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Evaluation of cellulase production by endophytic fungi isolated from young and mature leaves of medicinal plants using maize cob substrate

Peter K. Mwendwa[1]✉, George Isanda Omwenga[1], John M. Maingi[1,2] & Anncarol W Karanja[1,2]✉

Endophytic fungi in medicinal plants aid in producing useful therapeutic compounds and enzymes. Among the most useful enzymes are cellulases. However, cellulase enzyme production in endophytic fungi of *Azadirachta indica* and *Aloe secundiflora* has not been comprehensively explored. The objective of this study was to; isolate and identify endophytic fungi from the leaves of young and mature plants of *A. indica* and *A. secundiflora*, determine colonization frequency of the endophytic fungi, and evaluate and optimize the cellulase production by the endophytic fungi on maize cob media. Eleven fungal endophytic isolates were obtained from the leaves of both *A. secundiflora* and *A. indica*, collected in Kitui and Kiambu Counties in total: Six from Kitui County and five from Kiambu County. *Penicillium* Sp. had highest colonization frequency in Kitui, while *Candida* sp. had highest in Kiambu. For enzyme optimization, isolates *Candida boidinii*, *Galactomyces candidum*, and *Candida stellimalicola* produced the highest amounts of Fpases and endoglucanases on third, sixth and ninth days. High exoglucanase producers were *Colletotrichum gloeosporioides*, *Galactomyces candidum*, and *Candida stellimalicola*. The endophytic communities within the leaves of *A. indica* and *A. secundiflora* are diverse. Maize cob agrowaste media can be used to cultivate the production of cellulases successfully in fungal endophytic isolates of *A. indica* and *A. secundiflora*. The study concluded that the endophytes of *A. indica* and *A. secundiflora* can be harnessed and optimized to secrete cellulase enzymes for commercial use, and especially isolates *G. candidum* and *C. stellimalicola* which yield significantly high amounts of total cellulases, endoglucanases and exoglucanases.

Keywords Fpase, Exoenzymes, Endoglucanase, Exoglucanase, Avicel, Solid state fermentation, Colonization frequency, Enzyme extraction

Endophytic fungi are microorganisms that live in plants and are diverse in nature[1]. They are abundant and present in the leaves, fruits, roots, flowers, and stems. Endophytic fungi can be found in all parts of the plant and especially the leaves. In Thai orchids, fifty-two endophytic isolates were obtained from five different species of orchids, with the leaves having the most abundant number of isolates[2]. Fifty-six percent of the isolates produced cellulases on solid media, which were the second most abundant enzymes produced[2]. Endophytic fungi form mutualistic, neutralistic, or antagonistic relationships with the host[3]. They have been isolated from tropical and temperate plants, desert plants, mangroves, Tundra in the Arctic, croplands, savannas, and grasslands[4]. Examples of endophytic fungi that have been isolated from plants include *Fusarium* sp., *Colletotrichum* sp., *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., *Trichoderma* sp., *and Xylaria* sp., among others[5]. Species of endophytes isolated from A. *indica* include *Cladosporium* sp., *Aspergillus* sp., *Colletotrichum* sp., and *Fusarium* sp.[6].

According to Khalil et al.[4], medicinal plants are significantly unexplored when it comes to their functional associations with fungi. Medicinal plants contain endophytic fungi that secrete exoenzymes. The exoenzymes

[1]Department of Biochemistry, Microbiology, and Biotechnology, Kenyatta University, Nairobi, Kenya. [2]John M. Maingi and Anncarol Karanja have contributed equally to this work. ✉email: 27547.2018@students.ku.ac.ke; Karanja.anncarol@ku.ac.ke

hydrolyse structural compounds of pathogens and destroy them, providing plants with protection against these organisms[7]. These enzymes break down lignocellulosic materials[8]. They include amylases, pectinases, cellulases, proteases, lipases, and laccases[5]. These enzymes can have a direct antagonistic effect on pathogens. The amount and type of enzymes secreted by endophytic fungi vary and may depend on the source plant. According to[1], *Colletotrichum gloeosporioides*, *Cladosporium cladosporioides,* and *Aspergillus versicolor* are endophytic microbes with high amounts of cellulase activity.

According to[9], naturally, these fungi significantly aid in plant biomass degradation by producing carbohydrate-active exoenzymes dedicated to the degeneration of plant polysaccharides. The activities of the enzymes have been observed over time but their applications in the commercial world are recent[10]. In addition to their degrading activities, these enzymes have become prominently significant and essential in industrial application due to their ease of production, relatively low costs, efficiency, consistency, small spaces needed for production, and the ability to modify and optimize the production process[11]. Cellulases from microbes are among these commercialized enzymes. Cellulases refer to a group of enzymes that comprise endoglucanases, exoglucanases, and β-glucosidases[10]. The endoglucanases hydrolyse cellulose chains producing cello-oligosaccharides and glucose[10]. Exoglucanases degrade cellulose, yielding the primary product cellobiose[10]. β-glucosidases act on cellobiose to yield glucose[10]. Anoop Kumar et al.[10] state that cellulases have numerous applications in industries utilizing biotechnology and rank second in industrial use after proteases. In another study by[12], cellulases were the second most prominent group of enzymes produced by endophytic fungi. The industries using cellulases include textile, animal feeds, paper and pulp, wine and brewery, laundry, and food [10]. Industries showing very promising application of cellulases include the beverage, feed and food industries[10].

According to Ejaz et al.[13], the diverse uses of microbial-derived cellulases include clarifying fruit juices, tenderizing fruits, hydrolysing roasted coffee, and lessening roughage in dough, increasing taste and aroma in foods, and extracting essential oils and tea polyphenols from olives.[13] report that cellulase enzymes are utilized in hydrolysis of biomass into sugars such as hexoses and pentoses that are fermented to yield biofuels. Using microbial cellulolytic processes reduced the cost of processing biomass by forty percent[13]. As per Sopalun and Iamtham[14], there is an increase in industrial demand for novel sources of more potent enzymes. Reasons for these include the demand for more environmental-friendly bioprocesses and better thermostable enzymes[13]. According to Ejaz et al.[13], there is an increase in the use of enzymes in commercial markets but the applications of the catalytic potential of cellulases are yet to be fully explored, especially in the food industry.

The main roadblock in enzyme application in industry is the high cost associated with producing the enzymes[14]. The increased demand for enzymes calls for more cost-effective alternatives in producing the enzymes, such as using low cost and effective substrates[14]. Sopalun and Iamtham[14] report that agricultural countries such as Thailand produce large yields of agrowaste that pose challenges for disposal. These agrowastes include sugarcane bagasse, rice husk and corncob among others. The agrowastes are useful in producing biogas, biofuel and providing raw materials research work through solid state fermentation[15]. However, most of the agrowaste is underutilized and untreated, and usually destroyed through burning, landfilling or dumping[15]. Agrowaste media is beneficial because of its ease of availability, low cost, and eco-friendly utilization[14]. It is a great way to utilize raw material that would otherwise cause pollution[14]. This research study utilized maize (corn) cob as a substrate for the evaluation and optimization of cellulase enzymes. Maize is among the most commonly produced crops worldwide[15]. Such abundance means that it generates sufficient amounts of agrowaste that can be utilized for enzyme production at low costs. Moreover, El-Tayeb et al.[16] determined that corn cob has the highest amount of cellulose based on dry weight, at 61.2% w/w, which makes it the most preferable substrate for producing cellulase enzymes. According to[15], maize or corn provides commonly used substrate for solid state fermentation. The advantages of using solid state fermentation include low economic cost associated with the process, the ease of product recovery, reduced downstream processing, smaller size of the fermenter needed, and reduced energy needed for sterilizing and stirring[15]. The maize agrowaste medium and solid state fermentation are thus highly practical for research and industrial purposes.

The increase in demand for novel sources of thermostable and highly potent enzymes in industrial applications, the high demand for cellulases and the potential for cellulase application in various industries, the need for cheaper, available and eco-friendly processes, and the significant potential for research into medicinal plants and their endophytes are areas that need further exploration and research. Thus, the objective of this study was to isolate and identify fungal endophytes from medicinal plants *A. indica* and *A. secundiflora* by morphological and molecular characterization, and evaluate their potential for the production of cellulases under solid state fermentation using maize cobs as substrate.

Results

Morphological identification of all endophytic fungi

All colonies of the fungal isolates appeared smooth, except for isolate G1AL001. All isolates except M2L002 and G2AL001 appeared circular in Petri dish cultures. Isolates G1AL002 and G2L001 were non-filamentous, white, had aerial mycelia, and were septate. Isolate G2L001 was green and feathery in appearance. Isolates M2L001 (34 mm) and G1AL001 were fluffy, white, septate, with aerial mycelium (Table 1). Images of some of the fungi are shown below in Fig. 2.

The isolates were morphologically divided into three main clusters (A, B, and C). Clusters B and C were closer to each other than cluster A. Cluster A had two sub-clusters, I and II. Sub-cluster I was subdivided into two groups which showed close relationships between the isolates M2L001 and G1AL001 and M1L002 and M1L001. Sub-cluster II showed a close relationship between G2L001 and G1AL002. Cluster B had sub-clusters III and IV. Sub-cluster III had two sub-divisions, with isolates M2L002 and G2AL001. Sub-cluster IV had isolate M2L004. These isolates showed similar morphological traits. Cluster C had isolates M1L003 and G2AL002 (Fig. 1).

Isolates	Mycelia	Margins, Form	Elevation	Colour	Diameter (mm)
G1AL001	Septate, aerial, filamentous, fluffy	Lobate, Circular	Flat	White	52
G1AL002	Septate, aerial, non-filamentous	Entire, Circular	Raised	White	46
G2AL001	Septate, spreading, filamentous	Lobate, Irregular	Flat	Grey	45
G2AL002	Septate, spreading, filamentous	Entire, Circular	Raised	White	42
M1L001	Aseptate, aerial, filamentous, fluffy	Entire, Circular	Flat	White	37
M1L002	Aseptate, aerial, filamentous, fluffy	Entire, Circular	Flat	Grey	42
M1L003	Septate, spreading, filamentous	Entire, Circular	Flat	White	35
M2L001	Septate, aerial, filamentous, fluffy	Entire, Circular	Flat	White	34
M2L002	Septate, filamentous	Lobate, Irregular	Flat	Green	32
M2L004	Septate, filamentous	Entire, Circular	flat	White	39
G2L001	Septate, aerial, non-filamentous, feathery	Entire, Circular	raised	Green	40

Table 1. Morphological features of the fungal endophytic isolates.

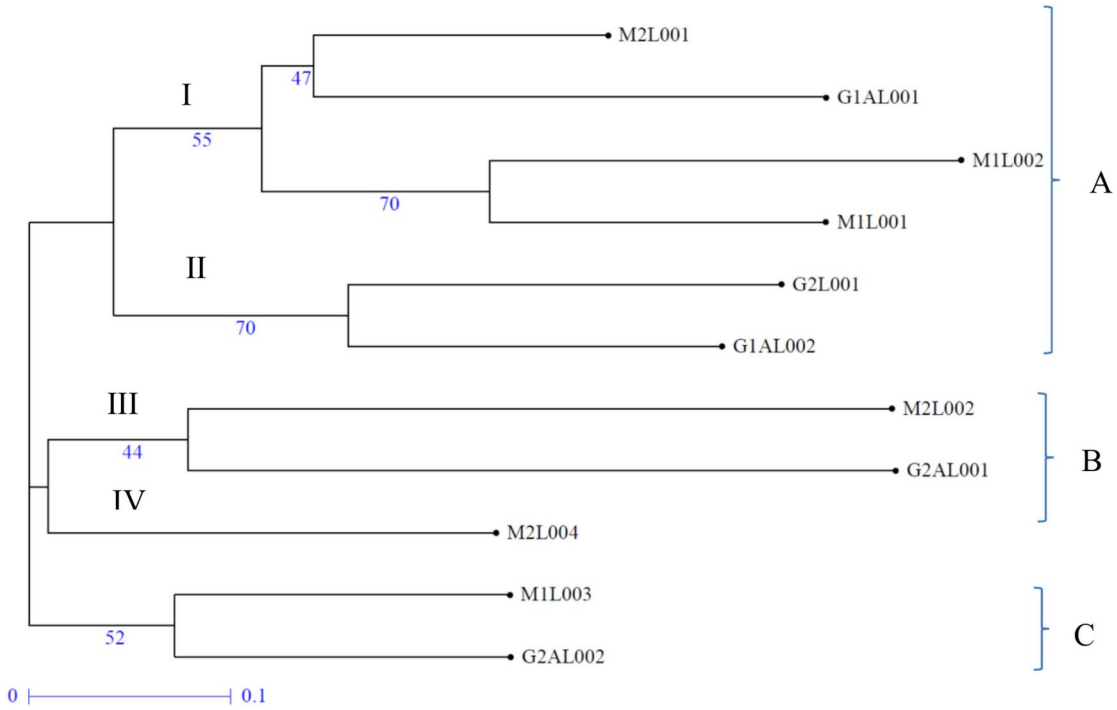

Fig. 1. Dendogram showing clusters of all morphologically similar fungal endophytic isolates. There are three main clusters A, B and C, and four sub-clusters I, II, III, IV.

Genetic identification of the fungal endophytes

Based on nucleotide BLAST in the NCBI database, most isolates had a 100% sequence match with reference strains in the database (Table 2). Isolates G1AL001, 99.5%, G1AL002, 90.98%, and G2AL002 were grouped in the Genus *Candida* with similarity indices of 99.5%, 90.98%, and 100%, respectively. Isolates G2AL001 had 90.95% identity with isolates in the Genus *Galactomyces*, while isolates (Fig. 2); M1L001 and M1L002 had 100% similarity with reference sequences for the Genus *Colletotrichum*. Isolate M1L003 had 100% similarity with the reference sequence for Genus *Fusarium*. The isolate M2L001 matched with the reference sequence of Genus *Trichoderma* with a 100% match. Isolates M2L004 and M2L002 matched with reference sequences of Genus *Penicillium*, with 100% similarity, and isolate G2L001 had 99.78% similarity with the reference sequence of Genus *Cladosporium* (Table 2).

Phylogenetic analysis

A phylogenetic tree based on the maximum likelihood method clustered the isolates into two main clusters (A and B). Cluster B had many isolates and two subclusters (i and ii). Subcluster I consisted of isolates related to the *Candida* genus and included *C. boidinii* and *C. stellimalicola*. Subcluster II was divided into two groups (1 and 2). Group 1 consisted of two main sub-groups; the first sub-group consisted of *Clad. cladosprioides*, and the second

Isolate	Accession number	Genus	NCBI Reference match	Query cover (%)	Percentage identity
G1AL001	ON077142	*Candida*	*Candida boidinii*	100	99.50
G1AL002	ON077143	*Candida*	*Candida stellimalicola*	90	90.98
G2AL002	ON077145	*Candida*	*Candida stellimalicola*	100	100
G2AL001	ON515749	*Galactomyces*	*Galactomyces candidum*	96	90.95
M1L001	ON077144	*Colletotrichum*	*Colletotrichum gloeosporioides*	100	100
M1L002	ON077151	*Colletotrichum*	*Colletotrichum fructicola*	100	100
M1L003	ON077146	*Fusarium*	*Fusarium luffae*	100	100
M2L001	ON077147	*Trichoderma*	*Trichoderma lixii*	100	100
M2L002	ON077148	*Penicillium*	*Penicillium* sp.	100	100
M2L004	ON077149		*Penicillium* sp.	100	100
G2L001	ON077150	*Cladosporium*	*Cladosporium cladosporioides*	96	99.78

Table 2. Isolates, their scientific names, query cover, and percentage identity.

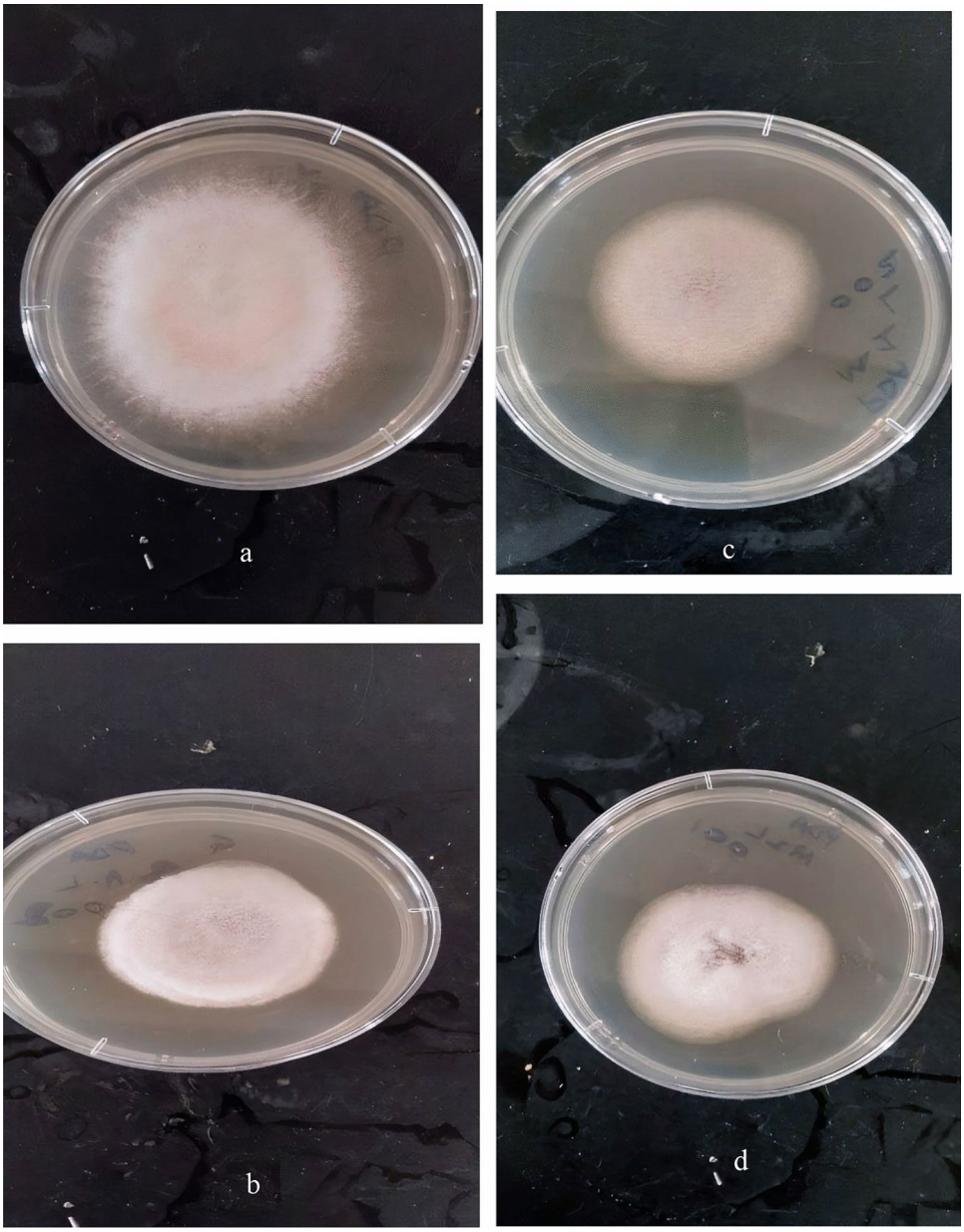

Fig. 2. Day 3 images after inoculation of fungal isolates: (**a**) M2L001, (**b**) G1AL001, (**c**) G2AL002, (**d**) M1L002.

sub-group contained *F. luffae*. Group 2 consisted of isolates of the *Colletotrichum* Genus (*Col. gloeosporioides* and *Col. fructicola*), *T. lixii,* and *Penicillium* Genus (*Penicillium* sp. voucher AC11) (Fig. 3).

The optimal tree with the sum of branch length = 2.19115597 is shown (Fig. 3). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[17] and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 968 positions in the final dataset. Evolutionary analyses were conducted in MEGA X[17];[18].

Colonization frequency of endophytes in the sampled plant tissues

The colonization frequency of the different isolates was determined based on the formula in Sect. "Sampling". Twenty-six segments were used, divided into thirteen for each plant per County. These were further divided into seven segments for young plants and six mature plants. The colonization frequencies were presented in the form of percentages for each isolate. The isolates of leaves of mature *A. secundiflora* plants in Kitui County consisted of *Penicillium* sp. voucher AC11 (66.67%) and *T. lixii* (50%). *F. luffae* (42.86%) was isolated from young plant leaves of *A. secundiflora*. In *A. indica*, the mature plants were colonized by *Penicillium* sp. voucher AC11 (83.33%), while the young plants were colonized by *Col. fructicola* (14.29%) and *Col. gloeosporioides* (71.43%) (Fig. 4).

In Kiambu County, the leaves of mature plants of *A. secundiflora* hosted *G. candidum* (83.33%), while the young plant leaves contained *C. boidinii* (85.71%). The mature plant leaves of *A. indica* were colonized by *C. stellimalicola* (50%) and *Clad. cladosporioides* (16.67%). The young plants had leaves colonized by *C. stellimalicola* (42.86%) (Fig. 5).

Cellulase assays

Total cellulase enzyme production (FPase)

For FPase, most of the isolates showed decreased enzyme production from day 3 to day 6. Isolate G2AL001 (*G. candidum*) showed an increase in enzyme production from day 3 to day 9. Isolates G1AL001 (*C. boidinii*), M2L001 (*T. lixii*), MIL003 (*F. luffae*), M2L002 (*Penicillium* sp.), and M2L004 (*Penicillium* sp.) showed a decrease in enzyme production from day 3 to day 9. Isolates G1AL002 (*C. stellimalicola*), M1L002 (*Col. fructicola*), and M1L001 (*Col. gloeosporioides*) had a decrease in enzyme production from day 3 to day 6 and an increase in enzyme production from day 6 to day 9. Isolate G2L001 (*Clad. cladosporioides*) had an increase in enzyme production from day 3 to day 6 and a decrease in production from day 6 to day 9. Isolate G1AL002 produced

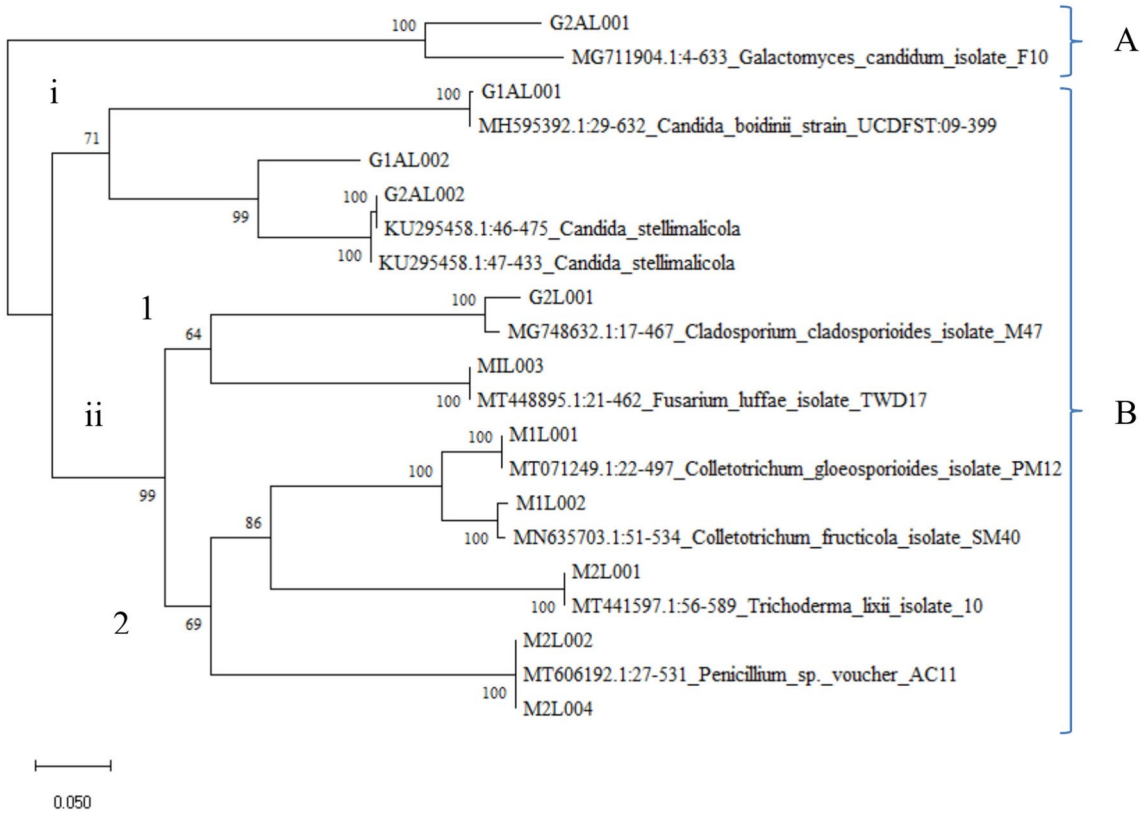

Fig. 3. The phylogenetic tree of the evolutionary history inferred using the Neighbour-Joining method.

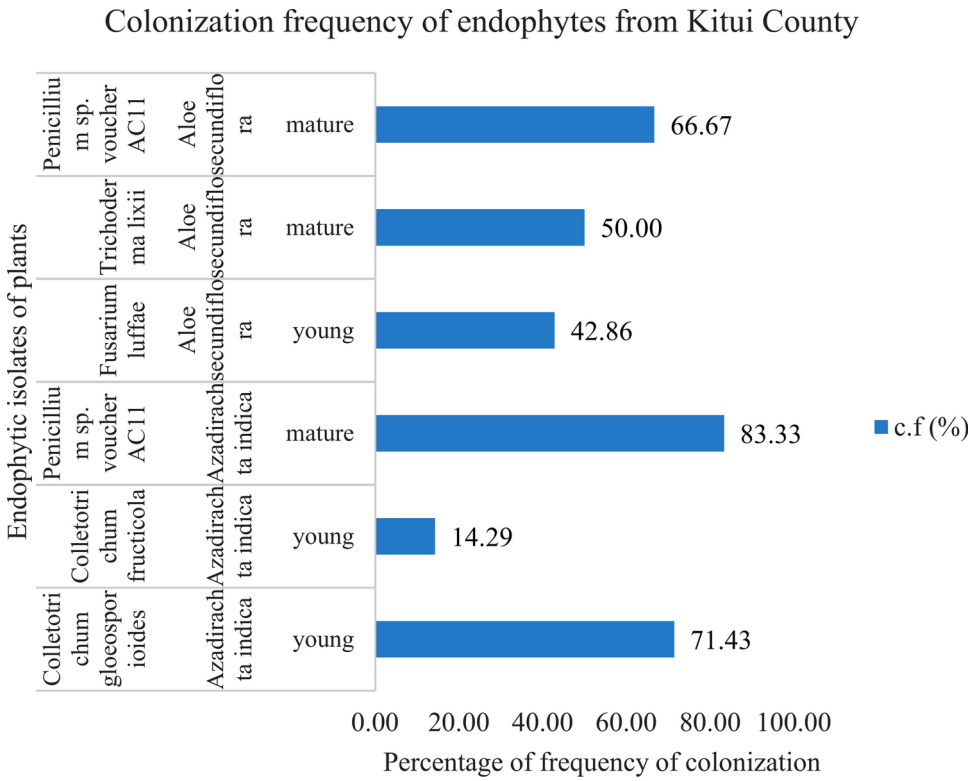

Fig. 4. Percentage colonization frequency of isolates from Kitui County.

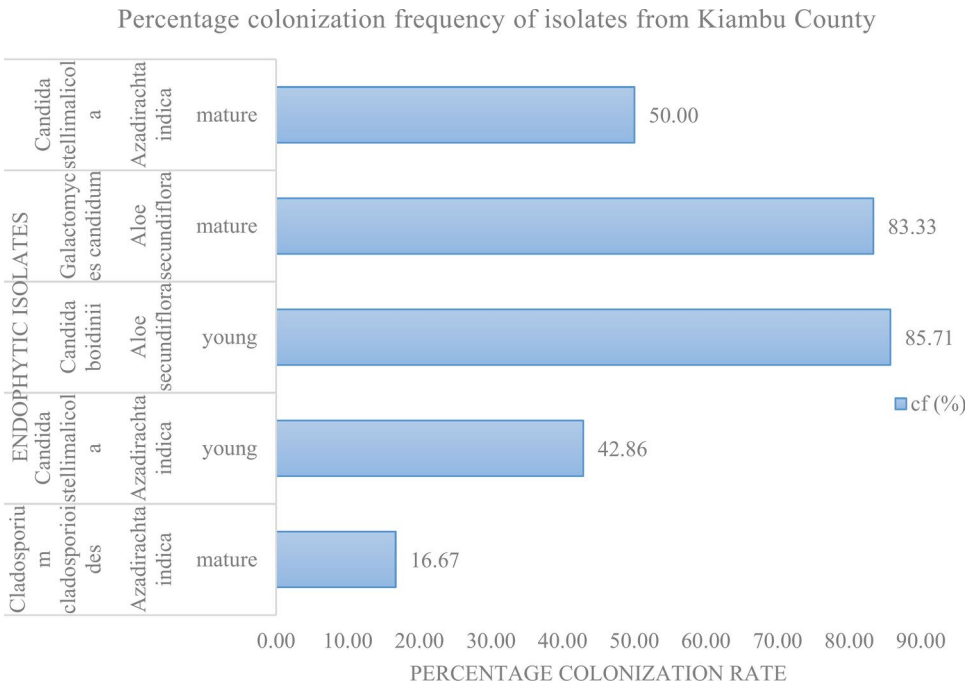

Fig. 5. Colonization frequency of isolates from Kiambu County.

the highest amount of total cellulase enzymes of 15.9 ± 0.28 IU/mL on day 3 which was not significantly higher than total cellulase enzymes produced by isolate M1L001 (14.9 ± 0.45 IU/mL). Isolate G2L001 produced the lowest total cellulase enzymes of 5.08 ± 0.02 IU/mL after three days of incubation. Isolate G2AL001 produced 18.9 ± 0.23 IU/mL on the 6th day of incubation which was significantly higher than FPase produced by all other fungal endophytes, while isolate M1L001 produced the lowest total cellulases on the 6th day of incubation at

Fungi	Day 3 Enzyme Units (IU/mL)	Day 6 Enzyme Units (IU/mL)	Day 9 Enzyme Units (IU/mL)
C. boidinii	9.25 ± 0.03^{c}	6.7 ± 0.27^{de}	5.63 ± 0.61^{de}
C. stellimalicola	15.9 ± 0.28^{a}	15.0 ± 0.38^{a}	19.8 ± 0.31^{a}
G. candidum	12.9 ± 0.22^{b}	18.9 ± 0.23^{b}	11.6 ± 0.07^{b}
Clad. cladosporioides	5.08 ± 0.02f.	6.24 ± 0.2^{de}	5.61 ± 0.17^{de}
Col. gloeosporioides	14.9 ± 0.45^{a}	5.29 ± 0.11^{e}	5.64 ± 0.09^{c}
Col. fructicola	6.52 ± 0.13^{e}	5.67 ± 0.34^{de}	8.46 ± 0.28^{c}
F. luffae	6.78 ± 0.38^{de}	6.01 ± 0.59^{de}	3.65 ± 0.31f.
T. lixii	7.93 ± 0.05^{d}	6.57 ± 0.42^{de}	6.38 ± 0.21^{de}
Penicillium sp.	10.3 ± 0.19^{c}	8.77 ± 0.48^{c}	6.82 ± 0.24^{d}
Penicillium sp.	10.0 ± 0.11^{c}	7.32 ± 0.52^{cd}	4.98 ± 0.42^{ef}

Table 3. Total cellulase enzyme production by fungal endophytic isolates on days 3, 6 and 9 of incubation. Values are means of 3 replicates ± SEM and are expressed as IU/mL. For each day means are expressed in the same column and different superscript letters are statistically different at $p \leq 0.05$.

Fungi	Day 3 Enzyme Units (IU/ml)	Day 6 Enzyme Units (IU/ml)	Day 9 Enzyme Units (IU/ml)
C. boidinii	8.11 ± 0.431^{e}	10.4 ± 0.62^{cd}	8.67 ± 0.66^{c}
C. stellimalicola	16.0 ± 0.36^{abc}	20.5 ± 0.58^{b}	23.7 ± 0.29^{a}
G. candidum	18.9 ± 0.34^{a}	23.9 ± 0.43^{a}	22.5 ± 0.32^{a}
Clad. cladosporioides	7.38 ± 0.59^{e}	9.15 ± 0.08^{d}	8.84 ± 0.39^{c}
Col. gloeosporioides	12.9 ± 0.61^{d}	8.39 ± 0.51^{d}	8.74 ± 0.59^{c}
Col. Fructicola	9.53 ± 0.87^{e}	8.82 ± 0.81^{d}	10.2 ± 0.33^{c}
F. luffae	8.66 ± 0.63^{e}	5.25 ± 0.52^{e}	5.73 ± 0.16^{d}
T. lixii	13.3 ± 0.75^{cd}	9.31 ± 0.18^{d}	9.62 ± 0.4^{c}
Penicillium sp.	17.4 ± 0.79^{ab}	12.7 ± 0.22^{c}	12.4 ± 0.12^{b}
Penicillium sp.	15.0 ± 0.08^{bcd}	9.20 ± 0.39^{d}	8.91 ± 0.25^{c}

Table 4. Endoglucanase enzyme production by fungal endophytic isolates on days 3, 6 and 9 of incubation. Values are means of 3 replicates ± SEM and are expressed as IU/mL. For each day means are expressed in the same column and different superscript letters are statistically different at $p \leq 0.05$.

5.29 ± 0.11 IU/mL. The highest FPase on the 9th day of incubation was 19.8 ± 0.31 IU/mL produced by isolate G1AL002 which was significantly higher than FPase recorded by all other fungal isolates (Table 3).

For total cellulase enzyme assay over time, there was a significant difference in the IU/mL (enzyme units) for days three, six, and nine ($p < 0.05$). Day 9 had the highest total cellulase enzyme production (11.82 ± 2.57 IU/mL), followed by day 3 (9.97 ± 0.64 IU/mL), and lastly day 6 (8.64 ± 0.81 IU/mL). *G. candidum* produced the highest amount over time (27.68 ± 5.94 IU/mL), while *F. luffae* had the lowest output over time (5.48 ± 0.52 IU/mL) at $p < 0.05$. The interaction between day of incubation and the fungal isolate was significant ($p < 0.05$).

Endoglucanase enzyme production

For endoglucanases, most of the isolates showed a decrease in enzyme production from day 3 to day 6. Isolate G1AL002 (*C. stellimalicola*) progressively produced more enzymes from day 3 to day 9. Isolates M1L001 (*Col. gloeosporioides*), M1L002 (*Col. fructicola*), M2L004 (*Penicillium* sp.), M1L003 (*F. luffae*), and M2L001 (*T. lixii*) had a decrease in enzyme production from day 3 to day 6, followed by an increase in production from day 6 to day 9. Isolates G1AL001 (*C. boidinii*), G2L001 (*Clad. cladosporioides*), and G2AL001 (*G. candidum*) peaked in enzyme production on day 6 and had the lowest enzyme production on day 3. Isolate M2L002 had a decrease in enzyme production from day 3 to day 9. Isolate G2AL001 produced 18.9 ± 0.34 IU/mL of endoglucanase enzyme which was only not significantly higher than endoglucanase enzyme produced by isolate G1AL002 on the 3rd day of incubation. On the 6th day of incubation, isolate G2AL001 produced 23.9 ± 0.43 IU/mL which was significantly higher than that produced by all other endophytic fungi. Isolate G1AL002 produced the highest endoglucanase enzymes of 23.7 ± 0.29 IU/mL on the 9th day of incubation, which was not significantly higher than that produced by isolate G2AL001 but was significantly higher than that produced by all other endophytic fungi (Table 4). Isolate G2L001 produced the lowest amount of endoglucanase enzyme on the 3rd day (7.38 ± 0.59 IU/mL), while isolate MIL003 produced the lowest amount of endoglucanase enzymes on the 6th day of incubation (5.25 ± 0.52 IU/mL) and on the 9th day of incubation (5.73 ± 0.16 IU/mL) (Table 4).

There was a significant difference ($p < 0.05$) in IU/mL for endoglucanase assays over time. The endoglucanase assay enzyme production was highest on day 3 (12.71 ± 0.74 IU/mL), while the enzyme production on days 6 (11.76 ± 1.03 IU/mL) and 9 (11.91 ± 1.07 IU/mL) showed no significant difference. *G. candidum* had the

Fungi	Day 3 Enzyme Units (IU/ml)	Day 6 Enzyme Units (IU/ml)	Day 9 Enzyme Units (IU/ml)
C. boidinii	32.8 ± 0.4^{ab}	10.9 ± 0.42^{bc}	5.87 ± 0.28^{bcd}
C. stellimalicola	29.6 ± 0.68^{c}	28.5 ± 0.41^{a}	28.4 ± 0.25^{a}
G. candidum	29.4 ± 0.27^{c}	28.9 ± 0.34^{a}	28.7 ± 0.39^{a}
Clad. Cladosporioides	12.7 ± 0.33f.	8.25 ± 0.25^{d}	6.10 ± 0.06^{bc}
Col. Gloeosporioides	34.8 ± 0.36^{a}	12.3 ± 0.04^{b}	6.02 ± 0.3^{bcd}
Col. Fructicola	22.0 ± 0.29^{e}	10.5 ± 0.32^{c}	6.09 ± 0.13^{bcd}
F. luffae	25.0 ± 0.91^{d}	6.62 ± 0.21^{e}	6.62 ± 0.08^{b}
T. lixii	23.1 ± 0.31^{de}	7.28 ± 0.39^{de}	5.16 ± 0.13^{cd}
Penicillium sp.	24.1 ± 0.31^{de}	10.5 ± 0.29^{c}	4.82 ± 0.41^{d}
Penicillium sp.	30.3 ± 0.72^{bc}	12.0 ± 0.31^{bc}	5.83 ± 0.22^{bcd}

Table 5. Exoglucanase enzyme production by fungal endophytic isolates on days 3, 6 and 9 of incubation. Values are means of 3 replicates ± SEM and are expressed as IU/mL. For each day means are expressed in the same column and different superscript letters are statistically different at $p \leq 0.05$.

highest endoglucanase enzyme output over time (21.75 ± 0.75 IU/mL), while *F. luffae* had the lowest amounts of endoglucanases over time (6.55 ± 0.58 IU/mL). The interaction between day of incubation and the fungal isolate was significant ($p < 0.05$).

Exoglucanase enzyme production

All fungal isolates peaked in enzyme production for exoglucanase activity on day 3. Isolates G1AL001 (*C. boidinii*), G2L001 (*Clad. cladosporioides*), M1L001 (*Col. gloeosporioides*), M1L002 (*Col. fructicola*), M2L001 (*T. lixii*), M2L002 (*Penicillium* sp.), and M2L004 (*Penicillium* sp.) had a decline in enzyme production from day 3 to day 9. Isolates MIL003 (*F. luffae*), G2AL001 (*G. candidum*), and G1AL002 (*C. stellimalicola*) had a decrease in enzyme production from day 3 to day 6 and a slight increase in enzyme production from day 6 to day 9. Isolates G1AL002 and G2AL001 produced the highest amounts of exoglucanase enzymes that were not significantly different but were significantly higher than those produced by all the isolates on the 6th and 9th days of incubation. Isolates M1L001 (34.8 ± 0.36 IU/mL) and G1AL001 (32.8 ± 0.4 IU/mL) produced the highest amounts of exoglucanase enzymes that were not significantly different but were significantly higher than the other isolates on the 3rd day of incubation. The lowest amounts of exoglucanase enzymes were produced by the isolate G2L001 (12.7 ± 0.33 IU/mL) on the 3rd day of incubation, isolate MIL003 (6.62 ± 0.21 IU/mL) on the 6th day of incubation, and isolate M2L002 (4.82 ± 0.41 IU/mL) on the 9th day of incubation (Table 5).

For exoglucanases, there was a significant difference in IU/mL over time for days 3, 6, and 9 ($p < 0.05$). Day 3 had the highest amount of exoglucanase enzyme production (26.38 ± 1.13 IU/mL), day 6 had 13.57 ± 1.44 IU/mL, and day 9 had the lowest (10.35 ± 1.69 IU/mL). *G. candidum* (28.99 ± 0.20 IU/mL) and *C. stellimalicola* (28.83 ± 0.30 IU/mL) produced the highest amounts of exoglucanases over time, while *Clad. cladosporioides* produced the lowest amounts of exoglucanases over time (9.02 ± 0.98 IU/mL). The interaction between day of incubation and the fungal isolate was significant ($p < 0.05$).

Discussion

The choice of the leaves and maize cob as agrowaste medium

The leaves were chosen for endophytic isolation because they had fewer barriers to infection, lacking the outer bark, cuticle wax, and lignin layers compared to the other parts of the plant such as the stem and were thus prone to higher numbers of endophytic species, as observed by[14]. Bungtongdee et al.[19] also obtained similar results showing that leaves have higher numbers of endophytic isolates. In comparing the abundance of endophytes in the leaves and stems of *A. indica*, Al-Daghari et al.[20] found that the leaves had greater fungal taxa than the stems. The exposure of the leaves of medicinal plants *A. Indica* and *A. secundiflora* to external environment and infections meant that the endophytes within their leaves were greatly adapted to these external stressors. According to Chaudhary et al.[21], endophytes have been shown to help plants cope with biotic stress. They also help in remediating abiotic stress[22]. Over time, the development of biotechnology has given rise to the use of enzymes for commercial reasons. The high cost of commercial enzymes has turned the focus of researchers to microbial enzymes[23]. Moreover, the process of harnessing the microbial enzymes is costly, but the cost can be cut by using innovative techniques such as employing agrowaste medium as raw material for the necessary fermentation processes[15]. In addition to maize cob being easily and ubiquitously available at low cost as agrowaste media, it is also has the highest amount of cellulose based on dry weight, at 61.2% w/w, as observed by[16], which makes it the most preferable substrate for quantifying endophytic cellulase enzymes.

Fungal endophytic isolates of *A. indica* and *A. secundiflora*

The endophytes isolated are part of a wider endophytic community found in non-medicinal and medicinal plants. Different species of fungal isolates were isolated from *A. secundiflora* and *A. indica*. In concurrence, Amirita et al.[24] report that a single plant or plant part can contain different endophytic isolates. Some factors determine the composition of communities of endophytes in plants. They include environmental factors, specificity of the organism towards the host, and composition of chemicals within the plants[25,26]. These factors could explain the

occurrence of endophyte species in young or mature plants. As stated by[27] diversity and colonization potential of endophytes in *Coccoloba cerefeira* depend on the amount of water in the leaves and the ratio of the polyphenol to the fresh or dry weight. These factors may contribute to the occurrence of one species in either young or mature plants or both[27]. The variation in the composition of microbial endophytes has been attributed to the level of chlorophyll present in the leaves[27]. The leaves of mature plants show wider variations in species of fungi compared to the leaves of young plants. Mature plants have an extended period to interact with fungi relative to young plants. The increased number of endophytes in mature plants is associated with their excellent nutrient potential due to higher rates of photosynthesis[28].

Some species of fungi were observed in young plants but not in mature plants.[28] reasoned that the changes that plants undergo as they grow can interfere with the innate environment of leaves and may favour different endophytic species over others as they mature. More species were isolated in samples from Kitui County compared to Kiambu County. There are few studies on the impact of the environment on the diversity of endophytic microbes. However, the relationship between the ecosystem and the plants significantly determines diversity[29]. The geographical area of study and ecology affect the endophytic communities in plants[30]. The interaction between the plants and the environment potentially leads to the variation of the endophytic community in Kitui and Kiambu Counties. Most endophytic organisms are transmitted horizontally; thus, the location and environment significantly determine the diversity of endophytic fungal species[31].

Khalil et al.[4] demonstrated that medicinal plants produce endophytes with the capability to produce cellulases, with the majority of the isolates being *Penicillium* sp. Similarly, the results of this study show that *Penicillium* sp. is highly abundant in the mature leaves of the *A. indica* (83.33%) and *A. secundiflora* (66.67%). Another study by Qureshi et al.[32] showed that *Penicillium* sp. had the third highest colonization frequency (28.00%) out of eleven endophytic isolates from *A. indica*. Qureshi et al.[32] isolated *Clad. cladosporioides* with a colonization frequency (CF) of 15.78% from the leaves of *A. indica*, which was similar to the 16.67% CF of *Clad. cladosporioides* in *A. indica* leaves. Qureshi et al.[32] isolated *Colletotrichum* sp. from the leaves of *A. indica* (CF 13.33%), which concurred with the results obtained for *Col. fructicola* (CF 14.29%) in *A. indica* leaves. There are very limited studies on the endophytic communities of *A. secundiflora*, but the common endophytes from other *Aloe* sp. similar to those obtained in this study comprise of endophytes of *Penicillium* sp., *Trichoderma* sp., and *Col. gloeosporioides*[33–35]. *T. lixii* (CF 50.00%) is the second most dominant species in mature plant leaves of *A. secundiflora* in Kitui County. The results differ with those of Kiambu County, where *G. candidum* (CF 83.33%) is dominant in the mature plants of *A. secundiflora*. However, the mature leaves of *A. secundiflora* in Kitui County had more species that those in Kiambu County with only one species. *F. luffae* (CF 42.86%) is dominant in the young plants of *A. secundiflora*. *C. boidinii* (CF 85.71%) is the dominant species in the leaves of young *A. secundiflora* plants. In Kiambu County the dominant species of mature *A. indica* are *C. stellimalicola* (CF 50.00%) and *Clad. cladosporioides*. Wu et al.[29] reported that the ecosystem significantly affects diversity in plant species. Furthermore, Chauhan et al.[30] state that endophytic communities are affected significantly by ecology and geography. These variations in species may be the result of the ecological and geographical differences in Kitui and Kiambu counties.

Isolates of genera *Candida, Trichoderma, Cladosporioides,* and *Colletotrichum* belong to the phylum Ascomycota[36–39]. The isolates *C. boidinii* and *T. lixii* showed similarities in colony appearance, including filamentous, fluffy, white, aerial mycelium, circular and septate. Katoch et al.[40] observed that isolates of *T. lixii* had septate hyphae, aerial mycelium, and white, filamentous colonies in concurrence with the obtained results. Isolates *Col. gloeosporioides* and *Col. fructicola* were smooth, circular, filamentous, aseptate, had aerial mycelia, and belonged to Genus *Colletotrichum* sp. The observations concur with morphological traits observed in *Col. gloeosporioides* and *Col. fructicola*[38,41]. *C. stellimalicola* is filamentous yeast, in agreement with Dupont et al.[42]. The morphological trait was shared by the isolates identified as *C. stellimalicola* and *Clad. cladosporioides*.[36] observed varying degrees of septation in Genus *Cladosporium* depending on external factors. Fungal isolates identified as *G. candidum* and *Penicillium* sp. are filamentous fungi. According to Wang et al.[43], the colony colour of *Penicillium* sp. varies depending on factors such as growth media. However, the colonies remain filamentous, with circular, entire margins.[15] reported that the fungus *G. candidum* is filamentous, similar to the results obtained. The observations indicate that isolates of clusters B and C share morphological features. These include smooth margins, filamentous growth, and circular and septate colonies. *F. luffae* and *C. stellimalicola* (accession number ON077145) produced flat, spreading, and dense mycelia. Wang et al.[44] studied *Fusarium* sp. complex and isolated *F. luffae*. The cultures were white, with dense mycelia, flat, spreading, and fast-growing. The results concur with the findings of the study.

Comparison of the amount of enzymes produced by the endophytic isolates

G. candidum and *C. stellimalicola* had the highest yields of endoglucanases over time. The production of exoglucanase enzymes by *G. candidum* and *C. stellimalicola* were also relatively high. The findings concur with studies showing *G. candidum* has high cellulase activity and produces exoglucanases[45,46]). However, Borisova et al.[45] observed low endoglucanase production of *G. candidum* (1.86 IU/mL) in contrast to the high endoglucanase production of this study, with an overall production of 21.75 IU/mL. These differences may be linked to the differences in procedures for enzyme assay where Borisova et al.[45] targeted 3–5% conversion rates, at 37 °C and pH 5.0, and reading the spectrophotometer at 404 nm. In this study, the pH is 4.8 and the reading is done at 540 nm. Subjecting the fungi to the same measurements of substrate, inoculum, pH and temperature, allows for an accurate measure of the capability of each endophytic fungi in producing cellulases. Even with the procedural differences, Borisova et al.[45] acknowledge that *G. candidum* produced high amounts of cellulases and has huge potential in industrial application. *Candida* sp. evidently demonstrated their ability to produce highly active enzymes of degrading substrates such as cellulose and amylose[47,48]. According to Adelabu et al.[47], *Candida* sp. possessed very cellulolytic activity with the highest day 3 cellulase production of 174.76 IU/mL by *Candida*

tropicalis. Similarly, *Candida stellimalicola* had the highest production of total cellulases on day 3 at 15.9 IU/mL. The levels of cellulase activity vary among species even in the same Genus as observed in the study by Adelabu et al.[47].

C. stellimalicola makes a good candidate for use in industries that employ cellulolytic enzymes[46]. *Col. fructicola* exhibited high exoglucanase production on day 3 and the most increased production of endoglucanases on the 9th day. Similarly, *Colletotrichum* sp. has been shown to secrete cellulases, among other enzymes[1]. Venkatesagowda et al.[1] showed that *Col. gloeosporioides* had high endoglucanase production on a plate by exhibiting a zone of clearance of over 8.0 mm. The results of this study indicate that *Col. gloeosporioides* does produce relatively high amounts of endoglucanases, 12.9 IU/mL, on day 3, which is 6.0 IU/mL lower than the *G. candidum*. Venkatesagowda et al.[1] also show that isolates *Col. gloeosporioides* and *Clad. cladosporioides* produce similar amounts of endoglucanases at 8.0 mm. In concurrence, the Table 4 of this study shows no significant difference in endoglucanase production of these isolates on days 6 and 9.

The exoglucanases yield of *Clad. cladosporioides* on day 3 is relatively higher than endoglucanase enzymes. Vázquez-Montoya et al.[2] demonstrated that *Clad. cladosporioides* did produce exoglucanases in avicel-rich media (205 IU/L) and endoglucanases in CMC media (606 IU/L). Even though the *Clad. cladosporioides* in this study had the lowest amount of exoglucanases over time among all the isolates (9.02 IU/mL), it was higher than the amount obtained by Vázquez-Montoya et al.[2], who used *Moringa oleifera* straw as agrowaste media. It indicates that the maize cob substrate may be better at harnessing enzymes from microbes. *Clad. cladosporioides* showed relatively higher endoglucanases over time from day 3 to day 9 and decreased exoglucanases over time. Similarly,[49] reported significantly less cellulase activity when the substrates used were avicel or filter paper compared to CMC.

C. boidinii has higher activity in avicel relative to CMC and total cellulase activity. The activity of the enzymes in all three assays is higher on the third day and decreases over time. This yeast has been shown to degrade methanol and is a potential source of enzymes in the biotechnology industry[50]. The cellulase enzyme activity of isolate *T. lixii* was highest on the third day and decreased over time. *T. lixii* has been isolated as an endophyte[40]. Similar to the results, Li et al.[51] reported that *Trichoderma* sp. produced enzymes that degraded cellulose with the maximum secretion of enzymes detected within the first two days of the experiment and decreased over time. *T. lixii* has the potential to be used for industrial purposes, just like *Trichoderma harzianum* has been applied for biotechnological purposes for its ability to produce enzymes that degrade lignin and cellulose substrates[52,53]. M'barek et al.[54] showed that *Trichoderma* sp. possessed high cellulose-degrading activity, with *Trichoderma/Hypocrea lixii* exhibiting the highest cellulolytic activity with a blue halo zone of 172 mm^2. This study showed that *T. lixii* produces cellulase enzymes, although at a significantly lower amount than most of the isolates. In the study by[54].

Trichoderma sp. has higher cellulolytic activity than *Penicillium* sp. (94 mm^2). The results contrast to this study, where there are slight significant differences in cellulase production, with *Penicillium* sp. producing more cellulases. It can be a factor of using different media, differing methodologies, test conditions, and also different strains of the same Genus. According to Chaverri et al.[55], *T. lixii* is part of the species belonging to the *T. harzianum* complex. Most studies have been directed toward the potential of *Trichoderma* sp. as a biocontrol organism for phytopathogenic microbes[56,57]. Li et al.[58] detected significant activity of exoglucanases and endoglucanase in *T. harzianum*. Isolates M2L004 and M2L002 (*Penicillium* sp.) had optimal yields on day 3 for all assays and the lowest yields on day 9. In a study, endophytic strains of *Penicillium* sp. exhibited secretion of significant amounts of cellulases compared to other endophytic organisms[59]. M'barek et al.[54] showed that *Penicillium* sp. have high cellulolytic activity. However the strains used in those studies, the raw materials and methodologies differ from those used in this study. *Penicillium* sp. in this study produce significantly higher amounts of cellulases than a few isolates; they still produce lower amounts of cellulases than most isolates.

These differences are visible in different studies as well. The amount of FPase produced in this study was higher than that produced in the study by Syed et al.[8], at 1.2 IU/mL, but the amount of endoglucanases in the study by Syed et al.[8] (19 IU/mL) is higher than that obtained in this study. Nonetheless, the *Penicillium* sp. isolated from medicinal plants produces significant amounts of cellulases that can be harnessed. Production of endoglucanases and exoglucanases was higher relative to the FPase in concurrence with the study by Syed et al.[8], with FPase and endoglucanases. Panagiotou et al.[38] demonstrated the ability of *Fusarium* sp. strains to produce exoglucanases and endoglucanases. Isolate *F. luffae* had a higher exoglucanase activity than the other assays. The lowest activity of the isolate was observed in FPase. The results concur with those of[60], who report that *Fusarium* sp. secreted significantly higher quantities of endoglucanases (128 IU/g) than FPase (95.2 IU/g). *Fusarium* sp. is the prominent organism in producing cellulases in rice straw[61].

Fungal endophytic isolates of *A. secundiflora* has more enzyme activity for total cellulase, endoglucanases, and exoglucanases. It could result from the fact that the fungal endophytes isolated from *A. secundiflora* comprise *Candida* species, which, according to studies by[47,48], have been shown to produce high quantities of cellulases. *Galactomyces candidum* isolated from *A. secundiflora* has been shown to possess high cellulase activity[45,46]. Fungal endophytic isolates of *A. indica* had relatively lower enzyme activity. It could be attributed to substrate specificity in isolates such as *F. luffae*, which could produce more cellulases in substrates other than maize cobs. According to[38], *Fusarium* sp. had better exoglucanase and endoglucanase activity than FPase activity. Isolate *Clad. cladosporioides* performed better for endoglucanases and exoglucanases, although relatively lower than other isolates. Vázquez-Montoya et al.[2] demonstrated the selectivity of *Clad. cladosporioides* by showing a higher exoglucanase activity than endoglucanase activity.

There was a significant difference in enzyme units of cellulases assays over time. The enzyme outputs significantly differed on days 3, 6, and 9. It can be attributed to the growth rate of the endophyte and ability to utilize the substrate. Li et al.[51] shows that some species of fungi peak in enzyme production on the first two days of growth and then decline in production over extended periods of time. An example is *Fusarium*

sp. that produces significant cellulase amounts for CMC and FPase assays[60]. Similar activity was observed for *Penicillium* sp.[8]. According to Sunitha et al.[62] internal factors of the host, including age and external factors, such as climate and geographical location or habitat determine the requirements of the host and endophyte that facilitate enzyme production.

Conclusions

The present study confirms that the endophytic communities of *A. secundiflora* and *A. indica* vary from one region to another, for example, the mature leaves of *A. indica* in Kitui County are dominated by *Penicillium* sp., while those in Kiambu County are dominated by *Candida stellimalicola*. The communities within the leaves of these medicinal plants are diverse. The present study found maize cob agrowaste media can be used to cultivate the production of cellulases successfully in fungal endophytic isolates of *A. indica* and *A. secundiflora*. The study concluded that the endophytes of *A. indica* and *A. secundiflora* can be harnessed and optimized to produce cellulase enzymes in large scale for commercial use, and especially isolates *G. candidum* and *C. stellimalicola* which yield significantly high amounts of total cellulases, endoglucanases and exoglucanases. In addition, enzyme production peaks occur at different time intervals for different isolates. While some of the isolated organisms may belong to potentially pathogenic genera, the fungal isolates selected, under controlled conditions, are typically not pathogenic. For large-scale production, we recommend using of GRAS (genetically recognised as safe) strains or genetically modified non-pathogenic strains. The solid-state fermentation process coupled with genetic improvements of strains and adherence to strict safety protocols are essential in large-scale production. These strategies would ensure that the production of cellulase can be safely and efficiently scaled for industrial applications. More research should be directed towards identifying novel endophytic sources of enzymes in medicinal plants and optimizing the production of biofuels in these endophytes. Further research is needed to evaluate and optimize the endophytes as sources for other groups of enzymes. It is possible to combine different agrowaste raw materials to develop the optimal media for cellulase production.

Materials and methods

Sampling

Samples were collected from Kanyonyoo in Kitui County (1°11′ 3″ S, 37° 49′11″ E) and Gachie in Kiambu County (1° 13′ 0″ S, 36° 47′ 0″ E). The samples were collected in August 2021 during the dry season. The permission for collection of the samples was granted by the owners of the sampling locations. The samples were identified and deposited in the Kenyatta University Herbarium with the help of staff in the Plant Sciences Department. The voucher specimen numbers provided for *A. Secundiflora* and *A. indica* and are PKM 001 and PKM 002 respectively. The mature *A. secundiflora* leaves sampled were over 30 cm in length, with a base leaf width of 10 cm and above. The young leaves were less than 30 cm long, with a leaf base width of less than 8 cm. The young leaves of *A. indica* sampled were purplish or reddish. The mature leaves were green, with dented edges, and between 4 and 8 cm in length. The leaves of *A. secundiflora* and *A. indica* were transported to Kenyatta University Microbiology Laboratories in labelled plastic bags and processed separately for each county within 24 h.

The leaves were washed with running tap water and allowed to air dry before being sterilized by immersing them in 70% ethanol for two minutes, followed by a minute in 3.5% sodium hypochlorite and rinsing five times using double distilled water. They were then dried using sterile blotting paper in a laminar flow cabinet. The leaves were cut aseptically using a sterile blade into 1 cm by 1 cm segments[4]. The segments prepared were inoculated on potato dextrose agar (PDA) containing streptomycin to inhibit bacterial contamination. They were incubated for five days at room temperature. The cut surfaces were placed in contact with the culture media to promote the growth of endophytes on the plate. The double-distilled water from the final rinsing was incubated on a control Petri dish on PDA to confirm that the surfaces of the leaves were sterile[63];[5]. The emerging fungal hyphae were sub-cultured on fresh PDA and incubated for five days at room temperature. The isolates were differentiated based on morphology, colour, and characteristics of the culture[64].

The colonization frequency by an endophyte was calculated using Eq. 1 below, as described by Vyawahare et al.[5].

$$\text{Frequency}\,\% = \frac{\text{Number of segments colonized by an endophyte}}{\text{Total number of segments}} \times 100 \quad (1)$$

Macroscopic and microscopic morphological identification of fungal isolates

The fungal endophytic isolates were studied macroscopically by observing features of the colony, including shape, colour, size, and mycelia. Small portions of the mycelia of each fungus were then loaded onto slides and stained using Lacto phenol cotton blue and observed using a compound microscope for spores, as described by[65].

DNA isolation

The mycelia of the fungal isolates were transferred to sterile microcentrifuge tubes containing 500 µL of sterile normal saline. The tubes were centrifuged to pellet the hyphae, and the normal saline was decanted. This process was repeated thrice to get rid of extracellular polymeric substances[66]. This was followed by cell lysis. Cell lysis buffer pH 8.0 containing (2% w/v CTAB, 1.4 M NaCl, 100 mM Tris–HCl pH 8.0, 20 mM EDTA pH 8.0, and 0.3% Mercaptoethanol) was added to the tubes [66]. The samples were vortexed for 1 min and then incubated at 65 °C for one hour. They were then centrifuged for 5 min at 13,000 rpm. The liquid phase was transferred to sterile microcentrifuge tubes, and an equal volume of chloroform:isoamyl alcohol (24:1) was added [66]. The mixture was gently mixed by inversion and incubated at – 20 °C for 30 min [66]. Centrifuging was done at 13,000 rpm for 4 min, and the supernatant was transferred to a fresh tube. DNA was precipitated by adding 500 µL of chilled

absolute ethanol to the supernatant, gently inverted, and left to for 1 h at room temperature [7]. This mixture was centrifuged at 12,000 rpm for 4 min to pellet the DNA. The supernatant was discarded, and the pellet was washed with 110 µL of chilled 70% ethanol and centrifuged at 12,000 rpm for 2 min [66]. After decanting the 70% ethanol, the pellet was air dried by inversion on sterile paper towels, followed by elution of the DNA using 50 mL TE buffer pH 8.0[66].

DNA amplification

The extracted DNA was amplified using ITS 1 (5' – TCCGTAGGTGAACCTGCGG – 3') and ITS 4 (5' – TCCT CCGCTTATTGATATGC – 3'), using a 25 µL One Taq Quick-load 2X master mix with a standard buffer from New England BioLabs. The reaction mixture contained 1.25 µL of each primer (10 µM), 12.5 µL, 2X master mix with buffer, 9.0 µL PCR water, and 1.0 µL of isolate DNA. The PCR was set for amplification for 35 cycles[66]. Initial denaturation was set at 94 °C (2 min), followed by 35 cycles of denaturation at 94 °C (45 s), annealing at 52 °C (45 s), and extension at 72 °C (2 min)[66]. The final extension was set at 72 °C (5 min). The amplification was a modification of the amplification perimeters used by[66].

DNA gel electrophoresis, sequencing, and identification of endophytic fungal isolates

After DNA amplification, the PCR products were subjected to gel electrophoresis. DNA loading buffer (3 µL) containing SYBR GREEN dye was mixed with 3 µL aliquots of the PCR product and pipetted into the 1% agarose gel wells in 0.5X Tris borate-EDTA (TBE) buffer and run at 80 V for 30 min and visualized with a UV trans-illuminator[67], to determine the quality and quantity of the PCR products. The amplified fragments were sent to MACROGEN in the Netherlands, Europe for standard DNA sequencing using the sanger dideoxy method and identified with the aid of the National Centre for Biotechnology Information genome sequence database.

Solid state fermentation (SSF) for cellulase enzyme production

Preparation of the substrate

Cellulase enzymes were produced under SSF using maize cobs as substrates. To remove moisture, the maize cobs were dried for three days at room temperature. The maize cobs were then ground and sieved using a 2 mm sieve. The maize cob powder was used as a carbon source in the solid-state fermentation (SSF) process. According to Kanengoni et al.[68], the carbon source composition of maize cob is primarily made up of cellulose (45–55%), hemicellulose (25–35%), and lignin (20–30%). These polysaccharides are the key substrates for cellulase enzyme production by the endophytic fungi. Furthermore, maize cob contains small quantities of lipids, proteins, and essential minerals that support growth and enzyme secretion in fungi. Five grams of the substrate were dispersed into 350 mL bottles, and 10 mL of the Bushnell-Haas solution (g/L) with constituents as described by[69], (0.1 g of $MgSO_4.7H_2O$, 0.5 g of Na_2HPO_4, 0.5 g of KH_2PO_4, 0.5 g of NH_4NO_3, 0.05 g $FeCl_3.6H_2O$, and 0.02 g $CaCl_2$) was dispersed into each bottle. The bottles were plugged with cotton wool and sterilized at 121 °C, 15 psi, for 20 min. The bottles were cooled to room temperature and inoculated with 2.5 mL of inoculum for each fungus[69].

Preparation of the inoculum and fermentation

The endophytic fungal isolates were inoculated in PDA for 5 days. The inoculum was adjusted to 1×10^6 CFU, and 2.5 mL were dispersed into 350 mL bottles with maize cobs prepared as in Sect. "Preparation of the substrate".[23] Filamentous fungi cultured on PDA for 5 days were scrapped off the media and blended in 5% sucrose solution for 30 s, and 2.5 mL of the blended inoculum was used to inoculate 350 mL bottles prepared as in Sect. "Preparation of the substrate"[23]. The inoculum was thoroughly mixed with the substrate, and the bottles were incubated at room temperature. Samples were taken in triplicates at 3, 6, and 9 days after inoculation for enzyme extraction for each fungus[23].

Enzyme extraction

The cellulase enzymes were extracted by adding 50 mL of 50 mM citrate buffer, pH 4.8, to each bottle and left to stand for 2 h at room temperature. The contents of the bottles were filtered and strained using clean cheesecloth. The filtrate was collected and centrifuged at 10,000× g for 10 min[23]. The supernatant containing the enzymes was collected and stored at – 20 °C awaiting bioassays[23].

Enzyme assays

The crude enzyme extracts were assayed for the total cellulase (FPase), endoglucanase, and exoglucanase activities.

Total cellulase activity (FPase)

Filter paper assay was adopted to determine the total cellulase enzyme activity according to[70]. Strips of Whatman Filter Paper No. 1 measuring 6 cm by 1 cm were cut, folded, and placed into 2.0 mL labelled microcentrifuge tubes. Five hundred microliters of 50 mM citrate buffer pH 4.8 were added to the tubes, and then five hundred microliters of the harvested enzymes (the supernatant obtained in Sect. "Enzyme extraction") were added. The control tubes had five hundred microliters of citrate buffer added instead of the enzyme extract. For the standards, one gram of glucose was dissolved in citrate buffer pH 4.8 in a 100 mL volumetric flask. Different concentrations of glucose were prepared by serial dilution in thirteen microcentrifuge tubes. A modified version of the method by[70] was used to determine glucose concentration range. The range of glucose concentration was from 0 to 2.4 mg/mL (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, and 2.4). The tubes containing the glucose standards and crude enzymes were incubated at 50 °C in a water bath for 1 h, and the amount of reducing sugars produced was determined using the DNS reagent (10 g of 3,5-dinitrosalycyclic acid, 0.5 g sodium sulfite, and 10 g NaOH) as described by[70]. Seven hundred microliters of DNS were added to all the enzyme bioassay

activity tubes and standard tubes to stop the reaction[14]. The tubes were then boiled for 5 min and cooled to room temperature. Three hundred microliters of 400 g/L potassium sodium tartrate (PTS) were added to the tubes containing enzymes, glucose standards, and negative control. The absorbance of the reducing sugars produced was determined at 540 nm using a JENWAY 6300 spectrophotometer, quantified using a glucose standard curve, and converted to enzyme units using the Eq. 2 below, as described by Rahnama et al.[23]:

$$Enzyme\ units\ (IU/mL) = \frac{(f-c)}{m} \times \left(\frac{df}{Sv}\right) \times \left(\frac{1}{T}\right) \times \left(\frac{1000\mu g}{180.16\mu g}\right) \times \left(\frac{50}{5}\right) \quad (2)$$

where *f* refers to final absorbance, *c* refers to the intercept, *df* stands for dilution factor, *m* refers to the slope from the standard curve, *Sv* stands for sample volume, and *T* is time expressed in minutes[23].

Endoglucanases activity
Endoglucanase activity was determined using 1% Carboxymethylcellulose (CMC) prepared in 50 mM citrate buffer pH 4.8 substrate as described by Shuangqi et al.[71]. Five hundred microliters of the CMC solution were transferred into 2.0 mL microcentrifuge tubes, and five hundred microliters of the extracted enzyme were added, while 500 µL of buffer were added to the control tubes[71]. The glucose standards were prepared as described in Sect. "Total cellulase activity (FPase)". The tubes were incubated in a 50 °C water bath for 30 min. The amount of reducing sugars produced was quantified as described for FPase[71].

Exoglucanases activity
Exoglucanase activity was determined using 1.25% microcrystalline cellulose (avicel) prepared in 100 mM sodium acetate buffer, pH 4.8. Five hundred (500) µL of avicel solution and 500 µL of the enzyme extract were added to 2 mL microcentrifuge tubes[71]. The control tubes contained 500 µL of the buffer. The tubes were incubated in a 50 °C water bath for 2 h[71]. The reducing sugars produced were quantified as described for FPase.

Data analysis
Consensus for sequences generated using ITS1 and ITS4 primers were created using BioEdit Software 7.1 after base calling using Chromas Lite 2.0 Software. A BLAST (Basic Local Alignment Search Tool) search of the consensus sequence on the National Centre for Biotechnology Information database was used for molecular identification of the isolates. The isolate consensus and identity sequences were aligned using the MUSCLE algorithm in MEGA X software[72]. The phylogenetic tree was developed using the neighbour-joining method, and evolutionary distances were determined using the maximum composite likelihood of the MEGA X statistical tool. The R statistical tool was used to analyse enzyme bioassay data. Two-way ANOVA was used to determine any significant difference in enzyme production on the third, sixth, and ninth days of the study using Tukey's HSD. Differences were considered significant if the p-value ≤ 0.05[67].

Data availability
Sequence data that support the findings of this study have been deposited in the DNA Data Bank of Japan (DDBJ) with the link https://ddbj.nig.ac.jp/arsa/search?lang=en&cond=quick_search&sortTarget=score&sortOrder=desc&displayFields=PrimaryAccessionNumber%2CDefinition%2CSequenceLength%2CMolecularType%2COrganism&query=mwendwa&operator=AND&filterQuery=Division%3APLN.

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Author contributions

PKM: drafted the proposal, collected data, carried out the experiments, analysis, interpretation, and prepared the manuscript draft. AK and JMM: reviewed the proposal, analysis and interpretation, preparation and review of the manuscript draft. GO: enzyme assays and data analysis. All authors reviewed and approved the final draft version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to P.K.M. or A.W.K.