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Research Article
Morphological and Molecular Characterization of *Vicia faba* and *Phaseolus vulgaris* Seed-born Fungal Endophytes

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Abstract

Background: Fungal endophytes are heterotrophic microorganisms that occur inside plant tissues, with some showing adverse effects against insects, nematodes and plant pathogens. Initiatives are underway at the International Center of Insect Physiology and Ecology (ICIPE) to use these endophytes as a novel strategy for control of *Liriomyza* leafminer. **Objective:** The objective of this study was therefore to search for fungal endophytes from the *Liriomyza* major host plants that could be used in the management of this invasive insect pest. **Materials and Methods:** Bioprospecting was undertaken to isolate fungal endophytes from *Phaseolus vulgaris* and *Vicia faba* seeds collected from different local and super markets in Kenya. Fungal endophytes were isolated through seeds surface sterilization and characterized using morphological and molecular techniques. Fungal occurrence was analyzed using analysis of variance while Chi-square tests were performed to compare the various endophytes species occurring in the two host seeds. **Results:** Five fungal isolates were isolated from both *P. vulgaris* and *V. faba* seeds with no significant differences in their occurrence. However, there was significant difference between the endophyte species occurring in *V. faba* compared to *P. vulgaris* seeds ($p<0.0001$). Isolated fungi included *Beauveria bassiana*, *Phialemonium* sp., *Phanerochaete chrysosporium* and *Metarhizium anisopliae*. *Beauveria bassiana* ICIPE 693 occurred in all the *V. faba* seeds (100%) but not in *P. vulgaris* seeds. Similarly, *P. chrysosporium* had 66.7% occurrence in *V. faba* but absent in *P. vulgaris* seeds. The prevalence of *Phialemonium* sp. (55%) was only recorded in *P. vulgaris*, while that of *M. anisopliae* was recorded in both *P. vulgaris* and *V. faba* seeds with 55.4 and 70.8% occurrence, respectively. **Conclusion:** Fungal endophyte species occurrence in *V. faba* differed from *P. vulgaris* seeds. The characterization of these bean seed-born endophytes will not only create awareness but also facilitate studies on the role of these fungi in pest management strategies. The outcome of this study will stimulate further studies on the possible roles of these fungi in inhibiting the growth of artificially inoculated endophytes, assessing their pathogenicity and virulence effects on different sucking arthropod pests and evaluating the nutritional value of the seeds containing these endophytes.

Key words: Seed-bioprospecting, *Beauveria bassiana*, *Phialemonium* sp., *Phanerochaete chrysosporium*, *Metarhizium anisopliae*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Endophytes are heterotrophic microorganisms[1] that live inside plants primarily for nutrition, protection and reproduction. Some of them are beneficial by showing adverse effects against insects, nematodes and plant pathogens[2,3]. Fungal endophytes have been detected in hundreds of plant species, including important agricultural crops such as wheat[4], bananas[5-7], soybeans[8] and tomatoes[9]. Endophytes occur in virtually all tissues of the host plant, however, Baker and Smith[10] and Schardl *et al.*[11] suggested that seeds may serve as good sources of endophytes or pathogens. Seeds are of particular interest as they may transmit endophytes vertically from generation to generation[12]. Indeed, plant seeds usually fall into the soil, a microbially rich habitat and lie dormant waiting for environmental cues to germinate, possibly recruiting surface microbes to help protect them against degradation or predation[13]. As seeds begin to germinate, endophytes may colonize the seedling as shown in rice[14,15], eucalyptus[16], maize[17] and beans.

However, most recent studies on seeds born-fungi were mainly focused on the fungal pathogens that transmit plant diseases to the host plants and consequently determined the quality of the seeds as well as their effects on the seeds germination[18-21]. In addition, until recently, most of the studies on these seed-born fungi were targeting fungi or microorganisms that were found to compromise the quality of the seed in terms of nutritional value and oil quality[22] but not necessarily in the prospect of exploring possible entomopathogenic fungi inside the seeds and that could be used not only as plant growth promoters but also as biocontrol candidates under the umbrella of pest management strategies. Gouda *et al.*[23] considered endophytes as a treasure of bioactive compounds and reported that they act as reservoirs of novel bioactive secondary metabolites, such as alkaloids, phenolic acids, quinones, steroids, saponins, tannins and terpenoids that serve as a potential candidate for antimicrobial, anti-insect, anticancer and many more properties. Hence, exploring possible seed endophytes could give a new research route to explore their potential for insect pest's management.

Horticultural crops are highly valued due to their importance in terms of nutrition, income generation and employment[24-26]. In Kenya where this study was conducted, *Phaseolus vulgaris* and *Vicia faba* are among the high value horticultural crops, which are also attacked by the invasive Agromyzid leafminer species, *Liriomyza huidobrensis* (Blanchard) *L. sativae* Blanchard and *L. trifolii* (Burgess) (Diptera: Agromyzidae) the most damaging pests on these crops[27,28]. It is then important to assess the occurrence of fungal endophytes in *P. vulgaris* and *V. faba* seeds in the scope of developing a sustainable management strategy against these pests and indirectly, reduce the abusive use of insecticides.

Fungal endophytes have been recognized to play multiple roles such as protecting plants from pests and diseases and promoting plant growth[29,30] and are being considered as important component of IPM[7,31-34]. Some fungal endophytes protect host plants against plant pathogens[35,36] and herbivores, including insects[29,32,37-40]. For example, exposure of two aphid species, *Rhopalosiphum padi* and *Metopopophium dirhodum* (Hemiptera: Aphididae) and wheat stem sawfly, *Mayetiola destructor* (Diptera: Chloropidae) to wild barley infected with *Neotyphodium coenophialum* (Hypocreales: Clavicipitaceae) reduced their survival[41,42]. Wheat leaves colonized by either *B. bassiana* or *Aspergillus parasiticus* (Eurotiales: Trichocomaceae) reduced the growth rate of *Chortoicetes terminifera* (Orthoptera: Acrididae) nymphs[34]. Endophytic *B. bassiana* in banana significantly reduced larval survivorship of banana weevil, *Cosmopolites sordidus* (Coleoptera: Curculionidae), resulting in 42-87% reduction in plant damage[43,44]. Reduction in feeding and reproduction by *Aphis gossypii* (Hemiptera: Aphididae) has also been reported on cotton endophytically colonized by either *B. bassiana* or *Lecanicillium lecanii* (Hypocreales: Clavicipitaceae)[34]. Another possible role of the fungal endophytes includes plant growth promotion as well as impact on tritrophic interaction[7,29,45,46]. Fungal entomopathogens that become established as endophytes can therefore play an important role in the regulation of insect populations. Most recently, Akutse *et al.*[31] demonstrated the effects of fungal endophytes on life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae) leafminers and their associated parasitoids[47]. The researchers reported that endophytic fungal isolates of both *Hypocrea lixii* (F3ST1) and *Beauveria bassiana* (G1LU3, S4SU1 and ICIPE 279) could endophytically colonize *Vicia faba* and *Phaseolus vulgaris* plants through seeds inoculation and cause detrimental effects on survival, fecundity, oviposition, emergence and longevity of *L. huidobrensis*. Thus, this study focuses on assessing the occurrence of fungal endophytes in *P. vulgaris* and *V. faba* seeds to develop a sustainable management strategy against *Liriomyza* leafminer pests. The aim was therefore to search for fungal endophytes that can be developed further for the control of the invasive *Liriomyza* leafminer specifically and other crop insect pests and diseases. Bioprospecting was subsequently carried out for isolation of fungal endophytes from *P. vulgaris* and *V. faba* seeds and we report here on their morphological and molecular characterization.

MATERIALS AND METHODS

Seeds: Seeds of open pollinated varieties of *Phaseolus vulgaris* (Rose coco beans) and *Vicia faba* (Faba beans) were collected from local markets in Kenya (Nairobi, Kasarani, Eastleigh, Nyamakima, Gikomba and sometimes from Ethiopia to Kenya's markets) and sometimes procured from super markets (Naivas) since farmers often use these seeds for sowing.

Fungal isolation and culture: Seeds were surface-sterilized in 70% ethanol for 2 min followed by 1.5% sodium hypochlorite for 3 min after which they were rinsed three times with sterile distilled water[32]. Prior to incubation of seeds at the laboratory (25±1°C and 60% RH), half of the seeds were ground in a sterile motor and the other half used as a whole for detection of variations in the fungal endophyte species occurrence and diversity. Three to five seeds were plated onto 9 cm petri dishes containing either Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) or Yeast Extract Agar (YEA). A total of 320 seeds were used and incubated in 40 plates per seed species and replicated 5 times.

To determine the presence of fungal endophytes in both ground and whole seeds, the latter was placed on PDA, SDA and YEA plates (at a distant of 4 cm apart) containing 0.05% antibiotics (Streptomycin sulfate salt and chloramphenicol)[33,34]. Plates were incubated at 25±1°C for 14 days, after which the presence of endophytes was determined. Prior to incubation of the seeds, the last rinse water was also plated to assess the effectiveness of the surface sterilization procedure[32]. The presence or occurrence of fungal endophytes in the seeds was recorded by counting the number of seeds of the different varieties that showed fungal growth/mycelia according to Koch's postulates[48]. This assessment of the fungal endophytes occurrence was repeated daily from the 1st day of incubation until 14 days post-incubation.

Isolated fungi were sub-cultured 4-6 times to obtain pure cultures and preserved in the ICIPE'S Arthropod Germplasm Center.

Morphological identification: Morphological identification was carried out using the procedures described by Burgess *et al.*[49], Humber[50] and Goettel and Inglis[51]. Fungal colony features, such as appearance, texture (colonization pattern) and pigmentation on both the top and reverse plates were observed[52,53].

Slide culture preparations were used for observation of the following characteristics: (1) Presence or absence of microconidia and macroconidia, (2) Shapes and sizes of conidia, (3) Type of phialides bearing microconidia, (4) Presence or absence of chlamydospores, microconidial chains and sporodochia and (5) Type of fungal mycelia. The variability of colony appearance, pigmentation, growth rate, length of chains, production of bluish sclerotia and concentric ring aerial mycelium were also observed.

Morphological data analysis: Fungal occurrence was expressed as a percentage of the total number of seeds that were plated out. Percentages were square root transformed [$\sqrt{(x+1)}$] before applying analysis of variance (ANOVA) in R (2.13.1). Tukey HSD multiple comparisons of means was used to separate the means. The chi-square tests were used to compare the various endophytes species occurring in *V. faba* and *P. vulgaris* seeds. All the analyses were performed using R (2.13.1) statistical software packages[54], while relying heavily on the epicalc package[55].

Molecular characterization

Preparation of fungal material: Conidia were harvested by scraping the surface of 2 week old cultures with a sterile spatula. Genomic DNA was extracted from the harvested conidia/mycelia using the Fast DNA Spin® kit for soil following the manufacturer's instructions (MP Biomedicals). Mycelia/conidia were freeze-dried for 24 h to facilitate easy grinding. Isolates of *B. bassiana* ICIPE 279 and *M. anisopliae* ICIPE 30 and S4ST7, previously used in the endophytic colonization study[31] were included as references in the phylogenetic trees for sibling or related isolate species characterization. These were obtained from the ICIPE'S Arthropod Germplasm Center.

Amplifications using ITS 5 and 4 and TW81 and AB28 primers: Amplifications were carried out for the rDNA region of the fungal isolates using[56] the ITS 5 and 4 and the TW81 and AB28[57] primers. The isolated DNA was amplified in 30 μL^{-1} PCR mix. The reaction mixture consisted of 3 μL^{-1} 10X PCR buffer (GenScript USA Inc), 1.5 μL^{-1} 25 mM $MgCl_2$, 0.6 μL^{-1} 10 mM dNTP mix, 1.5 μL^{-1} 10 µM of the primers (Table 1), 1 unit Taq polymerase (GenScript USA Inc) and 20 ng μL^{-1} genomic DNA. For the ITS region, the thermo-cycling conditions involved initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 58°C for 40 sec and primer elongation at 72°C for 1 min followed by a final extension at 72°C for 10 min giving a product range of between 550-600 bp.

Table 1: Primer sequences used in the DNA amplification

Primer name	Sequence (5'-3')	Expected size of PCR product
ITS 5	F5' GGA AGT AAA AGT CGT AAC AAG G 3'	550-600 bp
ITS 4	R5' TCC TCC GCT TAT TGA TAT GC 3'	
TW81	F5' GTT TCC GTA GGT GAA CCT GC 3'	500-550 bp
AB28	R5' ATA TGC TTA AGT TCA GCG GGT 3'	

The same procedure was applied during amplification using the TW81 and AB28 primers with the exception that the reaction was set for 40 cycles with the annealing temperature of 57°C for 40 sec. This gave a target region ranging between 500-550 bp. Both reactions were done in a PTC 100 thermocycler (MJ Research, Gaithersburg).

DNA purification and sequencing

Agarose gel electrophoresis and purification: The PCR products were resolved through 1% agarose gel for 1 h at 70 V (Bio-Rad model 200/2-0 power supply and wide mini-sub cell GT horizontal electrophoresis system, Bio-Rad laboratories, Inc., USA), followed by visualization of the DNA under UV-illumination. The gel was photographed, analyzed and documented using KODAK Gel Logic 200 Imaging System software (Raytest, GmbH, Straubenhardt).

The products were then gel purified using the QuickClean 5M Gel Extraction Kit II from GenScript (GenScript Corporation, Piscataway, NJ), following the manufacturer's instructions and subsequently sequenced in both directions using ABI 3700 genetic analyzers.

Sequencing and molecular data analysis: The sequences obtained were assembled and edited using Chromas version 2.13 (Technelysium Pty ltd, Queensland, Australia). Consensus sequences from both the forward and reverse strands were generated and were then queried through BLASTN in the GenBank database provided by the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm. nih.gov/) for identification purposes and to check for similarity with organisms already identified. Any isolate exhibiting ≥95% sequence similarity to NCBI strains were considered as the correct species for that isolate[58].

Moreover, the consensus sequences were aligned using[59] ClustalX version 1.81. These alignments were used for phylogenetic and molecular evolutionary analyses that were conducted[60] using MEGA version 6. Neighbour-joining trees were constructed[61] with bootstrapping and using the Kimura 2 distance matrix[62,63] for both sets of sequences of the 2 gene regions. Tables of between species distances were also constructed using[60] MEGA version 6. The tables of distances were used to generate the principal component plots using the program[64] GenAlEx 6.41.

RESULTS

Morphological identification of seed-born fungal endophytes: No fungal growth was observed in any of the plated washing water. There were no significance differences ($F = 0.97$, $df = 3, 20$, $p = 0.423$) in the growth of fungal endophytes in ground and whole seeds of both *V. faba* and *P. vulgaris*. A total of 5 fungal isolates were obtained from the 320 seeds of *V. faba* and *P. vulgaris* using morphological features and they belonged to genera *Metarhizium*, *Beauveria*, *Phialemonium* and *Phanerochaete* (Fig. 1). Among the key morphological features used to identify the isolates in the study that belong to the *M. anisopliae* included (1) The type of conidia formed that appeared either in short to long chains, (2) The short conidiogenous cells, with rounded to broadly conical apices (not having a distinctly narrowed and extended neck), (3) The conidiogenous cells borne at the apices of broadly branched and densely intertwined conidiophores that form a compact hymenium while the conidia are borne in parallel chains that are usually green in mass and (4) The cylindrical or ovoid conidia, forming chains that usually aggregated into a solid mass and appear as pale to bright green to yellow-green in colour (Fig. 1). For the *B. bassiana* isolates, the conidia were produced singly on many separate denticles on each conidiogenous cell or, if they were growing in some sort of slime, they were either singly or in small groups in a slime droplet. Their conidiogenous cells had globose bases with extended denticulate raches/apex and each rachis normally bore a single conidium per denticle. The conidia were usually white in colour (Fig. 1).

While the genus *Phialemonium* isolate was characterized by its abundance of adelophialides and few discrete phialides with no signs of collarettes. It also had distinct grayish white to brownish colonies pigmentation (Fig. 1) and allantoid conidia which had a cylindrical to bean-shaped structure.

Lastly, the white rot fungus *Phanerochaete chrysosporium* isolate had colonies which were white in colour when cultured on Potato Dextrose Agar (Fig. 1). The isolate also had hyaline mycelia which were well branched and lacked clamp-connections. Likewise, three types of conidia were observed, the aleuriospores, arthrospores and chlamydospores.

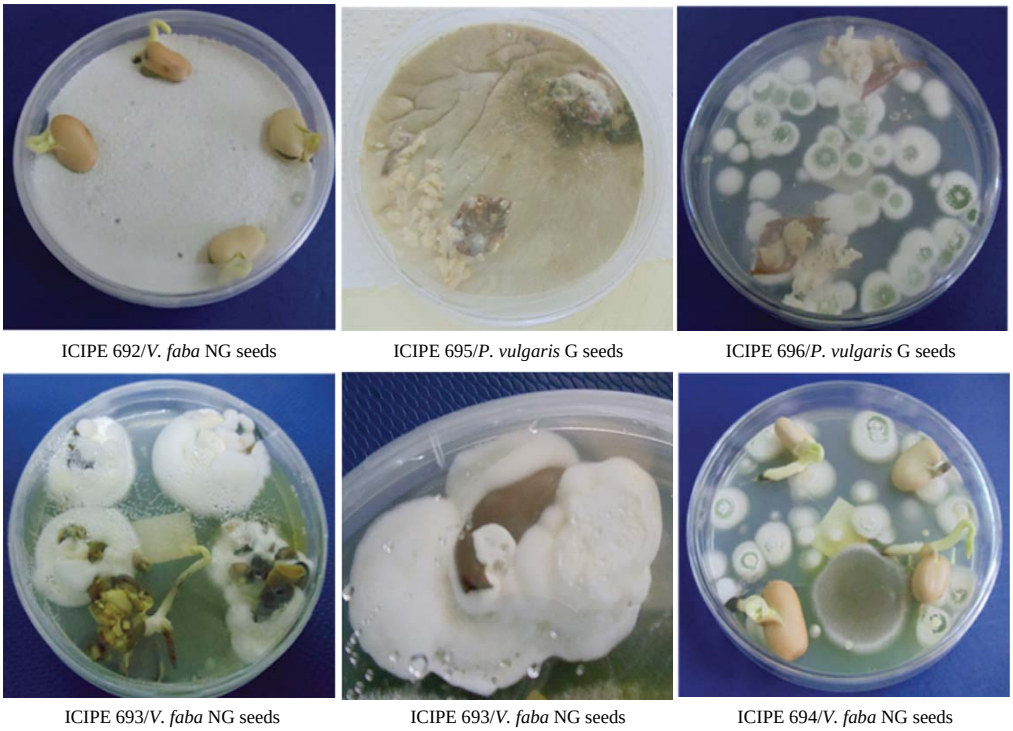

Fig. 1: Fungal species isolated from *Phaseolus vulgaris* and *Vicia faba* seeds 2 weeks after incubation. ICIPE 692: *Phanerochaete chrysosporium*, ICIPE 695: *Phialemonium* sp., ICIPE 696: ICIPE 694: *Metarhizium anisopliae* and ICIPE 693: *Beauveria bassiana*, G: Grinded seeds, NG: Non grinded seeds

These isolates were given ICIPE'S Arthropod Germplasm Centre's accession numbers as follows: *Phialemonium* sp., isolate ICIPE 695, *Phanerochaete chrysosporium* isolate ICIPE 692, *Beauveria* sp., isolate ICIPE 693 and *Metarhizium* sp., isolates ICIPE 694 and ICIPE 696, in addition to the existing standards isolates (ICIPE 30, ICIPE 279 and S4ST7) (Table 2). GenBank accession numbers provided for the nucleotide sequences of the fungal isolates for both primers pairs are as follows: For ITS 5 and 4, S4ST7 = KM463106, ICIPE 279 = KM463107, ICIPE 694 = KM463108, ICIPE 696 = KM463109, ICIPE 692 = KM463110, ICIPE 695 = KM463111, ICIPE 693 = KM463112, ICIPE 30 = KM463113 and for TW81 and AB28, ICIPE 693 = KM463114, ICIPE 696 = KM463115, ICIPE 695 = KM463116, ICIPE 30 = KM463117, ICIPE 694 = KM463118, ICIPE 279 = KM463119, S4ST7 = KM463120, ICIPE 692 = KM463121 (www.ncbi.nlm.nih.gov/books/NBK51157/, GenBank Submissions Handbook).

There were no significant differences in the prevalence/occurrence of fungal endophytes between *V. faba* ($F = 0.81$, $df = 3, 20$, $p = 0.502$) and *P. vulgaris* seeds ($F = 0.47$, $df = 3, 20$, $p = 0.707$) (Fig. 2). However, there was significant difference between the endophyte species, which occurred in *V. faba* compared to *P. vulgaris* seeds ($\chi^2 = 31.11$, $df = 1$, $p<0.0001$). For instance, *B. bassiana* ICIPE 693 which occurred in all the *V. faba* seeds (100%) did not occur in *P. vulgaris* seeds (Fig. 2). Similarly, the occurrence of *Phanerochaete chrysosporium* ICIPE 692 was 66.7% in *V. faba* whereas, the prevalence of *Phialemonium* sp. ICIPE 695 was of 55% in *P. vulgaris* only. However, the prevalence of *M. anisopliae* was recorded in both *P. vulgaris* (ICIPE 696, with 55.4% occurrence) and *V. faba* seeds (ICIPE 694, with 70.8% occurrence) (Fig. 2).

Molecular characterization of seed-born fungal endophytes: The molecular identification of the endophyte isolate species after sequencing is consolidated in Table 2. All fungal isolates identified using the ITS 5 and 4 primer sets were also confirmed by the TW81 and AB28 primer sets. The two sets of primers depicted isolate species identities with 99-100% similarity and 0.0 E values (Table 2). Like the morphological identification, the molecular characterization also yielded four isolates species identities: *Metarhizium anisopliae* (isolates ICIPE 694, ICIPE 696, S4ST7 and ICIPE 30), *Beauveria bassiana* (isolates ICIPE 693 and ICIPE 279), *Phanerochaete chrysosporium* (isolate ICIPE 692) and *Phialemonium* sp. (isolate ICIPE 695) (Table 2). Thus the molecular identification corroborates with the morphological identification for the endophyte isolates (Fig. 1, Table 2).

Table 2: Identified fungal endophytes species using ITS 5 and 4 and TW81 and AB28

Sample codes	Length (base pair)	Accession No.	Identified fungal samples	E-value	Identities (%)
ITS 5 and 4					
ICIPE 30	541	FJ545302.1	*Metarhizium anisopliae* isolate CNXJ2 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	99
S4ST7	541	FJ545302.1	*Metarhizium anisopliae* (same isolate CNXJ2 18S ribosomal RNA gene)	0	99
ICIPE 694	541	FJ545302.1	*Metarhizium anisopliae* (same isolate CNXJ2 18S ribosomal RNA gene)	0	99
ICIPE 696	541	FJ545302.1	*Metarhizium anisopliae* (same isolate CNXJ2 18S ribosomal RNA gene)	0	99
ICIPE 279	551	JQ266208.1	*Beauveria bassiana* strain MTCC_6286 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	99
ICIPE 693	551	AJ560668.1	*Beauveria bassiana* ITS1, 5.8S rRNA gene and ITS2, isolate IMI 386701	0	100
ICIPE 692	621	GU966518.1	*Phanerochaete chrysosporium* strain TS03 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	99
ICIPE 695	538	KF746155.1	*Phialemonium* sp., 1 AE-2013 strain F5070 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	99
TW81 and AB28					
ICIPE 30	507	FJ545302.1	*Metarhizium anisopliae* isolate CNXJ2 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	99
S4ST7	507	FJ609312.1	*Metarhizium anisopliae* strain M1311 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, partial sequence	0	100
ICIPE 694	507	FJ609312.1	*Metarhizium anisopliae* strain M1311 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	100
ICIPE 696	507	FJ609312.1	*Metarhizium anisopliae* strain M1311 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	100
ICIPE 279	553	JQ999974.1	*Beauveria bassiana* strain YNSK1106 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	99
ICIPE 693	517	AJ560668.1	*Beauveria bassiana* ITS1, 5.8S rRNA gene and ITS2, isolate IMI 386701	0	100
ICIPE 692	587	GU966518.1	*Phanerochaete chrysosporium* strain TS03 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	99
ICIPE 695	504	KF746155.1	*Phialemonium* sp., 1 AE-2013 strain F5070 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence	0	99

Phylogenetic trees

Phylogenetic tree using the ITS 5 and 4 gene region: The evolutionary history was inferred using the Neighbor-Joining method[61]. The optimal tree with the sum of branch length = 0.68264451 is shown in Fig. 3. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches[65]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Fig. 3). The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 506 positions in the final dataset.

Four groups resulted from this analysis (Fig. 3). The first group consisted of the *M. anisopliae* isolates where each of the sample (ICIPE 696 and ICIPE 694) and the standards (ICIPE 30 and S4ST7) isolates branched separately. Furthermore, all the *Metarhizium* isolates linked to a *Metarhizium* sp., of accession number FJ545302.1 during blasting (Table 2). The second group consisted of *B. bassiana* isolates ICIPE 693 and ICIPE 279, likewise branching from the same node but occupying different branches, a clear indication that the ICIPE 693 from *V. faba* seeds is different from the standard ICIPE 279 isolate. The last two clusters of the phylogenetic tree consisted of *Phialemonium* sp., isolate ICIPE 695 and *P. chrysosporium* isolate ICIPE 692, respectively (Fig. 3).

The genetic distances between the isolates were also inferred using Kimura 2-parameter model. The estimates of evolutionary divergence between sequences of the various endophyte isolates ranged between 0.0 and 0.472 (Table 3). Comparison of *M. anisopliae* isolates ICIPE 694 and

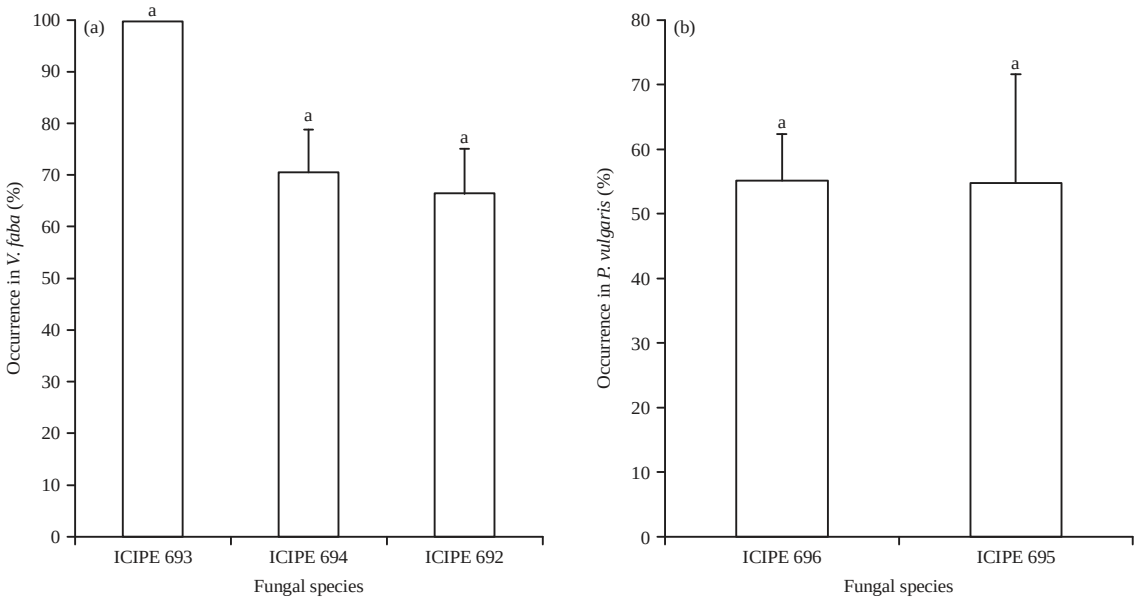

Fig. 2(a-b): Fungal endophytes species prevalence (%) in *Vicia faba* (left) and in *Phaseolus vulgaris* (right) seeds. ICIPE 692: *Phanerochaete chrysosporium*, ICIPE 695: *Phialemonium* sp., ICIPE 694: ICIPE 696: *Metarhizium anisopliae* and ICIPE 693: *Beauveria bassiana*

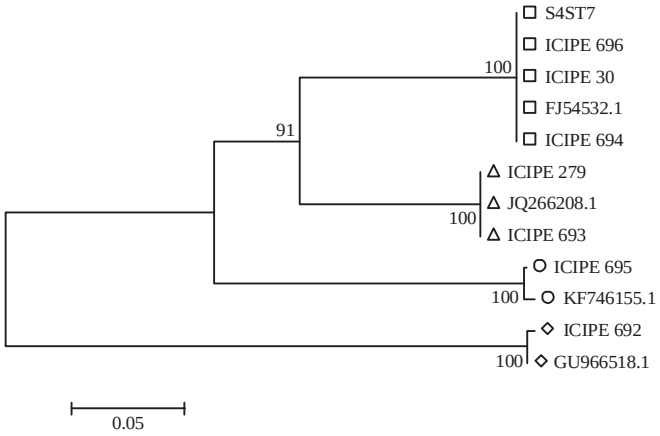

Fig. 3: Phylogenetic tree using ITS 5 and 4 regions showing the evolutionary relationships of fungal endophyte isolates from *Vicia faba* and *Phaseolus vulgaris*

ICIPE 696, isolated from *V. faba* and *P. vulgaris* with the standards ICIPE 30 and S4ST7 gave a square distance of 0.0. Similarly, comparison of *B. bassiana* isolate ICIPE 693 isolated from *V. faba* with the standard ICIPE 279, gave a square distance of 0.0 (Table 3). Table 3 was used to generate principal component plots. In the Principal Coordinate Analysis (PCA) plot, the 1st two axes explained 76.9% of the variation (the 1st axis 49.8% and the second axis 27.1%) (Fig. 4). The PCA separated the nine isolates into four distinct clusters. Each cluster was occupied by the isolates belonging to the different genera i.e., *Metarhizium*, *Beauveria*, *Phialemonium* and *Phanerochaete*, respectively (Fig. 4).

Phylogenetic tree using the TW81 and AB28 gene region: The evolutionary history was inferred using the Neighbor-Joining method[61]. The optimal tree with the sum of branch length = 2.17571022 is shown in Fig. 5. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches[65]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Fig. 5). The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding.

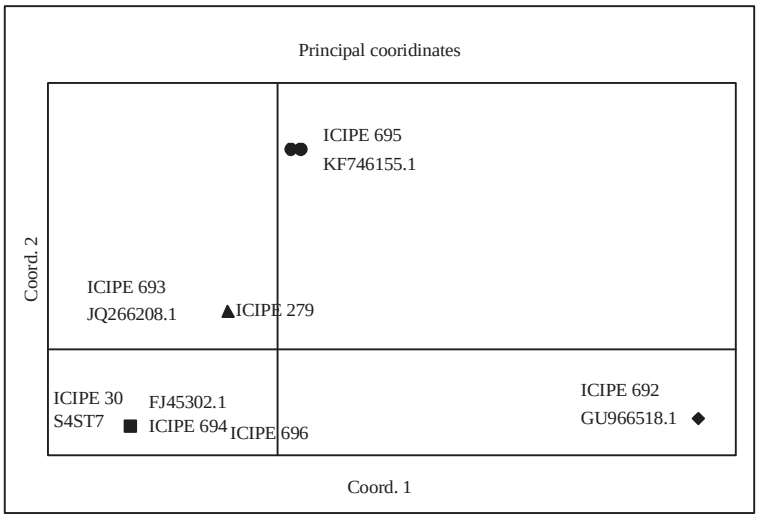

Fig. 4: Plot of Principal Components Analysis (PCA) via the covariance matrix with data standardization calculated using GenAlEx for the various endophytes species isolated from *Vicia faba* and *Phaseolus vulgaris* using the ITS 5 and 4 regions (PC1 = 49.78 and PC2 = 27.11%)

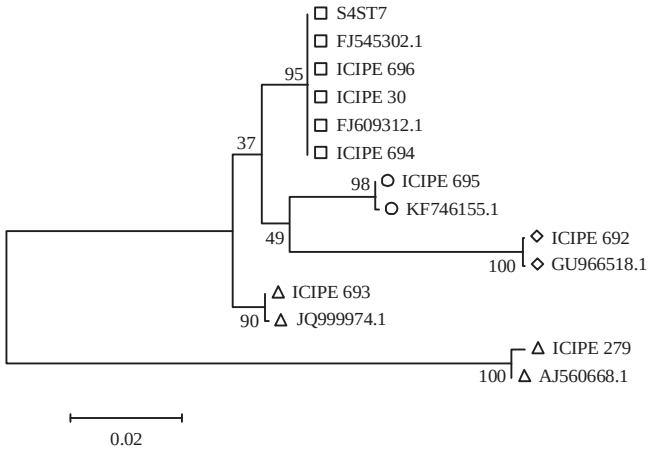

Fig. 5: Phylogenetic tree using TW81 and AB28 regions showing the evolutionary relationships of fungal endophyte isolates from *Vicia faba* and *Phaseolus vulgaris*

Table 3: Estimates of evolutionary divergence between sequences using ITS 5 and 4

Fungal isolates	ICIPE 279	JQ266208.1	ICIPE 693	ICIPE 694	FJ545302.1	ICIPE 30	S4ST7	ICIPE 696	ICIPE 695	KF746155.1	ICIPE 692	GU966518.1
ICIPE 279												
JQ266208.1	0.000											
ICIPE 693	0.000	0.000										
ICIPE 694	0.176	0.176	0.176									
FJ545302.1	0.176	0.176	0.176	0.000								
ICIPE 30	0.176	0.176	0.176	0.000	0.000							
S4ST7	0.176	0.176	0.176	0.000	0.000	0.000						
ICIPE 696	0.176	0.176	0.176	0.000	0.000	0.000	0.000					
ICIPE 695	0.268	0.268	0.268	0.269	0.269	0.269	0.269	0.269				
KF746155.1	0.274	0.274	0.274	0.274	0.274	0.274	0.274	0.274	0.006			
ICIPE 692	0.431	0.431	0.431	0.463	0.463	0.463	0.463	0.463	0.472	0.461		
GU966518.1	0.427	0.427	0.427	0.459	0.459	0.459	0.459	0.459	0.468	0.457	0.002	-

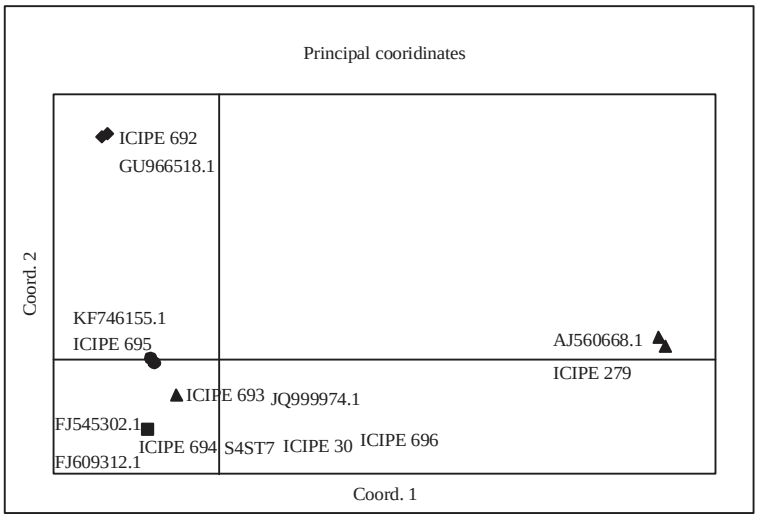

Fig. 6: Plot of principal components analysis (PCA) via the covariance matrix with data standardization calculated using GenAlEx for the various endophytes species isolated from *Vicia faba* and *Phaseolus vulgaris* using the TW81 and AB28 regions (PC1 = 61.48% and PC2 = 20.99%)

Table 4: Estimates of evolutionary divergence between sequences using TW81 and AB28

Fungal isolates	ICIPE 695	KF746155.1	ICIPE 693	JQ999974.1	ICIPE 694	FJ609312.1	ICIPE 30	ICIPE 696	S4ST7	FJ545302.1	ICIPE 692	GU966518.1	ICIPE 279	AJ560668.1
ICIPE 695														
KF746155.1	0.007													
ICIPE 693	0.314	0.321												
JQ999974.1	0.321	0.328	0.005											
ICIPE 694	0.292	0.298	0.196	0.202										
FJ609312.1	0.292	0.298	0.196	0.202	0.000									
ICIPE 30	0.292	0.298	0.196	0.202	0.000	0.000								
ICIPE 696	0.292	0.298	0.196	0.202	0.000	0.000	0.000							
S4ST7	0.292	0.298	0.196	0.202	0.000	0.000	0.000	0.000						
FJ545302.1	0.292	0.298	0.196	0.202	0.000	0.000	0.000	0.000	0.000					
ICIPE 692	0.587	0.582	0.576	0.581	0.553	0.553	0.553	0.553	0.553	0.553				
GU966518.1	0.586	0.581	0.586	0.591	0.558	0.558	0.558	0.558	0.558	0.558	0.005			
ICIPE 279	1.565	1.593	1.409	1.417	1.476	1.476	1.476	1.476	1.476	1.476	1.952	1.923		
AJ560668.1	1.514	1.539	1.367	1.374	1.455	1.455	1.455	1.455	1.455	1.455	1.890	1.864	0.011	

All positions containing gaps and missing data were eliminated. There were a total of 444 positions in the final dataset.

This analysis also clustered the *Metarhizium, Phanerochaete*, *Phialemonium* and *Beauveria* isolates into four distinct groups (Fig. 5). The first cluster consisted of all the *M. anisopliae* isolates included in the study. The ICIPE 696 and ICIPE 694 isolates branched separately from the standards (ICIPE 30 and S4ST7). The second group consisted of *B. bassiana* isolates ICIPE 693 likewise branching separately from the standard ICIPE 279. This is a clear indication that the samples are different from the standards. The last two clusters consisted of the *Phialemonium* sp., ICIPE 695 and *P. chrysosporium* ICIPE 692, respectively (Fig. 5).

The genetic distances between the isolates were also inferred as for the other primer region and were used for principal component analysis. The estimates of evolutionary divergence between sequences of the various endophyte isolates when using this primer region ranged between 0.0 and 1.952 (Table 4). Comparison of *M. anisopliae* isolates (ICIPE 694 and ICIPE 696) isolated from *V. faba* and *P. vulgaris* with the ICIPE standards, gave a square distance of 0.0, showing that the two are genetically closely related. However, comparison of *B. bassiana* isolate ICIPE 693 isolated from *V. faba* with the standard ICIPE 279, gave a square distance of 1.409 (Table 4), meaning that the 2 isolates are far apart. In the PCA plot, the first two axes explained 82.48% of the variation (the 1st axis 61.48% and the second axis 20.99%) (Fig. 6). The PCA separated the 9 isolates into 4 discrete clusters as observed with the ITS 5 and 4 gene region. Each cluster was occupied by isolates belonging to the 4 different genera i.e., a cluster consisting of the *Metarhizium* isolates, a cluster consisting of *Beauveria*, *Phialemonium* and *Phanerochaete*, respectively (Fig. 6).

DISCUSSION

Early approaches to fungal taxonomy or identification relied on morphological characterization at the macro and microscopic levels. The presence or absence of microconidia and macroconidia, shapes and sizes of conidia, type of phialides bearing microconidia, presence or absence of chlamydospores, microconidial chains and sporodochia, as well as the type of fungal mycelia as described by Burgess *et al.*[49], Humber[50] and Goettel and Inglis[51] have contributed to the morphological identification of especially the *M. anisopliae* and *B. bassiana* isolates. The variability of colony appearance, pigmentation, growth rate, length of chains, production of bluish sclerotia and concentric ring aerial mycelium[52,53] were other additional features which also assisted in the morphological identification of the isolated endophytes.

The key features used to identify *M. anisopliae* isolates (ICIPE 30, S4ST7, ICIPE 694 and ICIPE 696) are the conidia formed in short to long chains, short conidiogenous cells and with rounded to broad conical apices. Johnston[66] also proposed the morphological classification of *M. anisopliae* into long and short-spored forms. These features are also in line with the morphological idendification keys of Humber[50].

In *B. bassiana* isolates (ICIPE 279 and ICIPE 693), the conidia and conidiogenous shapes as well as the pigmentation (white in mass) corroborate with the features used by Humber[50] to describe *B. bassiana*. According to Thomas *et al.*[67], Viaud *et al.*[68], Alves *et al.*[69] and Kirkland *et al.*[70], the entomopathogen *B. bassiana* produces three distinct features *in vitro*, single cell propagules: Aerial conidia, blastospores and submerged conidia. Different infectious *B. bassiana* propagules can be isolated and selected for host targeting. Thus, in addition to mycelial and hyphal growth, *B. bassiana* produces a number of mono-nucleated single cell types, including aerial conidia, blastospores and submerged conidia[71,72]. These cells display distinct morphological, biochemical and pathological characteristics and attempts are being made to exploit these properties in pest targeting and/or the enhancement of virulence.

Phialemonium sp., isolate ICIPE 695 was mainly characterized by its abundance of adelophialides and few discrete phialides with no signs of collarettes as well as its grayish white to brownish colonies pigmentation. These morphological features were also used by Gams and McGinnis[73] when describing *P. curvatum.* Perdomo *et al.*[74] when describing *Phialemonium* species, focused on key morphological features such as smooth or finely floccose, white colonies, becoming brown at maturity, short and cylindrical adelophialides and less commonly, long, tapering discrete phialides. Inconspicuous collarettes could also be observed on types of phialides, hyaline and cylindrical to curved conidia[74].

The white rot fungus *Phanerochaete chrysosporium* isolate ICIPE 692 was identified morphologically based on the white floccose colonies, hyaline mycelia and the three types of conidia (aleuriospores, arthrospores and chlamydospores). These features observed in *P. chrysosporium*, were also described by Burdsall and Eslyn[75] and their conidial productions were abundant[76].

However, identification using morphological (macro and micro) and physiological (growth rates, media) characters alone of fungi were not only frequently criticized among mycologists but also are time-consuming and sometimes hard to interpret due to ambiguous responses by some isolates in the media tested and some unclear expressed features[77]. Morphological identification was then combined with molecular characterization approach to confirm the identities of the seed-born fungal endophytes isolated in this study.

Results from the amplification of the rDNA region using the two sets of primers in this study confirmed the morphological identification of the isolates which belong to the genera *Metarhizium*, *Beauveria*, *Phialemonium* and *Phanerochaete.* They also linked the standards (ICIPE 30 and S4ST7) to the *M. anisopliae* species while isolate ICIPE 693 to *Beauveria* spp. Similar to the identity of *M. anisopliae* standard (S4ST7) using both primers, a study by Akello[78] reported the isolate S4ST7 as a *M. anisopliae* species using molecular characterization with the following primers: IGS [PNFo (CCCGCCTGGCTGCGTCCGACTC) and PN22 (CAAGCATATGACTACTGGC)] and ITS [ITS1 (TCCGTAGGTGA ACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC)]. These results confirm the identity of our standard using different primers sets. In addition, Fernandes *et al.*[79] also used molecular-based techniques [AFLP and rDNA (ITS1, ITS2 and 5.8S) gene sequencing] to characterize morphologically identified *Metarhizium* spp., isolates from a wide range of sources.

Beauveria bassiana ICIPE 279 was reported earlier to be endophytic in *V. faba* and *P. vulgaris* by Akutse *et al.*[31]. Since, *B. bassianna* ICIPE 693, isolated from *V. faba* seeds, belongs to the same cluster as ICIPE 279, it may also be endophytic, not just in seeds of *V. faba* but also in the plants through vertical transmission or when artificially inoculated. *Beauveria bassiana* (Ascomycota: Hypocreales) has previously been reported as an endophyte in seeds and needles of *Pinus monticola* Dougl. ex. D. Don[80]. A number of studies have also reported on the presence of endophytes in seeds[81,82].

Rivero *et al.*[83] and Rao *et al.*[84] have also used ITS1 and ITS4 primers for molecular identification of *Phialemonium curvatum*. A species of *Phialemonium* (KF746155.1) has been reported as an endophyte[58] and having potential bioactivity as both anti-parasitic and anti-bacterial[85,86]. This is widely distributed in the environment, having been isolated from air, soil, industrial water and sewage[73]. Similar to the molecular characterization in this study, Lim *et al.*[76] have also identified 2 species of *Phanerochaete*, *P. chrysomonium* and *P. sordida* using ITS gene regions. *Phanerochaete chrysosporium* is known to be saprophytic capable of organic breakdown. Although, dying and dead plants serve as optimal substrate for *P. chrysosporium,* it has also been isolated from soil samples of petroleum refinery and was used for degradation of 5 polycyclic aromatic hydrocarbons (PAHs: Acenaphthene, anthracene, phenanthrene, fluoranthene and pyrene), simultaneously and individually in sterile and unsterile soil[87]. *Phanerochaete chrysosporium* has also been reported to be thermo-tolerant[88]. The white rot fungus *P. chrysosporium* is the only microbe capable of efficient depolymerization and mineralization of lignin. Furthermore, *P. chrysosporium* also has several features that might make it very useful. For instance, unlike some white rot fungi, it leaves the cellulose of the wood virtually untouched and it has a very high optimum temperature (about 40°C), which means it can grow on wood chips in compost piles, which attain very high temperatures. These characteristics point to some possible or potential roles of this fungus in biotechnology. The endophytic fungi isolated from *V. faba* and *P. vulgaris* seeds, may occur and propagate/accumulate naturally inside seeds under farming conditions or during seeds processing.

Although, plants are commonly colonized by a diverse array of endophytic organisms, it was previously reported that hardly any information exists on interactions between endophytic fungi and other ecological groups of fungi co-occurring in the same plant tissues/seeds[89]. It can therefore be expected that antagonistic interactions between these fungi are the rule rather than the exception. When entomopathogenic endophytic fungi are starting to grow systemically from the point of inoculation (from seeds for example) to other plant parts, they inevitably have to confront other fungi already established. Since, seeds are likely to already harbor endophytic fungi at the time of artificial inoculation, thus adding a component of interspecific interaction to the already complex set-up become important to clarify. In line with this hypothesis, a recent paper by Yan *et al.*[90] reported almost no systemic growth of endophytic fungi in *Silene dioica* (L.) Clairv., a non-mycorrhized forb, because most of the fungi were starting to exhibit antagonistic interactions when plated together in a kind of competitive setting on a growth medium. Vidal and Jaber[91] also underlined the possible interaction effects of the existing endophytes and the inoculated ones where they reported that these microorganisms may impact the effect of the endophytic entomopathogenic fungi on the herbivorous insects.

Therefore, the results of this study would create awareness on seed endophytes and facilitate/stimulate studies on the role of these fungi in inhibition/suppression of the growth of artificially inoculated endophytes used for pest control in general and for *Liriomyza* leafminer species in particular, explore the additive, symbiotic or synergic effects of these seeds endophytes in the management of *Liriomyza* leafminers and other pests. Regarding the exploration of possible inhibition of the growth of the artificially inoculated fungi, Kerr[92] has reported similar results where *Pseudomonas aeruginosa* was found to significantly suppress or inhibit the growth of *Candida albicans* and other eleven strains of fungi *in vitro*. The presence of these endophytes in *V. faba* and *P. vulgaris* seeds shows a high chance that the seedlings of these seeds may be colonized by the same fungal endophytes either naturally or artificially through inoculation. *Beauveria bassiana* and *M. anisopliae* are two key entomopathogenic fungi species isolated from these seeds, suggesting their potential use in biological control of arthropod pests. Entomopathogenic fungi are generally applied in inundative approach in the crops[93]. However, other strategies are currently being considered and include auto-dissemination[94] and endophytic colonization[30]. Fungal pathogens can endophytically colonize host plants and confer resistance against insect pests[30]. The pathogenicity and virulence effects of the isolated endophytes can also be assessed on different sucking arthropods *in vitro* and *in vivo* especially for the entomopathogenic fungi *M. anisopliae* and *B. bassiana* isolates. For instance, endophytic *B. bassiana* in banana significantly reduced larval survivorship of banana weevil, *Cosmopolites sordidus* (Coleoptera: Curculionidae), resulting in 42-87% reduction in plant damage[43,44]. Reduction in feeding and reproduction by *Aphis gossypii* (Hemiptera: Aphididae) has also been reported on cotton endophytically colonized by *B. bassiana*[34]. Vega *et al.*[29] reported similar effects of an endophytic *B. bassiana* SPCL 03047 on adult coffee berry borers, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) with an average survival period of 4.8±0.2 days compared to 15.0±0.6 days for the un-inoculated plants. The pathogenicity and virulence of fungal isolates against *Liriomyza* leafminer adults was also reported by Migiro *et al.*[95] using *M. anisopliae* through

auto-dissemination approach. Akutse *et al.*[31] also reported the effects of fungal endophytes on life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae) leafminers and their associated parasitoids[47]. The researchers reported that endophytic fungal isolates of *B. bassiana* (G1LU3, S4SU1 and ICIPE 279) could endophytically colonize *V. faba* and *P. vulgaris* plants through seeds inoculation and cause detrimental effects on survival, fecundity, oviposition, emergence and longevity of *L. huidobrensis*. However, no significant detrimental effects were observed on the development, survival and parasitism ability of the associated parasitoids *Diglyphus isaea* Walker (Hymenoptera: Eulophidae) and *Phaedrotoma scabriventris* Nixon (Hymenoptera: Braconidae)[47].

Assessment of the quality and the nutritional value of the seeds colonized by the endophytes can also be undertaken not only having reference to plant pathogens[18-22], but also when considering the possible presence of the entomopathogenic fungi in the seeds. For example, Van Du *et al.*[96] reported that some fungi species, such as *Fusarium graminum*, *Cephalosporium oryzae*, *Alternaria padwickii*, *Fusarium moniliforme*, *Fusarium pallidoroseum*, *Fusarium subglutinans*, *Phoma* sp. and *Sarocladium oryzae* found in rice seeds, affect the quality of the seeds through their effects on grain discoloration, milling recovery, cooking and palatability as compared to healthy seeds. In addition, McGee and Nyvall[97] reported that more than 30 fungi are seed-born on soybeans. Although, some cause quality problems[98] and reduce seed viability, most fungi that are associated with soyabean seeds are not known to cause diseases. Furthermore, the effects of seeds derived from endophytically inoculated plants on stored products insects can also be investigated.

CONCLUSION

Beauveria bassiana isolate ICIPE 693, *M. anisopliae* isolates ICIPE 694, ICIPE 696, *Phanerochaete chrysosporium* isolate ICIPE 692 and *Phialemonium* sp., isolate ICIPE 695 were isolated as seed fungal endophytes. These isolates were clearly different from the standards *B. bassiana* ICIPE 279 and *M. anisopliae* ICIPE 30 and S4ST7 despite clustering into the same groups. Results of the study showed that bean seeds do not only bear in their inside plant pathogens but also entomopathogenic fungi. The outcome of this study will create awareness on seed endophytes and stimulate further studies on the role of these fungi in (1) Inhibiting or suppressing the growth of artificially inoculated endophytes used for pest control in general and *Liriomyza* leafminer species in particular, (2) Assessing the pathogenicity and virulence effects of these endophytes on different sucking arthropods, (3) Evaluating the nutritional value of the seeds containing these endophytes and finally (4) Assessing the effect on stored products insects when storing seeds derived from endophytically inoculated plants. Furthermore, since other fungi may block the systemic growth and colonization of the artificially inoculated fungi in the host plant parts distant to the point of inoculation, then there is need to know the already existing species of fungi in the seeds and study their interactions with the inoculated ones, not only for seedling growth promotion, but also for better biological pest management strategies development using endophytic fungi. And this study established some of the key entomopathogenic fungal endophytes that naturally exist in the bean seeds. Knowing that bean seeds contain already these fungi, the results of this study will also help to develop specific markers to trace the fate of the artificially inoculated endophytes when released in the ecosystem as biocontrol agents.

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