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Note

Molecular identification of *Botrytis cinerea*, *Botrytis paeoniae* and *Botrytis pseudocinerea* associated with gray mould disease in peonies (*Paeonia lactiflora* Pall.) in Southern Chile

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ABSTRACT

Background: In Chile, the peony is the most important ornamental flower exported from the country. Gray mould is a phytopathological problem of this crop. This disease is caused by *Botrytis cinerea* and *Botrytis paeoniae*.
Aims: We carried out the first survey of *Botrytis* species associated with peony gray mould in Southern Chile to estimate the diversity of these pathogens.
Methods: Diseased peony leaves were collected from seven locations in Southern Chile covering a distance of 300 km. The *Botrytis* isolates obtained were studied by morphological and molecular methods. Finally, a PCR assay using primers based on the necrosis and ethylene-inducing protein gene (*nep*1) was used to specifically identify *B. paeoniae*.
Results: Seventeen isolates belonging to *Botrytis* genus were obtained, and all of them were pathogenic to peonies when inoculated in plants grown in a greenhouse. Morphological analyses showed that four isolates shared common characteristics, which distinguish them from the rest. Homology and phylogenetic analysis of G3PDH, as well as determination of the *Bc-hch* allele, allowed us to identify 12 isolates as *B. cinerea*, 4 as *B. paeoniae* and one isolate as *Botrytis pseudocinerea*. The PCR assay was found to be specific to *B. paeoniae*, amplifying a single band of 470 bp.
Conclusions: Three *Botrytis* species involved in peony gray mould disease are present in Chile. This is the first time that both *B. paeoniae* and *B. pseudocinerea* have been reported to be present in the country and also that they affect peonies. Finally, to our knowledge, the PCR based method herein described is the first of its kind to be used to identify *B. paeoniae*.

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Identificación molecular de *Botrytis cinerea*, *Botrytis paeoniae* y *Botrytis pseudocinerea* asociadas a la pudrición gris de peonías (*Paeonia lactiflora* Pall.) en el sur de Chile

Palabras clave:
Peonía
Pudrición gris
Botrytis
Identificación molecular

RESUMEN

Antecedentes: La peonía es la principal flor ornamental de exportación en Chile. La pudrición gris, causada por los hongos *Botrytis cinerea* y *Botrytis paeoniae*, es uno de los problemas fitopatológicos más importantes de su cultivo.
Objetivos: Realizar una primera estimación de la diversidad de especies del género *Botrytis* asociadas a la pudrición gris de la peonía en el sur de Chile.
Métodos: Se recogieron hojas de peonías con síntomas de pudrición gris en siete lugares del sur de Chile, cubriendo una distancia de 300 km. Se estudiaron morfológica y molecularmente los aislamientos de

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Botrytis obtenidos. Finalmente, se desarrolló un ensayo de PCR específico para identificar *B. paeoniae* basado en el gen *nep*1.

Resultados: Se obtuvieron 17 aislamientos del género *Botrytis*; todos ellos causaron pudrición gris en plantas de peonía en ensayos de invernadero. Los análisis morfológicos mostraron que cuatro aislamientos compartían características comunes que los distinguen del resto. La homología y el análisis filogenético basado en el gen G3PDH y la determinación del alelo *Bc-hch* permitieron identificar 12 cepas como *B. cinerea*, 4 como *B. paeoniae* y un aislamiento como *Botrytis pseudocinerea*. Finalmente, el ensayo de PCR resultó específico para *B. paeoniae* al amplificar una sola banda de 470 pb.

Conclusiones: Tres especies de *Botrytis* están presentes en Chile asociadas a la pudrición gris de las peonías, y se describe por primera vez en este país la presencia de *B. paeoniae* y *B. pseudocinerea* como especies patógenas de los cultivos de esta planta. Hasta donde conocen los autores, se describe por primera vez un ensayo de PCR para identificar específicamente *B. paeoniae*.

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Peony (*Paeonia lactiflora* Pall.) is an ornamental plant, native to China, and due to its high ornamental value it has become popular and has gained attention around the world.[25] In Chile, peonies were introduced as a new option of economic activity in the south of the country leading this commercial crop to represent 46% of the total income value of all exported flowers.[2]

Gray mould is one of the most important diseases of the peony and it is caused by two fungal species: the non-host specialized plant pathogen *Botrytis cinerea* and the peony-specific pathogen *Botrytis paeoniae*.[3] Both species have been reported to be widely distributed around the world such as in the USA,[3] Iran[16] and China,[26] but the relative importance of these pathogens is still unknown. Despite the availability of taxonomic guides, there is a lack of alternative, efficient and reproducible methods for the identification of *Botrytis* species.[9,16] However, several genes have been used to evaluate the phylogeny and evolution of *Botrytis* and could be used for molecular identification.[20,21,24] In the case of *B. cinerea*, the presence or absence of two transposons, *Boty* and *Flipper*, in the genome of the pathogen allows for the establishment of the genotypes named *vacuma* (isolates without either transposon) and *transposa* (isolates with both transposons).[8] Both genotypes are associated with differences in their ecology and population biology, such as in their host range and their genetic diversity, despite being frequently found in sympatry.[4,8,15,17–19] Studies based on multiple gene genealogies have shown that *B. cinerea* is in fact a complex of at least two genetically different groups, Group I and Group II, which were proposed to be phylogenetic species.[6,7] Group I isolates belong exclusively to the *vacuma* genotype and were formally described as a new species called *B. pseudocinerea*.[24] Group II isolates include both *vacuma* and *transposa* genotypes and are referred to as *B. cinerea*.[6,7]

Due to the exportation increase of this species, peony cultivation has become more intensive, which has increased the risk of phytosanitary problems. Therefore, we conducted the first survey of its pathogens to characterize and to identify the species involved in the peony gray mould found in the main production area of Southern Chile. For this study, infected peony plants were collected from November to March during 2008 to 2010 in seven commercial fields of peonies located at seven sites covering a distance of 300 km between Collipulli and Pilmaiquen (Table 1). Leaves with gray mould symptoms were incubated in a humid chamber until profuse sporulation was observed. Conidia were collected and plated onto half strength PDA plates (Potato-Dextrose Agar, Gibco/BRL, 1.5% agar) in order to obtain monosporic isolates.

To characterize the isolates morphologically, mycelium was inoculated in the center of half strength PDA plates and was cultivated at 25 °C in darkness. Observations of three replicate plates per isolate were made daily considering mycelium color and texture, the beginning of sporulation (determined as the time at which it was possible to observe the first conidiophores with conidia under the stereo microscope), the onset of sclerotia formation (considered as the time at which it was possible to detect the initial stage of sclerotium, as white or gray hyphae aggregated into small knots), and conidia and sclerotia size (determined by recording length and width of 50 conidia and 20 sclerotia under a light microscope or a stereo microscope using a micrometer installed in one of the ocular lenses).

Pathogenic assays were carried out in a greenhouse using 3-month-old peony cv. "Boule de Neige" plants potted in natural fertile soil. Each isolate was inoculated into two peony plants. Three leaves per plant were punctured and inoculated with a 4 mm diameter PDA disk containing conidia and mycelium of the isolate. The fourth leaf (control) was inoculated with a PDA disk without the fungus. Plants were immediately sprayed with sterile distilled water and then were covered with a transparent plastic bag for 72 h and kept in a greenhouse at 22–26 °C for nine days. Then, the diameter of the necrotic lesion formed around each PDA disk was measured. Aggressiveness was determined using a scale of 0–4 defined as: **0,** absence of lesion; **1,** less than 2 mm lesion; **2,** 2–3 mm lesion; **3,** 3–5 mm lesion; and **4**, over 5 mm lesions.

All of the 17 isolates selected for this study produced the characteristic gray mould lesion in the peony leaves under greenhouse conditions (Table 1), demonstrating all of them were pathogenic to the host plant. The level of infection was high and fairly homogeneous among the isolates, most of them scoring an aggressiveness of 3–4. Morphologically, at least two groups were detected when cultivated on PDA plates (Table 1). One group composed of isolates IBx7, IBx9, IBx12 and IBx16 showed fairly similar phenotypes such as abundant white aerial mycelium, conidia produced after seven days, and sclerotia observed after 15 days. The remaining isolates presented a mycelium such as that observed in *Botrytis* species with sporulation developing after 4–7 days and sclerotia being produced after 6–9 days.

The identification of *Botrytis* species was done by sequencing and phylogenetic analysis. In this regard, genomic DNA was extracted from each isolate as previously described,[18] and a fragment of the G3PDH gene was amplified by PCR[20] and sequenced at Unidad de Secuenciamiento Automático de ADN (Pontificia Universidad Católica de Chile). The obtained sequences (GenBank accession numbers KF015582 to KF015598, Fig. 1) were first analyzed for homology to known sequences with the BLAST program and the nucleotide databases.[1] All sequences obtained showed corresponding matches to *B. cinerea* or *B. paeoniae* except for the isolate IB ×15 that was homologous to *B. pseudocinerea* (JN692414). For the phylogenetic analysis, the corresponding G3PDH gene fragment of *B. cinerea* (AJ705005, AJ705006, AM231156 and AM231160), *B. paeoniae* (AJ705027, AJ705028, EF36130 and EF677131) and other *Botrytis* species (*B. tulipae* AJ705042; *B. gladiorum* AJ705020;

Table 1
Origin, characterization and identification of *Botrytis* isolates collected from peonies in Chile.

Species identified	Location[c]	Isolate code	Mycelium color	Mycelium aspects[d]	Sporulation (days)[e]	Conidium size (μm)	Sclerotia formation (days)[f]	Number of sclerotia[g]	Sclerotium size (mm)	Genotype[h]	Aggressiveness[i]
Botrytis cinerea[a,b]	Collipulli	IBx8	Gray-greenish	Normal	5	9.1 × 7.2	7	7	3.5 × 1.8	*Transposa*	2
	Collipulli	IBx14	Gray-brown	Normal	4	9.2 × 8.5	6	65	2.8 × 1.8	*Flipper*	4
	Collipulli	IBx17	Gray-brown	Normal	4	10 × 7.9	6	53	3.7 × 3.2	*Transposa*	4
	Cunco	IBx1	White	Sparce	6	7.6 × 6.9	8	70	2.8 × 2.1	*Transposa*	3
	Freire	IBx3	Gray	Cottony	4	7.9 × 7.4	6	44	4.2 × 2.6	*Flipper*	3
	Freire	IBx13	Gray-greenish	Normal	4	9.5 × 7.3	9	49	2.8 × 2.6	*Transposa*	3
	Gorbea	IBx4	Gray	Normal	6	9.5 × 8.5	8	48	5.6 × 3.1	*Flipper*	4
	Gorbea	IBx5	Gray	Normal	6	8.2 × 7.2	8	28	6.2 × 4.2	*Flipper*	4
	Gorbea	IBx6	Gray-brown	Normal	5	12 × 10	9	6	4.2 × 1.9	*Flipper*	4
	Gorbea	IBx10	Gray-greenish	Cottony	7	8.3 × 6	9	45	4.3 × 3	*Flipper*	3
	Panguipulli	IBx2	Gray-brown	Normal	4	8.7 × 8.4	7	106	3.8 × 2.8	*Boty*	4
	Panguipulli	IBx11	Gray-greenish	Normal	5	9.3 × 10	7	48	3.8 × 3	*Flipper*	4
Botrytis paeoniae[a]	Vilcún	IBx16	White	Cottony	7	9.5 × 7.1	15	43	1.9 × 1.2	*Transposa*	4
	Vilcun	IBx9	White	Cottony	7	12 × 9.6	15	17	2.3 × 2.2	*Transposa*	3
	Panguipulli	IBx7	White	Cottony	8	9 × 7	15	37	2.2 × 2	*Boty*	4
	Pilmaiquen	IBx12	White	Cottony	7	8.9 × 7.6	15	16	2.5 × 1.7	*Transposa*	3
Botrytis pseudocinerea[a,b]	Gorbea	IBx15	White	Normal	4	8.8 × 7	6	182	1.6 × 1.3	*Flipper*	3

[a] Identification based on GPDH homology and phylogeny.
[b] Identification based on *Bc-hch* allele determination.
[c] Locations are listed from north to south.
[d] Mycelium was considered as "normal" when white, gray or greenish aerial hyphae, sporulating or sclerotium were observed; "sparse" when a thin layer of mycelium stacked on the agar surface were observed, and "cottony" when abundant and dense white aerial mycelium was observed.
[e] The time at which the first conidiophores with conidia were observed under the stereo microscope.
[f] The onset of sclerotia formation was considered as the time at which a white or gray hyphae aggregated into small knots was observed.
[g] Number of sclerotia formed per plate.
[h] Determined by PCR-based detection of *Boty* and *Flipper* transposons.
[i] 0, absence of lesion; 1, less than 2 mm lesion; 2, 2 to 3 mm lesion; 3, 3 to 5 mm lesion; and 4, over 5 mm lesions.

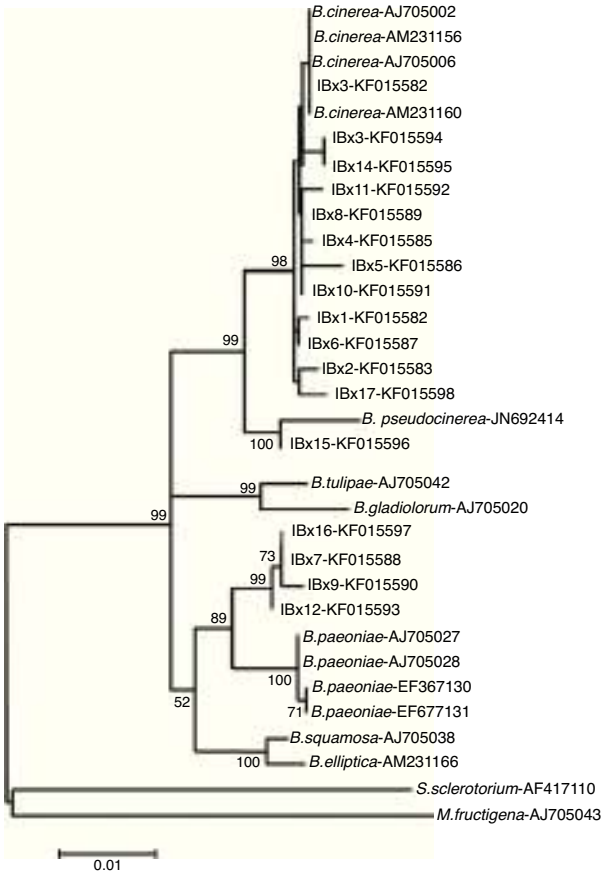

Fig. 1. Phylogenetic dendrogram constructed using neighbor-joining analysis of a GPDH fragments. The fungal species considered in this analysis and their corresponding GenBank accession numbers are indicated on the figure. The dendrogram was rooted with *Sclerotinia sclerotiorum* and *Monilinia fructigena*. Bootstrap values are given at each branch. The bar indicates the expected changes per site.

B. squamosa AJ705038 and *B. elliptica* AM231166) were considered. *Sclerotinia sclerotiorum* (AF417110) and *Monilinia fructigena* (AJ705043) were used as out-group controls. For the phylogenetic analysis, the sequences were first aligned and then analyzed using the neighbor-joining method of the distance matrix with 1000 bootstrap replicates using the MEGA4.0 program.[22] The dendrogram obtained (Fig. 1) showed that 12 isolates (IBx1, IBx2, IBx3, IBx4 IBx5 IBx6 IBx8 IBx10 IBx11 IBx13 IBx14 and IBx17) clustered with *B. cinerea* and four isolates (IBx7, IBx9, Bx12 and IBx16) formed a group with *B. paeoniae*. The species *B. cinerea* and *B. pseudocinerea* were confirmed by determination of the *Bc-hch* allele as previously described.[7]

Despite being expected, the presence of *B. paeoniae* has not been previously described in Chile. These isolates were collected in three different locations including Vilcún, Panguipulli and Pilmaiquen, thus suggesting that this pathogen is widely distributed throughout the country. Because peony production is a very recent economic activity in Southern Chile, and also due to the host specialization of this species, it is highly probable that *B. paeoniae* was introduced to the country along with the plant.

Detection of *B. pseudocinerea* was an unexpected finding and to our knowledge this is the first time this species has been reported in peonies and also in Chile. As previously described for other crops in the rest of the world,[5,10,11,13–15,19,24] *B. pseudocinerea* seems to be infrequent in peonies in Chile. However, a more detailed study is needed to establish the real frequency of this species.

In order to make comparisons with *Botrytis* populations previously described in Chile, isolates were genotyped using PCR to detect the presence of *Boty* and *Flipper* transposons as previously described.[17] Noteworthily, we found that the isolate IBx15 contained only the *Flipper* transposon (Table 1). In France, *B. pseudocinerea* populations have been described to be composed of only *vacuma* isolates.[6,7,24] Our result agrees with those reported in Hungary[5] and New Zealand,[11] where a wide diversity of transposon types have been found in the *B. cinerea* populations. In addition, a high frequency of the *Flipper* genotype (7 out of 12 isolates) was found in the isolates identified as *B. cinerea*, while the other genotypes were mainly *transposa* (4 out of 12 isolates) and only one isolate was identified as *Boty* (Table 1). The *Flipper* genotype has been nearly absent in most commercial crops studied[4,10,13,15,17] and has only been detected at significant levels in six plant hosts in Greece[19] and in vineyards in Hungary.[23] The particular distribution of these genotypes in the *B. cinerea* isolates suggests that the population of pathogens present in peonies might be different from those previously described in other crops in Chile.[4,17,18]

Finally, a specific PCR-based assay was established based on the necrosis and ethylene-inducing protein 1 gene (*nep1*).[21] *Nep*1 sequences corresponding to *B. paeoniae* (AM087032 and AM087033) were aligned with those corresponding to *B. cinerea* (AM087032, DQ211824 and JN672679) using the Clustal Omega program [http://www.ebi.ac.uk/Tools/msa/clustalo/help]. Primers BpNP1-F41 (5′-GCTACAGTCCCTCACGATTCG-3′) and BpNP1-R510 (5′-TAAGGTGGGGTTGGGGAGGG-3′) were designed using the FastPCR program.[12] DNA amplifications were done with GoTaq Hot Start Polymerase (Promega) under reaction conditions recommended by the manufacturer; primers were used at 200 nM, DNA ranged from 5 to 20 ng, and the final volume of the reaction was 20 μl. The program cycles were 2 min at 95 °C followed by 35 cycles of 20 s at 95 °C, 20 s at 58 °C and 20 s at 72 °C, and a final extension step of 5 min at 72 °C. Reactions were carried out in My Cycler™ thermal cycler (BioRad). Amplified products were visualized on 1% agarose gels stained with SafeView nucleic acid stain (NBS Biologicals Ltd), illuminated under UV light and digitally recorded.

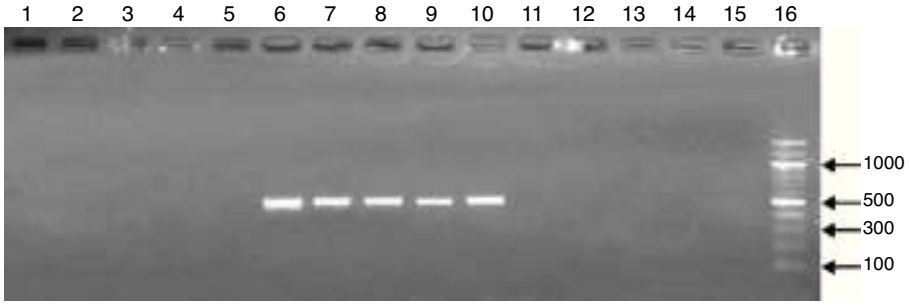

Fig. 2. PCR identification of *B. paeoniae* using BpNP1-F41 and BpNP1-R510 primers. Lanes 1–5 correspond to the DNA samples of *B. aclada*, *B. allii*, *B. byssoidea*, *B. squamosa* and *B. tulipae*. Lanes 6–10 correspond to the DNA of *B. paeoniae*, isolates 1908, IBx7, IBx9, IBx12 and IBx16. Lane 11 corresponds to isolate IBx15 of *B. pseudocinerea*. Lanes 12–15 correspond to DNA samples of *B. cinerea* isolates IBx1, IBx2, IBx6 and IBx16. Lane 16 represents the 1 kb ladder DNA.

Other *Botrytis* isolates included in these assays were *Botrytis aclada* 961, *Botrytis allii* 403, *Botrytis byssoidea* 94, *Botrytis squamosa* 1107, *Botrytis tulipae* 9701 and *B. paeoniae* strains 771 and 16084 (a donation from Jan Van Kan, Wageningen University). Our results showed that the designed primers amplified a single DNA band of the expected size specific to *B. paeoniae* isolates (470 bp, Fig. 2).

In conclusion, *B. cinerea*, *B. paeoniae* and *B. pseudocinerea*, are present in Chile and are involved in peony gray mould disease. This study represents the first report of *B. paeoniae* and *B. pseudocinerea* existence and also that they affect peonies in this country. Finally, to our knowledge, the PCR-based method herein described is used for the first time to identify *B. paeoniae*.

Disclosure statement

Authors have nothing to declare.

Conflict of interest

The authors declare that they have no conflict of interest.

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