimens were deposited at Beijing Forestry University (BJFU) with duplicates in the China General Microbiological Culture Collection Center (CGMCC) and the Mycological Herbarium of the Institute of Microbiology, Chinese Academy of Sciences (HMAS) (Table 1).

DNA extraction, PCR amplification and sequencing

DNA was extracted from mycelia grown on MEA plates with CTAB plant genome DNA fast extraction kit (Aidlab Biotechnologies Co., Ltd, Beijing, China). The internal transcribed spacer of rDNA (ITS) was amplified and sequenced with primers ITS-1 and ITS-4 (White et al. 1990). The translation elongation factor-1α (tef1-α) was amplified and sequenced with primers EF1-688F and EF1-1251R (Alves et al. 2008). The β-tubulin gene (TUB) was amplified and sequenced with primers Bt2a and Bt2b (Glass and Donaldson 1995). The actin gene (ACT) was amplified and sequenced with primers ACT-512F and ACT-2RD (Carbone and Kohn 1999, Sarr et al. 2014). PCR amplification and sequencing followed the protocols of Zhang et al. (2009).

Sequence alignment and phylogenetic analysis

DNA sequences of concatenated ITS, tef1-α, TUB, and ACT loci were analyzed to investigate the phylogenetic relationships among Macrophomina species with DNA sequences available from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), as well as the sequences generated herein (Table 1). A multiple alignment was conducted with MEGA v. 6 (Tamura et al. 2013) and analyses were performed in PAUP V. 4.0b10 (Swofford 2002). Prior to phylogenetic analysis, ambiguous sequences at the start and the end were deleted and gaps manually adjusted to optimize the alignment. Maximum parsimony (MP) was conducted with heuristic searches as implemented in PAUP with the default options method (Zhang et al. 2008). Analyses with gaps treated as missing data were conducted under different parameters of maximum parsimonious criteria as outlined
Table 1. GenBank accession numbers of isolates included in this study (newly generated sequences are in bold).

| Species                      | Sample number | GenBank accession number | ITS    | tef1-a  | TUB  | ACT  |
|------------------------------|---------------|--------------------------|--------|---------|------|------|
| *Botryosphaeria dothidea*    | CBS 115476    | AY236949                 | AY23698 | AY236927 | –    | –    |
|                             | CBS 110302    | AY259092                 | AY573218 | EU673106 | –    | –    |
| *Macrophomina euphorbiicola*| CMM 4045      | KU058928                 | KU058898 | MF457657 | MF457654 | –    |
|                             | CMM 4134      | KU058936                 | KU058906 | MF457658 | MF457655 | –    |
|                             | CMM 4145      | KU058937                 | KU058907 | MF457659 | MF457656 | –    |
| *M. phaseolina*              | CBS 162.25    | KF531826                 | KF531996 | KF531805 | KF951803 | –    |
|                             | CBS 227.33    | KF531825                 | KF532000 | KF531806 | KF951807 | –    |
|                             | CPC 21388     | KF951703                 | KF532074 | KF52165 | KF951843 | –    |
|                             | CPC 21392     | KF951705                 | KF532076 | KF52167 | KF951844 | –    |
|                             | CPC 21395     | KF951706                 | KF532077 | KF52168 | KF951846 | –    |
|                             | CPC 21399     | KF951707                 | KF532078 | KF52169 | KF951847 | –    |
|                             | CPC 21443     | KF951734                 | KF532104 | KF52194 | KF951872 | –    |
|                             | CPC 21444     | KF951735                 | KF532105 | KF52195 | KF951873 | –    |
|                             | CPC 21445     | KF951736                 | KF532106 | KF52196 | KF951874 | –    |
| *M. pseudophaseolina*        | CPC 21394     | KF951786                 | KF52148 | KF52228 | KF951913 | –    |
|                             | CPC 21402     | KF951789                 | KF52151 | KF52231 | KF951916 | –    |
|                             | CPC 21403     | KF951790                 | KF52152 | KF52232 | KF951917 | –    |
|                             | CPC 21417     | KF951791                 | KF52153 | KF52233 | KF951918 | –    |
|                             | CPC 21459     | KF951794                 | KF52156 | KF52236 | KF951921 | –    |
|                             | CPC 21501     | KF951796                 | KF52158 | KF52238 | KF951923 | –    |
|                             | CPC 21524     | KF951799                 | KF52161 | KF52240 | KF951925 | –    |
|                             | CPC 21527     | KF951801                 | KF52163 | KF52242 | KF951927 | –    |
|                             | CPC 21528     | KF951802                 | KF52164 | KF52243 | KF951928 | –    |
| *M. vaccinii*                | CGMCC 3.19503 | MK687450                 | MK687426 | MK687434 | MK687442 | –    |
|                             | CGMCC 3.19504 | MK687451                 | MK687427 | MK687435 | MK687443 | –    |
|                             | CGMCC 3.19505 | MK687452                 | MK687428 | MK687436 | MK687444 | –    |
|                             | CGMCC 3.19506 | MK687453                 | MK687429 | MK687437 | MK687445 | –    |
|                             | CGMCC 3.19507 | MK687454                 | MK687430 | MK687438 | MK687446 | –    |
|                             | CGMCC 3.19508 | MK687455                 | MK687431 | MK687439 | MK687447 | –    |
|                             | CGMCC 3.19509 | MK687456                 | MK687432 | MK687440 | MK687448 | –    |
|                             | CGMCC 3.19510 | MK687457                 | MK687433 | MK687441 | MK687449 | –    |

in Zhang et al. (2008). Clade stability was evaluated in a bootstrap analysis with 1,000 replicates, random sequence additions with the maxtrees set to 1,000 and other default parameters as implemented in PAUP. Maximum likelihood (ML) was also conducted using heuristic searches with the default options method as implemented in PAUP. For the ML analysis, best-fit model of nucleotide evolution (HKY+G) was selected by hierarchical likelihood ratio test (hLRT) in MrModeltest 2.3 (Posada and Crandall 2001). A bootstrap analysis with 1,000 replicates was used to test the statistical support of the branches. Trees were viewed in TreeView 1.6.6 (Page 1996). The nucleotide sequences reported in this paper were deposited in GenBank. Trees and alignments were deposited in TreeBase (https://treebase.org/treebase-web/home.html, submission ID: 24410).
Pathogenicity test

Three isolates of *Macrophomina vaccinii* (CGMCC 3.19503, CGMCC 3.19505, and CGMCC 3.19510) obtained in this study were used to conduct a pathogenicity test. The pathogenicity test was performed on 2-year blueberry stems (*cv. O’Neal*) in a humid chamber at 28 °C with semi-shaded conditions. Stems for inoculation were surface sterilized with 75% ethanol for 1 min before making a tangential cut (5 mm in length) on the bark (Espinoza et al. 2009). A 5-mm-diameter MEA medium with mycelial was taken from the 3-day colony, which was placed on to the wounded site, and subsequently covered with parafilm. Three replicates were conducted for each isolate. Noncolonized MEA agar plugs were used as negative controls. Pathogenicity was determined by the length of the necrotic lesion caused by the tested isolates three weeks after inoculation. Fungal isolates were re-isolated from the infected tissue, and morphological characterization and DNA sequence comparisons were conducted to fulfill Koch’s postulates. Mean comparisons were conducted using Tukey’s Honest Significant Difference test (HSD, α = 0.05) in R (Version 3.2.2, R Inc. Auckland, NZL).

Results

Phylogeny

Phylogenetic analysis of the concatenated ITS, *tefl-a*, *TUB* and *ACT* sequence dataset comprising 1,426 bp revealed 129 parsimony-informative characters. The outgroup taxon was *Botryosphaeria dothidea*. The heuristic search with random addition of taxa (1,000 replicates) generated 5,000 most parsimonious trees of 141 steps (CI = 0.972, RI = 0.990, RC = 0.962, HI = 0.028). In both analyses (MP and ML), *M. phaseolina* and *M. vaccinii* formed a well-supported clade (MP BS = 99%, ML BS = 91%). *Macrophomina pseudophaseolina* and *M. euphorbiicola* formed another clade which lacks of bootstrap support (MP BS = 68%, ML BS = 67%, Fig. 1).

Taxonomy

*Macrophomina vaccinii* Y. Zhang ter & L. Zhao, sp. nov.
Mycobank: MB830282
Figure 2

**Holotype.** CHINA, Fujian province, Nanping city, Jianyang district, Huilong village, from blighted stem of southern high bush (*Vaccinium corymbosum × V. darrowii*), 26 Feb. 2018, L. Zhao (HMAS 255479): ex-type living culture, CGMCC 3.19503.

**Etymology.** from “*Vaccinium*”, in reference to the host genus.
Figure 1. Maximum parsimony tree generated from sequence analysis of the concatenated ITS, tef1-α, TUB and ACT dataset. Designated out group taxa is B. dothidea. Maximum parsimony (MP) and maximum likelihood (ML) bootstrap support greater than or equal to 60% are shown above the nodes (* = value less than 60%). The positions of the Macrophomina vaccinii isolates are indicated in bold and red text.
Description. Sexual stage not observed. Asexual stage: Sclerotia developing on SNA, black, smooth, hard, 40–100 µm diam. Conidiomata pycnidial, dark brown to black, solitary or gregarious, up to 400 µm diam., each opening by a central ostiole. Conidiogenous cells lining the inner surface of the conidioma, hyaline, subcylindrical, each proliferating several times percurrently near the apex, 9–16 × 3–4 µm, young conidiogenous cells each covered by a mucous layer that extends over the apex of the developing conidium. Conidia ellipsoid to obovoid, smooth, (18–20–29(–33) × (8–)9–11(–12) µm (av. 24.8 × 10.1 µm, n = 60, L/W ratio = 2.5, range from 2.3 to 2.8), immature conidia hyaline, enclosed in a mucous sheath, that upon dehiscence encloses the top half of the conidium, transformed into two lateral tentaculiform, apical mucoid appendages (type C; Nag Raj 1993), no pigmented conidia observed after 30 days incubation. Microconidia aseptate, hyaline, smooth, guttulate to granular, straight to curved, ellipsoid to subcylindrical to irregular, 5–9(–10) × 3–5 µm.

Culture characteristics. Colonies on MEA at 25 °C in darkness, with even margins, sparse aerial mycelia. On MEA buff, turning pale olivaceous to olivaceous-black with dense, black sclerotial masses. Colonies reaching 58.6 mm on MEA after 2 d in the dark at 25 °C.
Additional specimens examined. CHINA, Fujian province, Nanping city, Jianyang district, Huilong village, from blighted stem of southern high bush (Vaccinium corymbosum × V. darrowii), 26 February 2018, L. Zhao (Paratype, HMAS 255480): living culture, CGMCC 3.19505; (HMAS 255481): living culture, CGMCC 3.19510.

Note. Based on phylogenetic analysis, *M. vaccinii* and *M. phaseolina* formed a well-supported clade. Morphologically, the wider conidia of *Macrophomina vaccinii* can be distinguishable from *M. phaseolina* ((8–)9–11(–12) µm (av. 10.1 µm) vs. (6–)8(–9) µm (av. 8 µm)) (Sarr et al. 2014). In addition, the larger-sized pycnidia of *M. vaccinii* are also distinguishable from *M. phaseolina* (up to 400 µm diam. vs. up to 300 µm diam.) (Sarr et al. 2014). A comparison of the 264 nucleotides across the *tef1-a* gene region of *M. vaccinii* and *M. phaseolina* (CBS 227.33) reveals 5 base pair differences (1.9%) (Table 3).
Table 2. Pathogenicity on 2-year blueberry stems (cv. O’Neal) using mycelia of *Macrophomina vaccinii* after 3 weeks.

| Species            | Isolate              | Blueberry stems inoculated with Mycelia ± SD (cm) |
|--------------------|----------------------|-------------------------------------------------|
| *Macrophomina vaccinii* | CGMCC 3.19503        | 12.63 ± 7.32 a                                  |
| *Macrophomina vaccinii* | CGMCC 3.19505        | 12.38 ± 0.48 a                                  |
| *Macrophomina vaccinii* | CGMCC 3.19510        | 10.75 ± 2.87 a                                  |
| Noninoculated control | –                    | 0.00 ± 0.00 b                                   |

Note: Data followed by different letters in each column are significantly different based on HSD tests at the P < 0.05 level.

Table 3. Major *tef1-a* and *TUB* and *ACT* base pair differences of *Macrophomina vaccinii*, *M. phaseolina*, *M. pseudophaseolina* and *M. euphorbiicola*.

| Species                        | Base pair difference | Position of nucleotides difference |
|--------------------------------|----------------------|-----------------------------------|
| *M. vaccinii* and *M. phaseolina* | G instead of A        | 11                                |
|                                 | C instead of T        | 41                                |
|                                 | C instead of G        | 48                                |
|                                 | A instead of C        | 75                                |
|                                 | A instead of G        | 160                               |
|                                 | T instead of C        | –                                 |
| *M. vaccinii* and *M. pseudophaseolina* | A instead of G        | 10, 24                            |
|                                 | C instead of T        | 27, 31, 48, 103, 186              |
|                                 | G instead of A        | 101, 144, 208                     |
|                                 | A instead of T        | 142                               |
|                                 | T instead of C        | 145, 197, 217, 227, 247           |
|                                 | T instead of A        | 219                               |
|                                 | C instead of A        | –                                 |
| *M. vaccinii* and *M. euphorbiicola* | C instead of T        | 14, 23, 33, 193, 221              |
|                                 | A instead of G        | 24                                |
|                                 | T instead of C        | 43, 250                            |
|                                 | C instead of G        | 48                                |
|                                 | C instead of A        | 106                               |
|                                 | G instead of A        | 144, 211                           |
|                                 | A instead of C        | 185                               |
|                                 | G instead of C        | –                                 |

Pathogenicity test

All the three isolates of *Macrophomina vaccinii* (CGMCC 3.19503, CGMCC 3.19505, and CGMCC 3.19510) were pathogenic on the blueberry stems. Brown lesions appeared on the inoculated spots after 3 days of inoculation for mycelia (Fig. 3). The diseased spots turned brown and lesion area enlarged after 7 days inoculation (Fig. 3). After inoculation for 3 weeks, the length of necrotic lesion reached up to 20 cm, and the infected xylem tissue turned light-brown (Fig. 3). The wounded area of the inoculated stems was the one that was most significantly higher than those of the control groups, while no significant difference was detected among these three inoculated treatments (Fig. 3, Table 2).
Koch’s postulates were performed by successful pathogen re-isolation from all the necrotic stems. The morphology and DNA sequences of these new isolates were consistent with the initial inoculate.

Discussion

*Macrophomina* is a cosmopolitan genus, with a broad host range and colonizing more than 500 crops and non-crop species, such as soybean, common bean, corn, sorghum, cowpea, peanut and cotton (Su et al. 2001, Ndiaye et al. 2010, Sarr et al. 2014, Sun et al. 2015). In this study, *Macrophomina vaccinii* was collected from the lesion of stem blight in Fujian province in China, a subtropical area in China. *Macrophomina phaseolina*, the most common species of *Macrophomina*, is considered as economically more important in subtropical and tropical countries with semi-arid climates, which tends to occur in hot and dry conditions (Wrather et al. 1997, 2001, Smith and Wyllie 1999, Radwan et al. 2014). Charcoal rot of beans is caused by *M. phaseolina*, however, this has frequently been reported in the northern part of China, with a disease incidence of 80% in Beijing and Tianjin (Zhang et al. 2009, 2011, Sun et al. 2015).

So far, seven species have been assigned within *Macrophomina*, viz. *M. euphorbiicola*, *M. limbalis*, *M. phaseoli*, *M. phaseolina*, *M. philippinensis*, *M. pseudeverniae* and *M. pseudophaseolina*. However, *M. limbalis* was transferred to *Dothiorella* (as *D. limbalis*), *M. pseudeverniae* to *Didymocyrtis* (as *D. pseudeverniae*), while *M. phaseoli* and *M. philippinensis* were treated as the synonym of *M. phaseolina*. Thus, only three species, viz. *M. euphorbiicola*, *M. phaseolina* and *M. pseudophaseolina* are currently accommodated within *Macrophomina*. Morphologically, wider conidia of *M. vaccinii* ((8–)9–11(–12) μm) are distinguishable from *M. phaseolina* (6–)8(–9) μm) and *M. pseudophaseolina* (7.5–)8(–9) μm) (Sarr et al. 2014). The larger-sized pycnidia of *M. vaccinii* (up to 400 μm diam.) can also be distinguishable from *M. phaseolina* (up to 300 μm diam.) and *M. pseudophaseolina* (up to 300 μm diam.) (Sarr et al. 2014). In addition, the smaller-sized sclerotia of *M. vaccinii* (40–100 μm diam.) also differs from *M. phaseolina* (100–400 μm diam.) and *M. pseudophaseolina* (100–400 μm diam.) (Sarr et al. 2014). *Macrophomina euphorbiicola* lacks morphological descriptions, and only DNA sequences are available for species comparison (Machado et al. 2019).

Phylogeny based on concatenated ITS, *tef1-a*, *TUB* and *ACT* DNA sequences indicated that the subclade comprising eight isolates of *Macrophomina vaccinii* are closely related to *M. phaseolina* (Fig. 1). A comparison of the *tef1-a* regions DNA sequence data of *M. vaccinii* and *M. phaseolina* revealed a 1.9% base pair difference. A comparison of the 266 nucleotides across the *tef1-a* gene region of *M. vaccinii* and *M. pseudophaseolina* (CPC 21417) reveals 17 base pair differences (6.39%). Although the morphological characteristics of *M. euphorbiicola* cannot be obtained, a comparison of the 269 nucleotides across the *tef1-a* gene region between *M. vaccinii* and *M. euphorbiicola* (CMM 4134) shows 13 base pair differences (4.83%) (Table 3). Following the
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recommendations of Jeewon and Hyde (2016) and Tennakoon et al. (2018), there is sufficient evidence to justify our taxon as a new species.

Pathogenicity tests conducted on 2-year blueberry stems (cv. O’Neal) indicated that inoculation of *Macrophomina vaccinii* were pathogenic on blueberry stems which causes the stem turn brown with necrotic lesions. Similar symptoms caused by *M. phaseolina* have been reported on blueberry in Serbia, resulting in foliage death, and brown discoloration of internal vascular tissues at the basal part of the bush (Popović et al. 2018). The brown lesion caused by *M. vaccinii* and *M. phaseolina* on blueberries differs from the widely reported charcoal rot diseases caused by *Macrophomina phaseolina* and *M. pseudophaseolina* (Su et al. 2001, Salik 2007, Yang et al. 2005, Zhang et al. 2011, Sarr et al. 2014, Sun et al. 2015).

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