

ASSESSMENT OF GENETIC DIVERSITY BASED ON ISSR MARKERS IN NEOPESTALOTIOPSIS SPECIES COLLECTED FROM GUAVA (*PSIDIUM GUAJAVA* L.) PLANTS AFFECTED WITH CANKER DISEASE IN PAKISTAN

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Abstract. Guava is susceptible to various phytopathogenic fungi that could be associated with canker diseases. In Pakistan, farmer's economy is heavily affected by this devastating disease. One of the several strategies to managing it is to develop canker disease resistant guava varieties. For the complete understanding of the associated pathogen and its detailed characterization are required. In this study, *Neopestalotiopsis* spp accessions of Fungal Molecular Biology Culture Collection UAF (FMB-CC-UAF) isolated from canker affected guava plants were investigated to find genetic diversity among them based on ISSR markers. Eleven (11) ISSR primers were used to analyze eight (8) *Neopestalotiopsis* accessions. Genetic variation among these fungal accessions were estimated using unweighted pair group method with arithmetic mean (UPGMA) based genetic diversity analysis and Nei's genetic diversity. Among them FMB-Guv-JF2 and FMB-Guv-JF7 showed 13.01% genetic difference to each other. However, the maximum genetic distance was estimated to be 82.32% between FMB-Guv-B2A and FMB-Guv-B1. Generated dendrogram revealed the grouping of investigated fungal accessions into distinct clusters with considerable genetic diversity. It is also supported by principal coordinate analysis (PCoA) which showed dispersed pattern of these fungal accessions indicating diverse genetic base.

Keywords: *fruit crop, plant pathogen, biodiversity, characterization*

Introduction

Guava (*Psidium guajava* L.) originating from tropical America belongs to the genus *Psidium*, family Myrtaceae. Its cultivars vary in size and vigor but it's usually a small tree or shrub having 3 to 10 m height with shallow root system, (Malo and Campbell, 1994; Yadava, 1996; Gutiérrez et al., 2008). Guava is now cultivated in many tropical and subtropical countries worldwide. Guava has potential to grow in a variety of soils having different textures, wide pH range (usually 4.4-9.4), drainage, even in poor soil and can tolerate dry and humid climatic conditions (Keith et al., 2006). Guava fruits have spherical to pear shape with penetrating aroma. The fruit is a great source of nutrition having vitamin A, C, B complex, iron, calcium, and phosphorus (Pachanawan et al., 2008). Pakistan ranks fourth among guava producers in the world after India, China and Thailand. In Pakistan guava is at third position in terms of area while at fourth position in terms of production (Pervaiz et al., 2008). In Pakistan guava is cultivated on 665 thousand ha with yield 31.79 t/ha (FAO, 2011), annual production is reduced from 509 to 495 thousand tons during 2015-2016 (Pakistan Bureau of Statistics, 2016). Actual yield is less than potential yields as (10-12 t/ha) (Ul-Haq et al., 2013).

Worldwide growth and production of Guava is stressed by different biotic and abiotic factors. Among the biotic factors diseases are the major production constraint of Guava. So far, 177 pathogens found to be associated with different plant parts of guava. Out of these 167 are fungal pathogens. In Pakistan twenty-one fungal pathogens have been reported so far, which to infect guava plant (Abbas et al., 2016). Twenty-one (21) fungi have been reported to infect guava plants in Pakistan *Phytophthora parasitica*, *Capnodium* sp., *Glomerella cingulate*, *Alternaria alternate*, *Alternaria tenuissima*, *Cladosporium*, *Curvularia* sp., *Fusarium solani*, *Fusarium solani* f. f. sp. Psidii, *Fusarium oxysporum*, *Fusarium oxysporum* f. f. sp. Psidi, *Penicillium* sp. on tree bark in Pattoki and Sharqpur, *Pestalotiopsis brevista*, *Phoma psidii*, *Diplodia psidii*, *Stagnopsis psidii*, *Pestalotia psidii*, *Lasiodiplodia undulate*, *Botryodiplodia theobromae*, *Polyporus* sp. Among the important fungal destructive diseases of Guava in Pakistan, Anthracnose, Wilt, Dieback/decline, fruit canker and scab are of great importance.

While conducting to document the data regarding disease incidence and severity of Guava Anthracnose, dieback and fruit canker and sampling for to detect the association of fungal pathogens with the disease affected plants, the research team of “Fungal Molecular Biology Laboratory” Department of Plant pathology, University of agriculture Faisalabad, Pakistan has identified different isolates as *Neopestalotiopsis* associated with guava plants. However, evolution results in continuous changes to genetic background of phytopathogens, which ultimately brings new races from them elite cultivars have become susceptible (Malathi et al., 2010). Therefore, unequivocal characterization of newly emerged pathogens is crucial for disease control (Saksena et al., 2013). ISSR marker based genetic analysis for determining and assessing the genetic variation among fungal isolates has become more reliable and efficient approach. Therefore, in this study, genetic diversity among *Neopestalotiopsis* species accessions of Fungal Molecular Biology Laboratory Culture Collection University of agriculture Faisalabad, Pakistan (FMB-CC-UAF), collected from canker affected guava plants were investigated using ISSR markers.

Materials and methods

Fungal accessions

Eight accessions of *Neopestalotiopsis* species (*Table 1*) isolated from canker infected branches, leaves and fruits of guava plants were used in this study. These fungal isolates were collected from different regions of Pakistan. Pure culture of each isolate were made by single spore technique and were maintained on Potato Dextrose Agar (PDA) medium. However, the list of potential pathogens associated to guava canker disease is given in the *Table 2*.

Table 1. Detail of *Neopestalotiopsis* spp. accessions of guava plant collected from different regions of Pakistan for genetic diversity analysis

Sr. #	Isolate code	Strain number
1	FMB-Guv-B3	FMB0026
2	FMB-Guv-C4	FMB0027
3	FMB-Guv-MB	FMB0028
4	FMB-Guv-JF2	FMB0127
5	FMB-Guv-JF7	FMB0128
6	FMB-Guv-L2A	FMB0129
7	FMB-Guv-B2A	FMB0130
8	FMB-Guv-B1	FMB0013

Table 2. Fungal pathogen(s) associated with *Psidium guajava*

Sr. No.	Pathogen	Symptom(s)	Reference	Country	Disease
1	<i>Fusarium solani</i> f. sp. <i>Psidii</i>	Chlorosis, wilting of branches and twigs, browning of internal tissues	Bokhari et al., 2008 and Gupta et al., 2010,	Pakistan, India	Wilt, decline
2	<i>Fusarium oxysporum</i> f. sp. <i>Psidii</i>	Infected seedlings wilt and die at seedling stage, Older plants show vein clearing, leaf epinasty, yellowing of lower leaves, stunting and defoliation. In case of severe decline quick wilting of leaves giving out burning appearance	Prasad et al., 1952; Pandey and Dwivedi, 1985; Ansar et al., 1994 and Lim and Manicom, 2003	India, Australia, Pakistan	Wilt, decline
3	<i>Phytophthora parasitica</i>	Reported to be associated with guava	Safdar et al., 2015	Pakistan	-----
4	<i>Curvularia lunata</i>	Reported to be associated with guava	Safdar et al., 2015	Pakistan	-----
5	<i>Glomerella cingulate/ Colletotrichum gloeosporioides/</i>	Twigs began to die back, twig blight, formation of brown which turns silvery grey on lateral stages, wither tip, bark splitting and roots began to rot at basal region	Ansar et al., 1994; Tandon and Agarwal, 1954; Lim and Manicom, 2003 and ul haq et al., 2013	All guava growing regions	Wilt, dieback and Decline
6	<i>Pestalotiopsis psidii/ Pestalotia psidii</i>	Symptoms appeared as small necrotic spots of blackish gray and brittle usually appeared on leaf apices. Formation of brown spots and corky lesions on fruit epidermis with elevated margin	Lim and Manicom, 2003; Misra and Prakash, 1986 and Rahman et al., 2003	Australia, India, Burma, Malaysia, Venezuela, Zambia, Bangladesh	Fruit canker and scab
7	<i>Gloeosporium psidii/ Colletotrichum gloeosporioides</i>	Numerous small, water soaked and shallow lesions are produced on ripened fruits, later on these lesions become enlarged and dispersed and fuse with each other to form larger spots which are irregular in shape	Srivastava and Tandon, 1969a	India	Anthraxnose or Fruit rot
8	<i>Botryodiplodia theobromae</i>	Infection starts as brown discoloration that usually begins at the stem end and moves downwards in irregular way	Srivastava and Tandon, 1969b	India	Soft watery rot
9	<i>Aspergillus awamori, A. wentii and A. niger</i>	Formation of small sized, round water soaked spots, rotted portion ooze that gives out fermented smell, fruit shrinks and reduction in fruit size occur and blackish mass cover the whole fruit surface	(Gupta et al., 1979 and Lal et al., 1980	India	<i>Aspergillus</i> soft rot
10	<i>Pestalotia psidii/ Pestalotiopsis psidii</i>	Dark brown to grey colored spots appeared on leaf margins or tips. On the upper surface of leaves fruiting pustules may also develops	Bilgrami and Purohit 1971	India	Leaf spot
11	<i>Puccinia psidii</i>	Formation of rust pustules on leaves, shoots, flower and fruits	Misra, 2004	Brazil	Guava rust
12	<i>Rhizopus stolonifer</i>	On infected fruits develop oily and water soaked lesions that are slightly sunken with distinct lesion margins	Ooka, 1980	Hawaii	<i>Rhizopus</i> fruit rot
13	<i>Mucor haemalis</i>	Development of water soaked lesions on infected portion, yellow and fuzzy mass of the fungus may cover the whole fruit surface and finally rotted fruits give off yeast like smell	Kunimoto et al., 1977	India	<i>Mucor</i> fruit rot
13	<i>Rhizoctonia solani</i>	Water soaked discoloration of infected seeds as well as seedlings during pre-emergence stage. During post emergence upper leaves develops yellow to brown color, move downward and cause rotting. Finally, seedlings hands down and eventually die	Gupta, 1978	India	Damping off of seedlings/ seedling blight
14	<i>Curvularia siddiquii</i>	Leaves develop dark brown spots that are restricted to leaf tips and margins at initial stage that covers the whole leaf and eventually defoliate	Srivastava, 1963.	India	Leaf spot
15	<i>Phytophthora parasitica/ Phytophthora citricola/ Phytophthora anicotianae</i>	Infected portion develops greyish brown, water soaked lesions with a greyish black centre, internal fruit surface becomes soft that emits unpleasant smell	Mitra 1929; Gupta et al., 1978 and Ko et al., 1982		<i>Phytophthora</i> fruit rot

Sr. No.	Pathogen	Symptom(s)	Reference	Country	Disease
16	<i>Alternaria tenuissima</i>	Reported to be associated with guava	Abbas et al., 2016	Pakistan	-----
17	<i>B. theobromae</i>	Yellowing of leaves, wilting and complete defoliation, infected trees bears no fruits or few fruits, fruits may become dry and wrinkled	Safdar et al., 2015	Pakistan	Guava decline
18	<i>Phoma psidii</i>	Small circular spots having dark brown center with reddish margin. In advance stage of the disease these spots enlarge and coalesce causing necrotic patches and blighted appearance	Sridhar and Ullasa, 1978	India	Leaf blight
19	<i>Phomopsis destructum</i>	Formation of small lesions of dark brown color and increase to 2 cm diameter. Infected tissues become soft and entire fruit becomes rotted within few days	Rao et al., 1976	India	Phomopsis rot

Genomic DNA isolation

Genomic DNA of these studied fungal accessions was extracted from the mycelia grown on PDA media. Mycelium was ground in liquid nitrogen to turn into fine powder. The fine powder of each sample was treated by CTAB method with some modifications, for genomic DNA isolation. The quality and quantity of extracted DNA was analyzed using agarose gel electrophoresis and spectrophotometer (8000 Spectrophotometer, ThermoScientific.) respectively. Purified DNA of each sample was stored at 4°C for further analysis.

PCR analysis

PCR analysis was performed in 96 well Veriti™ thermalcycler (Applied Biosystem). For genetic analysis of *Neopestalotiopsis* spp accessions, eleven (11) ISSR primers (Table 3) were used. PCR for ISSR was carried out in 25 µl reaction tubes containing primers, dNTPs, 10X Taq PCR buffer, Taq polymerase and genomic DNA. PCR products were separated on 2% high resolution agarose (ACTGene), visualized under Gel documentation System (BioRad) and photographs were saved.

Table 3. List of ISSR primers and statistical data obtained by ISSR primers among *Neopestalotiopsis* spp accessions

Sr.#	Primer code	Sequence (5'-3')	Total Loci	Monomorphic Loci	Polymorphic Loci	percent polymorphism	number of bands or alleles produced per primer	PIC
1	ISSR807	(AG) ₈ T	6	2	4	66.7	30	1.3037
2	ISSR823	(TC) ₈ C	5	0	5	100	22	1.7021
3	ISSR830	(TG) ₈ G	4	2	2	50	21	0.6635
4	ISSR845	(CT) ₈ RG	5	1	4	80	19	1.3432
5	ISSR848	(CA) ₈ RG	4	1	3	75	19	0.8745
6	ISSR857	(AC) ₈ YG	3	0	3	100	13	1.1088
7	ISSR860	(TG) ₈ RA	3	0	3	100	10	0.9287
8	ISSR4	(AGC) ₅ GC	3	0	3	100	13	0.8745
9	ISSR12	(GT) ₈	3	0	3	100	11	1.1088
10	ISSR15	(GT) ₇ AT	3	0	3	100	14	0.8583
11	ISSR19	GC (AG) ₇ A	2	1	1	50	15	0.1948
Total			41	7	34	----	187	----
Average			----	----	----	----	----	0.996445

Data analysis

DNA bands were counted from DNA banding profiles of each ISSR primer and scored as, “1” for presence and “0” for absence. The data were analyzed using different software packages such as; PAST v.3.16 for dendrogram based on unweighted pair group method with arithmetic mean (UPGMA) algorithm, PopGen32 v. 1.32 for similarity matrix, DARwin6 v. 6.0 software for Principal Coordinate analysis (PCoA), Power marker v. 3.25 for computing polymorphic information content (PIC).

Results

DNA markers are used to assess genetic variability among different individuals. This study was carried out with eight different *neopestalotiopsis* spp accessions to detect genetic variation among them. PCR amplification using eleven (11) ISSR primers yielded total 41 scorable loci of which 7 were monomorphic loci (*Table 2*). The number of scorable loci varied from 2 (ISSR19) to 6 (ISSR807). However, maximum number of alleles or bands (30) was obtained from primer ISSR807, while only 10 bands or alleles were produced by ISSR860. Of these 11 (eleven) ISSR markers, six primers (ISSR823, ISSR857, ISSR860, ISSR4, ISSR12, ISSR15) showed 100% polymorphism. The highest value of polymorphic information content (PIC) was 1.7021 of primer ISSR823, whereas the lowest value 0.1948 was from primer ISSR19 with average PIC value per primer was 0.996445.

The dendrogram clearly indicated the two clusters, of which cluster I comprising of four fungal accession, FMB-Guv-B3, FMB-Guv-MB, FMB-Guv-C4 and FMB-Guv-B1. However, cluster II was possessing four accessions viz., FMB-Guv-JF2, FMB-Guv-JF7, FMB-Guv-L2A and FMB-Guv-B2A (*Fig. 1*). This dendrogram showed that these all accessions are diverse and distinct to each other. The fungal accession FMB-Guv-B2A was shown to be highly diverse to FMB-Guv-B1 by scoring the 0.8232 that revealed they are 82.32% dissimilar followed by FMB-Guv-C4 to whom FMB-Guv-B2A is 76.91% dissimilar (*Table 4*). While fungal accessions FMB-Guv-JF2 and FMB-Guv-JF7 are closely related as compared to others with similarity co-efficient 0.1301, which showed, they have 13.01% dissimilarity, so they are more close to each other. Principal Co-ordinate analysis (PCoA) showed comparable results that were obtained in UPGMA based dendrogram (*Fig. 2*) and indicating considerable genetic variability among *Neopestalotiopsis* spp accessions. Principal coordinate analysis (PCoA) also indicated dispersed pattern of these fungal accessions demonstrating diverse genetic base.

Discussion

Studies at the genomic level are necessary and crucial to understand population genetics and genetic diversity and relationships among individual. There is no report for molecular marker assisted characterization of species of *Neopestalotiopsis* genus. Therefore, in this study, we have attempted the characterization of different isolates of *Neopestalotiopsis* spp collected from canker affected guava plants using ISSR molecular marker. Fungal Molecular Biology Culture Collection, University of Agriculture Faisalabad Pakistan, has accessioned these *Neopestalotiopsis* spp isolates. For identification of polymorphism in terms of genetic diversity among these studied fungal isolates, ISSR markers were used to assess intrapopulation genetic variation in

Neopestalotiopsis spp accessions collected from different region of Pakistan. The inherent genetic variation in this study on *Neopestalotiopsis* spp isolates revealed their genetic distinctness unambiguously. Dendrogram based on UPGMA, Nei's original measures and principal Coordinate analysis (PCoA) showed that they all are genetically diverse. These markers yielded percent polymorphism ranged from 50% to 100%, which is in accordance to the findings of Patel et al. (2018). In this study average polymorphic information content (PIC) values was 0.996445 that indicating the discriminating power and potential of these selected ISSR primers to determine genetic diversity among these fungal accessions. This finding is also similar to the findings of Ijaz et al. (2018 and 2019) and Patel et al. (2018).

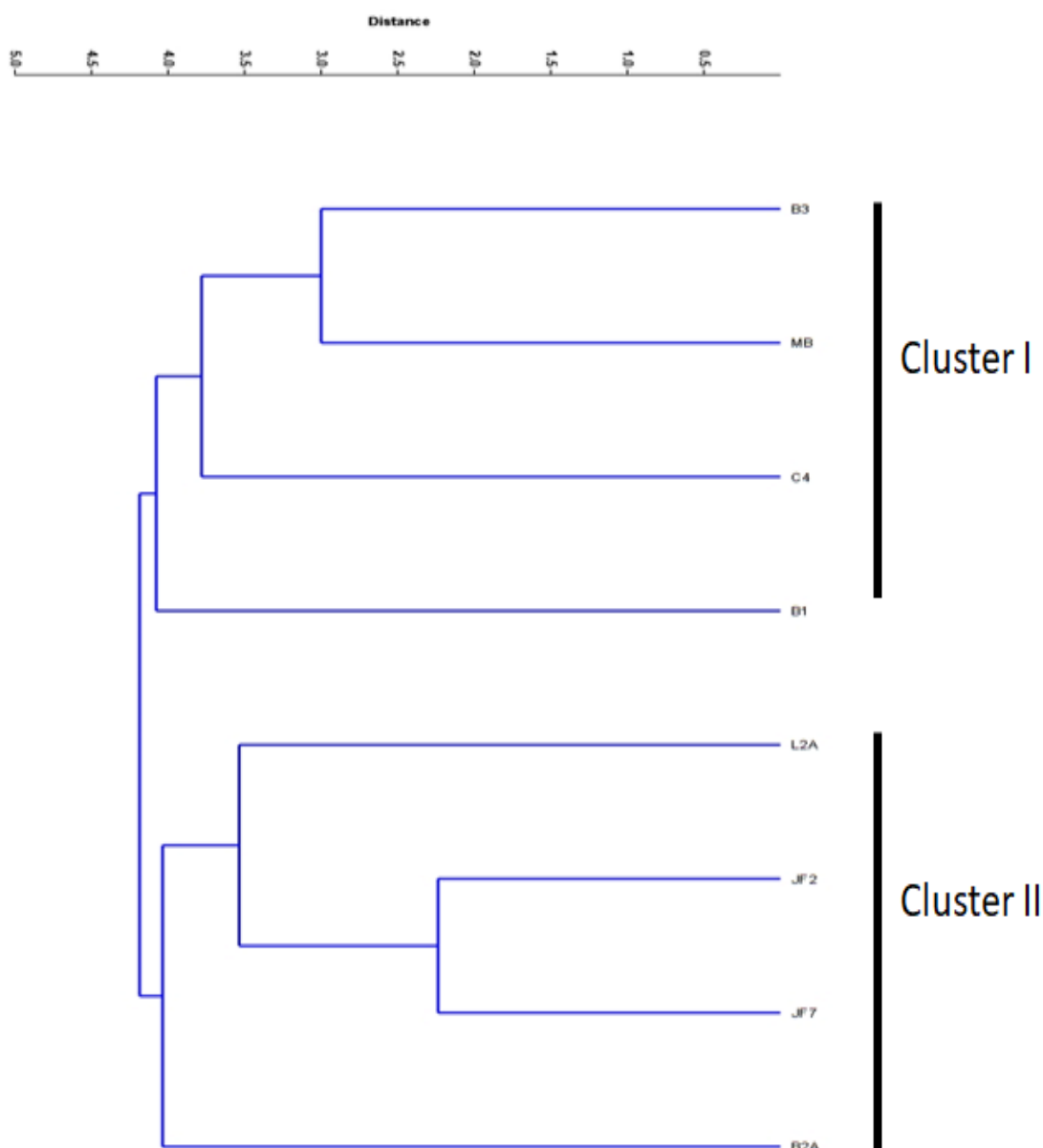


Figure 1. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based dendrogram of eight *Neopestalotiopsis* spp accessions collected from canker affected guava plants

Table 4. Similarity Matrix table based on Nei's original measures: Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Pop ID	B2A	L2A	JF2	JF7	B3	MB	C4	B1
B2A	****	0.6098	0.5366	0.6585	0.6585	0.6341	0.4634	0.4390
L2A	0.4947	****	0.6829	0.7073	0.6098	0.5366	0.6098	0.5366
JF2	0.6225	0.3814	****	0.8780	0.5366	0.6098	0.6829	0.5610
JF7	0.4177	0.3463	0.1301	****	0.4634	0.5854	0.6098	0.5854
B3	0.4177	0.4947	0.6225	0.7691	****	0.7805	0.5610	0.5366
MB	0.4555	0.6225	0.4947	0.5355	0.2478	****	0.7317	0.6098
C4	0.7691	0.4947	0.3814	0.4947	0.5781	0.3124	****	0.6341
B1	0.8232	0.6225	0.5781	0.5355	0.6225	0.4947	0.4555	****

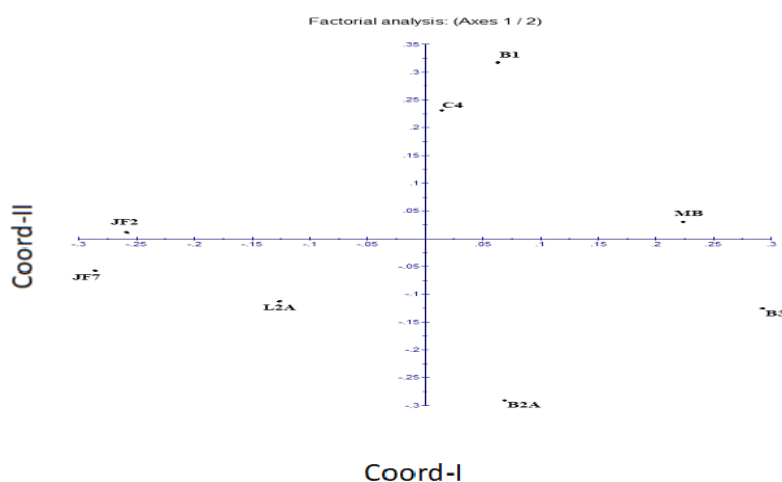


Figure 2. Two-dimensional plot of eight *Neopestalotiopsis* spp accessions collected from canker affected guava plants using Principal Coordinate analysis (PCoA)

Conclusions

This research study was based on the investigation of *neopestalotiopsis* species collected from canker affected guava tree. By considering these fungal isolates belonging to *neopestalotiopsis* species, to be an associated pathogen of canker disease in guava. We determined their genetic diversity. The genetic diversity analysis revealed that these subjects are of diverse in their genetic background, though, FMB-Guv-JF2 and FMB-Guv-JF7 showed close relatedness to each other. These results suggested that FMB-Guv-JF2 and FMB-Guv-JF7 could be as monophyletic group in phylogenetic hierarchy. However, remaining isolates were shown as genetically diverse background. The maximum genetic diversity was observed between FMB-Guv-B2A and FMB-Guv-B1 *neopestalotiopsis* species fungal accessions. Further experimentation to undertaking their molecular taxonomy would be an imperative step to resolve the species boundaries of these studied isolates.

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