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Isolation and Molecular Identification of *Colletotrichum gloeosporioides* from Infected Peanut Seeds

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AR and RN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author NK managed the analyses of the study. Authors CYSY and MHI managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Peanut (*Arachis hypogaea* (L) cv. Margenta) seeds are currently, significantly considered as an important source of protein and vegetable oil. Anthracnose caused by *Colletotrichum gloeosporioides* is a fungal disease from seed that reduces seedling emergence and seed germination. The pathogenic fungi that isolated from peanut were identified using ITS 1 and ITS 4 primer with polymerase chain reaction and NCBI database using BlastN algorithm. From NCBI using blastN analysed was 95% identify with other *Colletotrichum* species in NCBI database search. The pathogenicity of *Colletotrichum gloeosporioides* on peanut seeds and seedlings at 14 days after sowing result was inoculated with pathogenic fungi with 32.3% and control was 83%. Hence, this study was experimented with the object to reveal seed-borne infections on tropical peanut in Malaysia.

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1. INTRODUCTION

Peanut (*Arachis hypogaea* (L) cv. Margenta) seeds are currently, significantly considered as an important source of protein and vegetable oil. Peanuts contain 30 essential nutrients. It is considered to be a healthy source of Vitamin B complex and Vitamin E, certain minerals like magnesium, phosphorous, folate, niacin and fibre. Such phytochemicals are proven to be highly protective against certain kinds of cancers, cardiac ailments, the degenerative nerve diseases like Alzheimer's disease, and viral/fungal infections [1].

Anthracnose caused by *Colletotrichum gloeosporioides* is a seed borne disease that reduces the germination as well as the emergence of seedling from a seed [2]. Germination in *C. gloeosporioides* follows two routes: "pathogenic" and "saprophytic" [3]. On plants as well as hydrophobic surfaces, pathogenic germination occurs and is characterized by quick cell division (mitosis) once the single germ tube is developed. The process takes place quickly and as a result of it appressoria is formed. On the other hand, rich medium induces saprotrophic germination [4]. Two germ tubes are formed and also it takes long germination time. The germ tube comes out from the opposing sides spore sides. In *Colletotrichum gloeosporioides,* the two germination routes are regulated by signaling routes like CAMP route which is seen in saprotrophic germination, and CAMP independent route in pathogenic germination.

C. gloeosporioides requires 25-28°C temperature, pH 5.8-6.5 for better growth. The pathogen remains dormant as long as the season is dry and becomes active when exposed to favorable conditions. It involves a hemibiotrophic mode of infection where both phases, biotrophic and necrotrophic phases occur sequentially [5]. Various medium preparations can be employed for the growth and sporulation of *C. gloeosporioides* including potato dextrose agar, lima bean agar, malt extract agar and oatmeal agar.

Colletotrichum species that cause serious plant disease are also commonly isolated as endophytes from healthy plants, and have been identified as saprobes in dead plant material [6]. The symptoms includes, dark lesions appear on leaves, fruits and flowers of the infected plant which finally produce a concentric ring pattern. However, limited information is available on the *C. gloeosporioides* in the seed tissues of peanut. Therefore, this study was attempted with the aim to isolate the ITS region gene from *C. gloeosporioides* and to study the phylogenetic relation on *C. gloeosporioides* on tropical peanut in Malaysia.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of *Colletotrichum* sp. in Peanut Seeds

Peanut seeds were purchased from the seed storage unit, Mardi (Serdang, Selangor). Seed moisture content was maintained throughout the storage period. A total of 400 seeds were used for the isolation of *Colletotrichum* sp. in peanut seed. Plating the seeds directly on moist blotter paper method was used for external seed-borne infection. Surface sterilization of seeds with 12% Clorox® for 2 min, followed by repeated washings with sterilized distilled water and then plating onto potato dextrose agar (PDA) was used to determine the internal seed-borne infection [7]. The plates were nurtured for 5–7 days at ambient temperature (26 ± 2°C) in alternating cycles of 12 h light and 12 h darkness. The plates were checked for fungal propagation using compound and stereo microscopes on until fully grown. Further, isolates of *Colletotrichum* sp. were selected from water extract method to growth in pure culture. Then, each isolate was cultured again in potato dextrose agar (PDA) at 20°C for 5 days, and the mycelial mass was collected for DNA extraction.

2.2 DNA Extraction

The method was used to extract and purify total genomic DNA [8]. The mycelia were collected from plates and ground in liquid nitrogen. Then, 50mg of ground mycelium powder was weighed and added into a 1.5 mL Eppendorf tube containing 400 µl CTAB extraction buffer. The mixture was mix and vortexed. Then, the mixture was combined with 400 µl of chloroform:isoamylethanol (24:1) and mixed vigorously by hand. Then the sample mixture was heated at 65°C for 5 min, and continued with vortexed. Then the sample mixture was centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant was transferred to a new 1.5 mL

Eppendorf tube and DNA precipitated at room temperature by added 400 µl of ice-cold iso-propanol and centrifuged at 13,000 rpm for 3 min at 4°C. Then, 70% ethanol was added to wash the pellet DNA and further centrifuged at 13,000 rpm at 3 min. Then the DNA pellet was air-dried, and suspended in 20 µl of sterilized distilled water. The concentration of DNA was calculated using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). This DNA suspension from each isolate was diluted to a final concentration of 10 ng/µl prior to use for PCR amplification of the ITS-rDNA region [9].

2.3 Amplification of ITS by PCR and Sequencing

Two (Internal Transcribed Spacer) ITS regions of *Colletotrichum* sp. were amplified using the universal primers, forward primer of ITS1: 5 'GCC GTA GGT GAA CCTG CGG 3' and reverse primer of ITS4: 5' TCC TCC GCT TAT TGA TAT GC 3' [10]. PCR amplification was performed in a total volume of 25 µl, containing 5 µl of 10X buffer with $MgSO_4$, 0.75 µl of 10mM dNTP mix, 1 µl of 10 µmol forward primer, 1 µl of 10 µmol reverse primer, 2 µl DNA template (10 ng/µl) 0.5 µl Pfu DNA Polymerase (3 U/µl), and 13.5 µl nuclease-free water. The reaction mixture was incubated in a Thermal cycler (Biometra). DNA was denatured at 94°C for 3 min, followed by 35 cycles consisting of 30 s at 94°C, 20 s at 58°C, and 1 min at 72°C and final extension was for 5 min. PCR products were separated on 1% agarose gels in 1X TBE buffer. Gels were stained with ethidium bromide and DNA bands visualized with UV light. The size of the DNA bands was estimated with the aid of a standard DNA ladder (QIAGEN Inc. Mississauga, Canada). Purification of the amplicons from the gel was performed with (Promega, USA) purification kit according to supplier's protocols and stored at -20°C. The purified amplicons were sent to the First Base laboratories Sdn. Bhd (Seri Kembangan, Selangor) for sequencing [11].

2.4 Phylogenetic Tree

Sequences of the ITS genes were aligned using the multiple sequence alignment program CLUSTAL W, prior to being imported into Molecular Evolutionary Genetics Analysis (MEGA), which used neighbor-joining (NJ) methods to generate trees for similarity analysis. The most parsimonious trees were generated by heuristic search with the bootstrap test (1000 pseudo replicates). All characters had equal weight and gaps were treated as "missing" values. A bootstrap 50% majority-rule consensus was used to separate isolates. Sequences for the rDNA-ITS segment were used to generate the most neighbor-joining (NJ) tree. For the analysis of individual ITS regions, sequences ranging from 600 to 700 base pairs (bp) were chosen to generate the most neighbor-joining (NJ), ITS1F and ITS4R trees, respectively. In addition, *Rhizopus oryzae* was chosen as an outgroup for phylogenetic analysis which used neighbor-joining (NJ) methods and maximum likelihood tree to generate trees.

3. RESULTS

3.1 Amplication of ITS Region from *Colletotrichum* sp. by PCR

Molecular diagnosis of seed-borne fungal infection of peanuts was performed through PCR by using fungal ITS primer. Total genomic DNA was extracted from the *Colletotrichum* sp. mycelium using modified CTAB method and further amplified using fungal specific primers, ITS1 F and ITS4 R. The results indicated the presence of bands with a size of nearly 650 bp (Fig. 1).

3.2 Characterization of *ITS* Genes of *C. gloeosporioides* of Peanut

Results of BLAST-n analysis on Table 1 and multiple sequence alignment analysis of Clustal O on Fig. 2 showed that the sequence share high homology with *ITS* sequences from other fungal species. It's are include, such as, *Colletotrichum gloeosporioides* with homology 95% and 93%, *Glomerellales* sp., *Colletotrichum viniferum, Colletotrichum fructicola, Colletotrichum siamense, Colletotrichum nupharicola, Colletotrichum alatae* are maximum identity were 94% and *Colletotrichum endophyticum, Colletotrichum ignotum* (93%). Total score and query coverage showed the comparison between the DNA sequences of *ITS* gene of *C. gloeosporioides* and other fungal ITS sequence of other fungal from NCBI database.

3.3 Analysis of Phylogenetic Study on Internal Transcript Spacer (*ITS*) in *Colletotrichum* sp.

The phylogenetic analysis of *ITS* regions of *Colletotrichum* sp. sequence isolated from this

study was constructed by using MEGA 7 with various fungal sources of *ITS* region sequences [12]. A neighbour-joining tree and maximum likelihood were constructed for the *ITS* regions of *Colletotrichum* sp. sequences isolated from peanut and other ITS sequences *Colletotrichum* sp. of host plant species. Its closet homologues for *ITS* region was identified from NCBI Genebank are shown in Table 1. According to Moncalvo [13], a bootstrap value is 70-80% is often taken to indicate strong support for a cluster of sequences.

The phylogenetic tree was plotted to estimate the relationship between *Colletotrichum* sp. isolated of peanut in this study with other species based on *ITS* region. The phylogenetic tree was generated based on neighbour-joining method and maximum likelihood method using 16 sequences with the bootstrap support by taking 1,000 replicates and the resulted tree showed three major clades (Fig. 2) and four major clades (Fig. 3). The bootstrapping values obtained were strongly supported the phylogenetic tree generated with ITS- rDNA

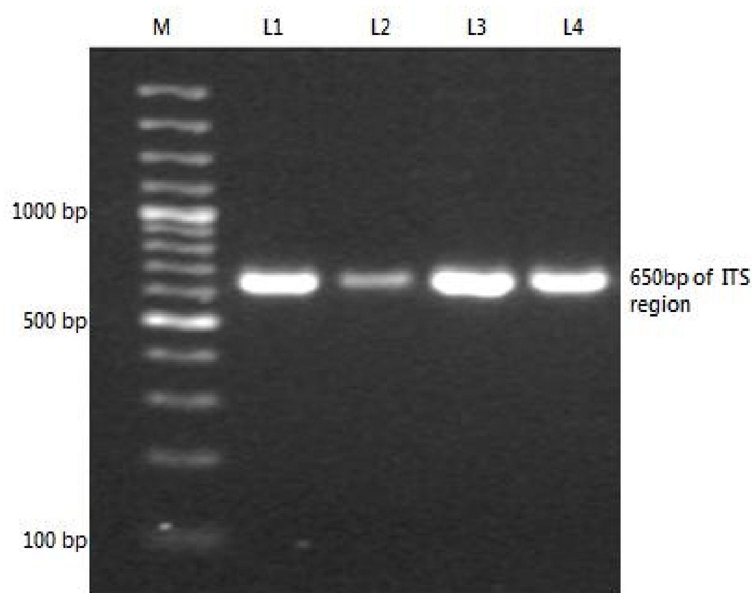

Fig. 1. PCR amplification of the partial ITS gene of *C. gloeosporioides* using primers ITS1F and ITS4R on a 1% agarose gel. Lane M, GeneRuler 100 bp DNA ladder; Lanes 1, 2, 3, and 4 showed the DNA quality of *C. gloeosporioides* form 4 replicates. PCR amplicons of about 650 bp obtained from amplification using genomic DNA extracted from *C. gloeosporioides*

Table 1. Result of Blast-n analysis of *ITS* gene of *C. gloeosporioides* from NCBI database

Accession	Fungal species	Total score	Query coverage (%)	*E*-value	Maximum identify (%)
KM117228.1	*Colletotrichum gloeosporioides*	574	76	3e-86	95
JQ814346.1	*Colletotrichum gloeosporioides*	535	73	3e-86	93
JX179230.1	*Glomerellales* sp.	572	77	1e-84	94
KX594378.1	*Colletotrichum viniferum*	547	77	6e-83	94
KX786433.1	*Colletotrichum fructicola*	567	77	6e-83	94
KX786432.1	*Colletotrichum siamense*	552	77	6e-83	94
KX020562.1	*Colletotrichum nupharicola*	565	77	6e-83	94
KU593526.1	*Colletotrichum alatae*	552	77	6e-83	94
KU251561.1	*Colletotrichum endophyticum*	554	77	8e-82	93
GU994367.2	*Colletotrichum ignotum*	561	77	8e-82	93

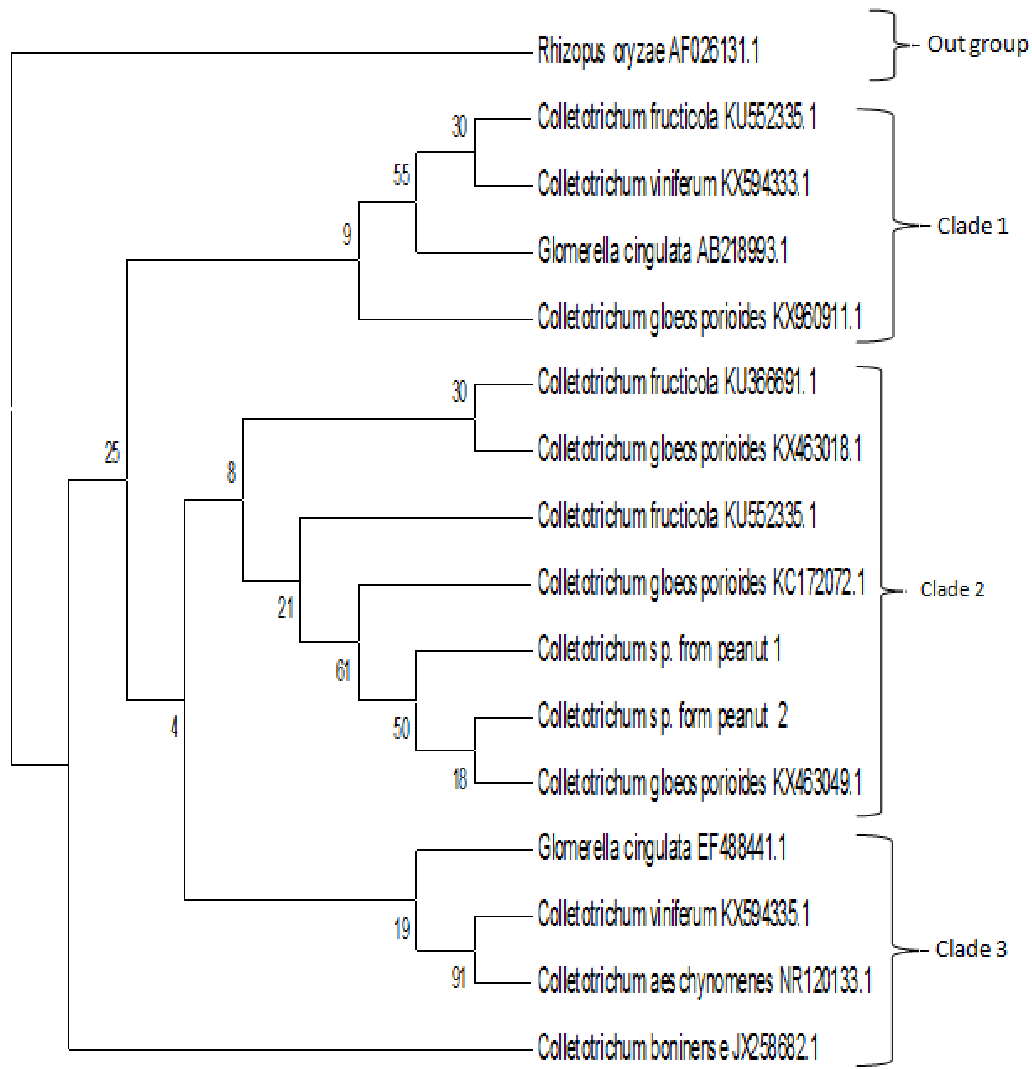

Fig. 2. A neighbor-joining tree displaying the phylogenetic tree with ITS nucleotide sequence of *Colletotrichum* sp. isolated from peanut and other ITS sequences *Colletotrichum* sp. of plants species using MEGA 7. Bootstrap values are indicated for each branch divergence of 1,000 replicates

sequence region. The ITS region isolated from peanut was supported by close related with *Colletotrichum gloeosporioides* (Figs. 2 and 3). In Fig. 2, the sequence with identical with 61 bootstrap value at clade 2. In Fig. 3, was showed similar identical *C. gloeosporioides* with isolated ITS region at clade 1 with 80 bootstrap values.

The clade was formed with *Colletotrichum fructicola* [KU366691.1], *Colletotrichum gloeosporioides* [KX960911.1], *Colletotrichum gloeosporioides* [KX463018.1], *Colletotrichum fructicola* [KU552335.1], *Glomerella cingulata* [EF488441.1], *Colletotrichum viniferum* [KX594335.1], *Colletotrichum boninense* [JX258682.1], Colletotrichum aeschynomenes [NR120133.1], *Colletotrichum gloeosporioides* [KC172072.1], *Colletotrichum fructicola* [KU552335.1], *Glomerella cingulata* [AB218993.1], *Colletotrichum viniferum* [KX594333.1], *Colletotrichum siamense* [KP703392.1] and *Rhizopus oryzae* [AF026131.1] as outgroup.

In the clade 2 in Fig. 2 and clade1 in Fig. 3 is though the least branch distance was observed between *Colletotrichum gloeosporioides* and *Colletotrichum* sp. form peanut but the notable

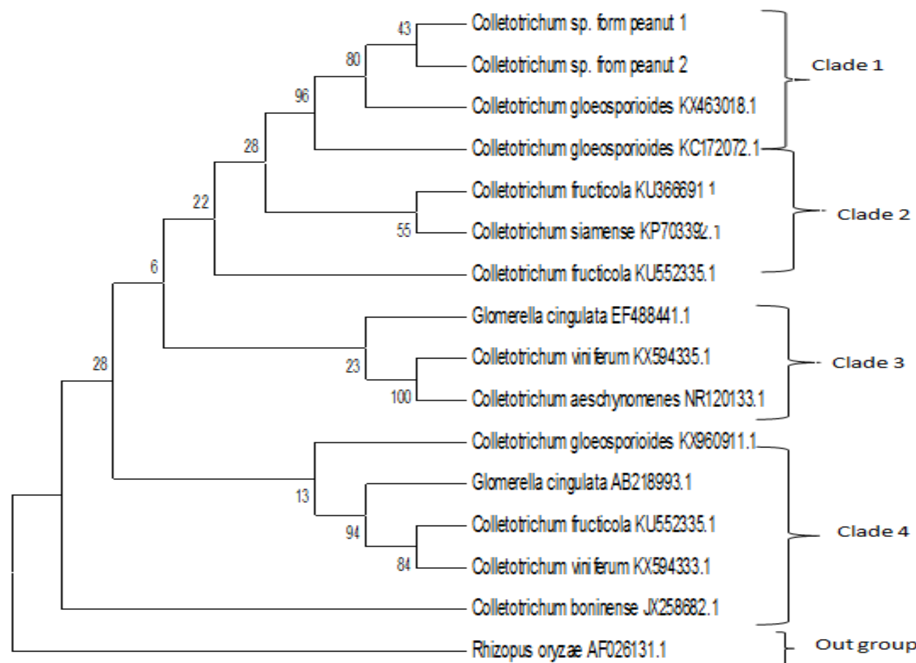

Fig. 3. A maximum likelihood tree displaying the phylogenetic tree with ITS nucleotide sequence of *Colletotrichum* sp. isolated from peanut and other ITS sequences *Colletotrichum* sp. of plants species using MEGA 7. Bootstrap values are indicated for each branch divergence of 1,000 replicates

observation was shown the similarity with supported by high bootstrap value with 80 and 61, which shows their genetic closeness.

4. DISCUSSION

Colletotrichum gloeosporioides is the pathogenic fungus causing anthracnose disease and results in several symptoms in certain leguminous and non-leguminous plants [14]. Previous study suggested that the seeds of peanuts can be colonized by a wide range of saprotrophic fungi as well as other pathogens. As a result of these observations, it can be suggested that these fungi are seen to have colonize the peanut seed.

The fungus *C. gloeosporioides* was isolated, among the isolated fungi, indicating that it is the most active and pathogenic fungus. It has been reported as the most important seed-borne fungi, causing widespread disease of peanuts in the tropics and subtropics [15,16], that always occurred in various peanut tested cultivars. Nuclear rDNA, with both its larger and smaller subunits, 5.8 S, and the Internal Transcribed Spacer (ITS) region, has proven to be perfect for molecular identification via PCR primer each sequence is variable at family, genus or species level [10]. Internal transcribed spacer (ITS) regions are used to distinguish fungal species which are closely related. When the target DNA is amplified via PCR with the help of taxon-specific primers, it is much sensitive and suitable way compared to other techniques of conventional microscopy. PCR technique does not need to have viable species present and work even in the conditions where the sample is limited.

In the research being conducted, a PCR based, highly sensitive diagnostic assay was formed with a prospect of detection of *Colletotrichum gloeosporioides* peanut anthracnose pathogen. For the very first time PCR based diagnostic assay is used to detect the presence of anthracnose pathogen of *C. gloeosporioides* in the peanut. *C. gloeosporioides* was pathogenic fungus was used with specifically designed universal primer to amplify the uniform band of 650 bp among all the isolates (Fig. 1).

C. gloeosporioides of ITS sequences give information that was analyzed using BLASTn. All annotations were based on BLAST searches with a score threshold of 200 for BLASTn and e-values 10-5 with a minimum of 85-100% identity

over at least 80% of the length of the nucleotide sequence which are the commonly used thresholds for reliable sequence annotation. For identification the sequences were confirmed using the repeated blast with the sequences which are already present for the taxonomy as well as identification of *C. gloeosporioides* making use of NCBI BLAST search for sequence identification of BLAST 2.0 (http:// www.ncbi.nlm.nih.gov/BLAST) (Table 1).

For fungi, the ITS region is the most widely sequenced region of DNA. At species level and within species, it has been useful for the molecular systematic. Because of its higher degree of variation than other genic regions of rDNA, variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. Along with the ITS standard primers which is used by majority of laboratories, several taxon primers which are specific have been explained which enable the fungal sequence's selective amplification [17].

A clear indication of probable phylogenic relationships of isolates characterized by conidial size and out group with *Rhizopus oryzae* [AF026131.1] is given by sequence analysis from ITS region gene. The studies in the field of molecular genetics have given very significant data for making the systematic of genus *Colletotrichum* clear [18]. On the other hand, the phylogenic studies revealed the ITS region sequence and its divergence among isolates. It found that there was an *Mvn*I specific site among ITS region of isolates of only *C. gloeosporioides* and other *Colletotrichum* that isolate in the studies. Subsequently, it was also confirmed by certain genuine sequence data and can be utilized for the differentiation of *C. gloeosporioides* from others.

5. CONCLUSION

The present investigation the universal primer-pair of ITS was used for isolation of plant pathogenic fungus, *Colletotrichum gloeosporioides* specifically for peanut. It has been validated and close relationship with other *Colletotrichum* species sequences that retrieve from NCBI database was analyzed with neighbour-joining tree and maximum likelihood by phylogenetic study. Thus, the sequence coding and phylogenetic study proved to be an efficient marker for species-specific primer pair development for detection of pathogenic fungus in peanut in future. It would be useful in developing a rapid and sensitive diagnostic PCR based assay using ITS region in this study for early detection and timely management of *Colletotrichum gloeosporioides* causing peanut anthracnose disease.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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