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First Report of *Diaporthe/Phomopsis* Complex Isolates in Soybean from Malaysia and Their Longevity in Stored Seeds

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ABSTRACT

Diaporthe/Phomopsis Complex (DPC) can infect soybean seed and reduce its germination in the field and also survive on the seed during storage. Ten soybean seed lots which were stored at 0°C for up to 13 years were used to evaluate the percentage of DPC infection on them and also to identify the DPC isolates. Morphological and molecular methods were used to determine the longevity and frequency of DPC isolates in stored soybeans seeds. Conventional and nested Polymerase Chain Reaction (PCR) was used for amplification of pure cultures and seed lots. The longevity of isolates in storage was found to be <9 years which suggested that *Diaporthe/Phomosis* sp., can survive up to 9 years in cold storage (0°C). Six isolates of DPC were detected, identified and characterized based on morphological and molecular methods in soybean seeds for the first time in Malaysia. Most of the isolates identified belonged to *Phomopsis longicolla* and only one isolate identified from *Diaporthe Phaseolorum* var. sojae. All isolates that were identified using morphological technique were confirmed using molecular method and registered in national center of biotechnology information (NCBI). The result of this study showed that DPC isolates can survive for long time in storage.

Key words: *Diaporthe/Phomopsis,* isolate, longevity, soybean, storage

INTRODUCTION

Diaporthe/Phomopsis Complex (DPC) as a major cause of seed decay is very common in a lot of soybean growing areas of the world that reduces the quality of soybean by reduction in germination (Zorrilla *et al.*, 1994).

Seed infected with DPC may not germinate or may die during germination. If the seeds were to germinate, infected seedlings are normally weak and the surviving plant produce low seed yield (Sinclair and Backman, 1989).

Three different fungi of DPC including *Diaporthe phaseolorum* var. sojae, *Diaporthe phaseolorum* var., *caulivora* and *Phomopsis longicolla* can cause decay in soybean (Roy *et al.*, 1994, 1997). *Phomopsis longicolla* is the most pathogenic among the three pathogen and is endemic in soybean production areas worldwide (Brown *et al.*, 1987; Hobbs *et al.*, 1985). All of these fungi, overwinter on infested soybean straw in the field or may be seed-borne (Mengistu *et al.*, 2007).

Severely infected seeds are usually elongated, shriveled, cracked and may appear white and chalky. Sometimes seed may be infected but does not show any symptoms (Hartman *et al.*, 1999). Some infected seeds may look normal after harvest but seed decay can occur during storage. The level of infection and storage condition will influence the activity of pathogen and the level of infection at the beginning and at the end of storage period. Higher levels of fungi infection were observed in stored soybean seeds after 16 weeks of storage in comparison with the beginning of storage period, whereas, the viability of seed had been reduced in that time (Yaja *et al.*, 2005). Other studies showed that *Phomopsis* infection significantly declines after a year or more of storage and seeds showed lower percentage of infection (Dorrance and Mills, 2009). It indicates that this fungus cannot survive at long dry conditions in storage but its survival depends on the preliminary level of infection at the time of storage.

Several isolates of *Diaporthe/Phomopsis* have been reported from different areas causing varying degrees of damage to crops. Zhang *et al.* (1997) observed varying levels of infection by *Diaporthe phaseolorum* and *Phomopsis longicolla* in soybean when inoculated with different isolates.

Morphological method is one of the most common ways for identifying isolates of fungi but this method requires long time to get result and the possibility of mistake is high due to slight differences in morphological appearance. Vrandecic *et al.* (2006) identified several isolates of *Diaporthe/Phomopsis* sp. from conspicuously infected soybean and velvetleaf by culturing onto potato dextrose agar. These isolates severely rotted the soybean seeds in pathogenicity test, the most identified isolates belonged to *Phomopsis longicolla*.

The molecular method such as sequencing by the amplified Internal Transcribed Spacer (ITS) is faster and the results indicate greater confidence and accuracy. Also molecular genetic differences can be determined among DPC clearly (Rehner and Ucker, 1994). Design of specific primers for this pathogen has also helped to identify different isolates of DPC conclusively. Mengistu *et al.* (2007) separated *Phomopsis longicolla* isolates (Stam 35) from soybean using morphological and molecular methods. Cui *et al.* (2009) identified *Phomopsis longicolla* isolates from soybean by morphological method and then confirmed them using molecular method and determined differences among the isolates by molecular method.

The isolates of this pathogen may be different among different growing season, so they should be identified for different soybean growing areas separately. The objectives of this study were to identify DPC isolates from soybean seed and evaluate their survival and longevity during storage condition.

MATERIALS AND METHODS

The seeds that were used for this study consisted of 17 lots (different seed production years) of seven soybean varieties that have been stored at 0°C and 8-10% seed moisture content in seed technology laboratory of Universiti Putra Malaysia (UPM) for 1-13 years. The earliest seed production year used was 1996 and the latest belonged to production year of 2008. The methods used to identify the species and isolates of DPC were based on morphological characteristics and molecular techniques.

Infection of *Diaporthe/phomopsis* complex in seed lots by culture plate method: The level of seed infection by DPC was determined based on morphological characteristics by plating the seeds in PDA media as outlined by McGee (1986). Seeds were surface sterilized in 0.5% NaOCl for 1 min and then rinsed in sterile water then were plated onto Potato Dextrose Agar (PDA) at pH 4.5,

under randomized complete design in three replications of 100 seeds per replication and incubated at 25°C in 12/12 h light and darkness. The percentage of seed infections were determined by counting the infected seeds at day 7 after planting.

Identification of DPC species in infected soybean seeds: For identifying different species of DPC, pure cultures were prepared from different fungal colonies preserved on infected seeds on PDA plates. A small slice of each colony was taken from the growing mycelium and placed in water agar and incubated at 25°C in 12/12 h light and darkness for making pure culture. After 2-4 weeks, different pure cultures were evaluated and separated based on forms of colonies and conidia type (i.e., α and β). Different species were separated and identified based on colony color and pigmentation.

DPC species were determined based on McGee (1986) specifications. *Phomopsis longicolla* were floccose, dense and white with occasional greenish yellow areas on PDA. In the lower side, the colonies were colorless, with black, spreading stomata. For *Diaporthe phaseolorum* var. cultures on PDA, the mycelium was floccose and rope-like, turning tan to brown with age. At the lower side of the colonies, the mycelium was tan to dark brown with black, pulvinate stromata.

Identification of DPC isolates based on morphological characteristics: Each species of DPC were evaluated for identification of different isolates. Different isolates were separated based on sporulating structure (i.e. pycnidia and/or perithecia), spore size and conidia type (i.e., α and β) (McGee, 1986).

Identification of DPC isolates in soybean seeds using molecular method: Molecular method was conducted for both seeds and pure cultures of the species obtained from culture plate that had infection of more than 10%. DNA extraction method was conducted on grounded seed, following the Delta portal protocol (Dellaporta *et al*., 1983). For amplification, nested PCR was used with two series of primers; ITS4 and ITS5 as universal primers and *Phom* I and *Phom* II as specific primers for *Phomopsis longicolla* and *Diaporthe phaseolorum,* that designed at *Phom*I (5¢-GAGCTCGCCACTAGATTTCAGGG-3¢) and Phom II (5¢-GGCGGCCAACCAAACTCTTGT-3¢). The primers had a GC content of 56 to 57% that located in ITS1 and ITS2 regions, respectively (Zhang *et al*., 1997). At first conventional PCR was run using universal primers space (ITS4 and ITS5), using a final volume of 25 μL, for 1 cycle at 96°C for 5 min followed by 45 cycles at 95°C for 30 sec, at 60°C for 30 sec and 72°C for 1 min for each cycle. For nested PCR specific primers were used, space (*Phom*I and *Phom* II) and reaction was run for 1 cycle at 96°C for 5 minute, followed by 35 cycles at 94°C for 45 sec of each cycle, at 53°C for 30 sec and 72°C for 1.5 min. The amplification efficacy was checked with 1% agarose gel electrophoresis using 5 μL of PCR products in TE buffer (Tris base, boric acid and 0.5 M EDTA [pH 8.0]) and stained with ethidium bromide (0.5 μL) and photographed. The 100-bp DNA Ladder set (Promega Inc, Madison, WI, USA) was used to adapt DNA bands of isolates based on marker size. The electrophoresis was run for 30-40 min at 100 V. DNA was purified from PCR products using the purification Kit 50 (Qiagen Inc., CA) and prepared for sequencing.

The pure cultures of *Phomopsis longicolla* and *Diaporthe phaseolurum* var. soja isolates that had been identified based on morphological characteristics also were used for molecular analysis. Pieces of agar with growing colonies were used to inoculate Potato Dextrose Broth (PDB) and cultures were grown at 25°C for 72 h. Cetyl Trimethyl Ammonium Bromide (CTAB) method was used for DNA extraction from pure cultures (Winnepenninckx *et al*., 1993).

The conventional PCR amplification using specific primers (*Phom* I, *Phom* II) was performed with 1 µL of extracted DNA that extracted from pure cultures of isolates. Reaction was run for 1 cycle at 96°C for 5 min followed by 45 cycles at 95°C for 30 sec, at 60°C for 30 sec and 72°C for 1 min for each cycle. The amplification efficacy was checked with 1% agarose gel electrophoresis using 5 µL of PCR products in TE buffer (Tris base, boric acid and 0.5 M EDTA [pH 8.0]) and stained with ethidium bromide (0.5 µL) and photographed. The 100-bp DNA Ladder set (Promega Inc, Madison, WI, USA) was included as size marker. The electrophoresis was run for 30-40 min at 100 V. The purified samples were sent to Medigen Company (Medigen Inc) for sequencing. The isolates were identified based on their sequencing results. For each isolate one access number was assigned by National Center of Biotechnology Information (NCBI, USA) based on sequence analysis and all isolates submitted with their access numbers and registered as a new isolate of DPC for soybean from Malaysia in NCBI.

RESULTS

DPC infection of stored seed: Percentage of seed infection showed significant differences between soybean seed lots and the varieties showed high variation of seed infection to Phomopsis seed decay (0-55%) in different years, (Table 1). The seeds of Palmetto variety produced in 2003 had the highest percent of seed infection (55%), whereas the seeds of this variety produced in 1996 and Pershing produced in 2006 did not show any infection. Deing variety produced in 2008 also had high infection (50%) and the AGS190 variety produced in 2003 had 36% infection to PSD (Table 1).

***Diaporthe/Phomopsis* species based on morphological identification of stored seed:** Two species of *Diaporthe/Phomopsis* complex were identified based on morphological characteristics. Most colonies showed similarity to *Phomopsis longicolla* species with floccose, dense and white mycelia on PDA at 14 days, the lower part of colonies were colorless with black spreading stomata

Table 1: Percentage seed infection by *Diaporthe/Phomopsis* on different soybean seed lots after different seed storage period

Variety	Production (year)	Storage (years)	Infection (%)
AGS 190	2000	9	7.3^{efg}
	2002	7	25.7^{cd}
	2003	6	36.0^{bc}
	2005	4	6.7^{efg}
	2006	3	18.7^{de}
Argomolio	2005	4	7.3^{efg}
	2006	3	19.7^{de}
Palmetto	1996	13	0.0^{g}
	2002	7	5.3^{fg}
	2003	6	55.0^{a}
	2007	2	17.3^{def}
Deing	2004	5	28.7^{cd}
	2008	1	50.0^{ab}
Pershing	2006	3	0.0^{g}
	2008	1	2.0^{g}
Willis	2004	5	2.6^{g}
Chikura	2006	3	18.6^{def}

Means followed by same letters are not significantly different

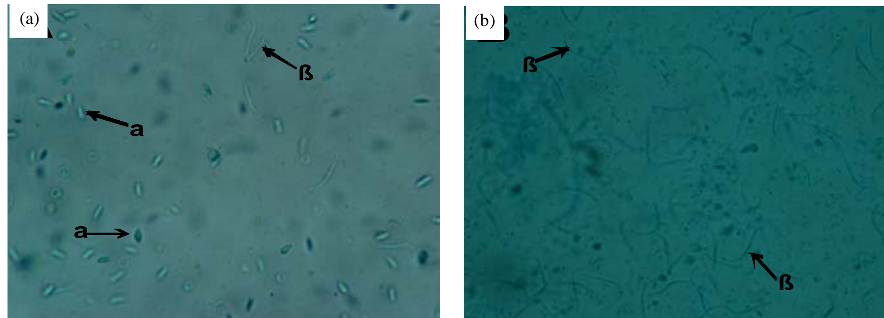

Fig. 1(a-b): Alpha and beta conidia in *Phomopsis longicolla* (a) and Beta conidia in *Diaporthe phaseolorum* var. sojae (b). Alpha conidia is indicated as α, beta conidia is indicated as **β**

Table 2: Identification of isolates based on morphological characteristics of different isolates

Isolates	Pycnidia morphological characteristics	Conidia type	Spore form	Spore size* (μm)
PL 1	Black, stromatic, Unilocular	α conidia	hyaline, ellipsoid	5-9×1.5-3
PL 2	Black, stromatic, Unilocular	α conidia	hyaline, ellipsoid	4-8×1-2.5
PL 3	Without clear pycnidia	α conidia	hyaline, fusiform	5-9×1.5-3
PL 4	Without clear pycnidia	α with a few β conidia	hyaline, guttulate	3.5-7.5×1-2.5
PL 5	Black, stromatic, aggregate	α conidia	hyaline, fusiform	5.5-9×1.5-3
Dps 1	Without clear pycnidia	β conidia	hyaline, filiform and hamate	18-28
Dps 2	Without clear pycnidia	β conidia	rod form	15-25

PL: *Phomopsis longicolla,* Dps: *Diaporthe phaseolorum* var. sojae, * For PL, spore size was based on length×width, For *Dps*, spore size was only based on length

after 25 days. Pycnidia formed were black, stromatic, solitary or aggregated, unilocular or multilocular. In some samples, pycnidia were not formed on PDA. All samples produced α conidia. In *Phomopsis longicolla* **β** conidia are uncommon, however in some samples a few beta conidia were formed, with hyaline, hamate and filiform shape (Fig. 1a). A few colonies on PDA showed morphological characteristics of *Diaporthe phaseolurum* var. sojae, with floccose and rope-like shape, when aged turning color from tan to brown. The bottom part of the colonies on the PDA was tan to dark brown with black, pulvinate stromata. The **β** conidia which were abundantly produced, were hyaline, filiform and hamate in shape (Fig. 1b).

***Diaporthe/Phomopsis* isolates based on morphological identification of stored seed:** Most of DPC species belonged to *Phomopsis longicolla*, therefore most of the isolates that were identified belonged to this species. Five isolates of *Phomopsis longicolla* were identified based on pycnidia morphological characteristics, conidia type, spore form and size (Table 2). These isolates were locally designated as PL UPM1, PL UPM2, PL UPM3, PL UPM4 and PL UPM5. The clear distinctions among the five isolates identified were based on pycnidia morphological characteristics and spore size. The α conidia were hyaline, oval to fusiform and with varying size, 4-10×1.5-3.5 μm. Only PL 4 isolate was found to produce few **β** conidia.

For *Diaporthe phaseolorum* var. sojae species, two isolates were identified. The DPC isolates had similarity in pycnidia morphological characteristics and conidia type. Therefore identification was based on spore form and size. These isolates were locally designated as Dps UPM1 and Dps UPM2 (Table 2).

Table 3: The isolates based on varieties production year and at different storage period

Variety	Production (year)	Storage (year)	Isolates
AGS190	2002	7	PL 1
	2003	6	PL 2, Dps1
	2006	3	PL 4
Palmetto	2003	6	PL 2, Dps1
	2007	2	PL4
Deing	2004	5	PL3
	2008	1	PL5
Argomolio	2006	3	PL4
Chikura	2006	3	PL4

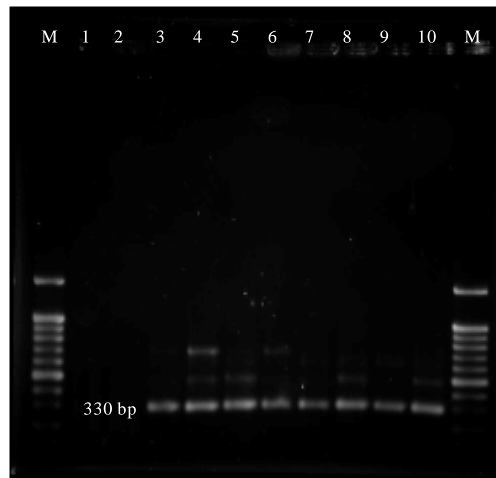

Fig. 2: Gel electrophoresis of amplified seed lots by Polymerase Chain Reaction (PCR) of the Internal Transcribed Spacer (ITS) region with ITS4 and ITS5 as universal primers and *Phom*I and *Phom* II as specific primers

Occurrence and persistence of *Diaporthe/Phomopsis* isolates of stored seed: It is interesting to note that only one isolate of *Phomopsis* was found to occur in each production year (Table 3). In 2003 production year, one *Diaporthe* sp. and one *Phomopsis* sp., isolates occurred together. Only PL 4 isolate was found to occur consecutively in two production years (2006 and 2007). The isolates of PL 1, PL2 and Dps 1 were found to survive for up to more than 6 years at 0°C storage.

***Diaporthe/Phomopsis* isolates based on molecular identification of stored seed:** Molecular identification of isolates was done for both batches of seeds and also for pure cultures. The seed lots with percentage infection of <10% did not show clear bands when amplified. Therefore, results of molecular identification and sequence analysis were presented only on seed lots with >10% seed infection.

By this technique, a 337 bp DNA fragment was amplified from some seed lots (Fig. 2). Lane M shows 100 bp ladder in the first and the end column of figure. Lanes 1 and 2 show disinfected seeds of Pershing and Palmetto varieties that did not show any infection in PDA culture based on

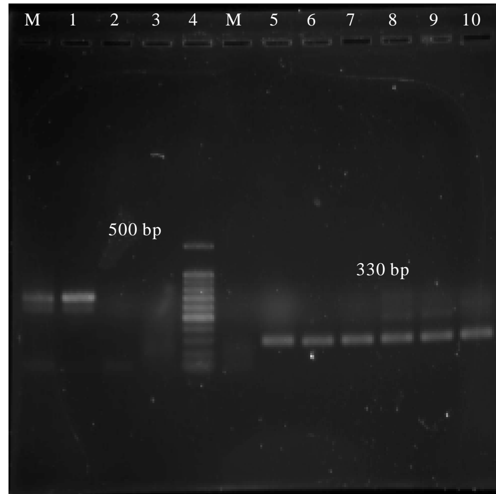

Fig. 3: Gel electrophoresis of pure cultures by the Polymerase Chain Reaction (PCR) with specific primers PhomI and PhomII

Table 4: The registration and submission of different isolates by NCBI

Isolate	Species of fungus	Local name	Access number	Genebank submitted name
No. 1	*Phomopsis longicolla*	PLUPM 1	1284469	GU217590
No. 2	*Phomopsis longicolla*	PLUPM 2	1286659	GU217591
No. 3	*Phomopsis longicolla*	PLUPM 3	1286658	GU217592
No. 4	*Phomopsis longicolla*	PLUPM 4	1286686	GU217593
No. 5	*Phomopsis longicolla*	PLUPM 5	1286685	GU217594
No. 6	*D. phaseolurum* var. sojae	DpsUPM 1	1286696	GU217595

morphological identification. These two samples were placed only as negative control samples for identification of isolates. The lanes of 3-10 belong to infected seed lots with *Diaporthe/Phomopsis* sp.

The pure cultures that have already been identified using morphological method are shown in Fig. 3 for their amplification using conventional PCR method in order to confirm the isolates.

The lanes 6-10 are isolates that were identified using pure cultures of *Phomopsis longicolla* isolates using specific primers, *Phom* I, *Phom* II. Lane 11 shows isolate of pure culture, *Diaporthe phaseolurum* var. sojae. Lanes 3-5 show disinfected seed lots as negative sample for DPC isolates and lane M is 100 bp DNA ladder.

The DNA fragment for isolates of DPC is very small (about 337 bp). Lanes 1 and 2 show infected seed lots with other fungus. These two lanes show the largest size of DNA fragments in comparison with DPC isolates.

The molecular identification results after sequencing analysis showed five isolates of *Phomopsis longicolla* that were identified earlier using morphological method. Therefore, based on morphological characteristics and sequencing analysis of molecular method, five isolates of DPC were identified from different varieties of stored soybean seeds. The two isolates (*Dps* 1 and *Dps* 2) of *Diaporthe phaseolurum* var. soja showed similarity for two samples based on the result of sequencing, therefore, they were identified as *Dps* 1 isolate. So, totally six isolates were identified

from soybean stored seeds for DPC pathogen. All DPC isolates were then submitted to NCBI based on their specification and registered (Table 3).

Total of five isolates from, *P. longicolla* and one isolates from *D. phaseolorum* identified based on morphological and molecular methods from stored soybean seeds (Table 4).

DISCUSSION

Diaporthe/Phomopsis sp. seed infection is detrimental to soybean seed quality in the field and during storage. The level of *Diaporthe/phomosis* sp. infection on soybean seed is dependent on field environments and variety. In this study, variety AGS190 seems to be a sensitive variety, because it showed high infection in most years. The seeds produced in 2003 showed high percentage of infection, could be due to favorable condition for incidence of *Phomopsis* sp., infection.

To obtain high quality seeds, the sensitive varieties should be determined and more tolerant varieties should be planted especially when the environment is favorable for infection. Although the long storage period commonly reduce the level of infection and pathogen may die during long storage but it depends on the severity of infection in the beginning of storage. In fact the percentage of infection on the seed plays the main role for survival of infection in storage and subsequently high seed quality in terms of seed viability.

In an earlier study, high level of fungi infection was observed on seed after 16 weeks of storage (Yaja *et al.*, 2005). Dorrance and Mills (2009) also reported that *Phomopsis* infection declined after one year of storage. In this study, the seeds which have been stored for 9 years (AGS190) also showed *Diaporthe/Phomosis* sp., infection which indicates that the fungi still can cause damage to the seeds but not virulent enough based on >10% infection on seeds. The results of this study suggest that *Diaporthe/Phomosis* sp. can survive up to 9 years in cold storage. As Lori *et al.* (2001) results, survival of pathogen in storage depends on the status of seed infection at the beginning of storage.

Only one *Diaporthe/Phomopsis* isolate occurred in a particular production year. This could be the antagonistic nature among the isolates, thus prohibiting the occurrence of several isolates in one production season. It may be due to limitation of planting area and varieties planted. In this study, the varieties were planted in a small area, so the possibility for several isolates to exist is low.

Although, the *Diaporthe/Phomopsis* isolates appear to survive up to 9 years of cold storage, the data could not conclusively determine which isolate can survive the longest storage period. It appears that all of the *Phomopsis* sp., isolates are equally pathogenic on soybean seeds. The variations of infection percentage in this study could be due to soybean variety resistance to the isolates and field environments. In earlier study (Roy *et al.*, 1997), all *Phomopsis* sp. isolates caused significant decrease in seed germination and seedling growth. The presence of *Diaporthe* isolate will cause higher infection to the seeds as was observed in AGS 190 and Palmetto seeds produced in 2003.

The inadequacy of morphological characteristics for isolates identification has been noted earlier (Uddin *et al.*, 1998). The use of molecular characterization of *Diaporthe* sp. (Pioli *et al.*, 2003) and *Phomopsis* sp. (Uddin *et al.*, 1998) revealed specific differences among fungal species.

Six isolates of *Diaporthe/Phomopsis* were identified in this study from seven pure cultures using specific primers. In this study, the use of two primers in the nested PCR, improved bands appearance on the gel, thus the molecular identification of the isolates had greater accuracy. In earlier study, Zhang *et al.* (1997) identified some isolates of *Diaporthe/Phomopsis* in soybean seed using similar primers (*Phom* I and *Phom* II). The nested PCR method used in this study appears to be a reliable and accurate method to determine DPC isolates in soybean seeds.

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