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Cryphonectria canker on *Tibouchina* in Colombia

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Summary

Cryphonectria canker, caused by *Cryphonectria cubensis*, has limited the development of new *Eucalyptus* plantations in tropical and subtropical regions. The pathogen is commonly found on *Eucalyptus*, but its occurrence on other hosts in the *Myrtaceae* has also been documented. In this study *C. cubensis* is reported as the causal agent of a serious canker disease on *Tibouchina* spp. (*Melastomataceae*) in Colombia. We used morphological studies, pathogenicity tests on *Eucalyptus* and *Tibouchina*, and a phylogenetic study using partial ribosomal DNA sequence data. This is the first record of *C. cubensis* on a host outside the *Myrtaceae*.

1 Introduction

Cryphonectria canker caused by *Cryphonectria cubensis* (Bruner) Hodges is one of the most important diseases of *Eucalyptus* grown in plantations (BOERBOOM and MAAS 1970; HODGES 1980; WINGFIELD et al. 1989). The disease is well known in tropical and subtropical areas of the world where relatively high temperatures and rainfall favour disease development (ALFENAS et al. 1982). Cryphonectria canker has severely limited plantation development in some of these areas. Clonal propagation of disease-tolerant genotypes, particularly using hybrids between species, has been necessary to reduce losses (ALFENAS et al. 1983; VAN ZYL and WINGFIELD 1999).

Although *C. cubensis* is most commonly found on *Eucalyptus* in the tropics and subtropics, it has also occasionally been found on other Myrtaceaeous hosts (HODGES 1980; HODGES et al. 1986). Likewise, *Endothia eugeniae* (Nutman and Roberts) Reid and Booth, a well-known pathogen of clove (*Syzigium aromaticum* (L.) Merr. and Perry), which also belongs to the *Myrtaceae*, is considered to be a synonym of *C. cubensis* (HODGES et al. 1986; MICALES et al. 1987). Although *C. cubensis* is found only on *Eucalyptus* in South Africa, it has been shown to be a virulent pathogen of exotic *Psidium guajava* L. in pathogenicity tests (SWART et al. 1991).

In 1995, a serious canker disease was first discovered in Colombia on *Tibouchina urvilleana* (DC). Logn. (*Melastomataceae*), which is native to Brazil. In a subsequent survey, the disease was also found on the Colombian native *Tibouchina lepidota* Baill. The aim of this study was to identify the causal agent of the disease found on *Tibouchina* using morphology and partial DNA sequence data. Pathogenicity tests were also conducted on *T. urvilleana* and *Eucalyptus grandis* (Hill) Maid. in Colombia.

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2 Materials and methods

2.1 Symptoms and collection of specimens

Girdling cankers were present on stems and branches of *T. urvilleana* trees (Fig. 1). The cambium was typically killed and pycnidia of an *Endothiella* sp. were produced abundantly on the surface of the cankers. The trees often had multiple cankers on the stems and branches and die-back associated with the disease was common.

Specimens for laboratory study were collected from *T. urvilleana* and *T. lepidota* in Buga, Colombia (3°32′N, 76°17′W, 1600 m a.s.l.) during 1999 (PREM 56913, PREM 56914, PREM 56915, deposited at the National Collection of Fungi, Pretoria, South Africa). Bark samples bearing pycnidia were transported to the laboratory for further study. The pycnidia were cut from the bark, sectioned vertically with a razor blade, mounted in lactophenol on glass slides and examined microscopically. To obtain good vertical sections, pycnidia were also embedded in Quetol 651 epoxy resin (Van Der Merwe and Coetzee 1992) using a modified method of Huhndorf (1991). These modifications were: fixation in a 2% formaldehyde/2.5% glutaraldehyde fixative in 0.075 M $NaPO_4$ buffer (pH = 7.4), the material was washed three times with dH_2O after the OsO_4 fixation step, and Quetol 651 was used as the embedding medium. Subsequent sectioning was carried out using a Reichart Ultracut E ultramicrotome (Setpoint Premier, Johannesburg, RSA). Approximately 10 measurements were made of each of the relevant fungal structures. The colour notations of Rayner (1970) were used.

Isolations were made by lifting conidial masses from the apices of the pycnidia with a sterile needle. These were transferred to malt extract agar (10 g malt extract, 15 g agar per 1000 ml water) and isolation plates were incubated at 25°C. The cultures were purified and representative isolates have been stored in the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa (Table 1).

Fig. 1. Girdling canker on stem of *Tibouchina urvilleana* caused by *Cryphonectria cubensis*

Table 1. **Isolates used in this study for the phylogenetic analysis and restriction fragment length polymorphisms**[a]

Isolate no.	Isolate identity	Host	Origin	Genbank Accession No.
CRY 0127[b]	*Cryphonectria cubensis*	*Eucalyptus camaldulensis*	China	AF 046890
CRY 0140[b]	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	AF 046892
CRY 289[b]	*Cryphonectria cubensis*	*Eucalyptus grandis*	Indonesia	AF 046896
CRY 268[b]	*Cryphonectria cubensis*	*Eucalyptus grandis*	Venezuela	AF 046897
CRY 1318[c]	*Cryphonectria cubensis*	*Eucalyptus grandis*	Colombia	AF 172656
CRY 82[b]	*Cryphonectria cubensis*	*Eucalyptus deglupta*	Thailand	AF 046899
CRY 0129[b]	*Cryphonectria cubensis*	*Psidium* sp.	Brazil	AF 046900
CRY 368[d]	*Cryphonectria cubensis*	*Tibouchina urvilleana*	Colombia	AF 265653
CRY 371[d]	*Cryphonectria cubensis*	*Tibouchina urvilleana*	Colombia	AF 265654
CRY 374[d]	*Cryphonectria cubensis*	*Tibouchina urvilleana*	Colombia	AF 265655
CRY 378[d]	*Cryphonectria cubensis*	*Tibouchina urvilleana*	Colombia	AF 265656
CRY 3723[d]	*Cryphonectria cubensis*	*Tibouchina urvilleana*	Colombia	AF 265658
CRY 794[d]	*Cryphonectria cubensis*	*Tibouchina urvilleana*	Colombia	AF 265657
CRY 66[b]	*Cryphonectria parasitica*	*Castanea dentata*	USA	AF 046901
CRY 44[b]	*Cryphonectria parasitica*	*Castanea dentata*	USA	AF 046902
CRY 67[b]	*Cryphonectria parasitica*	*Castanea dentata*	USA	AF 046903
CMW 2498[b]	*Diaporthe ambigua*	*Malus sylvestris*	Netherlands	AF 046906

[a]Culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa. CMW and CRY refer to the general and *Cryphonectria* culture collections, respectively.
[b]Isolate used in the phylogenetic study by MYBURG et al. (1999).
[c]Isolate used in the phylogenetic study by VAN DER MERWE et al. (2000).
[d]Isolates sequenced for this study.

2.2 Pathogenicity

Tibouchina urvilleana and *E. grandis* trees were grown from cuttings in order to undertake pathogenicity tests. Once rooted, the plants were established in a nursery near Buga in Colombia. For inoculations, 12 1-year-old trees (approximately 10-mm diameter) of each species were selected. An isolate of the *Endothiella* sp. (CRY 1477) that had previously been selected in a preliminary trial for virulence, was used for the inoculation test. This isolate was from *T. urvilleana.* Using a cork borer, wounds (5-mm diameter) were made on the stems of test trees to expose the cambium. Discs of agar from the edge of an actively growing culture were then placed, mycelium surface down, into the wounds so that the mycelium touched the freshly exposed cambium. In the case of controls, wounds were

treated with sterile malt extract agar discs. The treated wounds were covered with masking tape to prevent desiccation of the inoculum. After 4 months, the wounds were exposed and the lengths of the lesions were measured. The data were analysed using an unequal variance analysis (SAS 1989).

2.3 DNA isolation and amplification

The DNA was isolated as described by MYBURG et al. (1999). The internal transcribed spacer (ITS) region of the ribosomal RNA operon was amplified using the primers ITS1 and ITS4 (WHITE et al. 1990). Each 50 μl amplification reaction consisted of the following: 1 mM dNTPs (0.25 mM of each), 1 × reaction mix (supplied with the enzyme), 2.5 mM $MgCl_2$, 0.1 μM of each primer, 5 units of *Taq* Polymerase (Boehringer Mannheim, Mannheim, Germany) and DNA template. Amplification reactions were performed on a Perkin Elmer GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied Biosystems Inc., Foster City, CA, USA). The thermocycler was programmed as follows: an initial denaturation step (94°C for 1 min), was followed by 35 cycles of denaturing (94°C for 1 min), primer annealing (55°C for 1 min) and chain elongation (72°C for 1 min). A final chain elongation step (72°C for 5 min) followed the 35 amplification cycles. PCR products were visualized using an UV light source on a 1% (w/v) agarose gel containing ethidium bromide.

2.4 Sequencing and analysis of sequence data

Amplification products were purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). DNA fragments were sequenced in both directions with primers ITS1 and ITS4, respectively, using an ABI PRISM® Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, UK). DNA sequences were determined with an ABI PRISM 377® automated DNA sequencer. The DNA sequences were aligned using CLUSTAL W (THOMPSON et al. 1997) and checked manually.

Phylogenetic analyses were performed using PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b (SWOFFORD 1998). All constant and parsimony-uninformative characters were excluded prior to the heuristic search. Analyses were carried out using heuristic searches with tree-bisection-reconnection branch swapping and MULTREES (saving all optimal trees) effective. Gaps inserted during sequence alignment were treated as fifth characters (NEWSTATE) in the heuristic searches. A strict consensus tree was computed with the majority rule (50%) option effective. A partition-homogeneity test was conducted (100 replicates, heuristic search type) using PAUP to assess the combinability of the data partitions ITS1, 5.8S and ITS2. The tree-length distribution of 100 randomly generated trees was evaluated to assess the phylogenetic signal (HILLIS and HUELSENBECK 1992). The confidence levels of the tree branching points were determined by a bootstrap analysis (1000 replications). *Diaporthe ambigua* Nits. was used as the outgroup taxon to root the phylogenetic tree. Sequences were deposited in Genbank and accession numbers for the sequenced strains are listed in Table 1.

2.5 Restriction fragment length polymorphisms

Amplification products were digested with *Alu*I and *Cfo*I (Boehringer Mannheim) as described by MYBURG et al. (1999). The restriction fragment length polymorphism (RFLP) fingerprints generated were compared with the RFLP data in MYBURG et al. (1999) to confirm the identification of the fungus isolated from *Tibouchina*.

3 Results

3.1 Morphology

The pycnidia of the canker pathogen from *Tibouchina* in Colombia were superficial or slightly immersed in bark, pyriform, sometimes with the neck attenuated, no stromatal development, blackened except for the inside of the pycnidia and, for some, the tips of the necks were brownish luteous (19), 0.45–0.65 μm high, (0.3–)0.35–0.65 μm in diameter (Figs 2a, b). The necks were (93–)103–786 μm long and (93–)102–250 μm wide. The conidiophores were septate, sometimes branched, hyaline, 9.5–14.5(–15.5) μm long and (0.5–)1–1.5 μm wide at the midpoint (Fig. 2c). Conidia were hyaline, non-septate, oblong to oval with obtuse apices, 3–3.5 μm long and 1.5 μm wide (Fig. 2c). These characteristics made it virtually indistinguishable from the *Endothiella* anamorph of *C. cubensis* (HODGES 1980).

3.2 Pathogenicity

Inoculation of *T. urvilleana* and *E. grandis* stems with an isolate of the *Tibouchina* canker pathogen caused very severe disease on *T. urvilleana* (Fig. 3). Within 4 months, the fungus had killed three *Tibouchina* plants entirely and the lesion lengths could not be computed. In other cases, the stems had started to die and epicormic shoots were forming at the base of the cankers. The average lesion length of the nine remaining plants was 179 mm. All inoculated *E. grandis* stems were living at the termination of this study. The lesions on *E. grandis* differed from those on *T. urvilleana* in that they were exemplified by discoloured wood and were less obvious in the bark and cambium. The average lesion

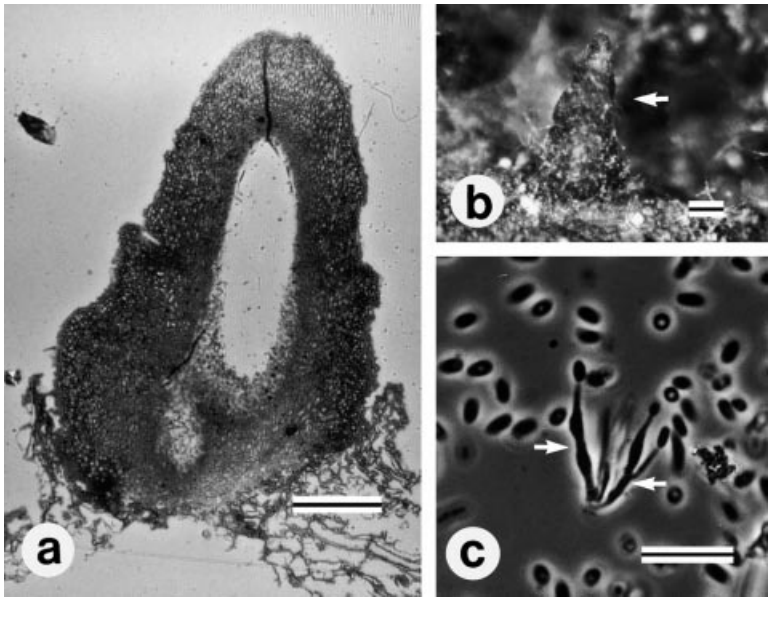

Fig. 2. Light micrographs of the anamorph of *Cryphonectria cubensis* isolated from *Tibouchina* in Colombia. (a) Vertical section of a pycnidium (bar = 100 μm); (b) pycnidium on bark surface (bar = 100 μm); (c) conidiogenous cells and conidia (bar = 10 μm)

Fig. 3. Lesion produced on *Tibouchina urvilleana* 4 months after inoculation with *Cryphonectria cubensis*

length on *E. grandis* was 86.9 mm and thus significantly smaller (Cochran value = 0.003) than those on the surviving *T. urvilleana*. No symptoms were observed on the control trees and the inoculation wounds were covered with callus tissue. A small discoloured area (average length 6 mm) was evident around the control wounds on both *T. urvilleana* and *E. grandis*. Pycnidia of the inoculated fungus were abundant on the surface of the lesions on inoculated plants, and re-isolations of the inoculated pathogen were easily made from these structures.

3.3 Sequencing and analysis of sequence data

Alignment of the sequences yielded a data set consisting of 540 characters. The total number of constant and parsimony-uninformative characters excluded were 447. Included parsimony-informative characters, having equal weight, totalled 93. A 50% majority rule consensus tree (length of tree = 122 steps, consistency index = 0.8770 and retention index = 0.9272), was generated from the 93 parsimony-informative characters (Fig. 4). A *P*-value of 0.73 was generated from the partition-homogeneity test, indicating that the data partitions (ITS1, 5.8S, ITS2) can be combined. The phylogenetic signal was also significant as indicated by the g1 value of 2.38.

From the phylogenetic tree (Fig. 4) it was found that the *C. cubensis* strains and the strains isolated from *Tibouchina* grouped together, but separately from *Cryphonectria*

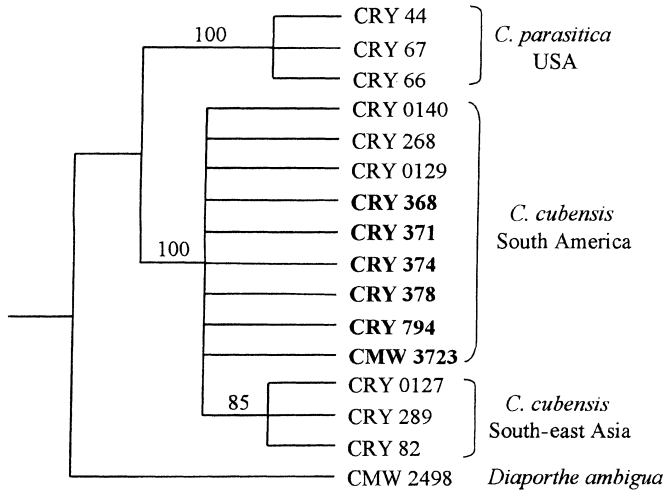

Fig. 4. Strict consensus tree (tree length = 122 steps, consistency index = 0.8770, retention index = 0.9272) generated during heuristic searches within PAUP 4.0b. Emboldened taxa (or isolates of *C. cubensis*) were sequenced for this study. Sequence data for the other taxa were obtained from MYBURG et al. (1999) and VAN DER MERWE et al. (2000). Confidence levels of the tree branching points (1000 bootstrap replications) are indicated. *Diaporthe ambigua* was used to root the tree

parasitica (Murr.) Barr and the outgroup *D. ambigua*. This separation was supported by a bootstrap value of 100%.

Within the *C. cubensis* clade, two geographical subclades are found. These represent a South-east Asian group and a South American group (bootstrap support 85%) as identified by MYBURG et al. (1999) and VAN DER MERWE et al. (2000). The strains isolated from *Tibouchina* in Colombia grouped more closely to *C. cubensis* isolates in the South American clade than those from South-east Asia (Fig. 4). Differentiation within the South American clade was not well resolved as bootstrap values were less than 50% and branches collapsed.

3.4 Restriction fragment length polymorphisms

The RFLP profiles generated for the strains isolated from *Tibouchina* were compared with the restriction map of MYBURG et al. (1999). It was found (data not shown) that the *Alu*I and *Cfo*I profiles for the *Tibouchina* fungus were the same as those found for *C. cubensis* (MYBURG et al. 1999).

4 Discussion

In this study we report, for the first time, a serious canker disease of *Tibouchina* caused by *C. cubensis* in Colombia. The disease is typified by girdling cankers that are very similar to those found on *Eucalyptus* in South Africa (WINGFIELD et al. 1989). In this sense, these symptoms are somewhat different to those on *Eucalyptus* in South America and South-east Asia where *C. cubensis* tends to penetrate the wood and often causes large stem swellings on trees (VAN HEERDEN et al. 1997). The fact that only pycnidia are found on the surface of cankers on *Tibouchina* also makes this disease more similar to Cryphonectria canker in South Africa, than to the disease in other parts of the world.

Based on morphological studies, the causal agent of the disease on *Tibouchina* spp. was clearly similar to the anamorph of *C. cubensis*. However, since the teleomorph of this fungus was not observed, it was necessary to confirm this identification using molecular data. Analysis of partial ribosomal DNA sequence data and RFLP profiles, compared with the data published by MYBURG et al. (1999), has clearly shown that the fungus causing girdling cankers on *Tibouchina* is *C. cubensis*. The six isolates from Colombia resided within the *C. cubensis* clade and also grouped more closely to isolates from South America and South Africa than to those from South-east Asia. The larger *C. cubensis* clade was distinctly different to the clade representing the *C. parasitica* isolates.

The fact that *C. cubensis* is causing a serious canker disease on *Tibouchina* in Colombia is intriguing. Previously, the fungus has been found only on members of the *Myrtaceae*. Its occurrence on a plant belonging to a totally different family is unexpected. However, based on morphological characteristics, it has been recognized that both families reside in a single order *Myrtales* (DAHLGREN and THORNE 1984; JOHNSON and BRIGGS 1984; RAVEN 1984). Recent phylogenetic studies based on sequence data have also confirmed the relatively close relationship between these families (CONTI et al. 1996, 1997). The occurrence of a *Eucalyptus* pathogen on *Tibouchina* confirms the relatedness of these families of trees. It could also suggest that these trees might share other fungal pathogens.

Cryphonectria cubensis is a virulent pathogen of *Tibouchina* in Colombia and has a wide distribution in that country. Given the serious damage that it causes to trees, it is surprising that it has not been recognized previously. The disease is most commonly encountered on the exotic *T. urvilleana* although it occurs on native *T. lepidota* in parks and gardens. Despite some considerable effort, we have not found the disease in native stands of *T. lepidota*. In preliminary inoculation tests, we have been able to cause large cankers on *T. lepidota* and there was little evidence to show that this species is less susceptible to *C. cubensis* after inoculation (M. J. WINGFIELD and C. RODAS, unpublished data).

Cryphonectria canker is well known on *Eucalyptus* in Colombia. In warmer parts of the country, it causes serious damage to plantations (VAN DER MERWE et al. 2001). The fungus from *Tibouchina* caused extensive lesions on *E. grandis* in this study and this suggests that isolates from *Eucalyptus* and *Tibouchina* are cross infective. However, the fact that symptoms on the inoculated plants were somewhat different, implies that this question deserves further study. Furthermore, naturally occurring cankers on *Eucalyptus* have both pycnidia and perithecia on them (VAN DER MERWE et al. 2001). The presence of only pycnidia on *Tibouchina* suggests that there could be some biological differences between the fungus on these hosts or that host response to infection differs.

At the present time, it is impossible to know whether *C. cubensis* on *Tibouchina* is native in Colombia. The origin of the fungus on *Eucalyptus* has been speculated to be in Indonesia where its native host could have been cloves (*Syzigium aromaticum*) (HODGES et al. 1986). There is also an unusual report of the fungus from native *Eucalyptus* in Western Australia (DAVISON and COATES 1991) that could imply an Australian origin. Studies by VAN ZYL et al. (1998), analysing the population diversity of a Brazilian population of the fungus, suggested that *C. cubensis* has been present in that country for a long time. Likewise, the phylogenetic analysis of isolates of the fungus from many parts of the world (MYBURG et al. 1999) gave no indication of an origin in Indonesia and suggested that *C. cubensis* could equally well have originated in South or Central America. If *C. cubensis* on *Tibouchina* is native to South America, it probably does not threaten the tree in native populations, although it is already doing significant damage to this tree where it is a prized ornamental. However, if it has been introduced, or if it has passed from *Eucalyptus* to *Tibouchina*, as *Puccinia psidii* Winter has passed from native *Myrtaceae* to exotic *Eucalyptus* in South America (COUTINHO et al. 1998), it could result in serious damage to *Tibouchina* in the future. Given the severity of the disease in Colombia, these questions deserve further study.

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Résumé

Un chancre à Cryphonectria sur Tibouchina en Colombie

Le chancre à *Cryphonectria cubensis* a limité le développement de nouvelles plantations d'Eucalyptus dans les régions tropicales et subtropicales. La parasite est couramment trouvé sur Eucalyptus mais son existence sur d'autres Myrtacées hôtes est aussi connue. Dans cette étude, *C. cubensis* est mentionné comme agent causal d'une grave maladie chancreuse sur *Tibouchina* spp. (*Melastomataceae*) en Colombie. Nous avons eu recours à la morphologie, à des tests de pouvoir pathogène sur *Eucalyptus* et *Tibouchina*, et à la phylogénie par séquençage partiel de l'ADN ribosomal. Il s'agit de la première mention de *C. cubensis* sur un hôte n'appartenant pas aux Myrtacées.

Zusammenfassung

Cryphonectria-Krebs an Tibouchina in Kolumbien

Der Rindennekroseerreger *Cryphonectria cubensis* begrenzt den Eukalyptusanbau in tropischen und subtropischen Gebieten. Dieses Pathogen tritt häufig an *Eucalyptus* auf, kommt aber auch an anderen Wirtspflanzen aus der Familie *Myrtaceae* vor. In dieser Untersuchung wird *C. cubensis* als Ursache eines Rindenkrebses an *Tibouchina* spp. (*Melastomataceae*) in Kolumbien beschrieben. Es wurden morphologische Untersuchungen, Infektionsversuche an *Eucalyptus* und *Tibouchina* sowie eine phylogenetische Studie anhand von partiellen RNA-Sequenzdaten durchgeführt. *Cryphonectria cubensis* wurde damit erstmals an einem Wirt ausserhalb der *Myrtaceae* nachgewiesen.

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