The fungus *Kalmusia longispora* is able to cause vascular necrosis on *Vitis vinifera*

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Abstract

Fungal diseases in agronomically important plants such as grapevines result in significantly reduced production, pecuniary losses, and increased use of environmentally damaging chemicals. Beside the well-known diseases, there is an increased interest in wood-colonizing fungal pathogens that infect the woody tissues of grapevines. In 2015, a traditional isolation method was performed on grapevine trunks showing symptoms of trunk diseases in Hungary. One isolate (T15142) was identified as *Kalmusia longispora* (formerly *Dendrothyrium longisporum*) according to morphological and phylogenetic analyses. To evaluate the pathogenicity of this fungus on grapevines, artificial infections were carried out under greenhouse and field conditions, including the CBS 824.84 and ex-type CBS 582.83 strains. All isolates could be re-isolated from inoculated plants; however, varying virulence was observed among them in terms of the vascular necrosis caused. The incidence and severity of this symptom seemed to be congruent with the laccase-producing capabilities of the isolates. This is the first report on the ability of *Kalmusia longispora* to cause symptoms on grapevines, and on its possible dependence on laccase secretion.

Introduction

*Vitis vinifera* L. is one of the most important perennial crops in agriculture, with high economic, cultural, and touristic impacts. The major threats to the sustainable management of grapevine plantations are the pests and diseases that severely affect the condition of the plants, as well as the yield and quality of the berries. Studies about the connection between climate and the distribution of the pests and pathogens of grapevines suggest that changes in the climate will create novel challenges for grape growers [1–3]. Fungal infections account for many of the diseases observed in grapevines [4]. Beside the well-known diseases such as powdery mildew and downy mildew and grey and black rot, vascular pathogens are receiving growing attention. These latter fungi cause the so called grapevine trunk diseases (GTDs). This group consist of Blackfoot disease, Botryosphaeria dieback, Eutypa dieback, Esca disease and Phomopsis disease [5]. Several fungal taxa are known to be associated with these diseases and their number is increasing rapidly. The characteristic symptoms are the necrotic lesions in the
wood, the impaired morphology of green shoots and the discoloration and necrosis of leaves. In its severe form, Esca disease can even cause the sudden death of the infected plant called apoplexy [5]. The management of GTD-associated pathogens is a great challenge for vine-growers. Since the ban of sodium arsenite there is no efficient chemical control against these fungi [6]. The application of well-known and satisfactory management procedures is hindered by several factors. The widely-used fungicidal sprays cannot reach the pathogens, which colonize the inner tissues of the trunks [7]. The unsatisfactory knowledge about the host-pathogen-environment interactions makes the development of alternative control methods against GTDs difficult. All the above-mentioned problems indicate that adequate information need to be collected about the causal agents of GTDs for the development of efficient management techniques.

The ascomycetous genus *Kalmusia* was established nearly 150 years ago [8] by the type species *Kalmusia ebuli*, and formerly consisted of more than 40 filamentous fungal species [9]. They are common members of the microbiome of various plants and their host range is wide; these fungi can be obtained from, for example, bamboo [10], oak [11], and raspberry [12]. The genus has been truncated, and nowadays, *Kalmusia sensu stricto* comprises less than 10 species [13,14]. In 2014, Verkley et al. [15] described the species *Dendrothyrium longisporum*, later assigned to the *Kalmusia* genus [16]. The morphological and molecular characterization of the original species was based on two strains, which were isolated from dwarf mistletoe (*Arceuthobium pusillum*) and common wheat (*Triticum aestivum*) [15]. Our knowledge about the lifestyle of the fungus is limited. Despite the fact that it seems to occur in plant material, the possible connection between *Kalmusia longispora* and plant diseases had not been examined before this study. A close relative of *K. longispora* is *Kalmusia varispora* (formerly *Dendrothyrium varisporum*); the strains used for the description of *K. varispora* (CBS 121517 and CBS 197.82) were isolated from a declined grapevine in Syria and from the dicotyledonous plant winter heath (*Erica carnea*) in Switzerland [15]. In addition, *K. varispora* was also reported on Persian oak (*Quercus brantii*) in Iran [11] and on symptomatic grapevine trunks in Croatia [17]. Recently, the pathogenicity of *K. varispora* was confirmed on grapevines [18]. It was shown that *K. varispora* isolates can develop necrotic lesions in the woody tissues of grapevine cuttings. Except for this study, we are not aware of any other confirmed case of phytopathogenicity in *K. sensu stricto*.

During the isolation-based examination of the mycobiota of grapevines, an isolate was found to be *K. longispora*, originating from a plant with symptoms of trunk diseases. The aims of the present work were to verify and confirm the identity of the isolate and to examine of the capability of *K. longispora* isolates to develop symptoms on grapevines.

**Results**

**Identification and phylogenetic analysis of the T15142 isolate**

During the collection of the isolate from the Kékfrankos grapevine variety from Helesfa, Pécs Wine Region, Hungary, one isolate (T15142) showed high internal transcribed spacer (ITS) sequence similarity with *K. longispora* strains using a BLAST search in GenBank. Based on the results of the phylogenetic analysis of the ITS, partial large ribosomal subunit (LSU), and partial β-tubulin (TUB), the T15142 isolate belongs to the pleosporalean family *Didymosphaeriaceae* and forms a well-supported clade with *Kalmusia* species, a sister group of *Alloconiothyrium aprootii* (Fig 1). Isolates of *K. longispora* and *K. ebuli* grouped together within the genus and T15142 showed the highest similarities with the two *K. longispora* strains. The ITS, LSU, and TUB sequences of this isolate showed 99.2%, 100%, and 99.8% similarity with...
the *K. longispora* ex-type strain CBS 582.83, respectively [15]. The results of the molecular phylogenetic analysis unambiguously show that the isolate T15142 represents *K. longispora*.

**Morphological characterization of the T15142 isolate**

To further characterize the T15142 strain, micro- and macromorphological examinations were performed. Colonies of the isolate were white with entire margins on potato dextrose agar (PDA), malt extract agar (MEA), and oatmeal agar (OA) media, while developed a pale-brownish color with undulated margins on water agar (WA) medium (Fig 2). The conidia of T15142 are cylindrical ellipsoids with dimensions of $4.21 \pm 0.3 \times 1.74 \pm 0.72 \mu m$ (Fig 3B). Conidiogenous cells were also observed in the disrupted pycnidia (Fig 3A).

**Pathogenicity tests of the *K. longispora* isolates on grapevines**

Artificial infections were performed to examine the pathogenicity of the *K. longispora* strains on grapevines. All three of the tested strains caused different incidences of symptoms on the
shoots (Fig 4A–4C). The isolate T15142 produced longitudinal, black necrotic lesions on all five infected shoots (Fig 4C), while the CBS 824.84 and CBS 582.83 strains developed necrosis on three and two shoots, respectively (Fig 4A and 4B). Non-symptomatic shoots showed brownish coloration at the inoculation point. The incidence of symptoms developed on canes (Fig 4D–4F) was somewhat similar. While both CBS 824.84 and T15142 caused black necrosis deep in the xylem on all five inoculated canes (Fig 4D and 4F), strain CBS 582.83 developed only slight surface discoloration at the inoculation point (Fig 4E). Radial necroses can be observed on cross sections of all the infected cuttings (Fig 4G–4I). However, this symptom was much more definite in case of CBS 824.484 (Fig 4G) and T15142 (Fig 4I), while CBS 582.83 caused only mild discolorations (Fig 4H). All of the tested strains were re-isolated frequently from all types of the inoculated plants. The isolate T15142 was identified in four out of the five inoculated shoots and in all five of the canes and cuttings. Both CBS 582.83 and CBS 824.84 could be re-isolated from all the infected shoots and canes and from four of the inoculated cuttings.

**Exoenzyme production of K. longispora isolates**

Examination of the digestive exoenzymes revealed differences between the *K. longispora* strains tested here (Fig 5). All strains showed equally large zones of clearance on cellulose-indicating medium (Fig 5A–5C). A very faint hydrolytic zone could be observed around the

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**Fig 2. Two-week-old colonies of the *K. longispora* isolate T15142 from Hungary.** The colonies were grown at 25°C on different media: (a) Potato dextrose agar (PDA); (b) malt extract agar (MEA); (c) oatmeal agar (OA); (d) water agar (WA).

https://doi.org/10.1371/journal.pone.0258043.g002

**Fig 3. Reproductive cells of the *K. longispora* isolate T15142.** Sporulation was induced on pine needles placed on PDA medium. (a) Conidiogenous cell (arrowhead); (b) conidia. Scale bars = 10 μm.

https://doi.org/10.1371/journal.pone.0258043.g003
colonies of all *K. longispora* strains on pectinase-detecting medium (Fig 5D–5F). None of the strains produced amylases according to Lugol’s staining of starch-containing medium, indicated by the absence of a yellowish zone around the colonies (Fig 5G–5I). On guaiacol-amended medium, the T15142 isolate was surrounded with a wide red-brownish halo (Fig 5L), indicating high laccase activity. This zone was very faint and narrow in the case of CBS 824.84 (Fig 5J) and completely absent in the case of CBS 582.83 (Fig 5K).

Comparison of the exoenzyme activities in liquid cultures of the *K. longispora* isolates by spectrophotometric methods showed similar results as observed on indicative media (Fig 6, S1 Table). The measured cellulase activities were high and slightly but significantly differed between the strains (Fig 6A). Pectinase activities were very low and no difference could be observed between the examined strains (Fig 6B). The highest laccase activity was measured in the culture filtrate of T15142 followed by a somewhat lower value in the case of CBS 824.84 (Fig 5L) and completely absent in the case of CBS 582.83 (Fig 5K).

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**Discussion**

Grapevines live together with a wide spectrum of fungi [19]. Recently, numerous studies have focused on the grapevine microbiome, including endophytic, pathogenic, and even saprotrophic fungi using different approaches and techniques [19,20], providing us with important information on the presence of potential pathogens. Although, there are broad-scale studies...
on considerable grapevine pathogens [21] and continuously increasing information on the fungi involved in trunk diseases [22,23]. In the present study, we introduced *K. longispora* as species associated with vascular fungal infections of grapevines. Experiments were carried out with a strain isolated from a grapevine trunk in Hungary, and all of the available strains were deposited into culture collections (CBS 582.83 and CBS 824.84), including the ex-type material on which the description of *K. longispora* was carried out [15]. The strain characterized in the present study (T15142) was isolated from the trunk of a symptomatic Cabernet Sauvignon grapevine, a variety highly susceptible to GTDs. The plantation was relatively old (15 years) and showed high incidence (18.6%) of GTD-related symptoms in the year of the isolation. The following well-known causal agents of different GTDs were also isolated from the same trunk: *Eutypa lata* (Eutypa dieback), *Neofusicoccum parvum* (Botryosphaeria dieback), and *Phaeoacremonium minimum* (Esca disease). Therefore, because of the diversity of the co-occurring pathogenic species, this fungus cannot be solely linked to and/or considered a solely causal agent of any of the above-mentioned infections.

The fungus *K. longispora* belongs to the family *Didymosphaeriaceae* (formerly *Montagnulaeaceae*), which includes saprobes, endophytes, and pathogens associated with a wide variety of
substrates worldwide [13]. The genus *Kalmusia* represents one of the basal lineages of the family and comprises endophytic or saprobic fungi mainly of the Santalaceae and Poaceae families [14]. *Kalmusia* species are reported to have *Coniothyrium*-like, *Cytoplea*, *Microsphaeropsis*, and *Paraconiothyrium* asexual morphs, which was also demonstrated here. The isolate T15142 showed all of the micro- and macromorphological characteristics of *K. longispora* described earlier by Verkley et al. [15].

All three *K. longispora* strains examined in the present study were originated from phylogenetically distant plant hosts, which fact suggests a general association of this fungus to plants. However, we are not aware of any studies on the possible relation of *K. longispora* to any plant disease. According to our results, *K. longispora* can cause symptoms on grapevines and its virulence shows high variances among strains. The most virulent strain was T15142 causing severe symptoms on all types of examined grapevine material and the least virulent was CBS 582.83 especially on canes and cuttings. The higher virulence of CBS 824.84 on canes and cutting rather than on the shoots could be explained by the longer incubation time between the inoculation and the observation. The same growing capacity of the tested strains in grapevines regardless of their virulence implies that the lifestyle of this fungus strongly depends on the host. Based on these results, we hypothesize that *K. longispora* can potentially act as a pathogen, depending on the strain and/or on the host plant. The pathogenicity tests did not show correlation between pathogen multiplication and virulence like it was demonstrated earlier in other types of fungal plant pathogens [24,25]. The most virulent T15142 and the least virulent CBS 582.83 strains were both isolated from dicotyledonous woody perennials, while CBS 824.84 with moderate virulence originated from a monocotyledonous herbaceous annual plant. This suggests that the very low virulence of CBS 582.83 is more likely due to the lack or loss of virulence rather than the inadequate adaptation of this strain to grapevines as hosts. Various biological background can result different virulence of conspecific strains. The different origin of the strains propose the role of host-pathogen coevolution [26], however the long-term preservation of the CBS strains should be also taken into account.

The results of the pathogenicity tests imply that some of the virulence factors were possibly synthesized in a lower amount by CBS 824.84 than by T15142, and these factors may be nearly absent in CBS 582.83. The high rate of re-isolation of all strains from the infected grapevines...
indicates that these virulence factors are responsible for the damage of plant tissues, rather than the growth ability of the fungi inside the plant. Since the wood necrosis in grapevines affected by trunk diseases is caused by the digestive exoenzymes of the pathogens [27–29], we also examined the *K. longispora* strains from this perspective. The three *K. longispora* strains were proven to be equally potent producers of cellulases, poor producers of pectinases and none of them showed amylolytic activity. The observed differences in the laccase secretion of the isolates seemed to be concurrent with the results of the pathogenicity tests; however, the varying isolation date and the preservation of the three strains under different circumstances may also have an effect on exoenzyme production. The high level of laccases secreted by T15142 could explain the high incidence of symptoms observed on the shoots and the capability of the isolate to necrotize the xylem of canes and cuttings. The moderate laccase activity in the case of CBS 824.84 may have contributed to the lower incidence of symptoms observed on the shoots and the restoration of virulence after a longer time of growing in canes and cuttings. The absence of laccase signal on indicative media and the very low activity measured in liquid cultures in case of CBS 582.83 may explain the low symptom incidence on the shoots and the inability of the strain damage the lignified tissues of canes and cuttings.

Herein, we showed, for the first time, the ability of *K. longispora* to cause vascular necrosis on grapevines, as well as the possible importance of laccases in the development of symptoms. This study contributes to the growing knowledge on an agriculturally important group of disease agents, the growing group of GTD-associated pathogens.

**Materials and methods**

**Isolation of fungal strains**

In 2015, five wine regions in Hungary (i.e., Eger, Neszmély, Pécs, Szekszárd, and Villány) were monitored for grapevines exhibiting symptoms of trunk diseases, and then the wood-colonizing fungi were isolated. Three thin discs were cut from the trunks. The traditional isolation protocol was carried out according to Váczy et al. [23]: The discs were surface-sterilized in 1% chloramine B solution for 5 min after the bark tissues were removed. The samples were rinsed in sterile distilled water and dried. Then, five wood chips were cut and placed on potato dextrose agar plates (PDA; Sigma-Aldrich, Germany). The plates were incubated at room temperature (21 ± 2°C) and the emerging mycelia were transferred to new PDA plates to obtain pure cultures for morphological and molecular works. In this study, three *K. longispora* strains, namely, CBS 582.83 (ex-type culture), CBS 824.84, and T15142, from Hungary were examined.

**Phylogenetic analysis**

For molecular identification of the chosen strains, DNA was extracted from the lyophilized mycelia grown on cellophane-covered PDA media using a DNeasy Plant Mini Kit (Qiagen, Germany). Polymerase chain reactions (PCRs) were performed to amplify the ITS region with the ITS1F [30] and ITS4 [31] primers. The LSU and TUB genes of this isolate were also amplified and sequenced using the LR0R and LR5 [32] and Btub2Fd and Btub4Rd [33] primer pairs, respectively. Sequences were compiled from electropherograms and edited using BioEdit version 7.1.9 software [34]. The ITS, LSU, and TUB sequences were deposited in NCBI GenBank under the accession numbers MN945157, MN945151, and MN939397, respectively. The isolate was deposited to the CBS-KNAW Culture Collection (Westerdijk Fungal Biodiversity Institute, the Netherlands) under the accession number CBS 144250.

The sequences were aligned with the sequences of respective loci from GenBank (Table 1) by the E-INS-i method of the online MAFFT program version 7 [35]. The alignments were
checked and edited in MEGA6 [36]. Multi-locus phylogenetic Bayesian analysis was performed with MrBayes 3.1.2 [37] using the GTR+G nucleotide substitution model implemented for the ITS, LSU, and TUB sequences. Four Markov chains were run for 10,000,000 generations sampled every 1000 generations, with a burn-in value set at 4000 sampled trees. Maximum likelihood (ML) phylogenetic analysis was carried out with raxmlGUI version 1.3 [38] implementation of RAxML [39]. The GTR+G nucleotide substitution model was applied with ML estimation of the base frequencies, and an ML bootstrap analysis with 1000 replicates was

Table 1. List of the isolates included in the phylogenetic analysis.

| Species                     | Former identification          | Isolate         | GenBank accession number | Host       | References |
|-----------------------------|---------------------------------|-----------------|--------------------------|------------|------------|
| Alloconiothyrium aptrootii  | Coniothyrium sp.                | CBS 980.95      | JX496234 JX496121 JX496460 | nd         | [15,16]    |
| Alloconiothyrium aptrootii  | Coniothyrium sp.                | CBS 981.95      | JX496235 JX496122 JX496461 | nd         | [15,16]    |
| Bimuria nova-zelandiae      |                                 | CBS 107.79      | AY016356 nd nd nd        | nd         | [16]       |
| Corynespora leucadendri     |                                 | CBS 135133      | KF251654 KF251150 KF252639 | nd         | [16]       |
| Deniguelia tabarringtoniae   |                                 | MFLUCC 110257   | KM213997 KM214003 nd    | nd         | [16]       |
| Didymocrea sadavashani      |                                 | CBS 438.65      | DQ384103 nd nd nd        | nd         | [16]       |
| Didymosphaeria rubi-ulmifoli|                                 | MFLUCC 140023   | KJ436586 KJ436586 KJ939277 | nd         | [16]       |
| Didymosphaeria rubi-ulmifoli|                                 | MFLUCC 140024   | KJ436585 KJ436585 KJ939276 | nd         | [16]       |
| Didymosphaeria sp.          | Paraconiothyrium brasiliense    | CBS 587.84      | JX496212 JX496099 JX496438 | Vitis vinifera | [15,16]    |
| Didymosphaeria sp.          | Paraconiothyrium brasiliense    | CBS 115.92      | JX496135 JX496022 JX496361 | Olea europaea | [15,16]    |
| Didymosphaeria variabile    | Paraconiothyrium variable       | CBS 120014      | JX496139 JX496026 JX496365 | Actinidia chinensis | [15,16]    |
| Kalmusia ebali              |                                 | CBS 123120      | JN644073 nd nd nd        | nd         | [16]       |
| Kalmusia italica            |                                 | MFLUCC 130066   | KP325441 KP325440 nd     | Sporium junceum | [13]      |
| Kalmusia longispora         | Dendrothyrium longisporum       | CBS 582.83      | JX496210 JX496097 JX496436 | Arceuthobium pusillum | [15,16]    |
| Kalmusia longispora         | Dendrothyrium longisporum       | CBS 824.84      | JX496228 JX496115 JX496454 | Triticum aestivum | [15,16]    |
| Kalmusia longispora         | Dendrothyrium longisporum       | CBS 144250      | MN945151 MN945157 MN939397 | Vitis vinifera | This study |
| Kalmusia sarothamni         |                                 | CBS 113833      | KF796671 KF796675 nd     | nd         | [9]        |
| Kalmusia variispora          | Dendrothyrium variisporum       | CBS 121517      | JX496143 JX496030 JX496369 | Vitis vinifera | [15,16]    |
| Karstenula rhodostoma       |                                 | CBS 690.94      | GU301821 nd nd nd        | nd         | [16]       |
| Letendraea cordylinicola    |                                 | MFLUCC 110148   | KM213995 KM214001 nd    | nd         | [16]       |
| Letendraea cordylinicola    |                                 | MFLUCC 110150   | KM213996 KM214002 nd    | nd         | [16]       |
| Letendraea padouk           |                                 | CBS 485.70      | AY849951 nd nd nd        | nd         | [16]       |
| Montagnula aloes            |                                 | CBS 132531      | JX069847 JX069863 nd    | nd         | [16]       |
| Montagnula opulenta         |                                 | CBS 168.34      | NG027581 nd nd nd        | nd         | [16]       |
| Neokalmusia brevispora      | Kalmusia brevispora             | KT 1466         | AB524600 nd nd nd        | nd         | [16]       |
| Neokalmusia scabrispora     | Kalmusia scabrispora            | KT 2202         | AB524453 nd nd nd        | nd         | [16]       |
| Paraconiothyrium fuckelii   | Coniothyrium rosarum            | CBS 764.71B     | JX496225 JX496112 JX496451 | Human      | [15,16]    |
| Paraconiothyrium fuckelii   | Coniothyrium rosarum            | CBS 508.94      | JX496209 JX496096 JX496435 | Rosa sp.   | [15,16]    |
| Paraphaeosphaeria angularis |                                 | CBS 167.70      | JX496160 JX496047 JX496386 | Saccharum officinarum | [15,16]    |
| Paraphaeosphaeria areacearum|                                 | CBS 158.75      | JX496156 JX496043 JX496382 | Elaeis guineensis | [15,16]    |
| Paraphaeosphaeria michottii |                                 | CBS 340.86      | JX496192 JX496079 JX496418 | Phragmites australis | [15,16]    |
| Paraphaeosphaeria viridescens|                                 | CBS 854.73      | JX681076 JX496085 JX496424 | nd         | [16]       |
| Phaeodothis winteri         |                                 | CBS 182.58      | GU301857 nd nd nd        | nd         | [16]       |
| Stagonospora paludosa       |                                 | CBS 135088      | KF251760 KF251257 KF252740 | nd         | [16]       |

CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; MFLUCC, Mae Fah Luang University Culture Collection

The ex-type and ex-neotype strains, respectively; LSU: Large subunits of the nuclear ribosomal RNA gene; ITS, internal transcribed spacers and intervening 5.8S nrDNA; TUB, partial β-tubulin gene. nd: No data.

https://doi.org/10.1371/journal.pone.0258043.t001
conducted to test the support of the branches. The phylogenetic trees were visualized and edited in MEGA6 [36].

**Morphological observations**

The isolate T15142 was grown on PDA, MEA (Sigma-Aldrich, Germany), and OA (Sigma-Aldrich, Germany) media, as well as on WA (agar from Sigma-Aldrich, Germany), at 25°C to examine its morphology. Conidia formation was induced by growing the fungus on sterilized pine needles placed on the surface of PDA medium at 25°C for 14 days [40]. The size of the conidia was defined from 100 measurements with ImageJ software [41]. Microscopic examinations were performed using an Alpha BIO-5f (Optika, Italy) microscope equipped with an Artcam-500MI camera (Artray, Japan).

**Pathogenicity tests**

Pathogenicity tests were carried out using *V. vinifera* cv. Cabernet Sauvignon to evaluate the virulence of *K. longispora*. Shoot sections (with one node and one leaf), one year old potted cuttings and canes under field conditions were artificially infected. Cuttings were prepared by potting two-bud cane sections in a 1:1 mixture of perlite and commercial soil and were grown in a greenhouse. Green canes growing on trunks with cordon canopy management were used for the field studies. The grapevines were injured after surface sterilization with 70% v/v ethanol and inoculated with agar plugs (3 mm in diameter) containing actively growing mycelia of the *K. longispora* strains CBS 582.83 (ex-type culture), CBS 824.84, and T15142. Mock inoculations were set up by placing agar plugs without mycelia. All inoculations were performed on five grapevines for each strain. The shoots were placed in water and kept in a greenhouse as well as potted cuttings. Inoculated grapevines were subjected to 21 days (shoots), 80 days (cuttings) or 90 days (canes) of incubation before the examination of symptoms. Alongside the observation of developed symptoms, fungal strains were re-isolated from the vascular tissues. Discs were cut 5 mm above the inoculation points and cut into five pieces. These chips were surface-sterilized in sodium hypochlorite (4% available chlorine w/v), rinsed in 70% v/v ethanol, dried, and then placed on PDA medium. After incubation at 25°C for a week, the emerging mycelia were transferred to new plates and used for DNA extraction. The identity of the re-isolated fungi was verified by sequencing the ITS region.

**Examination of exoenzyme production**

The secretion of digestive enzymes by the CBS 582.83, CBS 824.84, and T15142 *K. longispora* strains was compared. Minimal media (3% v/v glycerol, 0.15% w/v K₂HPO₄, 0.2% w/v KH₂PO₄, 0.1% w/v (NH₄)₂SO₄, 0.5% w/v MgSO₄, 0.2% w/v yeast extract, and 2% w/v agar) were prepared and supplemented with various substrates for the detection of different digestive enzymes. Ethyl cellulose (1% w/v) was used for cellulase, polygalacturonic acid (1% w/v) for pectinase, water-soluble starch (1% w/v) for amylase, and guaiacol (0.01% w/v) for laccase activity detection. The *K. longispora* strains were inoculated on these media as mycelial plugs of 3 mm in diameter growing on PDA medium. After 6–11 days of incubation at 25°C, the effects of the digestive enzymes were detected. Congo red staining was carried out for cellulase [42], precipitation with CTAB (hexadecyltrimethylammonium bromide) for pectinase [43], and Lugol’s staining for amylase [44] detection, while the visualization of laccase activity was based on the formation of a red-brownish reaction product from guaiacol incorporated into the medium [45].

Liquid cultures of the *K. longispora* strains were prepared for the quantification of exoenzyme activities. Strains were pre-grown on PDA medium for one week and mycelial plugs
with 10 mm in diameter were cut from the margin of the colonies. One mycelial plug was inoculated into 50 ml of liquid minimal medium in a 100 ml Erlenmeyer flask and incubated in a rotary shaker (25˚C, 180 rpm) for seven days. Sterile culture filtrates were obtained by the filtration of fermentation broths through a membrane with 0.45 μm pores. For the measurement of cellulase activity, 250 μl of culture filtrates were mixed with 250 μl cellulase assay solution (1%w/v carboxymethyl cellulose in 100 mM sodium citrate buffer pH 5.5) and incubated at 35˚C for two hours. Reducing sugars were determined spectrophotometrically by the dinitrosalicylic acid method using glucose as a standard [46]. Cellulase activities were expressed as mg of released reducing sugar equivalent to glucose per hour. Pectinase activities were determined as described above in the case of cellulase activity, except that 1%w/v pectin was used in the assay solution instead of carboxymethyl cellulose and a 16 h incubation period was applied before the dinitrosalicylic acid assay. Laccase activity was measured using guaiacol as a substrate [47]. 500 μl of culture filtrates were mixed with 1500 μl of 10 mM guaiacol in 100 mM acetate buffer (pH 5.0) and the change of absorbance at 470 nm was monitored spectrophotometrically at room temperature for 10 mins. Laccase activities were expressed as ΔA470×min⁻¹. Spectrophotometric measurements were done by the use of UV-1800 device (Shimadzu, Japan). All experiments were done in triplicates.

**Statistical analysis**

Statistical comparisons were done by GraphPad Prism 5 software (GraphPad Software, San Diego California USA, www.graphpad.com) using One-way ANOVA with Tukey’s post-hoc test.

**Supporting information**

S1 Table. Individual values of cellulase, pectinase and laccase activities, measured in liquid cultures of strains CBS 824.84, CBS 582.83 and T15142 in three measurements. GE: Glucose equivalent.

(DOCX)

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**References**

1. Angelotti F, Hamada E, Magalhães EE, Ghini R, Garrido LDR, Júnior P, et al. Climate change and the occurrence of downy mildew in Brazilian grapevines. Pesqui Agropecu Bras. 2017; 52:426–434.
2. Bois B, Zito S, Calonnc A. Climate vs grapevine pests and diseases worldwide: the first results of a global survey. OENO one. 2017; 51: 133–139.

3. Caffarra A, Rinaldi M, Eccel E, Rossi V, Pertor I. Modelling the impact of climate change on the interaction between grapevine and its pests and pathogens: European grapevine moth and powdery mildew. Agric Ecosyst Environ. 2012; 148: 89–101.

4. Kasssemeyer HH, Berkelmann-Löhnertz B. Fungi of grapes. In Biology of Microorganisms on Grapes, in Must and in Wine. König H, Unden G, Fröhlich J editors. (pp. 103–132). Springer, Heidelberg, Germany; 2017; pp. 61–87.

5. Bertsch C, Ramírez-Suero M, Magnin-Robert M, Larignon P, Chong J, Abou-Mansour E, et al. Grapevine trunk diseases: complex and still poorly understood. Plant Pathol. 2013; 62: 243–265.

6. Fussler L, Kobes N, Bertrand F, Maumy M, Grosman J, Savary S. A characterization of grapevine trunk diseases in France from data generated by the National Grapevine Wood Diseases Survey. Phytopathology. 2008; 98: 571–579. https://doi.org/10.1094/PHYTO-98-5-0571 PMID: 18943225

7. Waite H, May P. The effects of hot water treatment, hydration and order of nursery operations on cuttings of Vitis vinifera cultivars. Phytopathol Medit. 2005; 44: 144–152.

8. Niessl G. Beiträge zur Kenntniss der Pilze. Beschreibung neuer und wenig bekannter Pilze. Verhandlungen der Naturforschenden Vereins in Brünn. 1872; 10: 153–217.

9. Zhang Y, Zhang J, Wang Z, Fournier J, Crous PW, Zhang X, et al. Neotypification and phylogeny of Kalmsia. Phytoph. 2014; 176: 164–173.

10. Tanaka K, Harada Y, Barr ME. Bambusicolous fungi in Japan (3): a new combination; Kalmsia acbrispora. Mycoscience. 2005; 46: 110–113.

11. Gholbad-Nejhad M. Notes on some endophytic fungi isolated from Quercus brantii in Dena region of Kohgiluyeh and Boyer-Ahmad province; Iran. Mycol Iran. 2017; 4; 1–12.

12. Iznova T, Rukišénienė J. Ascomycete species new to Lithuania. Bot Lith. 2012; 18: 35–39.

13. Liu JK, Hyde KD, Jones EBG, Artyawansa HA, Bhat DJ, Boonme S, et al. Fungal diversity notes 1–110: taxonomic and phylogenetic contributions to fungal species. Fungal Divers. 2015, 72: 1–197.

14. Wijayawardene NN, Hyde KD, Wubet T, Glasneva IA, Mooney HA, Camporesi E, et al. Taxonomy and phylogeny of dematiaceous coelomycetes. Fungal Divers. 2016; 77: 1–316.

15. Verkley G, Dukik K, Renfurm R, Goker M, Stielow J. Novel genera and species of coniothyrium-like fungi in Montagnulaceae (Ascomycota). Persoonia. 2014; 32: 25–51. https://doi.org/10.3767/003158514X679191 PMID: 25264382

16. Artyawansa HA, Tanaka K, Thambugala KM, Phookamsak R, Tian Q, Camporesi E, et al. A molecular phylogenetic reappraisal of the Didymosphaeriaceae (= Montagnulaceae). Fungal Divers. 2014; 68: 69–104.

17. Lenderić J. 2016; Molecular identification of fungi isolated from diseased grapevine wood in Croatia. (Master’s thesis). University of Zagreb Repository. Retrieved from: https://repozitorij.ugr.unizg.hr/islandora/object/agr:428/preview.

18. Abed-Ashtiani F, Namani A, Arzanlou M. Analysis of Kalmsia varispora associated with grapevine decline in Iran. Eur J Plant Pathol. 2019; 154: 787–799.

19. Jayawardena RS, Purahong W, Zhang W, Wubet T, Li XH, Liu M, et al. Biodiversity of fungi on Vitis vinifera L. revealed by traditional and high-resolution culture-independent approaches. Fungal Divers. 2018; 90: 1–84.

20. Dissanayake AJ, Purahong W, Wubet T, Hyde KD, Zhang W, Xu H, et al. Direct comparison of culture-dependent and culture-independent molecular approaches reveal the diversity of endophytic grapevine communities in stems of grapevine (Vitis vinifera). Fungal Divers. 2018; 90: 85–107.

21. Guarinaccia V, Groenewald JZ, Woodhall J, Armengol J, Cinelli T, Eichmeier A, et al. Diaporthe diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. Persoonia. 2018; 40: 135–153. https://doi.org/10.3767/persoonia.2018.40.06 PMID: 30504999

22. Maharachchikumbura SSN, Larignon P, Hyde KD, Al-Sadi AM, Liu ZY. Characterization of Neopesalotiopsis; Pestalotiopsis and Truncatella species associated with grapevine trunk diseases in France. Phytopathol Medit. 2017; 55: 380–390.

23. Váczy KZ, Nemeth MZ, Csikós A, Kovács GM, Kiss L. Dothiorella omnivora isolated from grapevine with trunk disease symptoms in Hungary. Eur J Plant Pathol. 2018; 150: 817–824.

24. Imhoff MW, Leonard KJ, Main CE. Patterns of bean rust lesion size increase and spore production. Phytopathology. 1982; 72: 441–446.

25. Robert C, Bancal BO, Lannou C. Sheath leaf rust uredospore production and carbon and nitrogen export in relation to lesion size and density. 2002; Phytopathology. 92: 762–768. https://doi.org/10.1094/PHYTO-2002.92.7.762 PMID: 18943273
26. Restif O, Koella JC. Shared control of epidemiological traits in a coevolutionary model of host-parasite interactions. Am Nat. 2003; 161: 827–836. https://doi.org/10.1086/375171 PMID: 12858269

27. Marchi G, Roberti S, D’Ovidio R, Mugnai L, Surico G. Pectic enzymes production by Phaeomoniella chlamydospora. Phytopathol Mediterr. 2001; 40: 407–416.

28. Rolshausen PE, Greve LC, Labavitch JM, Mahoney NE, Molyneux RJ, Gubler WD. Pathogenesis of Eutypa lata in grapevine: identification of virulence factors and biochemical characterization of cordon dieback. Phytopathology. 2008; 98: 222–229. https://doi.org/10.1094/PHYTO-98-2-0222 PMID: 18943199

29. Stempien E, Goddard ML, Wilhelm K, Tarnus C, Bertsch C, Chong J. Grapevine Botryosphaeria dieback fungi have specific aggressiveness factor repertory involved in wood decay and stilbene metabolism. PloSOne. 2017; 12: e0188766. https://doi.org/10.1371/journal.pone.0188766 PMID: 29261692

30. Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol. 1993; 2: 113–118. https://doi.org/10.1111/j.1365-294x.1993.tb00005.x PMID: 8180733

31. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: a guide to methods and applications. Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. Academic Press: San Diego, California; 1990. pp. 315–322.

32. Vilgalys R, Hester M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. J Bacteriol. 1990; 172: 4238–4246. https://doi.org/10.1128/jb.172.8.4238-4246.1990 PMID: 2376561

33. Woudenberg J, Aveskamp M, Gruyters JD, Spiers A, Crous P. Multiple Didymella teleomorphs are linked to the Phoma clematidina morphotype. Persoonia. 2009; 2: 56–62. https://doi.org/10.3767/00315809X427808 PMID: 20198138

34. Alzohairy AM. BioEdit: An important software for molecular biology. GERF Bull Biosci. 2011; 2: 60–61.

35. Katoh K; Standley DM. MAFFT Multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30: 772–80. https://doi.org/10.1093/molbev/mst010 PMID: 23329690

36. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013; 30: 2725–2729. https://doi.org/10.1093/molbev/mst197 PMID: 24132122

37. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003; 19: 1572–1574. https://doi.org/10.1093/bioinformatics/btg180 PMID: 12912839

38. Silvestro D, Michalak I. raxmlGUI: A graphical front-end for RAxML. Org Divers Evol. 2012; 12: 335–337.

39. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014; 30: 1312–1313. https://doi.org/10.1093/bioinformatics/btu033 PMID: 24451623

40. Su YY, Qi YL, Cai L. Induction of sporulation in plant pathogenic fungi. Mycology. 2012; 3: 195–200.

41. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012; 9: 671–675. https://doi.org/10.1038/nmeth.2089 PMID: 22930834

42. Sazci A, Erenler K, Radford A. Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method. J Appl Microbiol. 1988; 61: 559–562.

43. Durrands PK, Cooper RM. Development and analysis of pectic screening media for use in the detection of pectinase mutants. Appl Microbiol Biotechnol. 1988; 28: 463–467.

44. Abd-Elha lem BT, El-Sawy M, Gamal RF, Abou-Taleb KA. Production of amylases from Bacillus amyloliquifaciens under submerged fermentation using some agro-industrial by-products. Ann Agric Sci. 2015; 60: 193–202.

45. Devasia S, Nair AJ. Screening of potent laccase producing organisms based on the oxidation pattern of different phenolic substrates. Int J Curr Microbiol Appl Sci. 2016; 5: 127–137.

46. Miller GL. Use of dinitrosalicyclic acid reagent for determination of reducing sugar. Anal Chem. 1959; 31: 426–428.

47. Vantamuri AB, Kaliwal BB. Purification and characterization of laccase from Marasmius species BBKAV79 and effective decolorization of selected textile dyes. J Biotechnol. 2016; 19: 1–10. https://doi.org/10.1007/s13205-015-0313-6 PMID: 28330071