Molecular identification and characterization of *Botrytis cinerea* associated to the endemic flora of semi-desert climate in Chile

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**Abstract**

*Botrytis cinerea* is a phytopathogenic fungus that infects over 200 plant species and can cause significant crop losses in local and worldwide agricultural industries. However, its presence in the endemic flora in the Coquimbo Region and its impact on local flora have not been studied yet. In order to determine whether *Botrytis* spp is present in the native plant in the Coquimbo Region, fifty-two field-samples were analysed. A total of 30 putative *Botrytis* spp were isolated and phenotypic and genetically characterized. The internal transcribed spacer (ITS) analysis of these isolates revealed that it corresponded to genus *Botrytis*. For further confirmation, nuclear protein-coding genes (G3PDH, HSP60, and RPB2) were sequenced and showed 100% identity against *B. cinerea*. Complementary to this, *Botrytis* can also be clustered in two different groups, group I (*B. pseudocinerea*) and group II (*B. cinerea*), based on DNA polymorphism, the *Botrytis* isolates were identified as member of group II. On the other hand, we investigated the presence and frequency distribution of the transposable elements *boty* and *flipper* in the isolates obtained. The results indicate that 83.3% of the isolates presented both transposable elements, *boty* and *flipper*, indicating that the most prevalent genotype was transpose. In addition, 16.6% of the isolates showed substantially reduced virulence in apple fruit in comparison to B05.10 strain. According to fungicide resistance studies, the results indicate that resistance to Fenhexamid or Bosalid was observed in the 22.6% of isolates. The results show for the first time that *B. cinerea* has not been described before in fourteen new host plants and contributes to our fundamental understanding of the presence of *B. cinerea* in the native plant in the Coquimbo Region and the possible ecological impact of this disease on native and endemic plants.

**1. Introduction**

*Botrytis cinerea* is classified as an anamorphic fungus and belongs to the family Sclerotiniaceae, it is a phytopathogenic fungus that causes gray mold disease on a wide range of dicotyledonous plants species, including fruits, vegetables and ornamental plants (Jarvis, 1977; Elad et al., 2012), as the second most important plant pathogen worldwide. The sequence of the internal transcribed spacer (ITS) is used for the genetic identification and fungus phylogenetic relationships (White et al., 1990). For further confirmation, a classification of the genus constructed based on DNA sequence data of three nuclear protein-coding genes (G3PDH, HSP60 and RPB2) is used (Staats et al., 2005). In addition, *B. cinerea* is a phytopathogenic fungus with significant genetic diversity. Diolez et al. (1995) identified a retroelement from *B. cinerea* named boty, a 6-kb gypsy-like retrotransposon, later on, the transposable elements *flipper* (an 1872-bp class II element) was identified in *B. cinerea* (Levis et al., 1997). Giraud et al. (1997) defined two sibling sympatric species based on the presence or absence of *boty* and *flipper: transposa* and *vacuama*. *Transposa* isolates possess both *boty* and *flipper*, whereas these elements are absent in *vacuama* isolates. Different studies have shown a significant differentiation in the frequency of...
distribution of transposable elements among Botrytis isolates collected from different host plant and/or geographic regions (Giraud et al., 1997; Giraud et al., 1999; Fournier et al., 2005; Kretschmer and Hahn, 2008; Tanovic et al., 2009; Fekete et al., 2012; Samuel et al., 2012; Tanovic et al., 2015; Wessels et al., 2016). Botrytis can also be clustered in two different groups, group I and group II, based on DNA polymorphism revealed by microsatellite markers (Fournier et al., 2003). Walker et al. (2011) carried out extensive research on Group I and named it B. pseudocinerea while Group II was named B. cinerea sensu stricto. Such information can help to characterize B. cinerea and the development of more effective strategies to control and manage the disease.

Fungicides with different modes of action are currently used to control B. cinerea such as Phenylpyrroles (Fludioxonil), Hydroxyanilides (Fenhexamid), Dicarboxamides (Iprodione), Succinate Dehydrogenase inhibitors (Boscalid), Demethyltransferase inhibitors (Tebuconazole) and inhibitors of methionine biosynthesis (Pyrimethanil). However, the intensive use of antifungal led to the rapid selection of resistant strains in various countries, in the field as well greenhouses (Faretra et al., 1989; Yourman and Jeffers, 1999; Moyano et al., 2004; Zhang et al., 2010). In the last decade resistance to Fenhexamid, Pyrimethanil, Fludioxonil. Anilinopyrimidine and Boscalid fungicides have been reported among populations of B. cinerea (Latorre et al., 2002; Moyano et al., 2004; Esterio et al., 2007; Fillinger et al., 2008; Fernandez-Onturino et al., 2012; Fernandez-Onturino et al., 2013; Fernandez-Onturino et al., 2014; Esterio et al., 2015). Molecular characterization of B. cinerea isolates that were sensitive or resistant to single-site fungicides showed the involvement of major genes of the fungus and a strong association between resistant phenotypes and point mutations (single nucleotide polymorphisms, SNPs) (De Miccoles Angeli et al., 2012). On the other hand, B. cinerea possesses multiple ABC (bcatrA, bcatrB and bcatrD) and MFS (bcmf51) transporter genes that play a role in protection against the plant defense and has also been associated to the transport of several drugs (Movahedi and Heale, 1999a; Movahedi and Heale, 1999b; Walton, 1994; Gomez-Gomez et al., 2002; de Waard et al., 2006).

This pathogen secretes an arsenal of enzymes, toxins, secondary metabolites to help facilitate penetration into the host (Doss, 1999; Choquero et al., 2007). Also, in the B. cinerea genome, 118 genes encoding putative Carbohydrate-Active Enzymes (CAZymes) have been identified and can be associated with plant cell-wall degradation to obtain the source of nutrition from dead tissue. This big enzymatic repertoire could explain, in part, the ability of B. cinerea to infect over 200 different plant species (Castillo et al., 2017). These CAZymes and other genes are essential for pathogenesis and infection process, some of them play a key role in degrading the cuticle and plant cell wall to enter the plant, others form specialized structures, such as appressoria, to penetrate the epidermis (Mendgen et al., 1996; Plaza et al., 2020). All these mechanisms contribute to B. cinerea being a good candidate to survive and spread in extreme climates such as the semi-desert climates in the Coquimbo Region in Chile (Plaza et al., 2018).

B. cinerea has been highly studied and characterized in crop to crops with an economic interest, such as, Vitis vinifera, Malus sp., Fragaria sp., Eustoma sp. Flowers, Rosa sp. flowers, Dianthus sp., Solanum sp., Phascolus sp., Zea sp., Beta sp., among others (Haydu and Legard, 2003; Dominguez et al., 2008; Salami et al., 2016; Yin et al., 2011; Leroch et al., 2013; Grant-Downton et al., 2014). Nevertheless, there are few studies focused on analyzing and characterizing the presence and infection of B. cinerea and other phytopathogenic fungi associated with native and endemic plants that have little commercial interest. The Region of Coquimbo in Chile is located in the southern limit of the Atacama Desert and presents a semi-arid climate, it extends from 29° 00‘ to 32° 10‘ covering an area of 40,462 km² (Novoa and Lopez, 2001). This Region is located in one of the 25 points with the greatest biodiversity worldwide (Myers et al., 2000; Arroyo et al., 2008), it contains a high percentage of native species of plants including 1478 species and 244 naturalized introduced plant species, these species of plants represent a 30% of the total species of the flora of continental Chile (Squeo et al., 2001), and the most predominant are herbs perennials (44.7%), followed by bushes (27.2%) and annual or bi-annual herbs (23%) (Squeo et al., 2001) and many of them are in the category of Vulnerable and in Danger of extinction (Squeo et al., 2001; Arroyo et al., 2008), due, possibly to water scarcity, human intervention and/or possibly due to viral or fungal infections. In Chile the studies about B. cinerea are concentrated mainly in plants of agricultural interest such as Vitis vinifera (Latorre et al., 2002; Munoz et al., 2002; Esterio et al., 2007; Esterio et al., 2011; Esterio et al., 2015; Latorre et al., 2015), however some efforts to identify and characterize of fungi associated with native plants or plants with low commercial interest have been done. Fungi associated with rocks of the Atacama Desert and endophytic fungi associated with the roots of Chenopodium quinoa have been identified (Goncalves et al., 2016; Gonzalez-Teuber et al., 2017). In addition, Plaza et al. (2018) report the presence of B. cinerea causing gray mold disease on the endemic plant Echinopsis coquimbana in the Coquimbo Region. Nevertheless, further studies are required to determine the distribution and ecological impact of this pathogen on endemic plants.

Our aims were to determine if there was a presence of phytopathogenic fungi B. cinerea, a highly pathogenic fungus on native and endemic plants in the Coquimbo Region. This manuscript presents a genetic and phenotypical study, where thirty filamentous fungus B. cinerea were characterized, while simultaneously obtaining a panoramic view about this fungal population found on these plants.

2. Materials and methods

2.1. Botrytis isolation from native plant and growth conditions

Native plant leaves were analyzed in 6 points in the semi-deserts climate in the Coquimbo Region, Chile: Caleta Hornos, Quebrada de Tulca, Punta Testinos, El Peñon, Totoralillo and Parque Nacional Fray Jorge 2014 to 2015 (Fig. 1). The samples were collected from tissue of symptomatic and non-symptomatic leaves of endemic plants. The samples were put on petri plates with 2% malt extract agar (MEA) for 4 days in the dark and only fungi similar to Botrytis cinerea were selected for analysis. Isolated Botrytis selected were grown on Petri plates with 2% MEA for 6 days at 20°C in the dark, then were incubated between 300 to 400 nm light spectrum for 24 hours, to induce sporulation (Tan and Epton, 1973), after which they were incubated in darkness for 24 to 48 hours again. The spores were preserved in glycerol 50% at -80°C.

2.2. DNA extraction, PCR amplification and sequencing

The spores were grown on malt agar for 48 hours at 20°C. Mycelium was harvested, submerged into liquid nitrogen and ground into a powder. Genomic DNA extraction was performed using standard protocols (Sambrook et al., 1989). DNA pellets were dissolved in 50 µL of TE (10mM Tris- HCl [pH 8.0], 1mM EDTA) and quantified using a Nanodrop spectrophotometer. To identify B. cinerea species we sequenced the ITS region and G3PDH, HSP60, and RPB2 genes (White et al., 1990; Staats et al., 2005). Primer combinations (Table S1) were designed to amplify the G3PDH, HSP60, and RPB2 regions. For amplifications of ITS region, primers IST1 and IST4 were used (White et al., 1990). The PCR reaction was conducted on a final volume of 25 µL, containing 1X Expand High Fidelity Buffer with MgCl2, 0.2 µM of each primer, 0.2 mM of each dNTP (GE Healthcare Lifesciences, USA). The following thermocycling pattern was used to amplify HSP60 and G3PDH gene fragments: 95°C for 60 s (1 cycle), 95°C for 30 s, 64°C for 60 s, 68°C for 90 s (15 cycles), 95°C for 30 s, 62°C for 60 s, 68°C for 90 s (20 cycles), and then 68°C for 5 min (1 cycle) (Table S2). To amplify RPB2 gene fragment the program was 95°C for 2 min (1 cycle); 95°C for 30 s, 58°C for 60 s, and 68°C for 4 min
2.4. Determination of group I and Group II of B. cinerea

To determine B. cinerea (group II) or B. pseudocinerea (group I) (Fournier et al., 2003) from the isolates collected from endemic flora we used the PCR-RFLP technique. We used the primers Bc-hch262 and Bc-hch520L (Table S1). We amplified it by PCR in a Axgyen MaxyGene II Thermal Cycler (Axygen a Corning Brand, USA), each PCR reaction containing a volume of 25 µL, 1X ThermPol Reaction Buffer with MgCl2, 0.2 µM of each primer, 0.2 mM of each dNTP, 1.25 U taq DNA Polymesase and 10 ng fungal DNA (taq DNA polymerase with ThermPol Buffer, New England BioLabs), using the program followed by 1 cycles of 4 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 1 minute at 55°C, 60 s at 68°C, finally 1 cycles of 5 minutes at 68°C (Table S2). Restriction digests were done for 1.5 h at 37°C, in a total volume of 20 µL containing 5 µL PCR product, 2 µL reaction buffer, and 1 U restriction enzyme Hhal (Fournier et al., 2003).

2.5. Sporulation and infection tests

The fungal isolates were grown on potato dextrose agar (PDA, AppliChem) and synthetic minimal medium (MM) containing 2% sucrose, 0.1% KH2PO4, 0.3% NaNO3, 0.05% KCl, 0.05% MgSO4.7H2O, pH 5.0 (Plaza et al., 2013). The spore production were measured from cultures on PDA, each strain was seeded with 0.5 mm circular section of PDA agar with mycelium of the fungus; the isolate was incubated in PDA for 2 weeks at 20°C under a 24 h photoperiod (12 h light/12 h darkness). The spores were collected and filtered with miracloth (Merck, USA) a resuspended in 10 mL water sterile and counted with Neubauer camera. For sclerotia formation, each isolate was seeded with 0.5 mm circular section of PDA agar with mycelium of the fungus in MM for 2 weeks at 17°C in darkness.

Infection tests of apple fruits were performed as described by Doehlemann et al. (2006). Prior to inoculation, the fruit tissues were wounded with a pinprick of a 21G syringe and surface-sterilized by immersion in 75% ethanol for 1 min. Inoculation of the fruits was performed with 5 µL droplets of 2.5×10⁴ conidia/mL conidial suspensions for 4 days at 20°C in Percival incubators (Percival, USA). The B05.10 wild-type strain was used as a control strain due its virulence on apple, tomato or grapevine as well as others fruit or plant (Nafisi et al., 2014; Plaza et al., 2015; Zhang et al., 2016; Plaza et al., 2018; Liu et al., 2019). The pathogenicity of B. cinerea isolates in this study was compared to the B05.10 strain.

2.6. Fungicide resistance test

To determine the antifungal susceptibility in samples isolated from field we tested six chemical groups of different antifungals: Fenhexamid, Iprodione, Fludioxonil, Tebuconazole, Pyrimethanil and Bosalid. Agar disk (8 mm diameter) were cut from malt agar 2% and previously inoculated with 10 µL of a conidial suspension 1×10⁶ conidia/mL and incubated for 24 hours at 20°C in the dark in Percival incubators (Percival, USA). These disks were then placed upside-down onto plates containing medium with different concentrations of antifungal and all cultures were incubated for 72 hours at 19°C in the dark in Percival incubators (Percival, USA). The Sisler synthetic medium composed of 2% KH2PO4, 1.5% K2HPO4, 1% (NH4)2SO4, 0.5% MgSO4.7H2O, 10% glucose, 2% yeast extract, 12.5% agar (Leroux et al., 1999) was used to Fenhexamide resistant assays. The Fenhexamide concentrations used were: 0, 0.3, 1, 3 and 10 µg/mL and Sisler medium with 0.5% ethanol was used as a control. Meanwhile, the culture medium used with the fungicide Iprodione was 2% Potato Dextrose Agar medium and the concentrations used were: 0.1, 0.3, 0.6, 1.25, 2.5 and 5 µg/mL and PDA medium and 0.5% ethanol was diluted in the medium and used as a control (Dennis and Davis, 1979). To Fludioxonil, Tebuconazole, Pyrimethanil and Bosalid fungicides were tested into nutrient medium composed with 10% glucose, 2% K2HPO4, 2% KH2PO4 and 10% agar. The

(30 cycles), and then 68°C for 10 min (1 cycle). For amplification of the ITS region, an amplification protocol was used as described by White et al. (1990). The presence of transposons was detected by PCR with the primers boty-F and boty-R to amplify transposon boty, and the primers F300 and F1500 to detect flipper (Table S1) (Giraud et al., 1999; Munoz et al., 2010). The amplification protocol consisted of an initial denaturation 95°C for 60 s (1 cycle); 95°C for 30 s, 58°C for 60 s, and 68°C for 90 s (32 cycles), and then 68°C for 10 min (1 cycle) (Table S2).

2.3. Molecular and phylogenetic identification

For the identification of native isolates Botrytis sp., analysis of HSP60, RPB2, and G3PDH gene were sequenced using the same primers by Macrogen (Seoul, Korea). The multiple alignments were performed using CLUSTAL W (Thompson et al., 1994). The phylogenetic trees were constructed based on the neighbor-joining method (Saitou and Nei, 1987) and the topology confirmed with the Maximum Likelihood and Maximum Parsimony methods by using the MEGA X software (Kumar et al., 2018). Distance matrices were calculated by the Kimura 2-parameter method and bootstrap analysis was performed based on 1000 re-samplings (Kumar et al., 2018). Nucleotide sequences of HSP60, RPB2, G3PDH, commonly used for differentiation of Botrytis species, were obtained from the GenBank website (Table S3). The partial sequences obtained in this study have been deposited in GenBank (Table S4).
concentrations used were: 0, 0.3, 1, 3, and 10 µg/mL. In a similar way to Fenhexamide and Iprodione, 5% ethanol was used as a control (Latorre et al., 2002; Weber and Hahn, 2011).

The EC50 value is the effective fungicide concentrations causing 50% inhibition of the germ-tube growth according to described by Weber and Hahn (2011). Fungicide sensitivity categories (sensitive and resistant) were defined according to the discriminatory doses that differentiate resistant from sensitive isolates as follows: Fenhexamid resistant (EC50 ≥ 7.68 µg/mL); Iprodione resistant (EC50 ≥ 2 µg/mL); Fluquinconazol (EC50 ≥ 3.2 µg/mL); Tebuconazole (EC50 = 1.65 µg/mL); Pyrimethanil (EC50 ≥ 9 µg/mL) and Boscalid (EC50 ≥ 6.4 µg/mL). The mean colony diameter minus the diameter of the inoculation disk was measured and expressed as the percentage of the mean colony diameter of the untreated control. Each test was performed three times.

2.7. Similarity analysis of B. cinerea isolates

To know the similarity in Botrytis isolates we performed an analysis of Multidimensional Scaling non metric, NMDS using the correlation matrix by index of similarity Bray-Curtis using PRIMER v6 program (Clarke and Gorley, 2006). This analysis was based on results of ITS sequencing, detection transposon boty and flipper, infection test and resistance boscalid antifungal.

3. Results

3.1. Isolations and fungal identification

Our studies were conducted in 2014/2015 and a total of Fifty-two field-sampled plants were processed (Fig. 1 and S1). From these, a total of 85 fungal strains were obtained. Thirty fungal colonies obtained were initially white, then became gray to brown after 6 days. Analysis of light micrographs displayed elliptical conidia on MEA. The overall morphology suggests the isolates are members of the genus Botrytis and a total of thirty putative Botrytis spp were isolated in 17 plant species (Table 1).

3.2. Sequence analyses

To determine their identity, all isolates were subjected to DNA sequence analysis. ITS analysis of this isolate revealed that all isolates correspond to genus Botrytis. For further confirmation, nuclear protein-coding genes (G3PDH, HSP60, and RPB2) were sequenced (Table S1 and S4). The partial sequences of the HSP60 (900 bp), RPB2 (926 bp) and G3PDH (888 bp) genes analyzed by Blast NCBi report a high percentage of identity of all isolates obtained with strains and isolates of Botrytis cinerea (Table S4). Therefore, based on these results, the phylogenetic analysis of genes was performed with strains belonging to species of the genus Botrytis, the phylogenetic position observed by the individual analysis of the genes (Fig. S2-S4) and concatenated genes (HSP60, RPB2 and G3PDH; 2714 bp) groups all isolates obtained in a single clade together with B. cinerea B05.10 (Fig. 2 and S5). Based on DNA polymorphism evidenced by the molecular marker Bc-hch and the restriction enzyme Hhal, a through the Restriction Fragment Length Polymorphism (PCR-RFLP) technique, two cryptic species can be differentiated, B. cinerea and B. pseudocinerea. The analysis showed that the 100% of isolates belong to the species of B. cinerea (group II) (data not shown).

To investigate the presence and frequency of the transposable elements in populations of the Botrytis isolates in the endemic plant, the retrotransposon (boty), and the transposon (flipper) was detected. PCR-based detection of the two transposable elements showed that, in all the sampled hosts, only three possible Botrytis types existed. The results showed that 25 of the 30 wild Botrytis isolates presented both transposases in their genome, indicating that they present the genotype transposa (83.3%). On the other hand, three wild Botrytis isolates had only the boty transposon (10%) in their genome, indicating that they present the genotype flipper, and two isolates belong exclusively to the vacuum genotype (6.7%), whereas the flipper genotype was absent (Fig. 3 and Fig S6). The results of these analyses indicate that the most predominant genotype in the isolates was transposa, where only Totoralillo and PNBFJ localities

Table 1

| Strain | Host Plant | Conservation | Geographic Origin | Year | Coordinates |
|--------|------------|--------------|-------------------|------|-------------|
| Bc.ad01 | Adesmia bedwelli | OD | PNBFJ Quebrada | 2015 | 30°70’43”S 71°67’41”O |
| Bc.ad02 | Adesmia bedwelli | OD | PNBFJ Quebrada | 2015 | 30°70’43”S 71°67’41”O |
| Bc.ar01 | Ariostochia chilensis | OD | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.ar02 | Ariostochia chilensis | OD | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.ar03 | Ariostochia chilensis | OD | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.ca01 | Cumulopuntia spharica | NE | Caleta de Hornos | 2015 | 30°37’54”S 71°17’03”O |
| Bc.eq01 | Equinopsis coquimbana | OD | Caleta de Hornos | 2014 | 30°37’54”S 71°17’03”O |
| Bc.eq01 | Euonymus acida | OD | Pienon | 2015 | 30°8’56”S 71°12’40”O |
| Bc.fu01 | Euonymus breviflora | OD | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.he01 | Fuchsia lyioides | OD | PNBFJ Bosque | 2015 | 30°6’19”S 71°68’81”O |
| Bc.he01 | Heliotropium stenophyllum | OD | Caleta de Hornos | 2014 | 30°37’54”S 71°17’03”O |
| Bc.he03 | Heliotropium stenophyllum | OD | Pienon | 2015 | 30°8’56”S 71°12’40”O |
| Bc.he04 | Heliotropium stenophyllum | OD | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.hi01 | Lithraea caustica | OD | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.ma01 | Malva nicaensia | IK | Quebrada de Talca | 2014 | 30°0’21”S 71°1’20”O |
| Bc.my01 | Myrcianthes coquimbensis | D | Punta Teatinos | 2014 | 29°50’24”S 71°65’56”O |
| Bc.na01 | Oxalis gigantea | OD | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.na02 | Oxalis gigantea | OD | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.na03 | Pleoctopus revolutus | OD | Pienon | 2015 | 30°8’56”S 71°12’40”O |
| Bc.pw01 | Portulania chilensis | VU | PNBFJ Quebrada | 2015 | 30°70’43”S 71°67’41”O |
| Bc.po01 | Portulania chilensis | VU | PNBFJ Quebrada | 2015 | 30°70’43”S 71°67’41”O |
| Bc.sc01 | Schinus molle | OD | Pienon | 2015 | 30°8’56”S 71°12’40”O |
| Bc.sc02 | Schinus molle | OD | Pienon | 2015 | 30°8’56”S 71°12’40”O |
| Bc.se01 | Senna cumingi | OD | PNBFJ Quebrada | 2015 | 30°70’43”S 71°67’41”O |
| Bc.se02 | Senna cumingi | OD | PNBFJ Quebrada | 2015 | 30°70’43”S 71°67’41”O |
| Bc.se03 | Senna cumingi | OD | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.sc01 | Trichocereus deserticola | NE | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.sc02 | Trichocereus deserticola | NE | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |
| Bc.sc03 | Trichocereus deserticola | NE | Totoralillo | 2015 | 30°4’6”S 71°22’27”O |

D= Danger, V= Vulnerable, OD= Out of danger, IK= Insufficiently known, NE= Not evaluated, PNBFJ= Parque Nacional Bosque de Fray Jorge.
presented vacuma and boty genotypes, meantime vacuma genotype was found only in the PNBFJ (Fig. 3).

3.3. Phenotypic characterisation of Botrytis isolates

Botrytis isolates sporulation was compared by counting the number of conidia formed by each one of them. The results showed significant variation in conidial production on MEA when compared to no isolates were multidrug-resistant. The 77.4% of isolates were susceptible to the six fungicides and none of the disease incidence were significantly lower in comparison to reference strain B05.10, where the strain Bc.ox01 was the least pathogenic when compared to the others isolates and control strain. This data supports the idea that B. cinerea isolates have not lost the ability to infect another host.

3.5. Resistance profiles of Botrytis isolates

Susceptibility to six active ingredients representing six chemical classes of fungicides, including Phenylpyrroles (Fludioxonil), Hydroxanilides (Fenhexamid), Dicarboxamides (Iprodione), Succinate Dehydrogenase inhibitors (Boscalid), DeMethylation inhibitors (Tebuconazole) and inhibitors of methionine biosynthesis (Pyrimethanil). In total, four different resistance profiles were detected for the 30 Botrytis isolates used in this study (Table 2). Resistance to Fenhexamid or Boscalid was observed in the 22.6% of isolates, whereas 77.4% of isolates were susceptible to the six fungicides and none of isolates were multidrug-resistant. The Bc.se01 isolate was moderately resistant to Fenhexamid, meantime Bc.ar03, Bc.he03, Bc.he04 and Bc.vi01 were slightly resistant, Bc.ar01 was moderately resistant and Bc.he02 highly resistant.

4. Discussion

Botrytis cinerea is a phytopathogenic fungus that can cause significant crop losses in the worldwide agricultural industries. However, its presence in the endemic flora in the Coquimbo Region has not yet been studied. In this present study, we conducted molecular and phenotypic analyses of thirty B. cinerea isolates from the native plants in the Coquimbo Region. Our analysis shows that ITS analysis of these isolates revealed that it corresponded to the genus Botrytis. For further confirmation, nuclear protein-coding genes (G3PDH, HSP60, and RPB2) were sequenced and the phylogenetic analysis of DNA sequence data showed 100% identity against B. cinerea. Classically, B. pseudocinerea and B. cinerea, two morphologically cryptic species, have been treated as B. pseudocinerea Group I, and B. cinerea sensu stricto Group II (Walker et al., 2011). Erroneously B. pseudocinerea has been referred to previously as B. cinerea Group I (Fournier et al., 2002; Martinez et al., 2005). Consistent with the results of the sequencing of the G3PDH, HSP60, and RPB2 genes, the PCR-RFLP characterization of the native isolates obtained in this study indicated all the isolates within group II or what is classically known as B. cinerea sensu strict, revealed by the Bc-hch locus. Coincidentally with the findings of Fournier et al. (2003) the isolates
This analysis shows that *B. cinerea* is present in seventeen species of native/endemic plants where only three genera have been described in the literature as hosts of this fungus, the genus *Fuchsia*, *Heliotropium* and *Echinopsis* (Fillinger & Elad, 2016; Plaza et al., 2018). Therefore, it is the first time that *B. cinerea* has been described in fourteen new host plant interactions. That could be explained because *B. cinerea* secretes numerous cell wall–degrading enzymes (CWDEs) and metabolites to breach the plant cell wall, obtaining the source of nutrition from dead tissue and also the ability to avoid plant resistance mechanisms (Staats et al., 2005; Choquer et al., 2007; van Baarlen et al., 2007; Hahn et al., 2014; Nakajima & Akutsu, 2014; Castillo et al., 2017; Plaza et al., 2020).

This strategy could explain, in part, why *B. cinerea* can attack a wide range of host plants, including Chilean native plants.

The presence and frequency distribution of the transposable elements *boty* and *flipper* in the isolates showed that the genotype *transposa* has more frequency with 83.3%, after transposon *boty* (10%) and *vacuma* (6.7%). This result is according to Munoz et al. (2002) where reported similar frequencies of *transposa*, *boty* and *vacuma* in grapes, tomato and blueberry in Chile, in addition they did not find any isolate with only transposable element *flipper*. Identical results have been found in this study or in others, this supports the idea that the predominant genotypes in Chile are *transposa* (Esterio et al., 2011). However, this frequency could be associated to the host, differences in genotype

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**Fig. 3.** Frequency distributions of transposable element types (*boty, flipper, transposa*, and *vacuma* type) in *Botrytis cinerea* populations collected from several hosts in Chile. 83% of the isolates presented both transposable elements (*boty* and *flipper*), indicating that the most prevalent genotype was *transposa*.

**Fig. 4.** Conidia production in *Botrytis cinerea* isolates in Coquimbo region. (a). Quantification of conidia production in the isolates grown for two weeks on MEA at 20 °C under a 24 h photoperiod (12 h light/12 h darkness). (b). Representative photographs of conidia suspensions in isolates strains. The experiments were performed in triplicates and the data was represented by means ± standard deviations. Asterisks indicate significant differences (p<0.05, Mann-Whitney U test) between the B05.10 WT strain and the isolates.
Fig. 5. Sclerotia production in *Botrytis cinerea* isolates in Coquimbo region. (a) (above) Sclerotia formation was induced by inoculating the strains on MEA plates in the darkness for two weeks. The form represents number of sclerotia and size sclerotia. (b) (down) Representative pictures are shown for some strains. The experiments were performed in triplicates and the data was represented by means ± standard deviations. Asterisks indicate significant differences (p < 0.05, Mann-Whitney U test) between the B05.10 WT strain and the isolates. Black asterisk represents significance in number of Sclerotia and gray asterisk represents significance in size sclerotia.

Fig. 6. Virulence assay of the *Botrytis* isolates. (a) Apple fruit tissues were wounded with a pinprick after which 5 mL droplets (2.5 × 10⁵ conidia/mL) were inoculated. After 4 dpi at 20 °C, sizes of the lesions were measured. (b) Representative photographs of apple fruits infected with isolates strains at 7 dpi. The experiments were performed in triplicates and the data was represented by means ± standard deviations. Asterisks indicate significant differences (p < 0.05, Mann-Whitney U test) between the B05.10 WT strain and the isolates.
Table 2
Sensitivity of *B. cinerea* isolates to the antifungal drug.

| Strain  | Host plant          | Fludioxonil EC<sub>50</sub> 3.2 µg/mL | Iprodione EC<sub>50</sub> 2 µg/mL | Fenhexamid EC<sub>50</sub> 7.68 µg/mL | Tebuconazole EC<sub>50</sub> 1.65 µg/mL | Pyrimethanil EC<sub>50</sub> 9 µg/mL | Boscalid EC<sub>50</sub> 6.4 µg/mL |
|---------|---------------------|---------------------------------------|-----------------------------------|---------------------------------------|---------------------------------------|-----------------------------------|-----------------------------------|
| Bc.ad01 | Adesmia bedwelli    | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.ad02 | Adesmia bedwelli    | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.ar01 | Aristolochia chilensis | S                              | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.ar02 | Aristolochia chilensis | S                              | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.ar03 | Aristolochia chilensis | S                              | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.ca01 | Camulopuntia sphaerica | S                              | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.eq01 | Equinopsis coquimbana | S                              | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.ea01 | Eulychnia acida     | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.ed01 | Eulychnia breviflora | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.fu.01 | Fuchsia lycoides    | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.he01 | Heliotropium sternophyllum | S                        | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.he02 | Heliotropium sternophyllum | S                        | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.he03 | Heliotropium sternophyllum | S                        | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.he04 | Heliotropium sternophyllum | S                        | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.hi01 | Lithraea caustica   | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.ma01 | Malva nicaeaensis   | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.my01 | Myrcianthes coquimbana | S                                | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.or01 | Oxalis gigantea    | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.or02 | Oxalis gigantea    | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.pl01 | Pleocarphus revolutus | S                             | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.po01 | Portiera chilensis  | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.po02 | Portiera chilensis  | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.sc01 | Schinus molle     | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.sc02 | Schinus molle     | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.se01 | Senna camingii    | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.se02 | Senna camingii    | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.se03 | Senna camingii    | S                                     | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.tr01 | Trichocereus deserticola | S                         | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.tr02 | Trichocereus deserticola | S                         | S                                 | S                                     | S                                     | S                                 | S                                 |
| Bc.tr03 | Trichocereus deserticola | S                         | S                                 | S                                     | S                                     | S                                 | S                                 |

S = Sensitive, LR = Slightly Resistant, MR = Moderately Resistant, HR = Highly Resistant, ND = Not Determined.
frequencies have been reported among populations in tomato, cucumber, grape, and strawberry, where *transposa* genotypes were predominant in the populations, while in kiwifruit and apple fruit the genotypes *vacuma* were prevailing (Samuel et al., 2012). Similar results were found in the frequency of the transposable elements in Raspberry fruit, or Pear blossoms (Fournier et al., 2005; Tanović et al., 2009; Wessels et al., 2016). Also, the frequency and distribution of transposon types varied markedly between geographic regions where India/Nepal and Australia were predominant by *boty* or *transposa* respectively, similar results have been reported in Europe (Kretschmer and Hahn, 2008; Fekete et al., 2012; Tanović et al., 2013), however in Bangladesh the predominant genotype was *flipper* (Isenegger et al., 2008). Until now there is no plausible explanation about these genetic differentiations between regions, however it has been hypothesized that the climate, the geographic region or the isolation of some areas could have played a key role in the predominance of some genotypes on a worldwide scale (Isenegger et al., 2008). Also, it has been proposed that change in the *vacuma* and *transposa* frequencies was most likely caused by differences in saprotrophic and pathogenic fitness (Martínez et al., 2005). This differential distribution also could be explained because *transposa* has showed themselves to be more virulent than the other genotypes, that could explain why in our study the frequency of *transposa* has been more successful in native plant (Martínez et al., 2003; Martínez et al., 2005).

Differences in phenotypes from *B. cinerea* isolates also were found in this study, that some isolates of this fungus lose the ability to produce conidia or sclerotia formation and revealed a great diversity among the isolates concerning both features. A decrease in conidial production was also found in the isolates and significantly more sclerotia but smaller in comparison to B05.10, suggesting different abilities of the isolates to survive in these hosts. The growth rate, virulence and fungicide sensitivity have been studied in *B. cinerea*, where diversity in conidia and sclerotia formation were also found (Nicot et al., 1996; Rebordisinos et al., 2000; Tanović et al., 2009; Tanović et al., 2015; Isaza et al., 2019; Fedele et al., 2020). The results of the pathogenicity test showed that disease incidence cause by isolates with *vacuma*, *transposa* or *boty* genotypes after wound inoculation of the apple fruit were similar. Although it has been suggested in *B. cinerea* that a relationship between genotypes and their pathogenicity. Martínez et al. (2005) demonstrated that *B. cinerea* with *transposa* genotypes were significantly more virulent than *vacuma* isolates, however other studies did not find differences among the isolates in each genetic group or correlation in isolates to different hosts, thereby making it the species where it is difficult to establish relationships between pathogenicity and genotype or phenotype characteristics (Rebordisinos et al., 2000; Samuel et al., 2012; Tanović et al., 2015; Isaza et al., 2019).

To control *B. cinerea* several synthetic fungicides are widely used in the agriculture like Iprodione, Fludioxonil, Fenhexamid, Tebuconazole, Pyrimethanil and Boscalid. Our results show different grades of resistance to Fenhexamid or Boscalid, but no antifungal resistance was found in the other fungicides used in this study.

Resistance in *B. cinerea* has been associated to target site modifications, *Hydroxysteroid Dehydrogenase* (HSD7). *B. cinerea* isolates from native plants in Chile were resistant to Fenhexamid and Boscalid. Further studies will be necessary to determine whether *B. cinerea* has far migrating spores, but one could imagine a human, insect or bird mediated transmission of the fungus that would favor the dispersion of infection between plant species.

5. Conclusions

In summary, this is the first study to provide evidence that *B. cinerea* can cause gray mold disease on a wide range of native and endemic plants in Chile. We provide evidence for phenotypes and genotypes diversity in these *B. cinerea* isolates. These findings are of great epidemiological importance for regions and the distribution and the possible ecological impact of this disease on native and endemic plants. Our results will help in the future for further studies toward improved target control strategies.

Ana Notte: Collection samples from native/endemic plant, Formal analysis, Investigation, review draft manuscript. Veronica Plaza: Formal analysis, Investigation, Methodology, Writing - original draft. Bárbara Marambio-Alvarado: Formal analysis and virulence data. Lila Olivares-Urbaña: Formal and Investigation analysis and Genetic analysis. Matías Pohlete-Morales: Informatic analysis in molecular and phylogenetic identification and assistance in the creation of Fig. 2 and Fig S2-S4. Evelyn Silva-Moreno: helped during the research work and helped for drafting the manuscript. Luis Castillo: The design concept, the content, original draft, as well as the visualization have been prepared and the manuscript revised.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ccmr.2021.100049.

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