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Isolation and molecular characterization of ginger soft rot pathogenic fungi in Aizawl district of Mizoram, India

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Ginger soft rot disease caused by fungal pathogens have become one of the most serious problems causing reduced production around the world. It has also caused a major problem among farmers of Mizoram state in India resulting in a huge decline in rhizome yield. However, the exact causative agents of this disease have not been identified in the state. Therefore, the present study was undertaken to isolate and characterized the causative agents of ginger soft rot disease from the diseased plants collected from five different villages of Aizawl district, Mizoram. Isolated fungi were cultured and morphological and molecular identification were done using internal transcribed spacer of rDNA. *Fusarium oxysporum, F. solani* and *Plectosphaerella cucumerina* were identified in ginger samples from five villages. *Fusarium* spp. were the most common and seem to be the major causative agents. It is suggested that further investigation is required to explore the diversity of ginger soft rot pathogenic fungi in the whole state which could be helpful in introducing effective and eco-friendly disease management programme.

Keywords: *Fusarium oxysporum, Fusarium solani,* ginger, internal transcribed spacer, soft rot disease.

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INTRODUCTION

Ginger (*Zingiber officinale* Roscoe), a member of the plant family Zingiberaceae, is grown worldwide in the subtropics and.[1] In India, it is one of the most common and important commercial crops cultivated by small and marginal farmers in Himachal Pradesh, Assam, Kerala, Meghalaya, Karnataka, Sikkim, Orissa and Mizoram. However, production of ginger is affected by plant pathogens such as *Fusarium* spp. *Rosellinia* spp. and *Pythium* spp. causing rhizome soft rot disease which is considered to be the most serious problem in many ginger producing areas around the world.[1,2] It has been reported that diseases caused by *Ralstonia solanacearum* (Smith) and *Pythium* spp. are among the major production constraints in ginger. *Pythium* spp. causing ginger soft rot resulting in reduced production has been reported nearly 100 years ago in India.[3]

Several other genera of fungi such as *Penicillium*, *Aspergillus*, *Mucor*, *Eurotium* and *Rhizopus* have also been isolated from ginger as causal agents of rhizome rot.[4] Among the many pathogenic species, *F. oxysporum* f. sp. *zingiberi* (Foz),[5,6] *F. solani*,[7] *F. equiseti*,[8] *F. semitechtum*,[9] *P. aphanider-*

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mantum and *P. myriotylum* were the most common species which adversely affects ginger rhizomes and crop production.[1,2,10-13] These fungi are seed- and soil-borne pathogen that cause serious damage during the growing period. The initial symptoms developed on leaves as slight fading of green colour followed by yellowing of tips. Chlorosis proceeds downwards resulting in withering and dead of leaves. The rhizome becomes soft and on pressing collapse easily. In advanced stage only skin is left. The internal tissues rot completely and the plant wilts and collapses resulting in serious yield reduction. The rotting continues in storage also. Literatures revealed that the disease can be managed by adopting organic amendments and fungicides.[14-18]

Mizoram is one of the eight states of northeast India, situated in the extreme end of the Himalayan ranges. It covers 21,081 sq km and lies between 92° 15' and 93°26'E longitude and 21°58' and 24°35'N latitude.[19] The state has two international borders, Bangladesh in the west and Myanmar in the east. Shifting cultivation is the major system of cultivation practices by the farmers in the rural areas of the state. Out of the many crops grown, ginger is the main cash crop in Mizoram taking the most important place due to its high market value. In north-east India, Mizoram ranks first in ginger productivity (8.4 t/ha).[20] However, over the last few years, despite of its contribution to the life of the cultivating family, its large scale production in the state is limited by problems especially rhizome/soft rot disease resulting in a huge decline of rhizome yield.

Our preliminary field survey during the rainy season in 2017 (July to September) revealed that rhizome rot disease outbreak occurred in almost everywhere in Mizoram. Among the farmers, knowledge on the causative agents of the disease is not well established. Due to this limited knowledge, farmers have started applying toxic chemicals like insecticides and herbicides to fight rhizome diseases without proper doses and guidance. This practice largely affected the health and quality of the crop and the soil reducing rhizome yield, eventually fails to fight the disease. Considering this, it is important for the farmers to be aware of ginger crop disease pathogens in order to improve in disease management as well as crop yield. However, there was no report and proper studies about the causative agents of ginger rhizome/soft rot disease in Mizoram.

Polymerase chain reaction (PCR) has become a very useful technique for researchers all over the world in identification and characterization of fungal species. The internal transcribed spacer (ITS) of ribosomal DNA has become the most commonly used region in identification of fungi at the species level.[11] Therefore, the present study was proposed to undertake isolation and a PCR-based identification of the causal pathogenic fungi from diseased plant of ginger from different villages in Aizawl District of Mizoram, India.

MATERIALS AND METHODS

Sampling of diseased ginger rhizomes

Foliar symptoms of soft rot disease appear as light yellowing of the tips of lower leaves which gradually spreads to the leaf blades. In early stages, the middle portion of the leaves remain green while the margins become yellow. The yellowing spreads to all leaves of the plant from lower region upwards and is followed by drooping, withering and drying of pseudo stems. Based on these criteria, rhizomes of diseased ginger were collected for direct isolation and identification of fungi. Samples were collected in August and September, 2017 from the field of ginger cultivation in five different villages of Aizawl district such as Tuirial, Sairum, Hualngo, Saitual and Ailawng. From each field, 7 diseased rhizomes were collected, for a total of 35 rhizomes.

Isolation of fungi from samples

Isolation of fungi was undertaken directly from diseased ginger with soft rot symptoms following the method described by Le *et al.*[21] Diseased plant

part (rhizomes) were thoroughly washed with running tap water, disinfected using 1% sodium hypochloride for 5 minutes and washed repeatedly with sterile distilled water to remove disinfectant and cut into 2-3 mm cube size using sterilized scissors. Isolation of fungal pathogens was done by using tissue-transplanting technique. Diseased and healthy ginger tissues were placed in a 1.5% water agar (WA) plates supplemented with 0.3 g/l ampicillin and gentamycin. All the inoculated plates were kept in an incubator at 25 ± 1°C. The cultures were periodically checked and the emerging single mycelial hyphae were transferred to potato dextrose agar (PDA, 3.9%) medium by means of hyphal tips transfer technique for purification.[22]

Identification through morphological characterization

Species identification was based on both morphological characteristic and molecular analysis. The morphological characteristics used in the present study were colony and spore characteristics.[22,23] For macroscopic observation, the mycelia disc (5 mm diameter) taken from the growing edge of colonies were cultured onto a potato dextrose agar (PDA) in darkness at 25°C for 10 days. Colony diameter was recorded daily (three replicates) for 10 days. Growth rate was calculated as the 10-day average of mean daily growth (mm per day). Colony colour and pigmentations were observed. For microscopic observations, parameters such as size and shape of macroconidia were used.

DNA extraction

Prior to extraction, pure fungal isolates were reactivated by sub-culturing on potato dextrose broth (PDB) growth media in a 1.5 ml microcentrifuge tubes and incubated at 25°C for 14 days to allow massive production of mycelia. DNA was extracted following the method described by Cenis (1992) with a slight modification.[24] Briefly, 100 mg wet wt. of mycelial mat was collected from the liquid culture by centrifugation at 13,000 rpm for 5 min. The mycelia pellet was washed with 500 ml of 1xTE buffer and centrifuge at 13,000 rpm for 5 min. Supernatant was discarded, 300 ml of extraction buffer [200 mMTris-HCl (pH 8.5), 25 mM EDTA (pH 8.0), 250 mMNaCl, 0.5% sodium dodecyl sulphate (SDS)] was added to the pellet and crushed the mycelium with the help of a conical grinder by hand for 10 min. A 150 ml of 3M sodium acetate (pH 5.2) was added and incubated at -20°C for 10 min. which was followed by centrifugation at 13,000 rpm for 5 min. The supernatant was transferred into a fresh 1.5 ml tube, added equal volume of isopropanol and leave undisturbed at room temperature for 15 min. The precipitated DNA was collected by centrifugation at 13,000 rpm for 10 min. and washed twice with 70% ethanol. The final DNA pellet was air-dried, re-suspended in 30 ml of 1xTE and store at -20°C until used.

Polymerase chain reaction

The internal transcribed spacer (ITS) regions 1 and 2, including the 5.8S rDNA, were amplified by PCR using Primers ITS1 and ITS4.[25] The PCR reaction was carried out using a ProFlex PCR System (Applied Biosystems, Life Technologies). A reaction volume of 25 ml containing 9.5 ml of nuclease free water, 12.5 ml of PCR MasterMix (Takara), 1 ml each of primer (10 pmole) [ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)] and 1 ml of DNA template was used. Amplification was performed in a thermal cycler programmed for initial denaturation at 95°C for 5 min., followed by 35 cycles of denaturation at 95°C for 30 sec., annealing at 56°C for 30 sec., extension at 72°°C for 45 sec. and a final extension at 72°C for 7 min. PCR products were separated by electrophoresis in 1.4% agarose gels, stained with SYBR Safe DNA Gel Stain (Invitrogen). Electrophoretic migration was carried out for 30 min. at 150 V. The amplified products were visualized and photographed under blue light in Safe Imager 2.0. A 100 bp DNA Ladder (GBiosciences) was used to estimate the size of PCR products. Each amplification reaction was replicated three times.

Sequencing and phylogenetic analysis

PCR products of ITS region were sent to Agrigenome Labs Pvt Ltd. for sequencing. The program BioEdit Sequence Alignment Editor, ver. 7.0.5.3 was performed to edit and align the sequence files. The aligned sequences were subjected to Blast search analysis at NCBI's GenBank nucleotide database for sequence similarity. Sequences with high degrees of similarity were downloaded from GenBank for construction of phylogenetic tree. Phylogenetic tree was generated using neighbor-joining method in MEGA 7.0. The phylogenetic tree was rooted to *Pythium aphanidermatum* (GenBANK accession number: MK158217).

RESULTS

In the present study, 35 isolates from 5 different study sites were examined and phylogenetically analyzed based on the ITS rDNA sequences. Based on the data of ITS rDNA sequences and morphological characteristics (Table 1), three species such as, *Fusarium oxysporum, F. solani* and *Plectosphaerella cucumerina* were identified.

From the microscopic characteristics, 14 isolates from Hualngo and Sairum villages were identified as *Fusarium oxysporum*. Slight differences were observed on the macroconidial sizes and shapes as well as the appearance of apical and basal cell morphology. Macroconidia was more common with straight, slender inshape with a curved apical cell while the basalcell was pointed (Figure 1C). The macroconidial septation ranged from 3 to 6. 3-septate macroconidia were commonly observed with 57% of the total (Table 1). The cultural appearances of the isolateson PDA showed sparse to abundant cottony mycelium with colony colour of white to white-violet aerial mycelium and the pigmentations were from pale violet to colourless (Figure 1A &1B). The isolates show a fast growth rate of 6.3±0.4 mm/day.

Based on morphological characteristics, 14 isolates from Saitual and Tuirial villages were identified as *Fusarium solani*. The isolates produced macroconidial septation from 3 to 7 with slight differences in their shapes and sizes (Table 1). Macroconidia with 7 septates showed slightly curved with ellipsoidal and pointed basal cells (Figure 2C). The cultural appearances of the isolates on PDA were sparse to abundant cottony mycelium with white-creamy aerial mycelium, and the pigmentations were white-creamy with a dark brown zonation (Figure 2A & B). The isolates show a slightly slow growth rate of 4.3±0.3 mm/day.

Based on morphological characteristics, 7 iso-

Table 1 | Morphological characteristic of fungal species.

Microscopic	*F. oxysporum*	*F. solani*	*P. cucumerina*
Microconidia-			
Length (µm)	3-septate = 27.4±1.2.	3-septate = 31.2±1.3.	1-septate = 8.2±1.8.
Width (µm)	3-septate = 5.6±0.6.	3-septate = 5.4±0.5.	3.2±0.7
Shape	Oval to kidney-shaped, ellipsoidal	Curved with pointed basal cells, ellipsoidal	Elipsoidal, hyaline and smooth
Conidial septation	3-6 septate	3-7 septate	0-1 septate
Common septation	3-septate (57%)	3-septate (61%)	1-septate (82%)
Macroscopic			
Colony colour	White to white-violet.	White-creamy.	Buff to salmon pink
Pigmentation	Pale violet to colourless.	White-creamy with dark brown zonation.	Pale violet with dark brown zonation
Growth rate[#]	6.3±0.4 mm	4.3±0.3 mm	3.8±0.2 mm

[#]Growth rates were taken after 3 days of inoculation at 25°C.

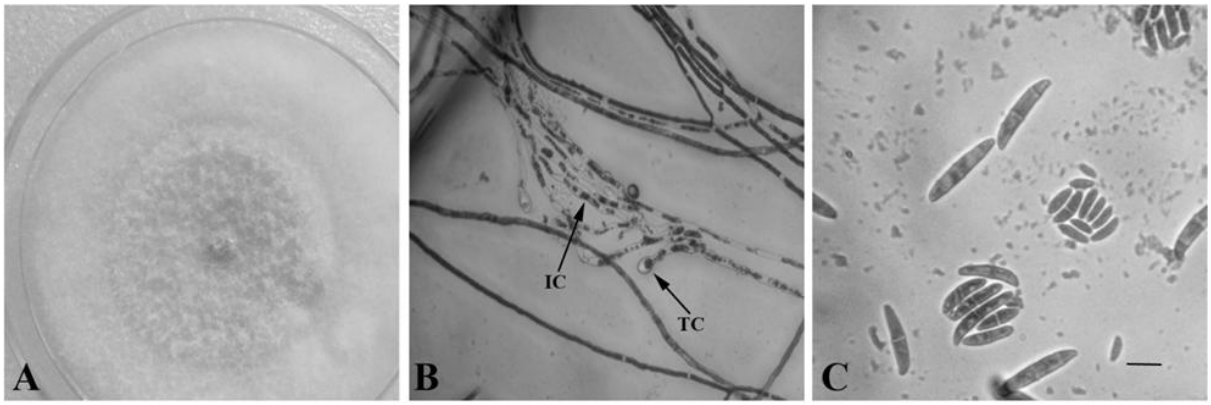

Figure 1 | *Fusarium oxysporum*. Colony on PDA after 10 days (A); hyphae with intercalary (IC) and terminal (TC) conidiophores (B); septate conidia (C). Scale bar = 10 µm.

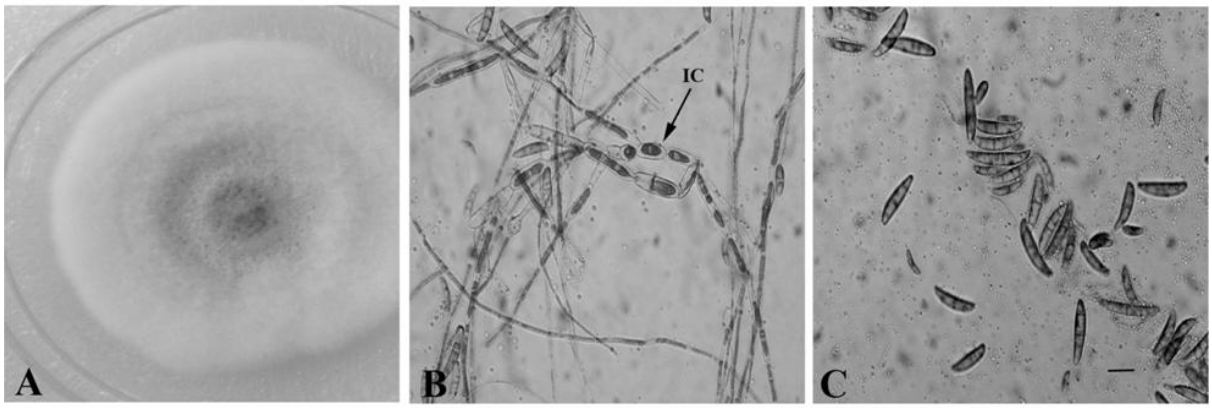

Figure 2 | *Fusarium solani*. Colony on PDA after 10 days (A); hyphae with intercalary (IC) conidiophores (B); septate conidia (C). Scale bar = 10 µm.

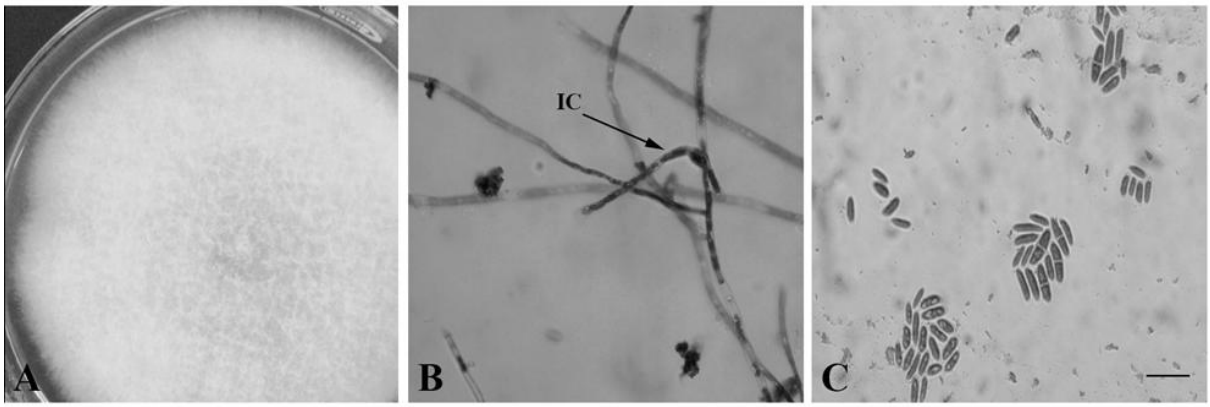

Figure 3 | *Plectosphaerella cucumerina*. Colony on PDA after 10 days (A); hyphae with intercalary (IC) conidiophores (B); non-septate and septate conidias (C). Scale bar = 10 µm.

Table 2 | Ten isolates of fungal species submitted to GenBank.

Species	Isolate number	Locality	Collector	GenBank accession number
P. cucumerina	Pc AL1A	Ailawng	G. Rosangkima	MK209106
P. cucumerina	Pc AL1B	Ailawng	G. Rosangkima	MK209107
F. oxysporum	Fo H1A	Hualngo	G. Rosangkima	MK209108
F. oxysporum	Fo H1B	Hualngo	G. Rosangkima	MK209109
F. oxysporum	Fo S1A	Sairum	G. Rosangkima	MK209110
F. oxysporum	Fo S1B	Sairum	G. Rosangkima	MK209111
F. solani	Fs ST1A	Saitual	G. Rosangkima	MK209112
F. solani	FsST1B	Saitual	G. Rosangkima	MK209113
F. solani	Fs T1A	Tuirial	G. Rosangkima	MK209114
F. solani	Fs T1B	Tuirial	G. Rosangkima	MK209115

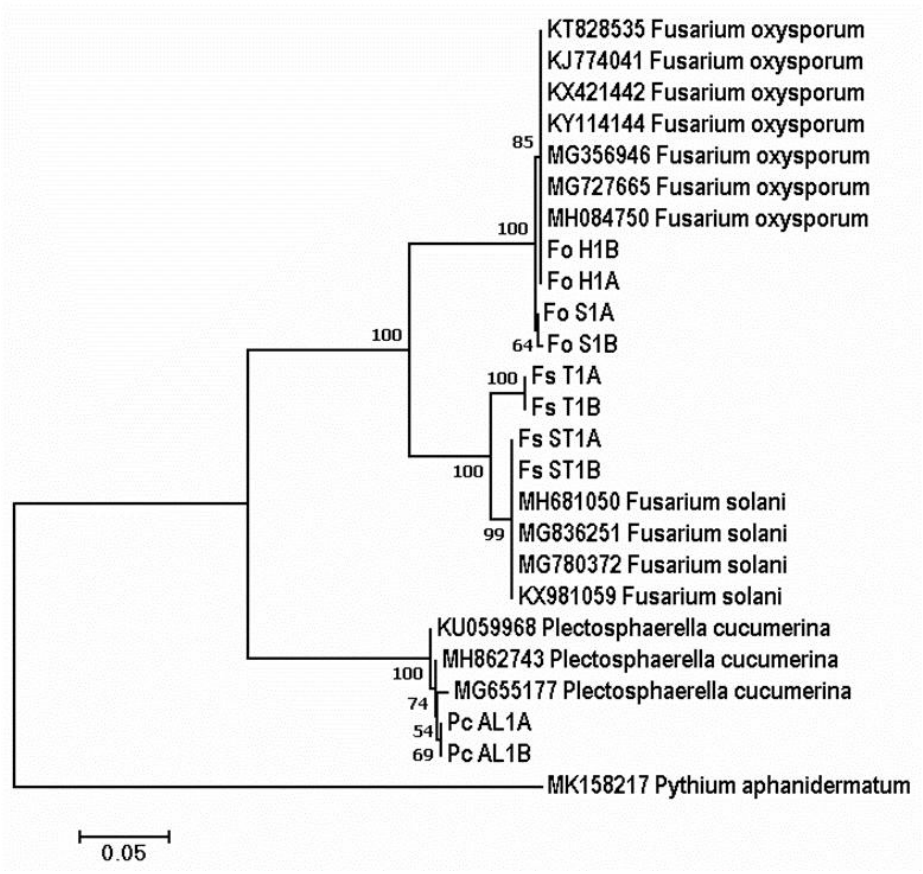

Figure 4 | Evolutionary phylogenetic relationship of the fungal isolates based on ITS ribosomal DNA sequences constructed using the neighbor-joining method. Bootstrap tests were performed with 1,000 replications; and the percentage value greater than 50 is considered a strong support for a branch. *Pythium aphanidermatum* (MK158217) obtained from GenBank was treated as the outgroup.

lates from Ailawng village were identified as *Plectosphaerella cucumerina*. Colonies on PDA were flat, slimy with sparse aerial mycelium; buff to salmon pink and pigmentation were pale violet with dark brown zonation (Figure 3A). Macroconidia were non-septate or 1-septate with 1-septate more commonly present (82%) (Figure 3C). 1-septate macroconidia were approximately 8.2 and 3.2 µm in length and width respectively (Table 1). The isolates show a slow growth rate of 3.8±0.2 mm/day.

Identification to species level was also conducted using sequence analysis of ITS rDNA. The total size of the ITS rDNA regions of the isolates studied ranges from 478 to 513 bp. Blast search of ITS rDNA data from 14 isolates of Hualngo and Sairum villages against GenBank nucleotide showed 98–100% similarity with *Fusarium oxysporum* isolates from different hosts. Blast search of ITS rDNA data from 14 isolates of Saitual and Tuirial villages against GenBank nucleotide also showed 99–100% similarity with *Fusarium solani* isolates from other hosts. Blast search results of 7 isolates from Ailawng village showed 99–100% similarity with *Plectosphaerella cucumerina* isolates from other hosts. Sequence of two isolates each from different study sites were submitted to GenBank and are available with the accession numbers MK209106, MK209107, MK209108, MK209109, MK209110, MK209111, MK209112, MK209113, MK209114 and MK209115 (Table 4). Phylogenetic inference based on the ITS rDNA sequence shows a clear distinction between the three species namely *Fusarium oxysporum, F. solani* and *Plectosphaerella cucumerina* (Figure 2).

In the phylogenetic tree, the representative isolates were highly clustered within the clades comprising their respective reference isolates. All the four *Fusarium oxysporum* isolates were clustered in one clade along with the seven reference isolates from GenBank (accession numbers KT828535, KJ774041, KX421442, KY114144, MG356946, MG727665 and MH084750). Four *Fusarium solani* isolates clustered in another clade also included four reference isolates from GenBank (accession numbers MH681050, MG836251, MG780372 and KX981059). *Plectosphaerella cucumerina* isolates were also clustered in a separate clade along with three reference isolates from GenBank (accession number KU059968, MH862743 and MG655177).

DISCUSSION

A large number of fungal species have been reported worldwide to cause soft rot disease in ginger. These fungi belong to different genus including *Fusarium, Pythium, Acremonium, Colletotrichum* etc.[26-28] In the present study, *Fusarium oxysporum, F. solani* and *Plectosphaerella cucumerina* were isolated and identified from soft rot diseased ginger rhizomes. *F. oxysporum* and *F. solani* were among the most common fungi responsible for soft rot disease of ginger causing drastic reduction in crop yield in different countries.[8, 21,29] They play the role of a silent assassin. By the time a plant shows any sign of infection, it is always too late, and the plant will die. They are effective pathogens and can cause disease in nearly every agricultural important crop.

P. cucumerina is a very common fungus found on decayed plant materials and in the soil.[30] In spite of the report on its occurrence on many crops; its pathogenicity is rarely recognized in agricultural production in many countries. In China, it was first reported on tomato and later on, it was reported as a pathogen of alfalfa and sunflower.[31-33] There is no report on its involvement in ginger soft rot disease. Therefore, our detection in the present study may be the first report as a causative agent in ginger soft rot disease. However, further investigation on its pathogenicity may be required. Literatures also revealed the pathogenicity of *F. oxysporum, F. solani* against ginger and *P. cucumerina* in other crops. In conclusion, the findings in the present study confirmed the presence of fungal species as a causative agent in ginger soft rot disease in Aizawl district of Mizoram state. It may be suggested that further investigation is required to study the diversity of ginger pathogenic fungi throughout Mizoram state which could be helpful in designing proper, effective and ecologically safe management

strategies particularly in Mizoram and other countries in general.

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