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ORIGINAL ARTICLE

Identification of *Elsinoë phaseoli* causing bean scab in Kenya and evaluation of sporulation using five adapted techniques

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Abstract

This research addresses the presence of *Elsinoë phaseoli* in Kenya, where information on the biology of this pathogen remains scarce. Employing a multifaceted approach, the study demonstrates the steps taken to isolate, identify, and characterize *E. phaseoli* as the pathogen responsible for scab on common bean. Field observations confirmed scab symptoms, particularly the prominent pod lesions. *Elsinoë phaseoli* was isolated from common bean using a targeted streaking method on older acervulus-bearing lesions. Morphological examinations revealed a notable diversity within *E. phaseoli* colonies, consistent with the characteristics of the genus. Molecular identification through ITS-rDNA sequencing confirmed isolate AscoSK1 obtained in this study as belonging to *E. phaseoli*, offering a robust species differentiation method. Assessing conidium production required the implementation of five different culture methods. An adaptation of the Scheper et al. (2013) method yielded the highest quantity of conidia from 25 colonies spaced at 1 cm apart, with a conidial yield of 5.0×10^6 conidia per 9-cm-diameter Petri dish. A higher conidial yield was attained after the colonies were pre-incubated on potato dextrose agar in the dark at room temperature for 28 days, followed by a transfer to corn meal agar for an additional 2 days at 20°C. This emphasizes the pivotal influence of incubation duration and pre-culture conditions on the process. This research provides insights into the biology of *E. phaseoli* and introduces an improved method for enhancing in vitro sporulation of the pathogen, setting groundwork for future research and handling.

KEYWORDS
aetiology, Elsinoë, Phaseolus vulgaris

1 | INTRODUCTION

Bean scab, caused by the fungus *Elsinoë phaseoli*, has been linked to significant yield losses in common bean (*Phaseolus vulgaris*) in Kenya. Our previous study (Masheti et al., 2024) first reported that *E. phaseoli* is the causative agent of bean scab in Kenya. Jenkins (1931) first implicated *E. phaseoli* in causing scab on lima bean (*Phaseolus lunatus*) in North America, and Phillips (1994) identified it as the cause of scab on common bean in South Africa. Additionally, its asexual form, *Sphaceloma* sp., has been recognized as the causal agent of scab on cowpea (*Vigna unguiculata*) in Africa, where both *Elsinoë* in common bean and *Sphaceloma* in cowpea are considered endemic (Allen et al., 1996; Mbong et al., 2012). In accordance with the 'one fungus = one name' concept, *Sphaceloma* was placed under the

genus *Elsinoë* (Fan et al., 2017), which falls within the Myriangiales order of the class Dothidiomycetes (Hyde et al., 2013; Li et al., 2011).

Elsinoë species are recognized for causing 'signature-bearing diseases', characterized by cork-like necrotic tissues—referred to as scabs. Notably, mycologists in the mid-20th century proposed new *Elsinoë* species based solely on disease symptoms, even without observing sporulation on specimens (Jenkins et al., 1946). On common bean, scab-like symptoms manifest on all above-ground plant parts and serve as a primary indicator of the disease. Symptoms on leaves manifest as round to irregular light-green lesions that evolve into raised patches of grey, brown, or brick-red necrotic tissue. On the stems, elongated, raised corky lesions develop, often leading to twisting and distortion of the stems. The most prominent symptoms of bean scab are pod lesions, appearing as raised, rough-textured, circular to ovoid or sub-circular lesions ranging in colour from grey to liver brown and dark red (Masheti et al., 2024; Oriama et al., 2024). Lesion colour varies with infection age and can even differ among lesions of the same age (Jenkins, 1931).

All members of the genus *Elsinoë* are specialized plant parasites known for their significant morphological diversity in vitro, with colonies originating from the same source often displaying various forms and colours (Fan et al., 2017; Masheti et al., 2024; Ujat et al., 2023). This diversity suggests that morphological features alone may be insufficient for species identification. Therefore, using genetic makers is recommended for molecular identification and characterization of these pathogens. In their study, Fan et al. (2017) highlight the effectiveness of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) as a locus for distinguishing various *Elsinoë* species. Further, the ITS region has successfully identified *Elsinoë ampelina*, the pathogen responsible for grapevine anthracnose (Li et al., 2018), and *Elsinoë australis*, the causal agent of spot anthracnose in poplar (Zhao et al., 2020). Spore production in vitro poses a significant challenge for many *Elsinoë* species, complicating tasks such as pathogenicity testing that require in vitro handling of these pathogens. Additionally, different *Elsinoë* species exhibit varied preferences for sporulation conditions, further complicating their study (Hyun et al., 2015; Li et al., 2018; Whiteside, 1975).

Building on the pivotal findings of Masheti et al. (2024), this study aims to refine and expand the methodologies for accurately identifying and characterizing *E. phaseoli*. It delves deeper into the symptoms and morphology of *E. phaseoli* and investigates the optimal conditions for its sporulation. By progressing from basic identification to detailed methodological and morphological analysis, this research enhances our understanding of bean scab and establishes a solid foundation for further studies into its epidemiology and management.

2 | MATERIALS AND METHODS

2.1 | Isolation and identification of *E. phaseoli* through symptoms and morphological traits

In January 2023, Bean parts displaying scab symptoms were collected from a bean field in Kakamega, Kenya, and transported to the laboratory for further processing. Distinctive symptoms were observed and documented on various plant parts. Infected bean plants were carefully brushed to eliminate soil particles before stereomicroscope examination, while light microscope analysis was done by thinly slicing infected plant samples into cross-sections. Stereo- and light microscope examinations were conducted at magnifications ranging from 1× to 5× and 40× to 400×, respectively. The thinly sliced infected sections were mounted in sterile water for fresh samples or 10% KOH for dry samples. Lacto-phenol cotton blue was applied to enhance clarity when necessary, and lesion features were observed and captured using a Motic™ microscope camera.

A streaking-based method was employed to isolate the pathogen responsible for bean scab. In this method, leaf lesions were first surface-sterilized by spraying them with 1% sodium hypochlorite solution from a hand sprayer until wet and then blot-dried with sterile tissue. A second spray of 95% ethanol followed and was also blot-dried using a sterile tissue. Scab lesions measuring not more than 5 mm each, three from the same leaf, and containing mature acervuli were selected under a dissecting microscope, cut using a sterile scalpel blade, and transferred to 1-mL Eppendorf tubes containing 50 µL of 0.005% Tween 20 solution. The lesions in the solution were left on the laboratory bench for 1–3 h while being periodically gently disturbed by a sterile dissecting needle. Ten microliters of the resulting suspension was then streaked using a 5-mm sterile wire loop onto 15-cm-diameter Petri dishes one-third filled with freshly poured potato dextrose agar (PDA) media. The space between streaks was kept at a minimum of roughly 1.5 cm to maximize the spread. The plates were then incubated in the dark at room temperature for 3 weeks to facilitate culture development. Sections were systematically excised from the expanding filamentous cultures for purification as they developed; single-tip sections were taken from standard cultures, and 1-mm^2 sections from denser, more compact cultures. These sections were then cultured onto 9-cm-diameter Petri dishes that were one-third filled with freshly poured PDA.

The process for inducing sporulation encompassed several distinct steps. Initially, the collected cultures were incubated for up to 30 days at room temperature, exposed to natural light and temperature cycles. For those cultures that did not sporulate, the Phillips (1994) method for *E. phaseoli* was employed. This involved excising 1-mm^2 sections of the culture with a sterile blade and placing them in 9-cm-diameter Petri dishes. Just enough sterile water was added to cover the tops of the agar plugs without submerging the micro-colonies. These dishes were then sealed with Parafilm and incubated overnight at 23°C.

Following this incubation period, any plates with isolates that had successfully sporulated were flooded with 50 mL of 0.005% Tween 20 solution. The plates were gently swirled and scraped to release the conidia into the solution. The spore suspension was then decanted by filtering through a double-layer lens paper, and the concentration of conidia was determined using a haemocytometer. Cultural characteristics were examined under both stereo- and light microscopes at the previously stated magnifications. The cultures were morphologically characterized based on colour, texture, form, elevation, margin and mycelial and conidial characteristics

(formation, type of fruiting bodies, shapes, colour, and size). The observed features were photographed.

2.2 | Identification of *E. phaseoli* through DNA barcoding and phylogenetic analysis

For each of the 16 morphologically identified isolates, precise sections were excised from 10-day-old filamentous cultures: single-tip sections for regular cultures and 1-mm^2 sections for denser, compact cultures. These samples were placed into 1-mL tubes containing 0.5 mL of potato dextrose broth. The tubes were then set on a mechanical rotator at 5 rpm and incubated at room temperature under natural light and temperature cycle for 10 days. Post-incubation, 150 mg of mycelia were harvested from the tubes, rinsed in sterile distilled water, and transferred to microfuge tubes for subsequent DNA extraction as described by Mahuku (2004). Following this method, acid-washed sterilized sand was added to the tubes to macerate the mycelia using a pestle, along with 150 μL of TES extraction buffer (0.2 M Tris–HCl, pH 8, 10 mM EDTA, pH 8, 0.5 M NaCl, and 1% SDS, along with proteinase K at a final concentration of 50 μg/mL). An additional 100 μL of TES extraction buffer was added after maceration to rinse off sample residue on the pestle. The mixture was vortexed and incubated at 65°C for 30 min with intermittent shaking at 5-min intervals. Subsequently, the samples were incubated for 20 min at room temperature, followed by the addition of 5 μL of RNAse A (concentration: 20 mg/mL) and an additional 30-min incubation.

The solution was then treated with 250 μL of ammonium acetate solution (7.5 M) and incubated for 10 min with periodic shaking. After centrifugation at 13,000 rpm for 15 min, the supernatant was transferred to new Eppendorf tubes. Ice-cold isopropanol was added to the supernatant, and the mixture was incubated for 2 h at −4°C before another centrifugation step at 13,000 rpm for 10 min to pellet the DNA. The pellet was washed with 150 μL of DNA washing solution (0.77 g/L ammonium acetate and 70% ethanol), incubated for 15 min at room temperature, and then centrifuged at 13,000 rpm for 5 min. The supernatant was discarded, and the DNA pellet was air-dried for 1–2 h before re-suspending it in 50 μL of 1× TE buffer. Amplification utilized ITS markers with primers ITS 4 and ITS 5. PCR was conducted with a final volume of 25 μL, including genomic DNA, reaction buffer, primers, dNTPs, $MgCl_2$, and Taq DNA polymerase.

Thermocycler conditions were programmed as follows: An initial denaturation step was carried out at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 1 min. The final extension was carried out at 72°C for 8 min, followed by storage at 4°C. Subsequently, electrophoresis was conducted using a 2% agarose gel pre-stained with 0.03 mg/mL ethidium bromide. Purified PCR products were directly sequenced in both directions at Inqaba Biotec, Pretoria, South Africa. Consensus sequences of isolates with each set of primers were obtained from alignments using BioEdit version 7.2.5. Basic Alignment Search Tool (BLAST) analysis was performed on the nucleotide sequences for isolate identification.

Evolutionary analyses were conducted using MEGA11 software, employing the Maximum Likelihood method and the Kimura 2-parameter model. To construct robust phylogenies for effective comparison and identification of *Elsinoë* species, multiple trees were generated using ITS sequences of *Elsinoë* isolates available in the GenBank database, whereby *Myriangium hispanicum* (CBS 347.33) was selected as the outgroup species as in Fan et al. (2017). Manual alignment and adjustments of both individual gene sequences and combined datasets were performed using BioEdit software. The initial trees for the heuristic search were generated automatically by the Neighbour-Joining and BioNJ algorithms, which utilized a matrix of pairwise distances estimated through the Maximum Composite Likelihood (MCL) approach. The tree topology that demonstrated the highest log likelihood value was selected.

2.3 | Completion of Koch's postulates

In total, 16 candidate isolates were used to validate Koch's postulates for disease causation via two replicate experiments, with each isolate inoculated onto a set of eight 3-week-old susceptible *P. vulgaris* 'GLP 2' plants. For the 11 isolates that successfully sporulated under benchtop conditions, the concentration of conidia was standardized to 5×10^6 conidia per mL. Of the five isolates subjected to the Phillips (1994) method, only AscoSK1 managed to sporulate, producing 6.5×10^5 conidia per 9-cm-diameter Petri dish. This yield was used without adjustment as it fell below the standard concentration.

To improve the likelihood of infection, particularly due to the low conidial output observed for isolate AscoSK1, a combined approach involving the cotton-plaster method and the spray method was utilized. The cotton-plaster method, which was adapted from Bruner and Jenkins (1933) for infecting *P. lunatus* with *E. phaseoli*, was implemented alongside the traditional spray method to enhance effectiveness. This approach involved first dipping a piece of wet absorbent cotton into the conidial suspension and then applying it to the upper surface of expanding primary or trifoliate leaves. In addition to this direct application, entire plants were also manually sprayed with additional inoculum at specified concentrations. This was done using a hand atomizer to create a fine mist, ensuring the leaves were thoroughly wetted but not dripping, to maximize the spread and contact of the inoculum. Afterwards, the plants were enclosed in polythene bags and kept in the dark at room temperature for 18–20 h to promote infection.

The polythene bags and cotton wool were then removed, and the plants were transferred to a screenhouse with a 12-h natural light and temperature cycle. Control plants, left uninoculated (distilled water without conidial suspension was used), were kept at a distance of 2 m away to prevent cross-infection. Symptom development was observed over a 21-day period, with successful infections identified by the presence of distinct scab lesions. The process of re-isolating and identifying the pathogen, necessary to complete Koch's

postulates, followed the procedures specified in the initial isolation process.

2.4 | Conidium production and viability

Five techniques, previously used with various *Elsinoë* species, were selected and tested to determine their effectiveness in inducing conidial sporulation in *E. phaseoli* in two independent replicate experiments. Among the five methods, two were based on Scheper et al. (2013), differing only in the duration of pre-incubation of *Elsinoë* colonies. For each protocol, five plates of 25 1-mm *E. phaseoli* colonies spaced at 1 cm apart were established using a similar batch of colonies. Based on the method of Phillips (1994), PDA plates were incubated at room temperature for 7 days. Twenty-five 1-mm^2 micro-colonies were then cut and placed in 9-cm-diameter Petri dishes at a spacing of 1 cm apart. An equal volume (5 mL) of sterile water was added to each plate until it reached the tops of the agar plugs without submerging the micro-colonies, and the plates were put in a sealable steel jar and incubated for 12 h (overnight) at 23°C. The plates were then swirled to dislodge the conidia.

Based on the Scheper et al. (2013) method, *Elsinoë* colonies were cultured on 9-cm-diameter PDA plates for 14 and 28 days at room temperature, after which 25 1-mm^2 pieces of colonies were subcultured from PDA onto 9-cm-diameter corn meal agar (CMA) Petri dishes at 1 cm apart. CMA plates with colonies were then incubated in the dark at 20°C for 2 days before the colonies were removed using sterile dissecting needles and placed in 50-mL Eppendorf tubes containing 5-mL 0.005% Tween 20 solution (5 colonies/mL) that was gently disturbed to dislodged conidia.

Based on the method of Hyun et al. (2015), 25 colonies of *Elsinoë* spaced at 1 cm apart were grown in 9-cm-diameter Petri dishes containing PDA for 21 days at room temperature. The colonies were mashed on a plate with a spatula to produce fungal fragments. These fragments were then placed into 50 mL of sterile rainwater at room temperature and agitated at 180 rpm in a shaking incubator for 24 h. Based on the Li et al. (2018) method, PDA medium was poured into 9-cm-diameter Petri dishes, and 25 10-day-old mycelial plugs of *E. phaseoli* each measuring 2-mm^2 and spaced at 1 cm apart were placed on the cooled PDA plates for 25 days in the dark at room temperature. PDA plates were then incubated at 21°C for 24 h in the dark, after which an equal volume (5 mL) of distilled water was added to each plate and colony surfaces were gently scraped with a brush to obtain conidial suspensions.

Following each procedure, the resulting suspension was first filtered through a double-layer sterile lens paper before being adjusted to a total volume of 50 mL by adding 0.005% Tween 20 solution, whereby the conidial concentration was measured with a haemocytometer. The viability and ability to infect conidia were assessed in two stages. Initially, a 100-µl suspension containing 10^3 conidia/mL was spread onto three replicates of CMA and PDA plates. These plates were then incubated at room temperature for 7 days in darkness. Subsequently, the virulence was tested through detached leaf inoculation using susceptible *P. vulgaris* 'GLP 2'. In the detached leaf procedure, three replicates, each comprising three expanding leaves of *P. vulgaris* 'GLP 2', were selected from 21-day-old plants grown in the screenhouse. The detached leaves were surface-sterilized with 70% alcohol for 10 s, rinsed three times with sterile water and placed in 15-cm-diameter Petri dishes lined with wet sterile tissue paper before being drop-inoculated with conidia in 0.005% Tween 20 solution at a density of 200 conidia per leaf. The control leaves were drop-inoculated with Tween solution without conidia. The plates were then covered to maintain high humidity and placed in the dark for 18 h before being transferred to the laboratory bench to be observed daily for symptoms of bean scab, from 1 to 14 days post-inoculation (dpi).

Statistical analysis was conducted using RStudio to evaluate the efficacy of different sporulation techniques through ANOVA and subsequent post-hoc testing. The analysis utilized the readxl package to import data from Excel files (Wickham & Bryan, 2019), the dplyr package for data manipulation (Wickham et al., 2021), and the ggplot2 package for visualization of the data (Wickham, 2016). For post-hoc testing, Sidak adjustment was used for pairwise comparisons between treatment groups after finding a significant result in the ANOVA, while mean comparisons were made at a 95% confidence level using the Kenward–Roger approximation.

3 | RESULTS

3.1 | Isolation and identification of *E. phaseoli* through symptoms and morphological traits

Bean scab symptoms were extensively observed on common bean leaves, stems, and pods, spanning various plant developmental stages (Figure 1). Typically, scab lesions exhibited a range of colours, starting from shades of grey to brown to liver brown and darkened with age. Scab symptoms were observed on both the lamina and veins of common bean leaves. On the lamina, symptoms appeared as slightly sunken light-green round to irregular lesions of up to 3 mm wide, which were predominantly visible on the upper side of the leaf. Over time, these lesions progressed to develop into superficially raised regions of grey, brown, or brick-red cork-like necrotic tissue. Occasionally, the lesions merged, forming necrotic regions that sometimes tore off, giving the leaves a tattered appearance. Early midrib and leaf vein symptoms manifested as elongated lesions visible on the lower side of the leaf that extended parallel to the leaf veins and midrib, without spreading to the lamina tissue. In some cases, infected veins twisted or bent, causing the whole or part of the leaf to curl inward, resulting in leaf distortion. Under favourable disease conditions, leaf distortion manifested in plants as early as 3 days after germination. Severely affected plants may undergo complete premature defoliation.

Scab symptoms on stems resembled those described for leaf veins, as they appeared mostly elongated and running parallel to the

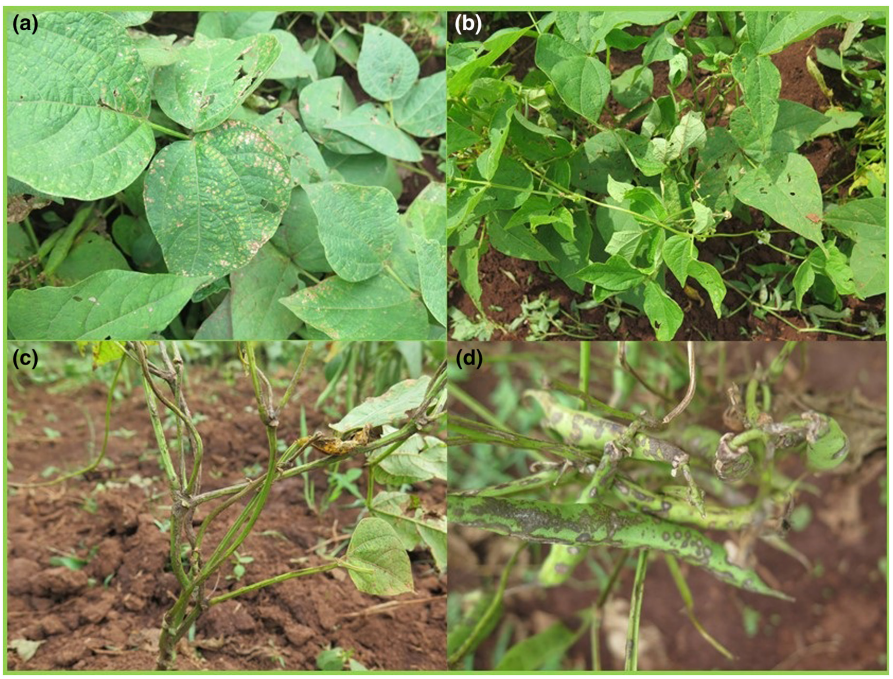

FIGURE 1 Scab symptoms observed on common bean plants. (a) Grey scab lesions evident on the leaf surface. (b) Scab-infected plant displaying stem twisting and distortion alongside leaf curls. (c) Severely infected stems featuring grey, cork-like scab lesions. (d) Scab lesions present on common bean pods.

stems, reaching a maximum length of approximately 2 cm, merging in severe cases to form superficial cork-like casts. Occasionally, the lesions appeared as irregular-shaped non-elongated corky bumps protruding on the stem's surface. Like in the leaves, severely infected developing stems frequently exhibited twisting and distortion, giving them a vine-like appearance, often accompanied by subsequent poor leaf formation or distortion. Pod lesions were the most easily recognizable bean scab symptoms. Initially, scab symptoms on green pods appeared as round to irregularly shaped light-green sunken watery spots of up to 3 mm wide. These spots gradually transformed into raised cork-like lesions of typical range of colours and mostly grey sunken centres. The edges of pod lesions occasionally turned brick red. Lesions without acervuli had a shiny-gelatinous surface, but when acervuli were present, they appeared as numerous superficial dark spots. Individual lesions could reach a maximum size of 10 mm but could coalesce and cover the entire pod surface or just a single valve. In severe infections, the pods became partially or completely enveloped in a uniform mummifying cast-like lesion. Young pods affected by symptoms often experienced severe malformation (twisting, distortion, or shrivelling) with subsequent poor seed formation. Heavy scab infection also occasionally caused slight opening of the pod suture exposing the seeds inside.

Microscopic examination of cross-sections of symptomatic plant tissue revealed erumpent epidermal ascomata exhibiting a range of colours, from grey to brown to brick red, occasionally with mixed coloration. These ascomata contained numerous locules, hosting bitunicate, globulose to sub-globulose asci irregularly arranged in single or multiple layers. The ascospores, measuring up to 10 μm × 5 μm, were hyaline to brownish, transeptate (1–3 septa) or muriform, irregularly positioned within the asci (Figure 2).

Pseudoparenchymatous acervuli, appearing on the surface of ascomata, contained hyaline to pale-brown conidiophores. These conidiophores produced hyaline, single-celled, oblong-elliptical conidia, mostly biguttulate and measuring up to 5 μm × 2 μm (Figure 2). Although this type of conidium was the most frequently observed, diversity in conidial forms was evident in some cross-sections. In certain cases, various types of conidia were found within the same lesions. Consequently, 16 morphologically distinct isolates were isolated from scab lesions. They ranged from filamentous fast-growing to compact slow-growing forms.

Elsinoë phaseoli was identified as one of the compact, slow-growing isolates and was tagged isolate AscoSK1. Distinctive *Elsinoë* characteristics were observed under a dissecting microscope after 4 days of incubation, with colonies measuring less than 1 mm in diameter. It featured irregular forms and extremely slow growth on PDA media as well as varied colorations ranging from orange to brown to dark red, at times displaying a combination of these tones (Figure 3). The colony surface exhibited cerebral or corrugated indentations, had undulated margins, and were either 'crusty' or 'leathery' in consistency. The crusty colonies tended to be predominantly compact, while the leathery colonies manifested in two distinct forms; they either appeared compact and solid or took on the shape of a bouffant cap, featuring an internal hollow structure. The hollow leathery colonies with a bouffant shape housed a cluster of gelatinous cells within their interior. When viewed under a light microscope, the crusty colonies were composed of irregular to ellipsoidal-shaped single cells that occasionally had one septum. These cells could multiply through budding or grow by forming germ-tube-like projections that stretched out to form hyphae (Figure 4). The gelatinous mass from underneath the leathery colonies showed similar characteristics, while the leathery surface was mostly mycelial.

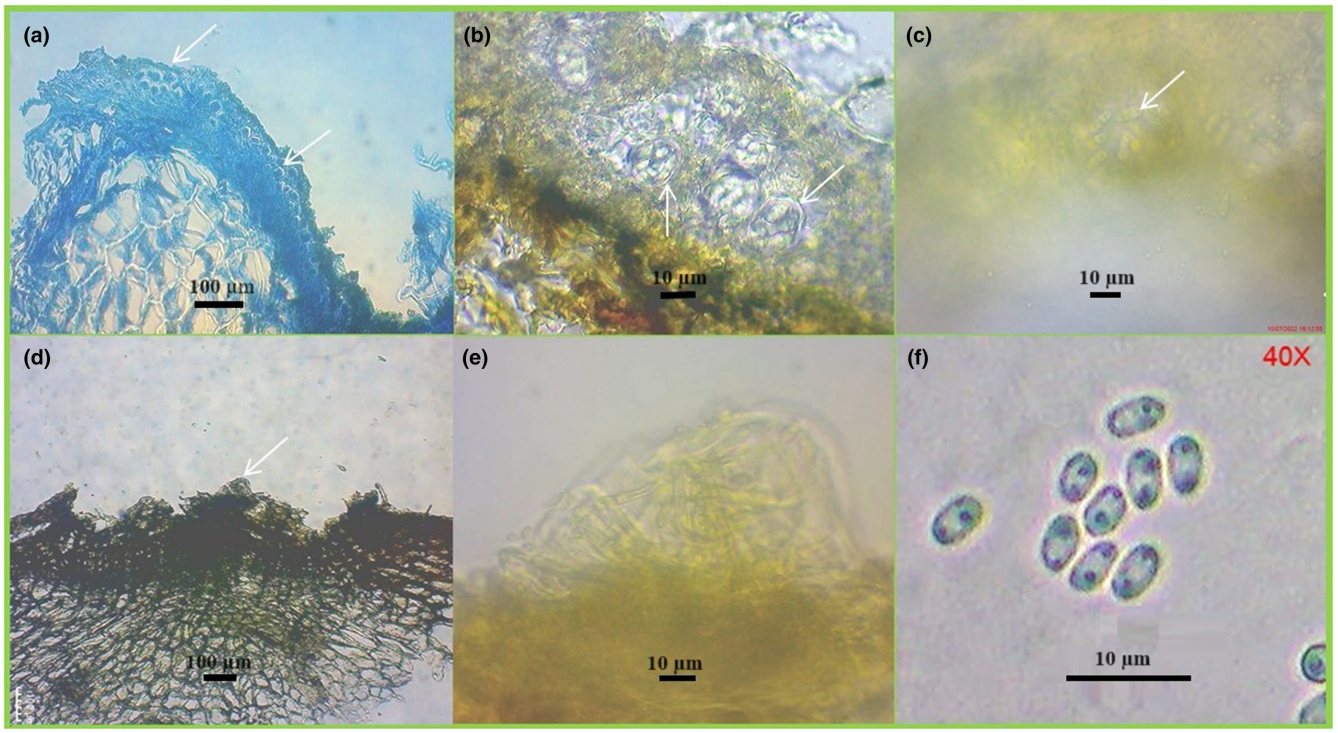

FIGURE 2 Sexual and asexual structures of *Elsinoë phaseoli*. (a–c) Sexual structures, with (a) stained cross-section revealing mainly epidermal ascomata, erumpent in nature, containing numerous locules, with arrows indicating locules containing asci. (b) Bitunicate, globulose to sub-globulose asci within the ascomata. (c) Multi-segmented ascospores irregularly arranged within the asci, indicated by arrow. (d–f) Asexual structures, with (d, e) superficial acervuli on scab lesions. (f) Biguttulate conidia of *E. phaseoli*.

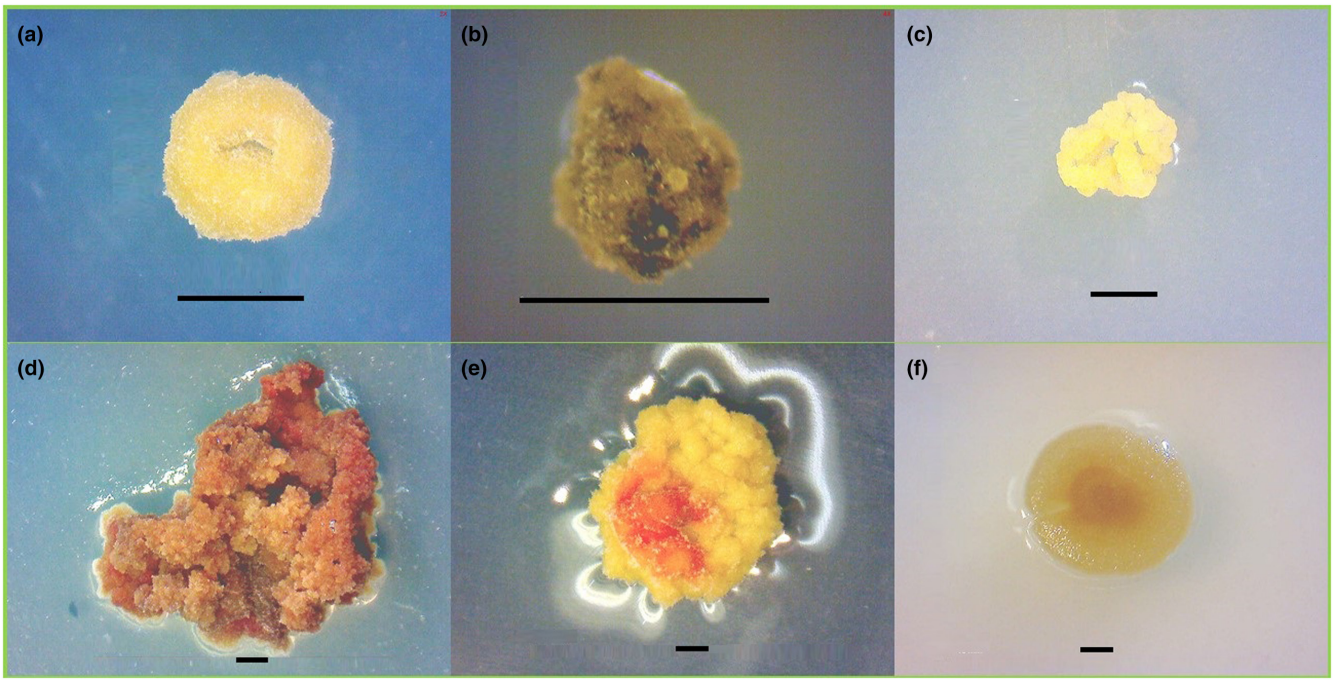

FIGURE 3 Colonies of *Elsinoë phaseoli*. (a–c) Four- to 7-day-old colonies of *E. phaseoli* grown on potato dextrose agar media. (d–f) Morphology of 35-day-old *E. phaseoli* colonies, with (d, e) showing colonies grown on potato dextrose agar media, and (f) showing colonies grown on corn meal agar media. Bars = 1 mm.

All AscoSK1 colony forms became compact approximately 4–7 days into their growth on PDA. These matured colonies exhibited irregular shapes, slow growth, and raised patterns with cerebral or corrugated surface features, accompanied by undulated margins and occasionally displaying a blend of orange, brown, and dark red hues that intensified with ageing. The shifts in colour seemed

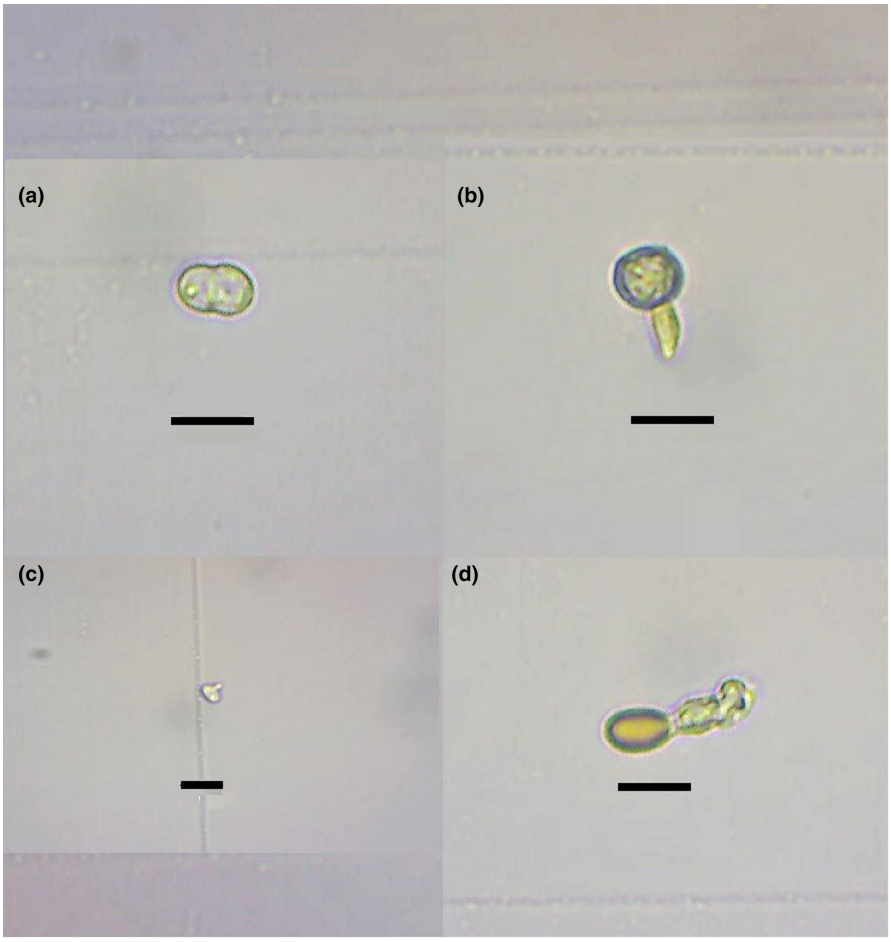

FIGURE 4 *Elsinoë phaseoli* growth and multiplication. (a) Early-stage budding of young *E. phaseoli* colony cells. (b) 'Germ-tube' like growth of mycelium from *E. phaseoli* colony cells. (c, d) Conidium germination. Bars = 10 μm.

haphazard and varied, even among colonies from the same source. However, colonies within the same plates tended to maintain consistent morphologies, even when unique combinations were observed. Upon transfer to CMA, all distinct colony types developed into a compact, slowly growing, cream-coloured, circular, raised mass of mycelia with entire edges. The colonies obtained a maximum diameter of 10 mm even after more than 3 weeks at room temperature on both PDA and CMA media.

The 11 isolates that underwent sporulation on the benchtop at room temperature successfully produced a large number of conidia. In contrast, among the five isolates subjected to the Phillips (1994) method, only AscoSK1 sporulated. The conidia from AscoSK1 were oblong or elliptical, hyaline, and mostly biguttulate, measuring up to 6 μm × 3 μm, originating at the tips of slightly pointed conidiogenous cells. The conidiophores were hyaline, unbranched, and compacted to form acervuli on the colony surface.

3.2 | Identification of *E. phaseoli* through DNA barcoding and phylogenetic analysis

The DNA of the 16 morphologically selected isolates was successfully extracted, and their ITS regions were sequenced. BLASTn search revealed species from seven distinct genera: *Aureobasidium*, *Cladosporium*, *Fusarium*, *Microdochium*, *Nigrospora*, *Colletotrichum*, and *Elsinoë* (AscoSK1). Notably, isolate AscoSK1 showed a 100% sequence identity with *E. phaseoli*, as referenced by GenBank Accession No. KX887266.1. Further phylogenetic analysis of AscoSK1 ITS sequences showed that the isolate clustered into the same clade with *E. phaseoli* species from GenBank, with a high bootstrap value of 95%, confirming AscoSK1 as *E. phaseoli*. The topology of the tree was congruent in terms of species grouping which included *E. leucospermi*, *E. protearum*, *E. punicae*, and *E. freyliniae* grouping as clearly separated from *E. phaseoli* and from each other. The outgroup species *M. hispanicum* had a clear separation from all *Elsinoë* species (Figure 5). The ITS sequence of *E. phaseoli* obtained from this study was deposited in GenBank (OR686938.1).

3.3 | Completion of Koch's postulates

When inoculated with AscoSK1, all eight plants exhibited typical scab symptoms, whereas the control plants remained symptom-free. This outcome was consistent across two independent replicate experiments. Additionally, none of the other 15 isolates induced any disease symptoms. Symptoms similar to those observed in the field, including scab spots, leaf curling, and stem twisting, appeared between 6 and 10 days post-inoculation. By 21 days, further symptoms such as chlorosis, defoliation, and stem breakage emerged,

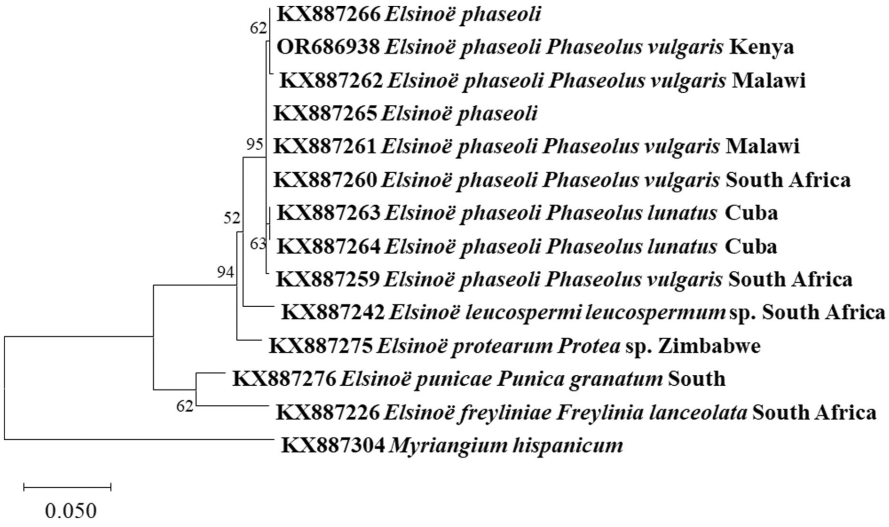

FIGURE 5 Phylogenetic tree with the highest log likelihood (−1291.10) drawn to scale. Isolate OR686938 represents *Elsinoë phaseoli* obtained in this study, while the rest of the sequences were obtained from GenBank. Branch lengths were measured in the number of substitutions per site. Analysis involved 13 nucleotide sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

with infected plants showing reduced vigour and stunted growth (Figure 6).

When cross-sections of the lesions were microscopically analysed, numerous locules containing bitunicate, globulose asci, and transeptate or muriform ascospores were detected, along with biguttulate, hyaline, oblong-elliptical conidia. Re-isolation using the streaking method yielded slow-growing, dark red, orange, or brown colonies with a corrugated surface. Additional sporulation, initiated via the Phillips (1994) method, produced conidia similar to those found in the AscoSK1 isolate, thereby satisfying Koch's postulates. This confirmation solidified AscoSK1 as *E. phaseoli*, the causal agent of bean scab.

3.4 | Conidium production and viability

The procedures outlined by Scheper et al. (2013) led to increased conidial production of *E. phaseoli*. Specifically, employing these methods with 25 colonies each spaced at 1 cm^2 within a 9-cm-diameter Petri dish achieved a conidial yield of 5.0×10^6 conidia per plate when pre-incubated in the dark at room temperature for 28 days before being transferred to CMA and incubated in the dark at 20°C for 2 days, compared with colonies pre-incubated for 14 days under similar conditions. However, even colonies aged 14 days in the Scheper et al. (2013) adapted protocols produced more conidia, with a total concentration of 2.35×10^6 conidia per plate, than the protocols adapted from Phillips (1994) and Li et al. (2018), which resulted in yields of 6.5×10^5 per plate and 2.75×10^5 per plate, respectively. Additionally, colonies did not undergo sporulation following the shaker-based protocol described by Hyun et al. (2015) (Figure 7).

To validate the viability and ability to cause disease of the harvested conidia, a 100-µL suspension containing 10^3 conidia/mL from each protocol was evenly spread onto CMA and PDA plates. Additionally, the suspension was applied to detached leaves of the susceptible *P. vulgaris* 'GLP 2' at a density of 200 conidia per leaf. Across all five protocols, the suspensions resulted in a dense colony mat covering more than 75% of the 9-cm-diameter PDA Petri dishes. Successful inoculation of detached leaves was observed for each successful protocol,

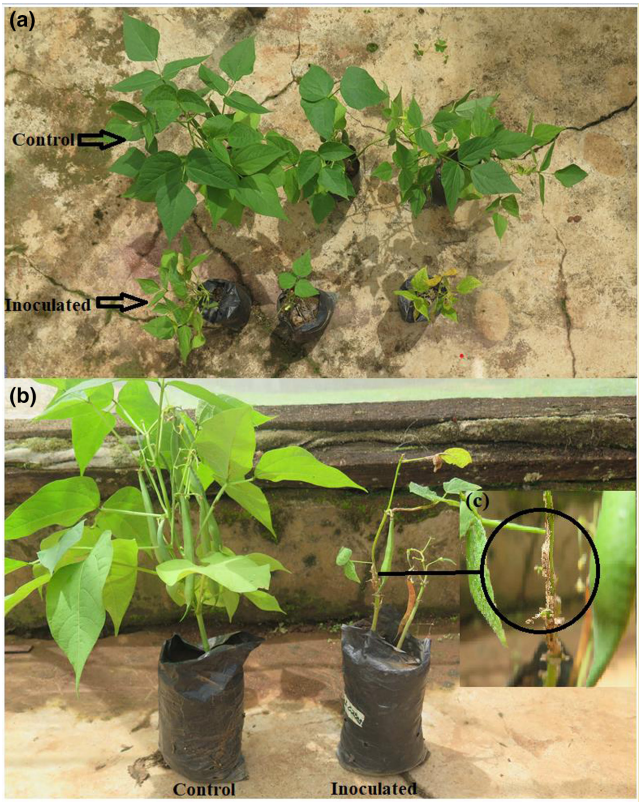

FIGURE 6 *Phaseolus vulgaris* 'GLP 2' in a screenhouse. (a, b) Contrast between healthy control and plants inoculated with *Elsinoë phaseoli* conidia. (c) Grey corky scab lesion on stem of inoculated bean plant.

with the most prevalent symptoms being the formation of lesions on the veins, occasionally accompanied by vein distortion.

4 | DISCUSSION

Easily recognizable 'signature' scab symptoms such as cork-like necrotic tissues, leaf curling, stem, and pod distortions were observed

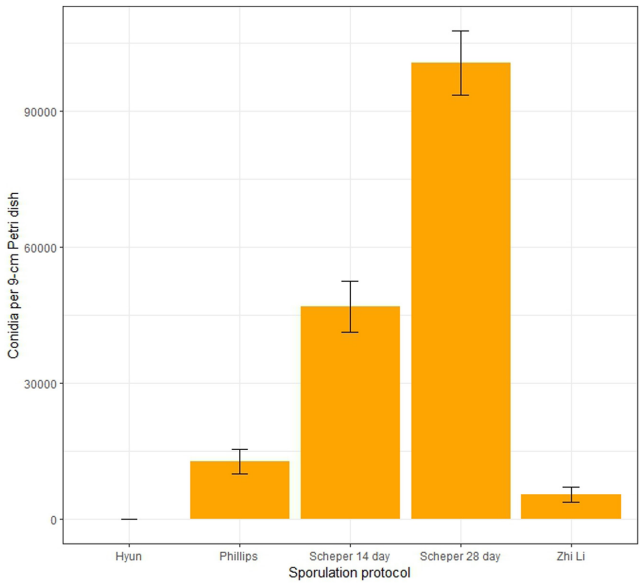

FIGURE 7 Conidial concentration obtained with five sporulation protocols adapted for *Elsinoë phaseoli*. Mean separation bars above the columns highlight significant differences ($p < .05$) between the conidial yields of the protocols. Values are means and standard errors of two replicate experiments.

on the leaves, stems, and pods of common bean plants. These symptoms occurred at various stages of development and affected the plants' vigour, structure, and pod quality with pod symptoms the most easily identifiable. The symptoms observed in this study matched those observed for *E. phaseoli* causing scab in lima bean (Bruner & Jenkins, 1933) and in common bean (Phillips, 1994) and are believed to be mostly universal for members of the genus *Elsinoë* (Fan et al., 2017; Ujat et al., 2023). Locules containing bitunicate asci containing irregularly arranged multi-segmented ascospores observed in lesion cross-sections were consistent with the sexual stage of the order Myriangiales in the class Dothidiomycetes (Hyde et al., 2013; Li et al., 2011), to which the genus *Elsinoë* belongs and has been reported in related species such as *E. ampelina* (Braga et al., 2020). Additionally, the hyaline, predominantly biguttulate conidia observed in this study, match those given by Jenkins (1931) and Phillips (1994) for *E. phaseoli*.

In many cross-sections, more than one type of conidia was observed within the same lesions. The presence of various organisms within scab lesions, extensively documented (Scheper et al., 2013; Whiteside, 1986), complicates in situ diagnosis of *E. phaseoli* as the cause of scab symptoms. Consequently, isolating and purifying potential pathogens for accurate identification was essential. Although the streaking method successfully isolated *E. phaseoli* from scab lesions, the success rate remained relatively low at about 25%, even under consistent conditions. Isolating *E. phaseoli* was particularly challenging; several other techniques, including those employed by Afutu et al. (2016) for *Sphaceloma* sp. and Zhao et al. (2020) for *E. australis* that involved whole tissue plating, were attempted but proved ineffective in reliably isolating the pathogen. A major challenge was the faster growth of opportunistic microorganisms, which often overshadowed the growth of *Elsinoë* colonies in the culture media. Scheper et al. (2013) raised the possibility of antagonistic interactions between *E. pyri* and yeast contaminants in media. The difficulty in isolating the pathogen might also stem from the lack of fertile structures in scab lesions, as noted by Whiteside (1986) and Fan et al. (2017). In this study, employing older lesions with acervuli and using the streaking method enhanced the likelihood of retrieving fertile structures and minimized the chance of faster-growing contaminants overshadowing the slow-growing *E. phaseoli* colonies. Scheper et al. (2013) failed in isolating E. pyri from leaves without acervuli.

There was remarkable morphological diversity of *E. phaseoli*, whereby colonies from the same origin displayed diverse forms and colours. Previous studies by Bruner and Jenkins (1933) and Phillips (1994) have documented the morphological diversity of *E. phaseoli*, and Fan et al. (2017) noted that such variations are common in cultures belonging to *Elsinoë* spp. The uniformity of colony morphology observed in this study across plates handled similarly suggests that aeration may be a key factor influencing the morphological variability of *E. phaseoli*. It is well known that aeration can induce morphological diversity in fungi, as highlighted by Wecker and Onken (1991). Furthermore, Cho et al. (2002) have linked aeration to the production of red pigment in *Elsinoë*. Additionally, Papagianni (2004) and Li et al. (2018) have demonstrated that aeration significantly affects colony formation in *Elsinoë*. This study recorded the initial single-celled stage of *E. phaseoli*, which includes cell division via budding and expansion through germ-tube formation, behaviours that Gabel and Tiffany (1987) previously noted in *E. panici*. This finding provides unique insights into the growth mechanisms of *E. phaseoli*.

Several isolates identified in this study displayed traits consistent with the photographic and textual descriptions of *Sphaceloma* sp. by Afutu et al. (2016). However, molecular analysis later confirmed these isolates as *Fusarium* sp. It is highly likely that the isolates described by Afutu et al. (2016) might also be *Fusarium* sp. Notably, the descriptions presented include traits such as multi-segmented conidia, white and pink cultures, and no mention of slow growth of the isolates. In fact, the photos presented show luxuriant growth of the pathogen, with 4-week-old cultures appearing to have completely filled the plates. This level of growth is highly unlikely for any member of the genus *Elsinoë* (Fan et al., 2017; Ujat et al., 2023).

The ITS region was sufficient for identifying *E. phaseoli*. A BLASTn search of the sequence from the *E. phaseoli* obtained in this study (GenBank Accession No. OR686938.1) showed a 100% match with the *E. phaseoli* sequence reported by Fan et al. (2017) (GenBank Accession No. KX887266.1). Further phylogenetic analysis confirmed its placement within the *E. phaseoli* clade. The ITS region is recognized as a dependable locus for distinguishing most *Elsinoë* species and differentiating between various genera, as highlighted by Pham et al. (2021). Fan et al. (2017) found that the ITS region could accurately identify up to 82.4% of species within the *Elsinoë* genus. Additionally, it has been effectively

used to identify *E. ampelina* (Li et al., 2018) and *E. australis* (Zhao et al., 2020), responsible for grapevine anthracnose and spot anthracnose in poplar, respectively.

While 16 isolates obtained from scab lesions were morphologically identified and subjected to Koch's postulates, only AscoSK1 induced disease reactions under the established conditions. Initial attempts to induce disease in bean plants using a hand atomizer to spray-inoculate them were not successful, likely due to insufficient sporulation of *E. phaseoli*. Drawing on Scheper et al. (2013), who noted that a substantial inoculum load of *E. pyri* is necessary for successful infection in apple, a different approach was necessary. Therefore, the cotton-plaster technique was adopted alongside spraying, which proved effective. Bruner and Jenkins (1933) had previously highlighted the suitability of the cotton-plaster method for *E. phaseoli*, particularly when conidia are scarce and the focus is on disease response. This method transfers mycelial cells directly to the plant, securing them in place to encourage further sporulation and enhance infection likelihood. However, this technique makes it challenging to precisely quantify the amount of *E. phaseoli* conidia that contact the plant. Morphological analysis of the re-isolated pathogen confirmed *E. phaseoli* as the pathogen responsible for scab of common bean in Kenya.

The most successful conidial yields (5.0×10^6 conidia per plate) were obtained using methods adapted from Scheper et al. (2013), which itself was derived from Jenkins (1932). Conversely, the shaker-based method by Hyun et al. (2015) did not yield any conidia. The protocol modified from Phillips (1994) produced fewer conidia than the methods of Scheper et al. (2013) However, it offered the benefit of shorter incubation periods, lasting only 7.5 days, as opposed to at least 16 days required by the quickest method from Scheper et al. (2013) Maintaining the recommended colony spacing of <1 colony per cm^2 using the Phillips method, which involved incubating micro-colonies in sterile water, proved challenging as the colonies often floated and clumped together or adhered to the plate walls. This may pose a challenge since colony-density-dependent conidium production has been observed in *Elsinoë* spp. (Kono et al., 2009; Scheper et al., 2013). Additionally, dislodging conidia from cultures using a brush as in the Li et al. (2018) method resulted in a suspension primarily composed of hyphal fragments with only a few conidia, making it challenging to determine the concentration of conidia in the suspension. Although the Hyun et al. (2015) method successfully induced sporulation in *E. fawcettii*, conidium production differed among isolates, likely due to variations in their colony characteristics. Consequently, this method may not be appropriate for *E. phaseoli*.

All pre-incubation was conducted in the dark at room temperature (23–26°C) in Kakamega, Kenya, where the experiment took place in May 2023. The average outside temperatures during this month was approximately 20°C, with a maximum day temperature of 27°C and a minimum night temperature of 16°C. Li et al. (2018) observed that the pre-culture temperature is less critical than the final incubation temperature for conidial formation in *E. ampelina*. Conidial production in *E. ampelina* occurs at temperatures of 2–40°C, with optimal sporulation at 20°C (Carisse & Lefebvre, 2011). Additionally, Scheper et al. (2013) reported that the optimal temperature for conidial germination is likely between 20 and 26°C, which fall within the range of room temperature in Kakamega, Kenya. Viability and virulence results were similar for each successful method, indicating that the selected sporulation treatments did not significantly affect conidium vitality.

Enhanced knowledge of *E. phaseoli* biology and the development of basic handling techniques for the pathogen lay the groundwork for analysing key epidemiological factors. These factors include the host range and diversity of *E. phaseoli* in Kenya, which are crucial for its effective management.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper. All authors have no financial or personal relationships that could inappropriately influence or bias the content of the work presented. This includes affiliations, funding sources, or involvement with any organization or entity with financial interest in the subject matter discussed in this manuscript. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All results and interpretations are based solely on the data and analysis performed by the authors.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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