

A detached leaflet assay to determine the pathogenicity of diverse *Botrytis* spp. causing chocolate spot disease in faba bean (*Vicia faba* L.)

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Research Article

Keywords: Detached leaflet assay, chocolate spot, disease screening, *Botrytis* species, moderate resistant, isolate-genotype interaction

Posted Date: June 2nd, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-6784656/v1>

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Additional Declarations: The authors declare no competing interests.

Abstract

Chocolate spot (CS) disease, caused by certain *Botrytis* species, is one of the most destructive diseases of faba bean worldwide. This research set out to determine whether different *Botrytis* species cause different responses in faba bean genotypes that display varied levels of CS susceptibility. Three faba bean inbred lines – Kontu, Mélodie/2, and ILB 938/2 – identified as highly susceptible, moderately susceptible, and moderately resistant, respectively, were chosen as hosts, and 21 *Botrytis* isolates, representing six species (*B. fabae*, *B. fabiopsis*, *B. cinerea*, *B. pseudocinerea*, *B. medusae*, and *B. euroamericana*), were used as disease agents. A PCR assay using primers targeting the 10 NEP protein genes was used to genotype *Botrytis* isolates. The *Botrytis* isolates were grown on half-strength PDA medium and treated with near-UV light to induce sporulation. Spore suspensions were inoculated onto detached, fully expanded leaflets. Results revealed a significant difference in virulence reactions among the inbred lines, isolates and their interaction. All the 21 *Botrytis* species/isolates tested induced CS symptoms. *B. fabae* 19B053-4 was the most virulent, causing the largest lesions, followed by *B. fabiopsis* 19B175, *B. fabae* 17B4, *B. fabiopsis* 17B24, and *B. cinerea* 19B048. At the other extreme, lesions developed most slowly with *B. cinerea* 17B10-1, *B. fabiopsis* 19B024, *B. fabae* 17B28, and *B. cinerea* 19B014. The DNA analysis revealed two main clusters and four sub-clusters among the *Botrytis* species, showing species-level groupings. The detached leaflet assay enabled a rapid and effective comparison of the pathogenicity of the studied *Botrytis* isolates.

Introduction

Faba bean (*Vicia faba* L.) is a cool-season grain legume crop with great potential to contribute to sustainable agriculture and protein security globally. Its nitrogen fixation ability is considered the strongest among cool-season grain legumes, which leads the crop to produce protein-rich seeds (Klippenstein et al., 2022). The crop is an attractive solution for providing sustainable plant-based protein for food and feed, while avoiding the growing global demand for chemical nitrogen fertilizer. Nevertheless, faba bean is considered to be very sensitive to chocolate spot (CS) disease, which is one of the most devastating diseases affecting faba bean globally (Rubiales and Khazaei, 2022), as it can cause yield losses of up to 90% in susceptible cultivars (Gorfu and Yaynu, 2001; Stoddard et al., 2010; Beyene et al., 2018; Barilli et al., 2025).

CS disease is caused by several necrotrophic fungi in the genus *Botrytis*. It is a polycyclic disease, with conidia serving as the primary source of inoculum, typically produced on residues from previous crops (Fan et al., 2015). These conidia are dispersed by wind and deposited on faba bean leaves, stems, flowers, and pods. Under favorable environmental conditions (~ 20°C, 98% RH) the conidia germinate, initiating infection and enabling rapid disease spread across the field. Infection is facilitated by the production of phytotoxins and cell wall-degrading enzymes such as polygalacturonases (Leisen et al., 2022). Symptoms of CS disease include oblong to elliptical lesions ranging in color from reddish to chocolate brown, often with a darker margin and concentric ring patterns (El-Metwally et al., 2010).

These symptoms typically appear on the upper surface of leaves, increasing in number and size, and may coalesce. In severe cases, extensive blackening occurs, leading to the death of the entire plant.

Botrytis fabae Sard. is the primary pathogen responsible for CS in faba bean. *B. cinerea* Pers. has also been reported to cause CS disease, either independently or as part of a pathogen complex (e.g., Harrison, 1988; Bilkiss et al., 2019). While *B. fabae* is host-specific, infecting only *Vicia* species, *B. cinerea* has a broad host range. Zhang et al. (2010) reported a third species, *B. fabiopsis*, from faba bean in Hubei Province, China, which produces symptoms similar to those caused by *B. fabae* and *B. cinerea*. *B. fabiopsis* may co-occur with *B. fabae* and *B. cinerea*, potentially contributing to complex infection dynamics (Teshome and Tagegn, 2013). The fourth species, *B. pseudocinerea*, closely related to *B. cinerea* (Plesken et al., 2015), has also been reported to infect faba bean (Bankina et al., 2021). Two further *Botrytis* species infect various legumes; *B. euroamericana* caused CS-like symptoms on faba bean, chickpea, field pea, lentil and soybean, while *B. medusae* infected narrow-leaved lupin, field pea and soybean (Brauna-Morževska et al., 2023). Recently, *B. eucalypti* has been reported to cause CS in faba bean in Qinghai Province, China (Ran et al., 2024).

Only a few sources of moderate genetic resistance to CS disease have been identified in faba bean (e.g., Villegas-Fernández et al., 2009; Maalouf et al., 2016), and resistant germplasm remains scarce. While screening large faba bean germplasm collections and genetic mapping populations for CS pathology and genetic studies are essential, the screening process is labor-intensive and typically requires large space in greenhouses or semi-controlled field environments. Indoor or outdoor screening of large germplasm collections for CS is often unreliable due to uneven pathogen infection. Detached leaves are often used for large-scale screening, and a detached-leaflet assay offers an even more efficient alternative, requiring less space and resources (Isenegger et al., 2011). This method enables rapid and effective comparison of the pathogenicity of a large number of *Botrytis* isolates, allowing the selection of candidates for further detailed studies. Hence, we set up an experiment using three faba bean inbred lines with proven response to CS disease: one highly susceptible line (selected from cv Kontu, Gela et al., 2022; Feyissa, 2022), one moderately susceptible line (Mélodie/2; Feyissa, 2022), and one moderately resistant line (ILB 938/2, Khazaei et al., 2018), to evaluate the virulence and pathogenicity of a set of 21 *Botrytis* isolates. This study further dissects the molecular diversity of *Botrytis* species by analyzing necrosis- and ethylene-inducing protein (NEP) gene markers, which play a key role in inducing necrosis and cell death during host infection and disease progression.

Materials and methods

Development of *Botrytis* isolates

A set of 21 isolates spanning six *Botrytis* species was obtained from Prof. Biruta Bankina at the Latvian University of Life Sciences and Technologies, Jelgava, Latvia. The isolates were cultured on half-strength PDA and maintained in darkness at room temperature (20°C) for 7 days, then put under near-ultraviolet light for 7 days to induce sporulation. Thereafter, the cultured plates were washed with sterile water and

scraped with a glass rod to dislodge the spores. The suspension was filtered through two layers of cheesecloth into a 250 ml conical flask, then 5 ml of Tween 20 (0.03% v/v) solution was added to keep the spores in suspension (Terefe et al., 2015). The concentration of spores was determined using a haemocytometer and adjusted to 5×10^6 spores per milliliter.

Plant material and growing conditions

Three faba bean inbred lines were chosen for this work. ILB 938/2 exhibits moderate resistance and Mélodie/2 is moderately susceptible to CS disease. These two inbred lines serve as the parental lines of a well-characterized recombinant inbred line population used to study CS disease response (Gela et al., 2022). The Finnish cultivar Kontu is highly susceptible to CS disease (Feyissa, 2022).

Seeds were surface sterilized with 70% ethanol for 3 min and rinsed three times with sterile distilled water. Sets of three seeds of each inbred line were planted in 2.5 L plastic pots filled with autoclaved peat soil (Kekkilä Coarse potting mix WR8494, Kekkilä-BVB OY, Vantaa, Finland). Plants were grown for 6–8 weeks in a controlled-environment growth chamber (Weiss-2000, Weiss Technik GmbH, Reiskirchen-Lindsruth, Germany). The temperature was set to 22°C during the day and 20°C at night, with a 12-hour photoperiod under visible light (150 $\mu\text{mol}/\text{m}^2/\text{s}$ photosynthetic photon flux density, PPFD).

Inoculation

Fully expanded leaves of six- to eight-week-old plants of the three inbred lines were collected from four to eight nodes two hours prior to inoculation and kept in sterile moist napkin paper. Leaves were washed with sterile distilled water, blotted dry, and placed in pairs on two layers of sterile filter paper in 90 mm petri dishes, which were arranged in a completely randomized factorial design with three replicates (five leaflets in each replicate per inbred line). Five ml of sterile water was applied in each petri dish to moisten the filter paper and to maintain high humidity within the dish. A piece of cotton moistened with sterile water was placed onto the petiole end to maintain the turgor pressure and prevent dryness. An experimental unit comprised three petri dishes containing 5 leaflets, inoculated with 20 μl suspension culture and a control was treated with sterile water in each replication for each accession. The petri dishes were organized in a plastic box (37.5 $\times$ 25.5 $\times$ 9.5 cm) and incubated in a growth chamber at 22°C and 12 h photoperiod with 170–188 $\mu\text{mol}/\text{m}^2/\text{s}$ PPFD. The fungal infection and necrosis were monitored every day to observe the first visible symptom, and after 5 days, leaflets were photographed against a measuring scale for image analysis.

Molecular characterization of *Botrytis* isolates using NEP protein gene markers

Botrytis isolates were genotyped using specific primer pairs for necrosis and ethylene-inducing proteins (NEP) (Table S1).

The *Botrytis* isolates were cultured on PDA containing a GVS Cellophane film (Diameter 76 mm, 0.1 µm; GVS, Bologna, Italy) on the medium and incubated in darkness at room temperature (20°C) for 14 days to produce enough mycelium. Mycelium was harvested using a sterile scalpel and put into a 1.5 ml Eppendorf tube; the lids of the tubes were opened for a few hours to allow the water from the mycelium to dry at room temperature. Subsequently, the air-dried mycelium was frozen into liquid nitrogen and grounded into powder using a plant DNA isolation kit (DNeasy® Plant Mini Kit, Qiagen). The extracted DNA was dissolved in 50 µl of TE buffer, quantified using a Nanodrop spectrophotometer (Thermo Scientific), and stored at -20°C.

PCR amplification was carried out in a 20 µl reaction solution, having 4 µl template (6 ng/µl) of fungal DNA, 10 µl of DreamTaq Green PCR Master Mix (2X, contains Dream Taq DNA polymerase, DreamTaq Green buffer, MgCl₂ and dNTPs, supplemented with two tracking dyes and a density reagent that allows for direct loading of the PCR products on a gel; ThermoFisher Scientific, Waltham, MA, USA), 0.8 µl of each primer, and 4.4 µl of sterile water. PCR reactions were conducted in a thermocycler (Eppendorf, Germany), using the amplification program: 1 cycle of 2 min at 94°C, 35 cycles of 30 sec at 94°C, and 30 sec at 50°C or 58°C for annealing temperature, 1 min at 72°C; and 10 min at 72°C for final extension. A PCR mixture without fungal DNA template was used as a negative control. The PCR products were separated and analyzed by electrophoresis using a 1% agarose gel and 1x TBE buffer, staining with 0.5 µg/ml ethidium bromide and visualized in a Molecular Imager, Gel Doc (Bio-Rad Laboratories, Hercules, CA, USA) for the presence or absence of the expected size of band.

Statistical analysis

The infected area of the leaves was determined using ImageJ software. The scale was fixed at 1 cm and the estimated distance was 50 pixels (Rha et al., 2015). The mean infected area of the 5 infected leaves in an experimental unit was calculated. The data were transformed to log (area + 1) to normalize the variance, and used in the analysis of variance (ANOVA) to determine the effects of isolate, inbred line and their interaction. Means were separated using Tukey-HSD multiple pairwise comparisons using the JMP Pro SAS statistical computing package (Grayson et al., 2015). Pairwise genetic distances were estimated using Nei's coefficient (Nei, 1978), and a dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm on Darwin v. 6.0.021.

Results

All 21 isolates caused CS symptoms. On average, Kontu showed the most susceptible reaction, ILB 938/2 the most resistant, and Mélodie/2 an intermediate reaction (**Figure S1**). The first characteristic water-soaked symptoms of CS disease were found using *B. fabae* 19B053-4 on Kontu after 24 h, on Mélodie/2 after 36 h and on ILB 938/2 after 72 h. In contrast, symptoms induced by *B. fabae* 17B4 were observed after 36 h on Mélodie/2 and Kontu and after 96 h on ILB 938/2. By 120 h after inoculation, symptoms were obvious and consistent blackish lesions on most combinations of isolate by inbred line

(Table 1). The ANOVA results showed significant variation among the *Botrytis* isolates, the faba bean inbred lines, and the isolate $\times$ inbred line interactions ($P < 0.0001$, Table 2).

Table 1
Time (h) to first visible symptoms induced by the *Botrytis* species on three inbred lines of faba bean. Sterile distilled water was used as a control.

<i>Botrytis</i> species (isolate)	Kontu	Mélodie/2	ILB 938/2
Hours to first symptom			
<i>B. fabae</i> (19B053-4)	24	36	72
<i>B. fabae</i> (17B4)	36	36	96
<i>B. fabae</i> (17B28)	96	120	144
<i>B. fabiopsis</i> (19B175)	36	48	72
<i>B. fabiopsis</i> (17B24)	36	48	120
<i>B. fabiopsis</i> (17F9)	48	60	96
<i>B. fabiopsis</i> (18B16)	60	72	96
<i>B. fabiopsis</i> (17F4)	72	96	132
<i>B. fabiopsis</i> (19B024)	96	120	132
<i>B. cinerea</i> (19B048)	48	60	96
<i>B. cinerea</i> (19B026)	48	60	96
<i>B. cinerea</i> (17B10-1)	96	120	144
<i>B. cinerea</i> (B05.10)	60	96	96
<i>B. cinerea</i> (19B009)	72	96	132
<i>B. cinerea</i> (19B009)	96	120	144
<i>B. cinerea</i> (18B3)	48	48	60
<i>B. pseudocinerea</i> (18B11)	48	60	110
<i>B. pseudocinerea</i> (18B2-3)	72	96	132
<i>B. pseudocinerea</i> (18B6)	48	48	60
<i>B. euroamericana</i> (19B053-3)	72	96	132
<i>B. medusae</i> (19B047)	60	60	72

Table 2
Analysis of variance showing the effects of fungal isolate, host inbred line, and isolate×inbred line on the areas of chocolate spot disease lesions.

Source	df	Sum of Squares	F Ratio	Prob > F
Isolate	20	144.16	24.58	< 0.0001
Inbred line	2	152.40	259.86	< 0.0001
Isolate × Inbred line	40	39.42	3.36	< 0.0001
Error	124	36.36	0.29	
df, degrees of freedom.				

B. fabae 19B053-4 was the most virulent isolate on two of the lines, developing the largest lesions on Kontu (10.10 cm² necrosis) and Mélodie/2 (9.30 cm² necrosis), and the second-largest on ILB 938/2 (1.92 cm²) (Fig. 1). *B. fabiopsis* 19B175 was the most virulent on ILB938/2 (2.16 cm²) and the second on both Kontu (7.70 cm²) and Mélodie/2 (5.87 cm²). Isolate 19B048 was the most virulent from *B. cinerea* and the fifth most virulent overall (5.73 cm² on Kontu, 2.42 cm² on Mélodie/2 and 0.53 cm² on ILB 938/2), and isolate 18B11 was the most virulent from *B. pseudocinerea* (4.40 cm² on Kontu, 0.84 cm² on Mélodie/2 and 0.15 cm² on ILB 938/2). The smallest lesions were induced by *B. cinerea* 17B10-1 on Kontu (0.43 cm²), Mélodie/2 (0.35 cm²), and ILB 938/2 (0.08 cm²), followed by *B. fabiopsis* 19B024 and *B. fabae* 17B28.

In every case except *B. fabae* 17B4, the lesion on Kontu was larger than that on Mélodie/2. Similarly, in every case except *B. fabiopsis* 19B024 and *B. euroamericana* 19B053-3, the lesion on Mélodie/2 was larger than that on ILB 938/2. Nevertheless, in these cases the apparent crossover was much smaller than that required for statistical significance, and the overall significance of the isolate × inbred line term in the Analysis of Variance is attributable to the lack of discrimination between inbred lines by some isolates while others clearly affected one more than another.

The NEP DNA marker analysis exhibited two main groups and four sub-clusters, with a few variations inside each group (Fig. 2). All *B. cinerea* clustered together with *B. fabae*. *B. medusae* and *B. euroamericana* isolates were grouped together. *B. fabiopsis* isolates and *B. pseudocinerea* mostly clustered together with some minor deviations (*B. fabiopsis* isolate 19B175 and *B. pseudocinerea* isolate 18B6).

Discussion

The *Botrytis* spp. used in this study showed varying reactions on detached leaves of ILB 938/2, Kontu, and Mélodie/2 in an *in vitro* assay using spore suspension cultures. Our results revealed a significant difference among the isolates, inbred lines, and isolate × inbred line interactions. ILB 938/2 showed

moderate resistance while Kontu performed completely susceptible and Mélodie/2 was moderately susceptible. These findings agreed with previous assessments of the quantitative CS resistance of these genotypes (Khazaei et al., 2018; Gela et al., 2022; Feyissa, 2022).

In our study, the first visible water-soaked CS symptoms appeared within 24 hours, and as the incubation period progressed, the lesion size increased, following a linear pattern over time (Villegas-Fernández et al., 2012). Brauna-Morževska et al. (2023) also observed the first visible symptom within 24 h, but Taffa et al. (2013) noticed the first CS symptoms two days after inoculation with *B. fabae*. Other workers have detected the first CS disease symptom within six to eight hours of inoculation (Bouhassan et al., 2004).

All the 21 *Botrytis* species/isolates used in this study caused CS symptoms. *B. fabae* isolates 19B053-4 and 17B4 were the quickest to cause symptoms and produced the largest lesions. These findings are in line with many previous studies showing that *B. fabae* is the main pathogen underlying CS (e.g., Bankina et al., 2021; Brauna-Morževska et al., 2023). The *B. cinerea* isolates were generally less virulent than the *B. fabae* isolates, with the exception of *B. fabae* 17B28, which was a very weak pathogen. This result aligns with the findings of Rhaïem (2020), who reported that *B. fabae* was generally more virulent than *B. cinerea* isolates when tested on both whole plants and detached leaves. In a detached leaflet assay of faba bean, *B. fabae* induced more progressive and larger necrotic lesions than *B. cinerea* (Omar et al., 1986), consistent with our findings. Brauna-Morževska et al. (2023) found that *B. cinerea* isolates were widely variable in pathogenicity across a range of legumes, and often appeared to be secondary pathogens or saprophytes on already infected tissues.

B. pseudocinerea and *B. fabiopsis* have not been extensively studied to date. *B. fabiopsis* isolates 17B24 and 19B175 developed aggressive lesions on Kontu and Mélodie/2 in our study, whereas *B. pseudocinerea* 18B11 and 18B6 were more aggressive on Kontu. Our results confirm that these organisms are part of the complex of *Botrytis* species causing CS (Zhang et al., 2010; Brauna-Morževska et al., 2023). The individual isolates of *B. medusae* and *B. euroamericana* produced smaller lesions than the other species, suggesting that they may not be major contributors to the disease, in agreement with Brauna-Morževska et al. (2023).

There was wide variation in pathogenicity within each species, with *B. fabae* 17B28, *B. cinerea* 17B10-1 and *B. fabiopsis* 19B024 all producing very small lesions. While previous comparisons have also found wide variation, these results are not well correlated with those of Brauna-Morževska et al. (2023), who found *B. fabae* 17B28 to be their most aggressive isolate on faba bean, although *B. cinerea* 17B10-1 was similarly weakly pathogenic in their study. Pathogens often lose virulence during culturing, and it may be that *B. fabae* 17B28 had done so, leaving the previously second most virulent isolate, 19B053-4, as the leader in the current experiment.

NEP1-like proteins (NLPs) are phytotoxic agents that cause cell damage and death, and elicit defence responses in most dicotyledonous plants (Staats et al., 2007; Arenas et al., 2010). NLPs were first found in *Fusarium oxysporum* f. sp. *erythroxyli*. The oomycetes as well as phylogenetically a wide variety of microorganisms including true fungi, gram-positive and gram-negative bacteria, carry NLP genes. A

differential expression pattern may be seen through the infection process by NLP-encoding genes (Arenas et al., 2010). Based on the NEP protein gene-based DNA markers we used in this study, *B. cinerea*, *B. fabae* and some *B. pseudocinerea* isolates showed genetic similarities. All *B. fabiopsis* isolates except the most virulent (19B175) clustered together. *B. euroamericana* and *B. medusae* also showed genetic similarities (Fig. 2). These findings are similar to those of Brauna-Morževska et al. (2023), who used RPB2, HSP60 and G3PDH gene sequences. Although *B. cinerea* and *B. pseudocinerea* are morphologically very similar, they are phylogenetically more distant from each other than *B. cinerea* is from *B. fabae*, and *B. fabiopsis* is very distant from *B. fabae* (Hyde et al., 2014). Our results are largely consistent with previous reports, with a few exceptions. These deviations might be due to the different DNA marker system we employed or growing conditions, mutation within long-lived somatic lineages, intense recombination and large population size of the *Botrytis* species (Fillinger and Elad, 2016).

Detached leaflet assays have been widely used to study the response of faba bean germplasm to *B. fabae* (e.g., Hutson and Mansfield, 1980; Bouhassan et al., 2004; Villegas-Fernández et al., 2012; Brauna-Morževska et al., 2023). Although these assays allow screening of germplasm under uniform growing conditions, they may have negative effects, with senescence triggered by leaflet detachment (Liu et al., 2007). Detached leaflet assays have shown moderate and positive correlations with whole-plant field phenotyping (Bouhassan et al., 2004; Feyissa, 2022). Our study, which evaluated the response of three known faba bean inbred lines to *B. fabae* under both greenhouse and field conditions (Gela et al., 2022; Khazaei et al., 2018; Feyissa, 2022), demonstrated the reliability of our developed detached leaflet protocol.

Conclusions

The detached leaflet assay provided a rapid and effective method for comparing the pathogenicity of the 21 *Botrytis* isolates. We employed faba bean inbred lines with known response to *B. fabae* as checks to validate the assay. The data showed interactions between inbred lines and *Botrytis* isolates, with the CS-susceptible lines displaying varying responses to different species. The detached leaflet assay is space- and resource-efficient, making it a practical tool for high-throughput, preliminary screening for CS disease. Here, it has allowed identification of pathogenicity extremes for use in further experiments. In the future, it could allow rapid screening in genetic mapping analysis, leading to the identification of genome regions associated with disease resistance that can be followed up with more precise, hypothesis-driven experiments. We are now adapting this detached leaflet assay for screening a large faba bean genetic population for genetic studies.

Declarations

Acknowledgment

We thank Prof. Biruta Bankina, Latvia University of Life Sciences and Technologies, Jelgava, Latvia, for the kind donation of the 21 isolates. We thank Markku Tykkyläinen and Eero Kuisma, technical assistants

of the glasshouse of the University of Helsinki for their kind assistance during the experiments. We acknowledge Marjo Kilpinen, Eija Takala, Katrin Artola, Dr. Teng Zhang, and Dr. Sylvain Poque for their assistance during the lab work and statistical analysis.

Authorship contribution

Maniruzzaman: Investigation, Formal analysis, Writing – original draft. **Hamid Khazaei:** Writing – review & editing, Formal analysis, Resources. **Minna Haapalainen:** Supervision, Writing - review & editing. **Frederick L. Stoddard:** Supervision, Writing – review & editing, Investigation, Conceptualization, Funding acquisition, Resources.

Funding

The work was part of the project “Fabanova – Climate-ready faba beans for the Nordic and Baltic region” funded under the NordForsk call “Sustainable agriculture and climate change”. H.K. was supported by the Research Council of Finland, Academy projects, funding decision 363375 (Fabagen).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

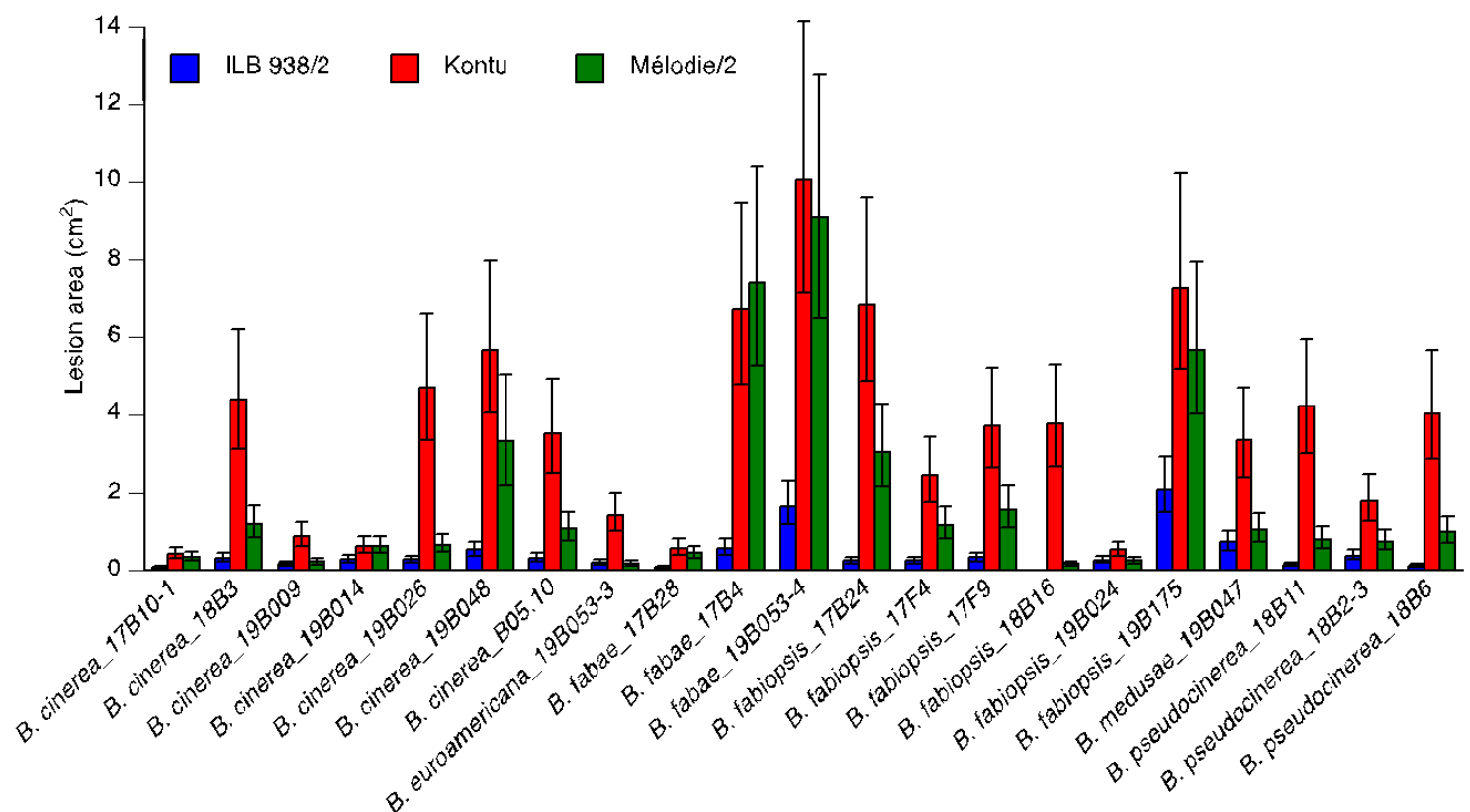


Figure 1

Mean lesion size of 21 isolates of *Botrytis* spp. on faba bean inbred lines Kontu, Mélodie/2, and ILB 938/2. Values are back-transformed from the analysed natural logarithms, $\pm$ RMSE standard error.

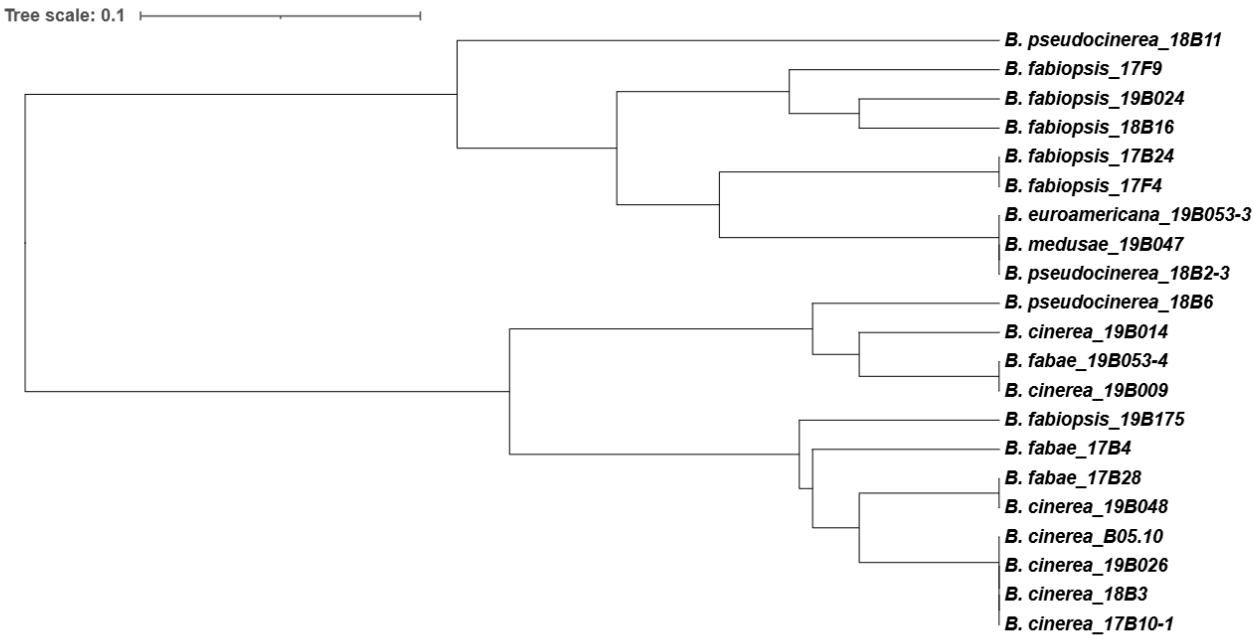


Figure 2

Unrooted dendrogram of the 21 *Botrytis* spp. isolates analyzed by 10 NEP DNA markers.

Supplementary Files

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