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Alejandra Larach * , Paulina Vega-Celedón , Eduardo Salgado , Aldo Salinas , Natalia Riquelme , Diyanira Castillo-Novales , Paulina Sanhueza , Michael Seeger , Ximena Besoain *

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Higher Virulence of *Diplodia seriata* on Vines of cv. Cabernet Sauvignon Associated with 10-Year-Old Wood Compared to Young Tissue

Alejandra Larach [1,2], Paulina Vega-Celedón [1,2], Eduardo Salgado [1], Aldo Salinas [1], Natalia Riquelme [1], Diyanira Castillo-Novales [1,2], Paulina Sanhueza [1], Michael Seeger [2] and Ximena Besoain [1,*]

[1] Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, Casilla 4-D, Quillota 2260000, Chile

[2] Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Chemistry Department & Centro de Biotecnología Daniel Alkalay Lowitt, Universidad Técnica Federico Santa María, Avenida España 1680, Valparaíso 2340000, Chile

* Correspondence: ximena.besoain@gmail.com

Abstract: In vineyards, Botryosphaeria dieback (BD) occurs in young and old plants. In the field, the prevalence and severity of the disease increase proportionally with the age of the vineyards. Among the pathogens that cause (BD), *Diplodia seriata* is the most prevalent species in Chile and other countries with a Mediterranean climate. To date, no information is available on the susceptibility of adult wood to infection by this pathogen since most of the pathogenicity tests have been carried out on 1- or 2-year-old shoots or detached canes. Therefore, a pathogenicity test was carried out on plants under field conditions, with inoculations in plants, on one-year-old shoots, and 2- and 10-year-old wood in grapevine cv. Cabernet Sauvignon. A pathogenicity test was carried out with two isolates of *D. seriata* selected in an assay in detached canes that were 2-year-old. The results on plants showed that *D. seriata* was significantly more aggressive on 10-year-old than on one- or two-year-old tissue. These results were compared with the lesions obtained from two-year-old canes. The results of this work are consistent with the damage observed in the field, highlight the importance of identifying each pathogenic species that causes (BD) and contribute to the knowledge of the epidemiology of this disease in Mediterranean climates.

Keywords: plant–pathogen interactions; grape trunk diseases; GTD; Botryosphaeria dieback; pathogenicity; Tissue age; virulence; *Vitis vinifera;* Cabernet Sauvignon

1. Introduction

Grapevine trunk diseases (GTDs) are the leading cause of vineyard deterioration, causing a decrease in the viability of vineyards, an increase in production costs, and major economic losses in the wine industry [1–6]. The three main GTDs are esca disease, Eutypa dieback, and (BD) (BD), which generally attack the structural parts of plants [2,6–10]. (BD) is present on all continents where wine or table grapes are grown [2,5,7,11–16]. Regarding the pathogens associated with BD, 26 species have been identified [10], mainly within the genera *Botryosphaeria*, *Diplodia*, *Lasiodiplodia*, and *Neofusicoccum* [7,10,11,17]. Some genera are more common in different climates, for example, *Diplodia* in temperate regions or those with cold winters and *Lasiodiplodia* in tropical and subtropical areas [18–24]. *Diplodia seriata* is the most prevalent species in vineyards in Central Chile [5,15,22] as well as in other parts of the world, such as New South Wales [25–27], Western Australia [28], California [29] and South Australia [26].

(BD) causes essential losses in the production of grapes for wine, with a decrease from 30 to more than 50% of production depending on the severity of the disease [5]. The loss of production is associated with the damage it causes to the vines due to the death of the wood where fruit develops, such as cankers in the wood, dead spurs, dead arms, and brown discolorations in the vascular area [2,5,7,15,16,30–34]. In the field, the prevalence and severity of (BD) increase proportionally with the

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age of the vineyards [5–7,10,15,33]. However, many available pathogenicity studies have been carried out on one-year-old shoots or two-year-old canes. Another critical issue to consider is the susceptibility and importance of some varieties for the wine industry. In this case, Cabernet Sauvignon is the most planted variety in Chile, and it is appreciated for good quality wines [35].

For this reason, in this study, a pathogenicity test was carried out with five Chilean isolates of *D. seriata* in 2-year-old detached canes of *Vitis vinifera* cv. Cabernet Sauvignon, and with the two most representative isolates, a pathogenicity test was carried out on vine plants under field conditions, with inoculations in 1-year-old shoots, 2-year-old wood, and 10-year-old wood of grapevine. In this study, we observed that *D. seriata* is more aggressive in old tissue of grapevine cv. Cabernet Sauvignon than in young tissue, which contributes to the knowledge of the epidemiology of this disease in Mediterranean climates.

2. Results

2.1. Pathogenicity Tests on Detached Canes (Nonactivated Isolates)

Ninety days after inoculation with *D. seriata* isolates PUCV 1472, PUCV 1549, PUCV 2120, PUCV 2142, and PUCV 2183, the vascular lesions and canker lengths on detached canes 2 years old were measured.

2.1.1. Damage Assessment and Recovery of Pathogens

The isolates PUCV 2120 and PUCV 2183 produced vascular discoloration, which extended up and down from the point of inoculation, with vascular lesions presenting variable lengths of 12.6 and 6.9 cm, respectively (**Figure 1**a). The isolates PUCV 1549 and PUCV 2142 produced dark brown canker lesions on inoculated canes, with lengths of 1.0 and 1.1 cm, respectively (**Figure 1**b). The isolate PUCV 1472 produced neither canker nor vascular discoloration (in this assay, in the assay with the activated isolate, there was some canker and vascular discoloration). The controls (noninoculated canes) did not present damage (no canker and no vascular discoloration). From the lesions produced by the isolates, the pathogen was recovered, and *D. seriata* was morphologically identified.

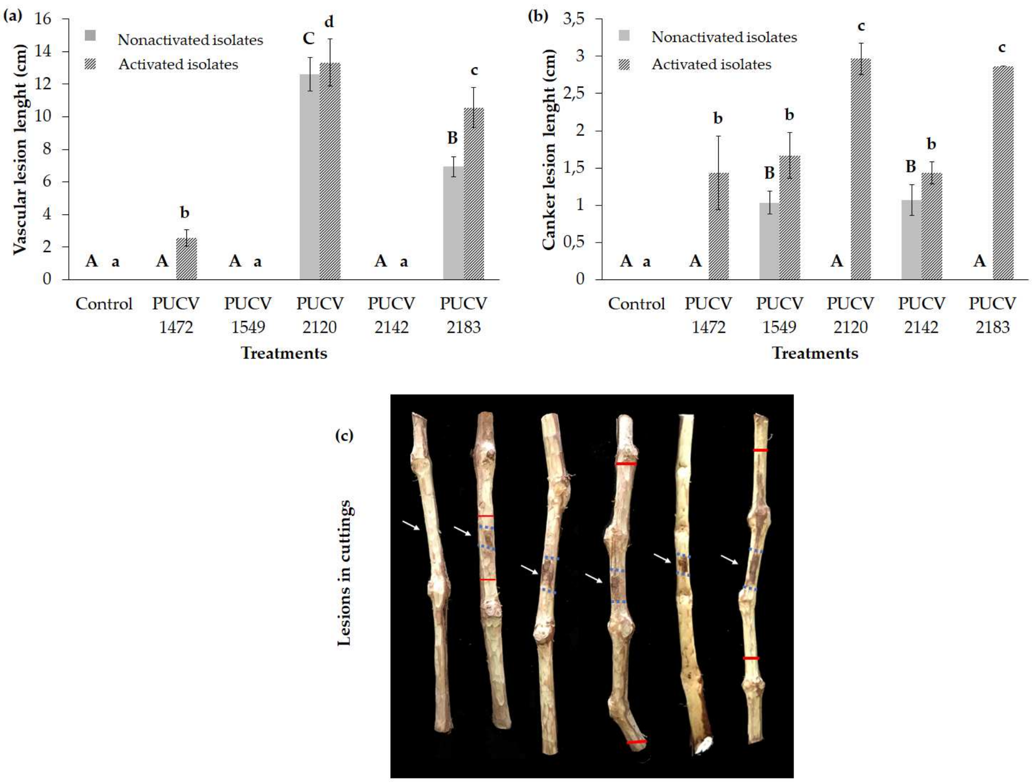

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Figure 1. Severity of the damage of the symptoms caused by *D. seriata* on detached canes of two-year-old *Vitis vinifera* cv. Cabernet Sauvignon. (a) Mean vascular lesion length. (b) Means canker lesion length. (c) Vascular and canker lesions on cuttings with activated isolates. Means with a common letter are not significantly different, according to Tukey's test ($P \leq 0.05$). The light blue arrow indicates the point of inoculation. The red line describes vascular discoloration.

2.2. Pathogenicity Tests on Berry Grapes

Fifteen days after inoculation with *D. seriata* isolates PUCV 1472, PUCV 1549, PUCV 2120, PUCV 2142, and PUCV 2183 on berry grape cv. Red Globe, the diameter of lesions was measured.

2.2.1. Damage Assessment and Recovery of Pathogens

All isolates caused circular soft rot around the point of inoculation on grape berries, with lesion diameters between 2.1 and 2.9 cm and the presence of grayish-white mycelium around the point of inoculation (**Figure 2**a). The inoculated berries were cut longitudinally, presenting rottenness on the pomace as well. From the zone of advancing lesions, the pathogen was recovered, and *D. seriata* was morphologically identified. The controls (noninoculated berry grapes) did not present damage (**Figure 2b**), thus corroborating that all isolates of *D. seriata* were pathogenic on berry grape cv. Red Globe.

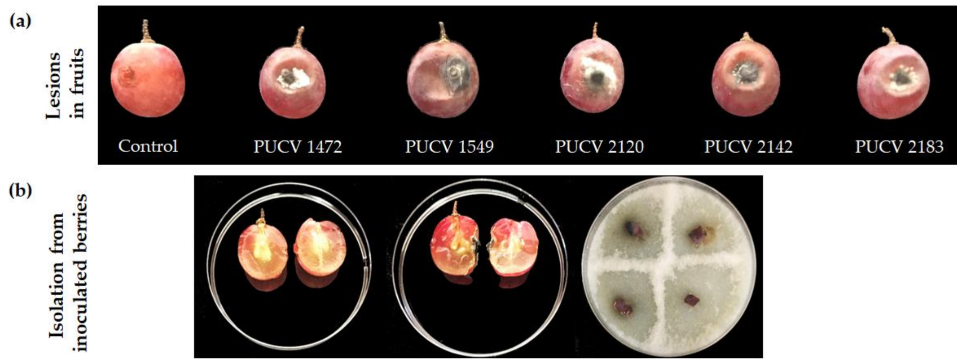

Figure 2. Soft rot lesions caused by *D. seriata* on *Vitis vinifera* cv. Red Globe. a) Circular and sunken rot around the point of inoculation with the different isolates. b) Internal damage of inoculated berry grapes, control berries (noninoculated) and berries isolated with recovery of *D. seriata* from inoculated berry grapes.

2.3. Pathogenicity Tests in Detached Canes (Activated Isolates)

Ninety days after inoculation with isolates of *D. seriata* PUCV1472, PUCV1549, PUCV 2120, PUCV 2142 and PUCV 2183 (previously inoculated in berries) in detached 2-year-old canes, vascular and canker lesion lengths were measured.

2.3.1. *Damage Assessment and Recovery of Pathogens*

All isolates produced dark brown canker lesions on inoculated canes, with lengths between 1.4 and 3.0 cm (**Figure 1**b,c), and isolates PUCV 2120 and PUCV 2183 causing the longest cankers. Isolates PUCV 1472, PUCV 2120, and PUCV 2183 also produced vascular discoloration, which was expressed up and down from the point of inoculation. The vascular lesions presented variable lengths between 2.6 and 13.3 cm (**Figure 1**a), and as with the cankers, the isolates PUCV 2120 and PUCV 2183 were the ones that caused the most significant lesions. The controls (noninoculated canes) did not present damage, no canker or vascular discoloration, thus corroborating that all isolates of *D. seriata* were pathogenic. From the lesions produced by the isolates, the pathogen was recovered, and *D. seriata* was morphologically identified. With the results obtained in both detached cane assays, the isolates PUCV 2120 and PUCV 2183 were selected for the field trial.

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2.4. Pathogenicity Trial in the Field

Five months after inoculation with *D. seriata* isolates PUCV 2120 and PUCV 2183 on tissues of three different ages (one-year-old, two-year-old and 10-year-old wood) of plants cv. Cabernet Sauvignon, the length of vascular lesions was measured.

2.4.1. Damage Assessment and Pathogen Recovery

Both isolates produced significant vascular lesions compared to controls (T0 and T00), as shown in **Figure 3**. In young tissue (one-year-old shoots and two-year-old wood), vascular lesions were significantly smaller than those in older tissue (10-year-old wood), **Figures 3** and **4**a. The vascular lesions developed in the 10-year-old wood were 4.2 and 4.3 cm for PUCV 2183 and PUCV 2120 isolates, respectively, with no significant differences between the aggressiveness of *D. seriata* isolates evaluated. In young tissue, vascular lesions caused by *D. seriata* isolates ranged from 1.9-2.7 cm, with no differences between age of tissue (one or two years old) or isolate (PUCV 2120 or PUCV 2183). Only from inoculated plants was the pathogen recovered, both from young and old tissue. This result shows that the vines used in this trial were healthy and that the lesions presented are attributable to inoculations with *D. seriata* isolates. Inoculated plants did not show foliar symptoms (**Figure 4**b,c).

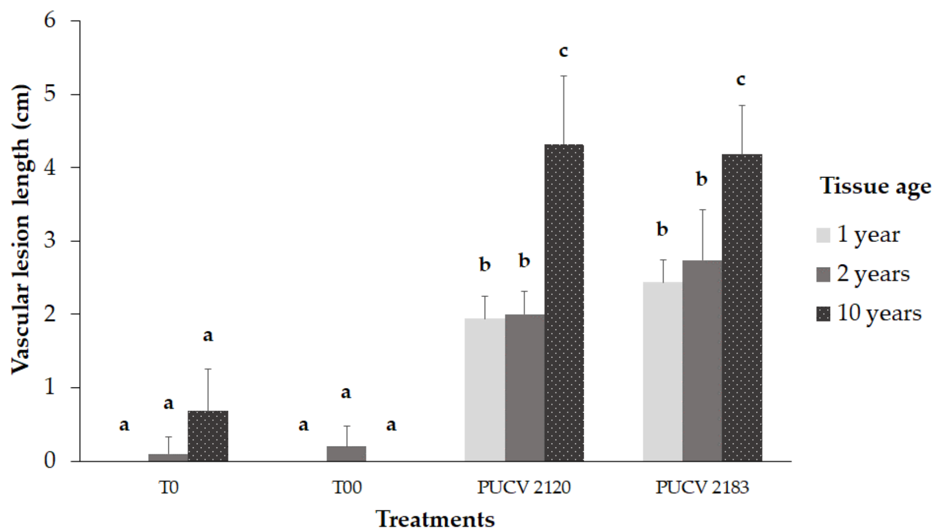

Figure 3. Damage severity of symptoms (vascular lesion length) caused by *Diplodia seriata* on a tissue of different ages of *Vitis vinifera* cv. Cabernet Sauvignon. T00: without cutting or inoculation; T0: cutting plus sterile distilled water. PUCV 2120: *D. seriata* isolate. PUCV 2183: *D. seriata* isolate.

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Figure 4. Symptoms developed on *Vitis vinifera* cv. Cabernet Sauvignon plants five months after inoculation with *D. seriata*. a) Left to right: two-year-old tissue vascular lesion and 10-year-old tissue vascular lesion caused by the PUCV 2021 isolate and 10-year-old tissue of noninoculated plants (T0). b) General view of the field trial five months after the inoculation of the plants. c) Plant control (T0: noninoculated and d) Plant inoculated with *D. seriata* (PUCV 2120) on 10-year-old wood, no visible foliar symptoms. The white arrow indicates the inoculation point. Red lines delimit vascular discoloration. Scale bar a-b = 2 cm.

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2.5. Maximum Parsimony Analysis

The concatenated ITS and BT phylogenetic analysis included 66 *D. seriata* isolates, all obtained from *V. vinifera*, showing that the five isolates used in this assay are in different clades within the species, regardless of virulence or origin. The analyses contained 802 nucleotides, of which 702 were constant and 22 were parsimony informative. The analyses yielded tree #1 out of the 10 most parsimonious trees, with tree length = 53; consistency index = 0.960000, retention index = 0.995781, and composite index = 0.976992 (0.955949) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the tree-bisection-regrafting (TBR) algorithm with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 79 nucleotide sequences. There were a total of 802 positions in the final dataset.

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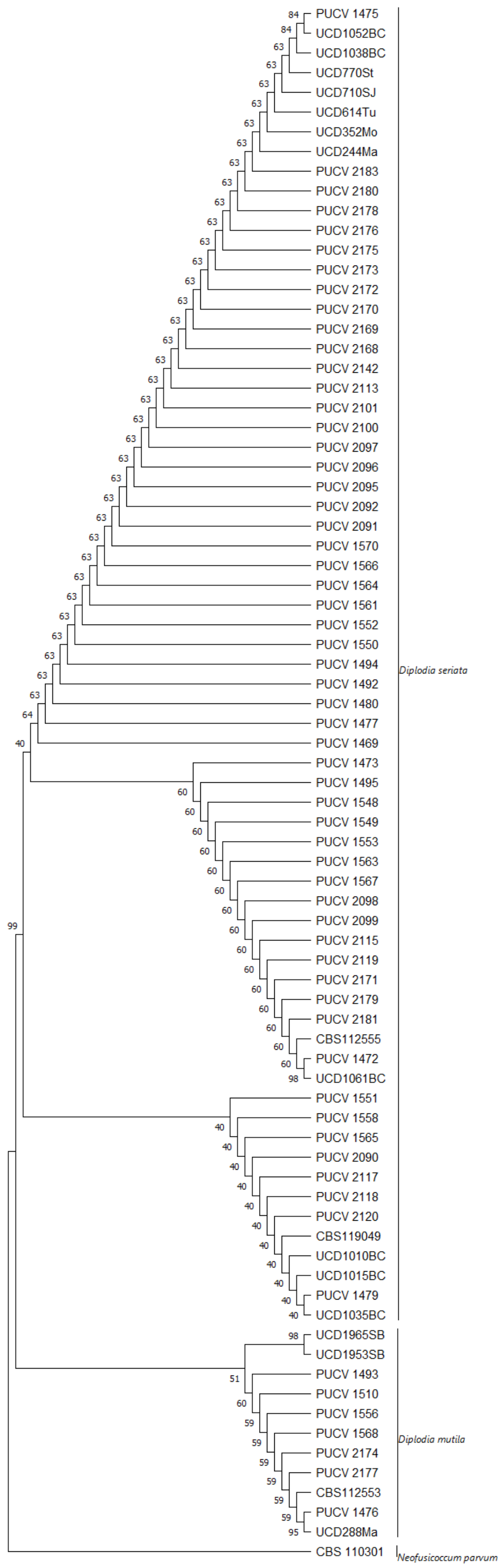

Figure 5. Phylogenetic tree of isolates of *D. seriata* based on maximum parsimony (MP) analysis of two loci (ITS and β-tubulin). Chilean isolates used in this work with PUCV code. MP bootstrap values are shown above the branches. *Spensecermartinsia viticola* isolates PD258 (Inderbitzin et al., 2010) and UCP105 (Adesemoye and Eskalen, 2011) were the outgroups used. Values were obtained with Mega X software.

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3. Discussion

This study reports for the first time the comparison of the pathogenicity of the fungus *Diplodia seriata* on tissues of different ages in cv. Cabernet Sauvignon plants and demonstrates its aggressiveness in old tissue under field conditions. *Diplodia seriata* is one of the most prevalent pathogens isolated from grapevines with symptoms of (BD), mainly in wine or table grape-producing areas with Mediterranean climates [5,6,15,17,23,25,26,28,36]. Of the isolates obtained from diseased tissue, *D. seriata* is the most widely distributed in studies carried out from more than 20 years to the present. In a sampling of 11 sites distributed in different vineyards in the Hunter Valley of New South Wales (NSW, Australia), *D. seriata* was isolated in 90% of them [25]. In California (USA), a survey on different varieties of grapevine (table grapes and wine) indicated that *D. seriata* had the broadest geographical distribution within the *Botryosphaeriaceae* found between the wine-growing areas located between Mendocino and Kem [12]. From collections in the wine-growing regions of Western Australia and the Hunter Valley and Mudgee of NSW [17,28], *D. seriata* accounted for more than 50% of the total number of isolates collected and present in all the regions surveyed. Pitt et al. [26] reported that *Diplodia* was the most predominant genus. The species *D. seriata* represented almost 80% of *Botryosphaeriaceae* isolates collected during the survey in NSW and South Australia. In Catalonia (Spain), the results of GTD-associated fungal isolates showed that *D. seriata* was isolated in up to 68.4% of the samples in diseased plants, being the most prevalent species isolated [36]. In central Chile, with a Mediterranean climate, results of studies carried out on table grapes and wine have been consistent with those of the above studies. Morales et al. [15] reported an 83.3% prevalence of *D. seriata* in table grape plants affected by (BD). Additionally, in Chile, Díaz et al. [16] reported that *D. seriata* (unlike other pathogens found) was present in all the valleys studied in a national survey of Chilean decayed vineyards. Valencia et al. [22] recorded the dissemination of *Botryosphaeriaceae* conidia, and *D. seriata* was the main species detected in vineyards of cv. Cabernet Sauvignon. Specifically, in cv. Cabernet Sauvignon, a sampling carried out in the main producing area of this wine variety in Chile (O'Higgins and Maule regions), reported that in 2010, *D. seriata* was the most prevalent species isolated from symptomatic vines (68%). Eight years later (2018), when sampling the same area, *D. seriata* continued to be the most prevalent species, increasing its prevalence (91%), and the overall yield losses associated with (BD) were 39% in 2010 and 46% in 2018 [5]. Based on the works mentioned above, most isolates of *D. seriata* were obtained from symptomatic old wood tissues. However, pathogenicity tests for *D. seriata* are mainly carried out on young tissue (shoots, one- or two-year-old canes, rotted plants, or young tissue on old plants). In these cases, *D. seriata* virulence was weak or less significant than that of other *Botryosphaeriaceae* species, such as *Lasiodiplodia theobromae*, *L. viticola*, *Neofusicoccum parvum* or *Diplodia mutila* [8,33,37,38], or when compared with other causal agents of GTDs, such as *Eutypa lata* [39]. This difference in virulence could be due to several factors, including the differential susceptibility of the vine varieties, the conditions, age and tissue type of the host plant, the inoculation method used, the geographic origin of the isolates, and the different incubation periods of the experiments [7,31,32,40,41].

In our work, the pathogenicity of *D. seriata* isolates was verified in plants, with inoculations on young tissue (one- or two-year-old tissue age) and on old tissue (10-year-old wood) in vine plants. The vascular lesions developed by *D. seriata* on old tissue (10-year-old wood) were more than twice as long as those in the young tissue. Our results coincide with those described by Morales et al. [15] in old table grape plants in a commercial vineyard. When inoculating shoots <one year, canes >five years old, and mature arms in a 25-year-old vines in commercial vineyards, *D. seriata* increased the damage by more than 40% in mature arms with respect to the other two ages evaluated, contrary to *D. mutila.* Additionally, our results are consistent with those reported in naturally affected vineyards. In the field, symptoms of (BD) increase with the age of the vineyard, both in incidence and severity [5,12,15,30]. The extension of the advance of this pathogen in the wood can be explained by the microenvironmental theory [42], where pathogen reaction zones can alone explain the progression of fungi within tree trunks, as fungi follow dehydrated, oxygen-rich zones that occur along the reaction zones near the wounds. The high moisture content and the associated aeration restriction limit the activity of mycelial fungi in felled wood. Additionally, the most significant damage in old tissue can

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be explained concerning the plant microbiome, which evolves over time and with plant tissues. Microbiome diversity is higher in young vines [1,43–45] and in visually healthy tissues than in diseased tissues [46].

In contrast, studies with *Neofusicoccum* on walnut plants show that the relationship between age and damage is inverse. One-year-old shoots were significantly more sensitive to *N. parvum* than two-year-old to four-year-old shoots wood [47]. Therefore, correctly identifying the causal agent and studying the interaction with the host is essential.

In our work, when comparing the results of pathogenicity tests on two-year-old tissue, detached cuttings or canes of plants (attached), our study shows a difference in the severity of *D. seriata* damage. This may be due to the type of inoculum, mycelium (in the first case) or conidia (in the second case), and/or the state of the tissue (detached or attacched). The vascular lesion was much more aggressive in detached cuttings inoculated with mycelium, reaching up to 12 cm, than in canes on plants, where the lesions were up to 5 cm tissue. In the literature, it is described that during the infection of the vine, a series of molecules are activated that provide protection against the spread of the pathogen [46]. The first active defense is the formation of tylosis within the vessels. Responses to GTD fungi are thought to occur due to PAMP-activated immunity. Phytoalexins, compounds of the phenylpropanoid pathway such as resveratrol or viniferin, are found in higher amounts in tissues after GTD fungal infection [48–52]. Phytoalexins can inhibit the growth and colonization of fungi, block some metabolites produced by fungi during infection, or interfere with oxidation–reduction reactions [52]. In addition to the active defense implemented by the vines, the anatomy of the wood could also be essential. The density of parenchymal rays and their arrangement in space could enhance active responses to infection [54] and the diameter of the vessels could also be a factor [55–57]. Contrary, Amponsah et al., [58], showed a faster rate of germination of *N. luteum* conidia on attached shoots and leaves wounded than on detached and unwounded leaf surfaces, this may be due to the ability of *Neofusicoccum* species to attack on spring and summer time associated to shoots and leaves damage, unlike *D. seriata* which mainly affects woody tissue. Regarding the type of inoculum, our results are similar to those reported by Moral et al. [59]. They found that inoculation with a suspension of *Botryosphaeria dothidea* conidia resulted in more severe disease than inoculation with a plug of mycelium in olive fruits, probably due to the presence of water in the inoculum. In addition, in other *Botryosphaeriaceae* species, *B. dothidea* in this case, the physiological characterization of the isolates that cause Dalmatian disease showed that the optimal temperature was 26°C for mycelium growth and 30°C for germination of conidia. Both factors can influence the aggressiveness of the symptoms, which should be studied for *D. seriata*.

The results obtained in detached canes (two years old) show that, in general, all the isolates increased their pathogenicity after activation on grape berry cv. Red Globe, being higher in the oldest isolates. For example, with nonactivated isolates, the PUCV 1472 isolate did not cause damage to the cuttings (no vascular lesions and no canker), and in the assay with activated isolates (after inoculation on grape berries), vascular lesions and canker developed. The PUCV 1549 isolate increased the length of the canker by 67% after inoculation in grapes (**Figure 1**a,b). Concerning this point, studies carried out with postharvest pathogens (i.e., *Botrytis cinerea*, *Colletotrichum*, and *Alternaria)* can explain these results. In this sense, pathogen enzymes are not all constitutive and may require induction by proper substrates [41]. Reveglia et al. [60] demonstrated that the *Botryosphaeriaceae* family associated with (BD) in Australian grapevine leaves generates secondary metabolites. Martos et al. [61] demonstrated that *D. seriata* can produce secondary metabolites with phytotoxic properties, and it is possible that it has other mechanisms besides mycelial growth in the host that could be related to its virulence and that this phytotoxicity was strictly dependent on acidic pH substrates. This could explain the lower virulence variability of *D. seriata* isolates when inoculated on fruits rather than on canes, although this requires further study. In addition, according to the phylogenetic tree obtained, the *Diplodia seriata* isolates analyzed, all isolated from *Vitis vinifera*, presented greater variability in the ITS-BT segment with the available *D. mutila* isolates (also from *Vitis vinifera*), requiring further analysis.

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4. Materials and Methods

4.1. Chemicals, Reagents, and Culture Media

The reagents and culture media used were sodium hypochlorite (SMF Ltda., Chile), agar papa dextrose acidulated (APDA): 20 g L^{-1} granulated agar (Algas Marinas S.A., Spain), 20 g L^{-1} mashed potatoes (Nestlé S.A., Switzerland), 22 g L^{-1} glucose (Vimaroni S.A., Chile), 7 drops of citric acid (Merck S.A., Germany) and 1 liter of sterile distilled water (SDW). Sterile distilled water was obtained by using a distiller (Pobel, Spain) and autoclave (Zonkia, China). The parafilm used was obtained from Bemis, USA.

4.2. Fungal Isolates

Five isolates of *D. seriata* were used (**Figure 6 and Table 1**). The isolates belong to the fungal collection of the Phytopathology Laboratory, School of Agronomy, PUCV. The isolates PUCV 1472, PUCV 1549, PUCV 2120, PUCV 2142, and PUCV 2183 were previously obtained from plants with cankers and vascular lesions from commercial vineyards in the wine-growing zone of central Chile, all with a history of BD that was previously molecularly identified [5]. The isolates were classified based on a phylogenetic analysis of the concatenated ITS-BT. A multilocus phylogenetic analysis was performed using maximum parsimony (MP) in MEGAX (Kumar et al., 2018). Bootstrap values were calculated using 1,000 replicates yielding the MP tree using Tree Bisection and Reconnection algorithms. The tree was rooted with *Neofusicoccum parvum* strain CBS 110302, and other reference isolates of different isolates of *D. seriata* and *D. mutila* were used (**Table S1**). The retention index, rescaled consistency index, and homoplasy index were calculated using MEGA X.

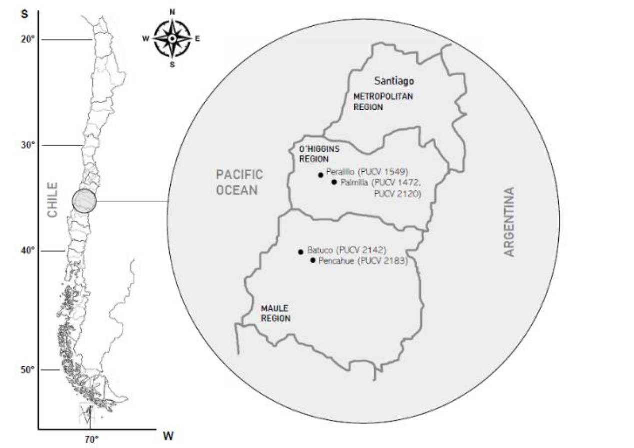

Figure 6. Locality origin of the *Diplodia seriata* isolates. On the left, the O'Higgins and Maule regions are highlighted on the map of Chile. On the right are the localities of origin of each isolate of *D. seriata* and its year of isolation.

4.3. Pathogenicity Tests on Detached Canes (Nonactivated Isolates and Activated Isolates)

The inoculations were performed on healthy detached 2-year-old canes (15 cm in length) of *Vitis vinifera* cv. Cabernet Sauvignon. The canes were obtained in the winter of 2020 from an experimental vineyard of the Experimental Station La Palma of Pontifical Catholic University of Valparaíso (PUCV) and maintained at 5°C for three months before use [62].

Nonactivated isolates: detached 2-years-old canes were disinfected (1% sodium hypochlorite for 5 min and 95% ethanol for 30 s) and then triple washed in SDW, according to Morales et al., [15], and an inoculum of 5-mm-diameter mycelial plugs from a 6-day-old on APDA culture was inoculated using an oblique cut made in the bark with a sterile scalpel at the middle of the canes. The inoculated area was covered with parafilm [15,47]. Three detached 2-year-old canes were used for each isolate, and three were used for the control (noninoculated canes, only APDA plug). The assay was

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performed in duplicate. The canes were incubated for 90 days in an individual humid chamber at 23°C and were distributed according to a completely randomized design (CRD).

Activated isolates: The pathogenicity assay was repeated with the five isolates previously activated in grape berry cv. Red Globe plants. After recovering the isolates from infected berries, we proceeded to repeat what was described above.

4.3.1. Damage Assessment and Pathogen Recovery

After three months of incubation, vascular lesions and canker lengths of the canes were measured up and down from the point of inoculation. For data analysis, the total length of the lesions was considered. Analysis of variance (ANOVA) and Tukey's test were used for data analysis. Tissue samples were taken from the zone of advancement of the lesions, disinfected with 1% sodium hypochlorite for 5 s, triple washed in SWD, and cultivated in APDA.

4.4. Pathogenicity Tests on Berry Grapes

The inoculations were performed on fresh berry grape cv. Red Globe of *Vitis vinifera.* Berry grapes were obtained in the summer of 2021 from the experimental vineyards of Estación La Palma of Universidad Católica de Valparaíso (PUCV) and immediately used for the test.

4.4.1. Fungal Isolates and Inoculation

Five isolates of *D. seriata* were used, the same isolates as in the previous section (**Table 1**). For the inoculation, berry grapes were disinfected with 1% sodium hypochlorite for 30 seg and then triple-washed in sterile distilled water. A 5-mm-diameter inoculum of a 5-mm-diameter mycelial plug from a 6-day-old grown in APDA culture was inoculated in the middle of the berry through a previously made wound with a sterile needle. The inoculated area was covered with parafilm. Five berry grapes were used for each isolate, and five were used for the control (noninoculated berry grape, only APDA plug). The berry grapes were incubated for 15 days in an individual humid chamber at 23°C and were distributed according to a completely randomized design (CRD). The pathogenicity assay in detached canes was repeated with the five isolates previously activated in grape berries.

Table 1. Chilean isolates of *Diplodia seriata* obtained from canker and vascular lesions of *Vitis vinifera* cv. Cabernet Sauvignon used for pathogenicity tests.

Isolate of *Diplodia seriata**	**Year of Collection**	**GenBank Access No.**		
		ITS	**BT**	**EF1-α**
PUCV 1472	2010	KM372581	KP762454	-
PUCV 1549	2010	KM580514	KP762464	-
PUCV 2120	2018	MT023573	MT063140	MT120827
PUCV 2142	2018	MT023574	MT063141	MT120827
PUCV 2183	2018	MT023587	MT063154	-

*Larach et al. **[5].**

4.4.2. Damage Assessment and Pathogen Recovery

After incubation of the berry grapes, fruit rot was measured around the point of inoculation. The diameter of the lesions was considered. Analysis of variance (ANOVA) and Tukey's test were used for data analysis. Tissue samples were taken from the zone of advancement of the lesions, disinfected with sodium hypochlorite for 5 seg, triple washed in ADE, and cultivated in APDA. The plates were incubated for 7 d at 24°C.

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4.5. Pathogenicity Trial in the Field

The experiment was carried out on 10-year-old not grafted cv. Cabernet Sauvignon plants in an experimental vineyard at Experimental Station La Palma of Pontifical Catholic University of Valparaíso (PUCV). The selected vineyard did not present previous GTD symptoms, and it was trained in the bilateral cordon system, with the spur pruned to 4 buds. The inoculations were performed on tissues of three different ages: one-year-old shoots, two-year-old wood, and 10-year-old wood, with each inoculation/age on a different plant (**Figure 7**).

Figure 7. Inoculation with conidia solutions of *D. seriata* in grapevine plants cv. Cabernet Sauvignon. a) Inoculation of two-year-old shoots on fresh pruning cut. b) Inoculation of 10-year-old wood (arm) freshly cut by chainsaw.

4.5.1. Fungal Isolates and Inoculation

Two isolates of *D. seriata,* PUCV 2120 and PUCV 2183 (**Table 1**), previously selected in pathogenicity tests on two-year-old detached canes, were used for the field experiment. The inoculation was carried out in July 2021, with 50 µl of inoculum at $1*10^4$ spores*ml^{-1} on fresh cuts on one-year-old shoots, two-year-old wood, and 10-year-old wood (arm) (**Figure 7**). The cuts in one-year-old shoots and two-year-old wood were made with pruning shears and in 10-year-old wood with a chainsaw (Stihl, Germany). Spore suspensions were prepared according to Úrbez-Torres et al. [38] and Larach et al. [5]: 5-day-old mycelial plugs of each isolate were placed in Petri dishes containing 2% agar water and needle-autoclaved pine. Plates were incubated in a chamber at room temperature (19–21°C) under near-ultraviolet light (λ = 320 nm) until pycnidium production and conidium development. Mature pycnidia were crushed in sterile distilled water, and the solution obtained was filtered through a sterilized cheesecloth. Five plants were used for each isolate/tissue age, with one inoculation per plant. Five absolute control plants (without cutting or inoculation, T00) and five control plants (cut plus sterile distilled water, T0) were used. Plants were distributed according to CRD.

4.5.2. Damage Assessment and Pathogen Recovery

Five months after inoculation of tissue, vascular lesions and canker length were recorded. For this, the damage produced in each tissue/age was evaluated in the field, and the plant material was cut to measure the lesions. The assay was performed in duplicate. Analysis of variance of two factors (ANOVA) and Tukey’s test were used for data analysis. To recover the fungus, tissue samples were taken from the zone of advancement of the lesions, disinfected, and cultivated in APDA.

5. Conclusions

In this study, we observed that *D. seriata* is more aggressive in old tissue of grapevines plants cv. Cabernet Sauvignon than in young tissue, verifying the pathogenicity of *D. seriata* and its role as a causal agent of the disease (BD). These results contribute to the knowledge of the epidemiology of this disease in Mediterranean climates. Additionally, the pathogenicity tests carried out to select the isolates for pathogenicity testing in the field showed different results for vegetative tissue or berry grapes, highlighting the importance of the type of tissue in the pathogenicity or virulence of the *D. seriata* pathogen. Finally, the damage observed in cuttings is probably due to the combination of the type of inoculum (mycelium or conidia) and the state of the plant material (cut or not cut), which should be studied in depth to determine the extent of its role in the severity of the damage.

Supplementary Materials: The following information can be downloaded at: Does not include.

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