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Host by pathogen interactions of six *Botrytis* spp. causing chocolate spot disease on faba bean (*Vicia faba* L.)

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Chocolate spot (CS) disease, caused by *Botrytis* species, severely damages faba beans. This study analyzed interactions between three faba bean genotypes with varying levels of resistance and 21 *Botrytis* isolates from six species that were genotyped using 10 NEP markers. The isolates were cultured, sporulated under near-UV light, and inoculated onto 6–8-week-old, fully expanded, detached leaflets. All isolates induced CS symptoms, revealing significant interactions among lines and isolates. *Botrytis fabae* 19B053-4 was the most virulent, followed by *B. fabiopsis* 19B175 and *B. fabae* 17B4. Conversely, *B. cinerea* 17B10-1 caused the slowest lesion development. Notably, more resistant hosts consistently remained less susceptible across all isolates, indicating no crossover interaction. AUDPC values confirmed *B. fabae* as the primary, highly aggressive specialized pathogen, whereas related species pose opportunistic threats. Tracking cumulative disease progress identified quantitative host resistance through slowed infection rates, offering a framework for crop protection.

Key words: detached leaflet assay, disease screening, *Botrytis* species, moderate resistance, isolate-genotype

Introduction

Faba bean (*Vicia faba* L.) is a cool-season grain legume crop with significant potential to contribute to sustainable agriculture and global protein security. Its nitrogen fixation ability is considered the strongest among cool-season grain legumes, and the crop produces 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 in conducive weather conditions (Gorfu and Yaynu 2001, Stoddard et al. 2010, Beyene et al. 2018, Barilli et al. 2025).

Several necrotrophic fungi in the genus *Botrytis* cause CS. 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 water and deposited on all aerial parts of a faba bean plant. 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 (Hutson and Mansfield 1980, 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 rapid defoliation and death of the entire plant.

Botrytis fabae is the primary pathogen responsible for CS in faba bean. *B*. *cinerea* 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 considered 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. 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; in an in vitro assay, *B. euroamericana* caused CS-like symptoms on faba bean, chickpea, field pea, lentil, and soybean, while *B. medusa*e infected narrow-leafed lupin, field pea, and soybean

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Agricultural and Food Science (2026) 35: xx–xx
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(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).

Botrytis infects plants by rapidly degrading host tissues with cell-wall-degrading enzymes and secreted effectors that promote cell death and facilitate nutrient uptake (Fillinger and Elad 2016). Within this effector repertoire, necrosis- and ethylene-inducing peptide (NEP) genes are useful molecular markers for studying population structure and pathogenic variability (Fillinger and Elad 2016). NEP1-like proteins (NLPs) are phytotoxic proteins that trigger membrane damage, cell death, and defense activation in most dicotyledonous plants (Staats et al. 2007, Arenas et al. 2010). They are widely distributed across oomycetes, fungi, and both gram-positive and gram-negative bacteria. A set of 22 *Botrytis* species all had two NLP paralogues with highly divergent sequences, and certain sites in each gene showed evidence of being the locations of positive, diversifying selection, contributing to host specificity (Staats et al. 2007). The two in *B. cinerea* share about 39% overall sequence similarity (Siewers et al. 2005). Differential expression of NLP-encoding genes is often observed during infection, suggesting roles at distinct stages of host colonization (Arenas et al. 2010).

Genetic resistance is the most effective way to manage this disease, but there has been little breeding success to gain an adequate level of resistance (Stuthman et al. 2007). 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 the resistant genotype remains scarce. ILB 938 was among the early identified sources of partial resistance (Khazaei et al. 2018), and more sources, such as Maris Bead, Nubaria 3, Nubaria 4, Nubaria 5, Sakha 4, Giza 3, and Triple White have since been identified (El-Abssi et al. 2024, Webb et al. 2024). More sources of resistance are listed in Maalouf et al. (2016).

The essential task of screening large faba bean genotype collections and genetic mapping populations for CS pathology and genetic studies is labor-intensive and typically requires plentiful 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 frequently used for large-scale screening, and a detached-leaflet assay offers the opportunity to evaluate in a uniform environment, requiring less space, without the impact of season, phenology, or microenvironmental impact around the leaves, stems, pods, and resources (Kohpina and Stoddard 2000, Herath et al. 2001, Liu et al. 2007, 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 known responses to CS disease to study the interactions between *Botrytis* species and inbred lines (genotype). This study further dissects the molecular diversity of *Botrytis* species by analyzing NEP gene markers.

Materials and methods

Development of *Botrytis* isolates

A set of 21 isolates spanning six *Botrytis* species was used in this study. Among them 20 were collected from the Institute of Soil and Plant Sciences, Latvia University of Life Sciences and Technologies and one was collected from University of Helsinki (Brauna-Morževska et al. 2023). The *Botrytis* isolates were first multiplied on potato dextrose agar (PDA) medium and after 7 days an agar block with freshly grown isolate was cultured on half-strength PDA and maintained in darkness at room temperature (20 °C) for 7 days, then grown for 7 days for 12h (20 °C) under near-ultraviolet light for UV radiation and 12h of darkness to induce sporulation (Masangkay et al. 2007). The culture plates were then 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, and 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 conidia was determined using a haemocytometer and adjusted to 4×10^6 conidia per milliliter.

Plant material and growing conditions

Three faba bean inbred lines were chosen for this work. ILB 938/2 exhibits moderate resistance (Khazaei et al. 2018), and Mélodie/2 is moderately susceptible to CS disease (Feyissa 2022). 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, Gela et al. 2022).

Agricultural and Food Science (2026) 35: xx–xx
https://doi.org/10.23986/afsci.184976

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 µmol m^{-2} s^{-1} 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 rinsed 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) (Bouhassan et al. 2004, Rhaiem 2020). Five ml of sterile water was applied to 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, each unit containing 5 leaflets, inoculated with 20 µl conidial suspension culture using a pipette, and a control was treated with sterile water in each replication for each accession. The petri dishes were arranged in a plastic box (37.5 × 25.5 × 9.5 cm) and incubated in a growth chamber at 22 °C with a 12-h photoperiod and a PPFD of 170–188 µmol m^{-2} s^{-1}. The fungal infection and necrosis were monitored every day to observe the first visible symptom, and after five days, leaflets were photographed against a measuring scale for image analysis.

AUDPC (area under the disease progress curve)

Using the four most virulent *Botrytis* isolates from the four major groups after their preliminary screening the AUDPC was carried out through a single evaluation in the controlled growth chambers applying the whole plant test on the three inbred lines. The severity of chocolate spot disease was monitored daily after infection. Randomly, 10 leaves per inbred line were scored everyday using the (0–9) rating scale in the growth chamber. The disease severity was subsequently converted into percentage severity index (*PSI*) (Simko and Piepho 2012, Alemayehu et al. 2024).

$$PSI = \frac{(\text{sum of numerical ratings})}{(\text{number of plants scored} \times \text{maximum scored on a scale})} \times 100$$

Using the Campbell and Madden (1990) formula, the trapezoidal rule was employed to numerically calculate the AUDPC.

$$AUDPC = \sum_{i=1}^{n-1} 1/2\{(X_{i+1} + X_i)(t_{i+1} - t_i)\}$$

where X_i is the cumulative disease severity at the i^{th} observation, t_i is the time of the i^{th} assessment, and n is the total number of observations.

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 (ø 76 mm, 0.1 µm; GVS, USA) 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 in liquid nitrogen, ground into powder, and the DNA was extracted using a plant DNA isolation kit according to the manufacturer's instructions (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^{-1}) of fungal DNA, 10 µl of DreamTaq Green PCR Master Mix (2X, contains Dream Taq DNA polymerase, DreamTaq Green buffer, $MgCl_2$,

Agricultural and Food Science (2026) 35: xx–xx
https://doi.org/10.23986/afsci.184976

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, 30 sec at 50 °C or 58 °C for annealing, and 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 1×TBE buffer, staining with 0.5 µg ml^{-1} 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 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 student Edition 18 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

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 hours after inoculation, symptoms were evident, with consistent blackish lesions observed on most combinations of isolate and inbred line (Table 1). 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).

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.

Botrytis isolates	Hours to first symptom		
	Kontu	Mélodie/2	ILB 938/2
B. fabae 19B053-4	24	36	72
B. fabae 17B4	36	36	96
B. fabae 17B28	96	120	144
B. fabiopsis 19B175	36	48	72
B. fabiopsis 17B24	36	48	120
B. fabiopsis 17F9	48	60	96
B. fabiopsis 18B16	60	72	96
B. fabiopsis 17F4	72	96	132
B. fabiopsis 19B024	96	120	132
B. cinerea 19B048	48	60	96
B. cinerea 19B026	48	60	96
B. cinerea 17B10-1	96	120	144
B. cinerea B05.10	60	96	96
B. cinerea 19B009	72	96	132
B. cinerea 19B014	96	120	144
B. cinerea 18B3	48	48	60
B. pseudocinerea 18B11	48	60	110
B. pseudocinerea 18B2-3	72	96	132
B. pseudocinerea 18B6	48	48	60
B. euroamericana 19B053-3	72	96	132
B. medusae 19B047	60	60	72

Agricultural and Food Science (2026) 35: xx–xx
https://doi.org/10.23986/afsci.184976

The main effects of *Botrytis* isolates and inbred lines were significant (Table 2). The interaction between the *Botrytis* isolates × Inbred lines was also significant ($p < 0.0001$, Table 2).

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 5 days after inoculation

Source	df	Sum of Squares	F Ratio	*p*-value
Isolates	20	144.24	24.28	<0.0001
Inbred line	2	152.49	256.72	<0.0001
Rep	2	0.13	0.21	0.8088
Isolate × Inbred line	40	39.47	3.362	<0.0001
Error	124	36.23		

df = degrees of freedom

B. fabae 19B053-4 was the most virulent isolate, developing the largest lesions on Kontu (10.1 cm^2 necrosis) and Mélodie/2 (9.3 cm^2 necrosis), and significantly smaller lesions on ILB 938/2 (1.9 cm^2) (Fig. 1). *B. fabiopsis* 19B175 was the most virulent on Kontu (7.7 cm^2) and the second on both Mélodie/2 (5.9 cm^2) and ILB 938/2 (2.2 cm^2). Isolate 19B048 was the most virulent from *B. cinerea* and the fifth most virulent overall (5.7 cm^2 on Kontu, 2.4 cm^2 on Mélodie/2, and 0.5 cm^2 on ILB 938/2), and isolate 18B11 was the most virulent from *B. pseudocinerea* (4.4 cm^2 on Kontu, 0.8 cm^2 on Mélodie/2, and 0.15 cm^2 on ILB 938/2). The smallest lesions were induced by *B. cinerea* 17B10-1 on Kontu (0.43 cm^2), Mélodie/2 (0.35 cm^2), and ILB 938/2 (0.08 cm^2), followed by *B. fabiopsis* 19B024 and *B. fabae* 17B28.

The lesion on Kontu was larger than that on Mélodie/2 in every case except *B. fabae* 17B4, where they were equal. 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 ANOVA is attributable to the lack of discrimination between inbred lines by some isolates, while others clearly affected one more than another.

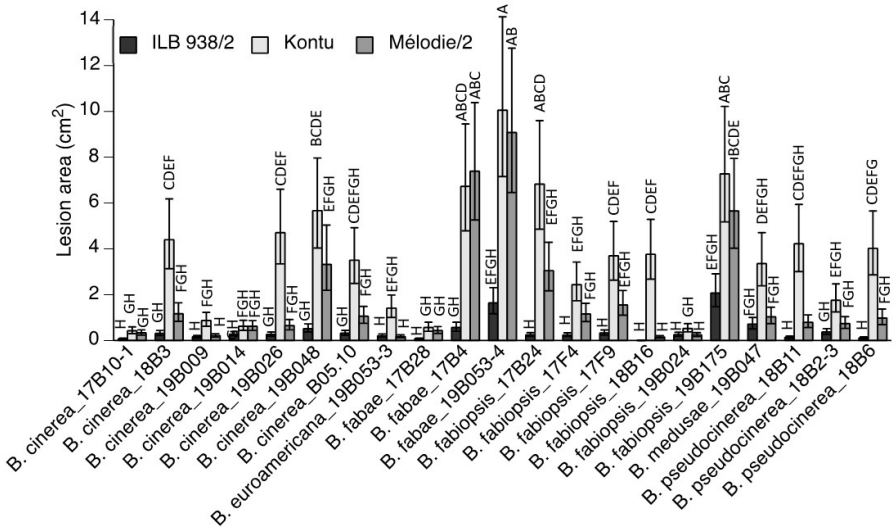

Fig. 1. Mean lesion size of 21 isolates of *Botrytis* spp. on faba bean inbred lines Kontu, Mélodie/2, and ILB 938/2. Values are back-transformed from the analyzed natural logarithms, ± RMSE standard error. Mean comparison done by Tukey HSD, α=0.05.

Significant differences in AUDPC values among the inbred lines were observed throughout the assessment period in a single evaluation ($p < 0.0001$). However, the interactions involving *Botrytis* species, days, and host inbred lines did not show significance because they were almost similar in virulence observed in the preliminary screening (Table 3).

Agricultural and Food Science (2026) 35: xx–xx
https://doi.org/10.23986/afsci.184976

Table 3. Analysis of variance showing the AUDPC of the five days of the disease progression period by four virulent *Botrytis* species

Source	DF	Sum of Squares	F Ratio	*p*-value
Isolate	3	0.29	28.15	<0.0001
Inbred line	2	0.57	82.85	<0.0001
Isolate × Inbred line	6	0.06	2.71	0.0304
Error	32	0.11		

df = degrees of freedom

The cumulative AUDPC values overall ranged from 255 to 379. *B. pseudocinerea* recorded the lowest AUDPC values of 255, 339, and 305 on ILB 938/2, Kontu, and Mélodie/2, respectively, whereas *Botrytis fabae* had the highest values of 332, 379, and 352, respectively, on the same inbred lines (Fig. 2).

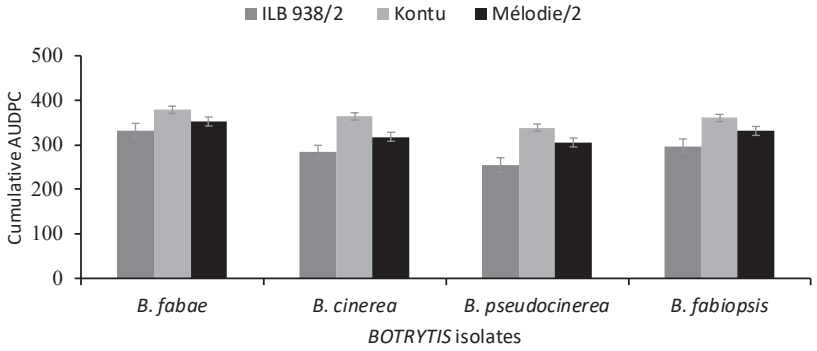

Fig. 2. Cumulative AUDPC of the five-day disease progression period by four virulent *Botrytis* species

Figure 3 shows the progression of AUDPC over five days period for four *Botrytis* isolates across three genotypes. AUDPC increased steadily with time for all isolates and inbreds, with the greatest increase occurring between days 2 and 3, followed by a slower rate of increase toward Day 5–6. Across all isolates, Kontu consistently exhibited the highest AUDPC values, while ILB 938/2 had the lowest, with Mélodie/2 showing intermediate responses.

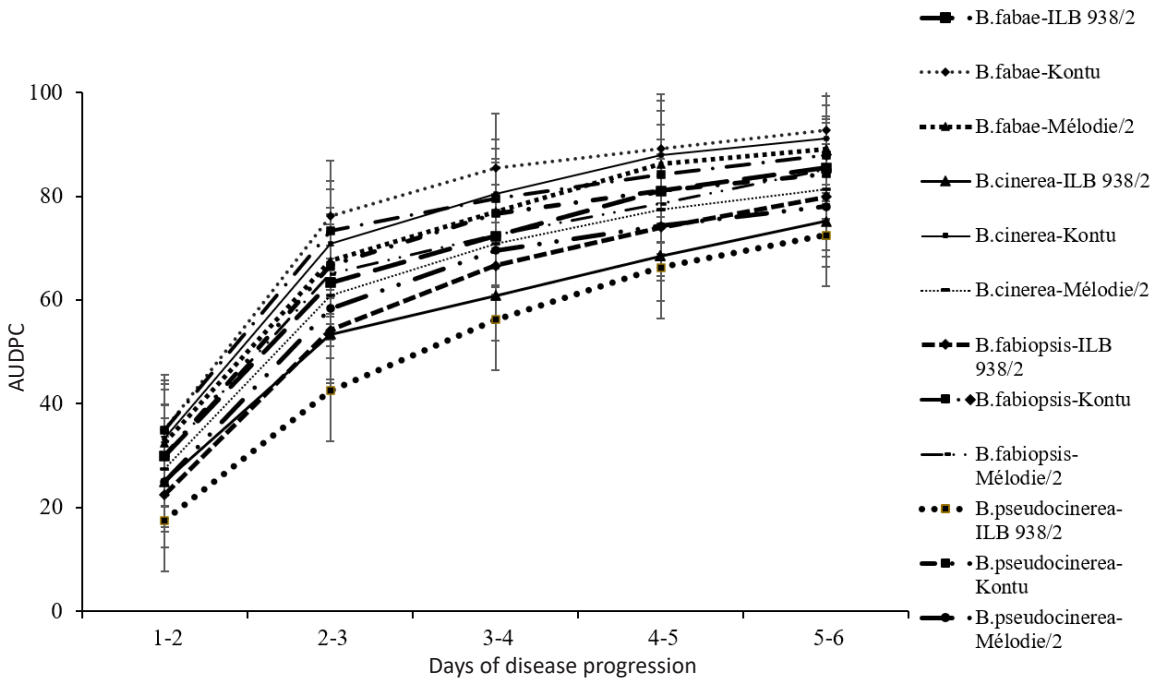

Fig. 3. AUDPC on each day of the five-day disease progression period by four virulent *Botrytis* species

Agricultural and Food Science (2026) 35: xx–xx
https://doi.org/10.23986/afsci.184976

The NEP DNA marker analysis exhibited two main groups and four sub-clusters, with a few variations inside each group (Fig. 4). 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).

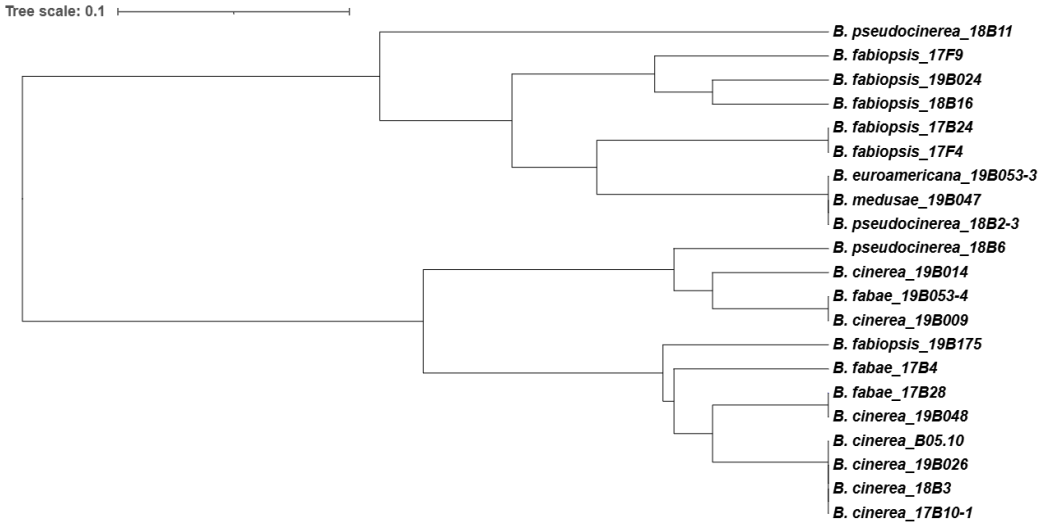

Fig. 4. Unrooted dendrogram of the 21 *Botrytis* spp. isolates analyzed by 10 NEP DNA markers

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 suspensions of conidia. The results were somewhat affected by leaf position and age, as they were collected from different nodes of the plants. Lower leaves develop earlier than the upper node's leaf and old leaf is more susceptible than the younger leaf to CS. Leaf position and age have significant impact on CS development in faba bean reported by Mahmoud et al. (2015). Our results revealed significant differences among the isolates, inbred lines, and isolate × inbred line interactions. On average, lesions were smallest on ILB 938/2, intermediate in size on Mélodie/2 and largest on Kontu, in line with previous assessments of these lines as moderately resistant, moderately susceptible and highly susceptible, respectively (Khazaei et al. 2018, Feyissa 2022, Gela et al. 2022).

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, as shown previously (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* 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 (Lee et al. 2020). This result aligns with the findings of Lee et al. (2020) and Rhaiem (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* isolates 18B11 and 18B6 were more aggressive on Kontu. Our results confirm that these organisms are part of the complex of

Agricultural and Food Science (2026) 35: xx–xx
https://doi.org/10.23986/afsci.184976

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.

A high AUDPC may indicate a shift in population dynamics due to selection pressure. *B. fabiopsis* causes symptoms that are indistinguishable from those of *B. fabae*. Comparing its AUDPC against *B. fabae* helps establish whether it poses a similar or distinct threat to regional faba bean production. Historically, *B. fabae* is recognized as the primary and most aggressive incitant of chocolate spot, and in the current experiment, it produced the highest AUDPC on each host. These results validate the status of *B. fabae* as the primary and most aggressive specialized pathogen of chocolate spot, in line with findings from Alemayehu (2024) and Haile et al. (2016). The lower AUDPC values found for *B. cinerea*, *B. pseudocinerea*, and *B. fabiopsis* highlight their differing ecological roles (Figs. 2 & 3). The limited role of *B. cinerea* affirms its classification as an opportunistic invader, rather than a primary agent of epidemic spread. These observations are in agreement with the results of Walker et al. (2011). However, literature shows that newly described species heavily distort these curves. For instance, *B. pseudocinerea* produces smaller lesions than *B. fabae* but is highly pathogenic in Northern Europe and China, significantly accelerating overall disease progress (Brauna-Morževska et al. 2023). Similarly, the high frequency and severe necrotic nature of the newly emerged *B. fabiopsis* in certain regions (Zhang et al. 2010) represents a potent driver that can sharply steepen the slope of the disease progress curve.

The variation in AUDPC across faba bean genotypes serves as a robust indicator of quantitative resistance. Unlike qualitative resistance, which may provide total immunity, the reduced AUDPC observed in specific genotypes suggests a host-mediated slowing of the infection rate or an extension of the latent period (Alemayehu et al. 2024). It captures the cumulative progress of the disease over time, offering a comprehensive view of the infection cycle. Identifying genotypes with lower AUDPC provides a framework for developing improved disease management strategies and enhancing long-term crop protection.

The NEP markers used in this study allowed the separation of many of the isolates that were indistinguishable by the earlier analysis of RPB2, HSP60 and G3PDH gene sequences (Staats et al. 2005, Hyde et al. 2014, Brauna-Morževska et al. 2023). The six *B. fabiopsis* isolates that were almost indistinguishable by the earlier sequences were separated into 5 branches of the NEP dendrogram, with only isolates 17B24 and 17F4 remaining together. Similarly, the six *B. cinerea* isolates that were almost indistinguishable by the earlier sequences were separated into 4 branches by NEP, with 17B10-1, 18B3 and 19B026 remaining together. All three *B. pseudocinerea* isolates were separated by NEP but not by the earlier analysis. In contrast, three clusters that were not separated by NEP (all containing more than one species: *B. fabae* 19B053-4 and *B. cinerea* 19B009; *B. euroamericana* 19B053-3, *B. medusae* 19B047, and *B. pseudocinerea* 18B2-3; *B. cinerea* 19B048 and *B. fabae* 17B28) were clearly distinguished by Brauna-Morževska et al. (2023). Thus, in the present set of isolates, RPB2, HSP60 and G3PDH sequences separated species, and PCR fragments of NEP genes separated isolates within species. Since 17F4 and 17B10-1 were much lower in virulence than their otherwise identical partners, the existing information on PCR fragment length is not detailed enough to allow any NEP genotypes to be associated with levels of virulence. Table S2 provided details on grouping *Botrytis* species according to the set of NEP protein markers we used in this study and the nuclear gene markers used by Brauna-Morževska et al. (2023).

Conclusions

We evaluated the pathogenicity and virulence of 21 *Botrytis* species on three inbred lines of faba bean with known CS responses through an *in vitro* detached leaf assay to study *Botrytis* by host interactions. The data showed significant interactions among the *Botrytis* species and the inbred lines, as some isolates were equally pathogenic on some pairs of lines that were separated by other isolates. *B. fabae* is the main aggressive pathogen causing CS in faba beans, while related *Botrytis* species are less aggressive. Monitoring the AUDPC helps quantify resistance across different faba bean genotypes, aiding long-term crop protection and yield prediction. The information

Agricultural and Food Science (2026) 35: xx–xx
https://doi.org/10.23986/afsci.184976

provided by NEP genotyping was complementary to that provided by RPB2, HSP60 and G3PDH gene sequences, as the sequences separated species well, while the NEP markers separated isolates within species. Our results will contribute to the understanding of the interactions of *Botrytis* and genotype in this species.

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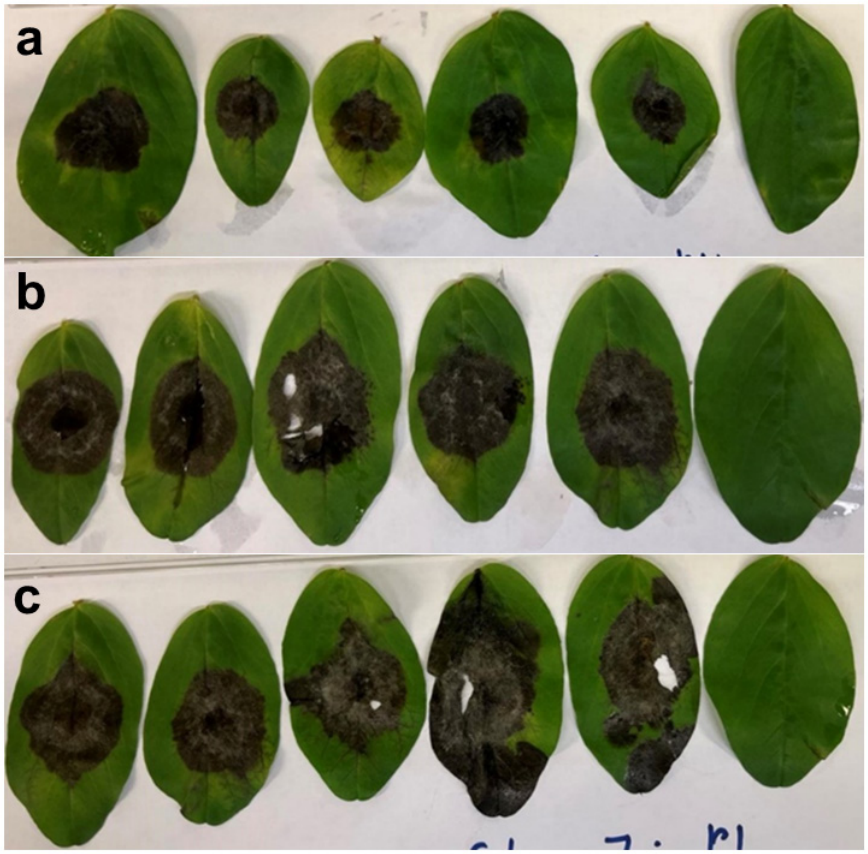
Supplemental

Fig. S1. Chocolate spot reaction in (a) ILB 938/2, (b) Mélodie/2, and (c) Kontu caused by *B. fabae* isolate 19B053-4. The rightmost leaflets show the sterile distilled water control.



Agricultural and Food Science (2026) 35: xx–xx
https://doi.org/10.23986/afsci.184976

Table S1. The NEP markers were used to genotype the 21 *Botrytis* isolates in this study.

Primer name	Primer sequence (5´-3´)	Reference
NEP1for	CCAACGCAAAATTCCTTTCTATCC	Staats et al. (2007)
NEP1revB	GTTGGCGAAGTTGTGGTCATTGAA	
NEP2forD	TTGCCTTCTCAAAATCATTACAGC	
NEP2revD	TCTAGAAAGTAGCCTTCGCAAGAT	
NEP1(-207)for	CACCTTGTGGGAGATTGTATGGGTGGATATACATC	
NEP1(+1124)rev	GGTCACCTAATTTTGGCTTTCAGGGTC	
NEP2 (-200)for	GAACTTTGAATAGTGGGCAGTTGGG	
NEP2 (+1147)rev	GAGTTTCAGGTATATTCGTTTGGTGGA	
NEP2forE	GTGACTGTAAAACGACGGCCAGTTCATCATGGTTGCCTTCTCAAGAT	
NEP2revE	GTGACCAGGAAACAGCTATGACCAAGTAGCAGCTGCAAGATTGTTTG	
NEP2forF	GTGACTGTAAAACGACGGCCAGTTTCGGTCTTGGCATCTACAGTCAT	
NEP1revA	GTGACCAGGAAACAGCTATGACCCTTGGCGAGGTTGTTGTTGAAGTT	
NEP2forE	GTGACTGTAAAACGACGGCCAGTTCATCATGGTTGCCTTCTCAAGAT	
NEP1revA	GTGACCAGGAAACAGCTATGACCCTTGGCGAGGTTGTTGTTGAAGTT	
NEP2forF	TTCGGTCTTGGCATCTACAGTCAT	
NEP2revE	AAGTAGCAGCTGCAAGATTGTTTG	
NEP2for	TGGTTGCCTTCTCAAAATCA	Mirzaei et al. (2008)
NEP2rev	GCCTTCGCAAGATTGTCTG	
$C_{729+}$for	AGCTCGAGAGAGATCTCTGA	Rigotti et al. (2002)
C_{729-}rev	CTGCAATGTTCTGCGTGGAA	

Agricultural and Food Science (2026) 35: xx–xx
https://doi.org/10.23986/afsci.184976

Table S2. Molecular diversity comparison between the NEP protein markers (this study) and nuclear gene markers (Brauna-Morževska et al. 2023)

Botrytis isolates	Separation by the NEP gene and nuclear gene markers	Pathogenicity and separation
B. fabiopsis 17B24, *B. fabiopsis 17F4*	Not separated by either method	*B. fabiopsis* 17B24 >*B. fabiopsis* 17F4
B. cinerea 17B10-1, *B. cinerea*19B026, *B. cinerea* 18B3	Not separated by either method	*B. cinerea* 19B026 = *B. cinerea* 18B3 > *B. cinerea* 17B10-1
B. fabiopsis 18B16, *B. fabiopsis* 19B175, *B. fabiopsis* 17F9, *B. fabiopsis* 19B024	Not separated by nuclear gene markers, separated by NEP	NEP separates these from *B. fabiopsis* 17B24 and *B. fabiopsis* 17F4
B. cinerea 19B048, *B. cinerea*19B009, *B. cinerea* 19B014	Not separated by nuclear gene markers, separated by NEP	NEP separates these from *B. cinerea* 18B3, *B. cinerea* 17B10-1, and *B. cinerea* 19B026
B. pseudocinerea 18B6, B. pseudocinerea 18B11, B. pseudocinerea 18B2-3	Not separated by nuclear gene markers, separated by NEP	*B. pseudocinerea* 18B6=*B. pseudocinerea*18B11>*B. pseudocinerea* 18B2-3
B. fabae 17B4, B. fabae 17B28	Not separated by nuclear gene markers, separated by NEP	*B. fabae* 17B4>*B. fabae* 17B28
B. fabae 19B053-4*, B. cinerea19B009*	Not separated by NEP, separated by nuclear gene markers	*B. fabae* 19B053-4>*B. cinerea* 19B009
B. euroamericana 19B053-3, B. medusae 19B047, B. pseudocinerea 18B2-3	Not separated by NEP, separated by nuclear gene markers	*B. medusae* 19B047>*B. pseudocinerea* 18B2-3>*B. euroamericana* 53-3
B. cinerea 19B048, B. fabae 17B28	Not separated by NEP, separated by nuclear gene markers	*B. cinerea* 19B048>*B. fabae* 17B28
B. cinerea B05.10	Not tested by nuclear gene markers	