Phylogenetic analysis and assessment ability of *Fusarium* isolates for the biodegradation of the pesticide carbofuran

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ABSTRACT. Carbofuran is a pesticide used against insects and nematodes. It is a common environmental pollutant associated with agricultural activities. Carbofuran has harmful effects on target and non-target organisms through the accumulation of acetylcholine at the junction of the nerve cell and the receptor sites system by inhibiting acetylcholine esterase. Since the Kingdom of Saudi Arabia is one of the countries that use the pesticide carbofuran in agricultural applications, it inevitably suffers from this worsening environmental problem. Therefore, removal of this compound from the environment is a necessity to ensure human health. In this study, 20 fungal isolates were isolated from pesticide-contaminated agricultural soils. Three fungal isolates (KKU-F5, KKU-F11, KKU-F17) were selected that have the ability to tolerate and utilise carbofuran. The exact identification of these isolates was determined using ITS1-5.8S rRNA-ITS2 region amplification and sequence determination. Alignment results and the comparison of ITS1-5.8S rRNA-ITS2 region sequences of the isolates to ITS1-5.8S rRNA-ITS2 sequences available in the GenBank database, as well as the phylogenetic analysis, confirmed the accurate identification of the isolates KKU-F5, KKU-F11 and KKU-F17 as *Fusarium polyphialidicum*, *Fusarium oxysporum* and *Fusarium verticillioides*, respectively. The three selected fungi demonstrated a high growth rate on 3 gm/L carbofuran, which is twice the recommended dose of carbofuran, after an incubation period of seven days. The percentage of carbofuran removal determined by HPLC analysis for the isolates KKU-F 5, KKU-F11 and KKU-F17 was 88%, 77% and 90.10% respectively,

within this period. RAPD-PCR fingerprinting was performed to determine the differentiation at the molecular level within the three carbofuran-degrading *Fusarium* isolates (KKU-F5, KKU-F11 and KKU-F17). The RAPD-PCR results exhibited different RAPD banding profiles indicating that the three *Fusarium* isolates were different from each other and could be clearly distinguished by their amplification patterns.

Key words: Pesticide; Carbofuran biodegradation; *Fusarium* fungi, ITS1-5.8S rRNA-ITS2 sequences; Phylogenetic analysis

INTRODUCTION

There is an urgent need to develop and spread agriculture across the planet to meet the food and feed needs for human and animals, respectively. In modern agriculture, millions of tons of pesticides applied annually are used to increase production by controlling harmful effects (Al-Arfaj et al., 2013)**.** The insecticide-nematicide carbofuran is a broad-spectrum pesticide used to control mites, nematodes, and a variety of insects on crops including corn, potatoes and strawberries (Chapalamadugu and Chaudhry, 1992). According to the United States Environmental Protection Agency (EPA, 2004), the first introduction of carbofuran onto the market was in 1965. The name Furadan is used predominantly in the field, while the common name (carbofuran) has become the accepted name for the formulation used in the laboratory (Flickinger et al., 1980). Carbofuran is a common environmental pollutant associated with agricultural applications and has harmful effects on target and non-target organisms through the accumulation of acetylcholine at the junction of the nerve cell and the receptor sites system by inhibiting acetylcholine esterase. (Yang et al., 2011). The adverse effects of carbofuran on the hypothalamus is likely to be due to its effects on the Paraventricular Nucleus (PVN) and Ventromedial Nucleus (VMN) because of their involvement in food and water intake (Alewu and Anuka, 2008). Exposure to this compound over a long period of time may result in its accumulation in organisms and poses chronic health effects, such as neurological and teratogenic effects and cancer (Pant 2013). Carbofuran is highly toxic to humans and wildlife through oral and inhalation routes as well as the predominant mode of contact of dermal absorption through the intact skin and stomach action (vomiting, nausea, abdominal cramps and diarrhoea) (Baligar and Kaliwal, 2002). Therefore, removing this hazardous pesticide from the environment using biodegradation strategies is of interest. Several physical and chemical methods have been used to remove carbofuran including oxidation with ozone, Fenton degradation, ozonation, member filtration, photodegradation and adsorption (Yang et al., 2011).

The use of microorganisms in the biodegradation of pesticide-contaminated sites may be an efficient alternative to physical and chemical methods to clean up the environment because a variety of microorganisms are known to utilise pesticides as their sole carbon or energy source(Yang et al., 2011). Several microorganisms, such as fungi, have the natural capability to degrade and transform many toxic chemicals. Fungi are very effective because they are robust organisms and can tolerate higher concentrations of pollutants than bacteria (Adelowo et al., 2014) However, these natural processes proceed at a relatively slow rate (Singh et al., 2008). Since these microorganisms exist in abundance in the environment, the biodegradation of contaminants by microorganisms is one of the most eco-friendly, safe and cost-effective methods (Adelowo et al., 2014). The identification of the local key microorganisms that play a role in pollutant degradative processes is one of the most effective and efficient ways to remove this contamination from the environment (Hesham et al., 2006a&b; 2009; Hesham and Mohamed, 2011). Since the Kingdom of Saudi Arabia is one of the countries that use pesticides in agriculture, it inevitably suffers from this worsening environmental problem. Therefore, there is an urgent need to remove pesticide contamination from the environment.

The objectives of this study were to isolate and molecularly identify the key local pesticide-degrading fungi, to perform a phylogenetic analysis of the fungi identified based on ITS1-5.8S rRNA-ITS2 gene sequencing and to determine the efficiency of these fungal isolates to degrade and utilise the hazardous pesticide carbofuran.

MATERIALS AND METHODS

Soil sampling

Soil samples extending from the ground surface to a depth of 10–15 cm was collected from different agricultural farms in Abha, KSA. The samples were preserved in plastic bags. They were then transported to the laboratory and stored at 4°C until they were used for fungal isolation.

Isolation, purification, and maintenance of carbofuran-degrading fungal isolates

Five grams of collected soil samples were suspended in 100 mL potato dextrose broth (PDB) and incubated in a shaker at 120 rpm at 28°C for two days. After all the soil debris had settled, the supernatant was streaked onto potato dextrose agar (PDA) medium and incubated for 6-7 days at 28°C. To avoid bacterial growth, 100 µg/mL penicillin-streptomycin solutions were added. Several different fungal isolates were selected for purification on PDA medium. Pure fungal isolates were maintained on slant agar at 4°C.

Evaluation and selection of carbofuran-degrading fungi

Fungal screening was carried out using the poisoned food method (Islam et al., 2012). A 5 mm inoculum disc of each fungal species was cut from the edge of an actively growing culture and placed in centre of each petri plate containing PDA. Fungal growth was monitored by measuring the radial growth diameter (cm) of each isolate. The growth capability of the fungal isolates was monitored at 3 gm/L of carbofuran in PDB medium. The treated and control flasks were incubated on a rotary shaker at 120 rpm at 28°C for 7 days with three replicates for each. The dry and wet weights of the fungal growth were measured and expressed in terms of grams of the mycelia (Leslie and Summerell, 2006)

MOLECULAR GENETIC IDENTIFICATION

Isolation of genomic DNA and PCR amplification of the fungal ITS region

Genomic DNA was isolated from the selected fungal isolates using a DNeasy plant mini kit (Qiagen, USA) according to the manufacturer's instructions. The purity and quantity of the DNA was determined by recording its UV absorption spectrum and analysing it using 1% agarose gel electrophoresis. For identification, the ITS region of fungal DNA was amplified using the fungal-specific primer sets: ITS1-F (CTTGGT CAT TTA GAG GAA GTA A) and ITS4 R (TCC TCCGCT TAT TGA TAT GC) as described by White et al. (1990). The PCR reaction was performed in a final volume of 50 µL containing GoTaq (Promega, Madison, WI, USA) Green Master Mix, 1 µL of DNA sample and 1 µL of each primer (at a concentration of 0.5 mM). The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min and holding at 4°C.

PCR products purification and Sequence determination

To verify the presence of appropriate sized amplicons, the PCR product for each isolate was subjected to electrophoresis using 1% agarose gel in TAE buffer according to the standard method. The product of the correct size was purified using a TaKaRa agarose Gel DNA Purification Kit Ver.2.0 and sequenced in both directions using an ABI 3730 automated sequencer (Macrogen, Seoul, Korea).

Alignment and phylogenetic analysis

The sequences obtained were aligned with known ITS sequences in the GenBank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/ BLAST/), and the percent homology scores were generated to identify the fungal isolates. The phylogenetic tree was constructed with MEGA version 4.0 using a neighbour-joining algorithm, and the Jukes-Cantor distance estimation method with bootstrap analyses for 1,000 replicates was performed (Hesham et al., 2012).

Nucleotide sequence accession number

Partial ITS regions of the rDNA sequences of the *Fusarium* isolates reported in this study were deposited in the DDBJ (www.ddbj.nig.ac.jp/), EMBL (www.embl.de/), and GenBank nucleotide sequence databases (http://www.ncbi.nlm.nih.gov) under accession numbers KU527420 (*Fusarium polyphialidicum* KKU-F5), KU527423 (*Fusarium oxysporum* KKU-F11) and KU527425 (*Fusarium verticillioides* KKU-F17).

Carbofuran degradation and HPLC analysis

The degradation of carbofuran was determined using high-performance liquid chromatography (HPLC). Each fungal isolate selected was cultivated in PDB medium supplemented with 3 gm/L carbofuran, which was twice the recommended dose. Control flasks of the growing media PDB with carbofuran were prepared in the same way but lacked the fungal isolates. The samples and controls were prepared in triplicate (100 mL for each). Flasks were incubated on a rotary shaker at 120 rpm for 7 days at 28°C (Leslie and Summerell, 2006). The broth culture medium was filtered through Whatman filter paper (1 × 0.5 µm). The liquid filtrates of the triplicate assays for each isolate were mixed and extracted three times in a separating funnel using chloroform. After that, potassium carbonate was added for 20 minutes, and the liquid was then filtered and left open overnight until the chloroform had evaporated. The controls were prepared in the same manner. The carbofuran in each flask was then dissolved in methanol, and the concentrations of the pesticide in each growth culture were determined by HPLC as described previously (Adelowo et al., 2014). Pesticide concentrations were analysed using a liquid chromatograph equipped with ultraviolet detection (Shimadzu SPD-6A UV spectrophotometric detector) at 254 nm. The samples were subjected to isocratic HPLC using a mobile phase of 60/40 (v/v) methanol/water.

RAPD-PCR and gel electrophoresis

RAPD profiles were generated using five decamer primers (OPA-2, 5'-TGCCGCGCTG-3'; OPA-3, 5'-AGTCAGCCAC-3'; OPA-9, 5'-GGGTAACGCC-3'; OPA-11, 5'-CAATCGCCGT-3' and OPA-15 5'-TTCCGAACCC-3') (Martorell et al., 2005). The amplification reactions PCR condition and gel electrophoresis were performed as described by Hesham et al. (2016).

RAPD fingerprinting analysis

All the fragments generated by RAPD-PCR using the primers were considered and analysed separately to determine the RAPD types. The size of each band was determined using Kodak Digital Science 1D (KSD1D 2.0, Rochester, USA) software, and the presence (1) or absence (0) of a particular band was recorded to generate a binary table. The data table was exported into Popgen32 software (Yeh and Boyle, 1997) for analysis. The normalized RAPD pattern was further analysed using Gel Compare II version 2.5 (Applied Maths, St Martens-Latem, Belgium). The level of similarity in the RAPD fingerprints was calculated using Pearson's correlation coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) method. The relationships between the pattern profiles were displayed as dendrograms and expressed as percentage similarities. The numerical discriminatory index (*D*) that is a measure of the discriminatory ability of the typing methods was calculated as described by Hunter and Gaston (1988).

RESULTS

Isolation of Carbofuran –Tolerating Fungal Isolates

Twenty fungal isolates were obtained from pesticide-contaminated soils that were sampled from different agricultural farms in the Abha area using PDA medium supplemented with 3 grams per litre of carbofuran. After isolation, all of the fungi were purified and maintained on PDA plates.

Evaluation of carbofuran-degrading fungal isolates on solid and liquid media

The 20 pure fungal isolates were evaluated for their growth in PDA medium supplemented with 0.75, 1.5 and 3 gm/L representing the lower, recommended and upper doses, respectively, of carbofuran using the poisoned food method (Islam et al., 2012). Growth started after 48 hours of incubation and continued gradually up to 144 hours of incubation (Table 1). In addition, the growth of the pure fungal isolates was monitored at 3 gm/L, which is twice the recommended dose of carbofuran in the PDB medium. For the growth assessment in the liquid media, the treated and the control flasks were incubated on a rotary shaker at 120 rpm at 28°C with three replicates each. The fresh and dry weights of the 20 isolates were measured after 7 days of fungal growth. Among the 20 isolates, three isolates grew much more effectively in the PDB liquid media. Thus, their fresh and dry weights were determined (Figure 1). These three isolates were designated KKU-F5, KKU-F11 and KKU-F17 and were selected for further analysis.

Table 1. Radial growth (cm) of the fungal isolates KKU-F5, KKU-F11 and KKU-F17 at different concentrations of the pesticide carbofuran after varying incubation periods

Fungal isolate	Carbofuran concentration (gm./L)	Radial growth (cm) after different incubation periods (hr)		
		48	96	144
KKU-F5	0.75	4.26±0.03	5.76±0.31	7.40±0.70
	1.5	4.33±0.33	4.93±0.65	5.93±0.46
	3	4.23±0.53	5.16±0.43	6.46±0.29
KKU-F11	0.75	4.06±0.46	5.50±0.96	6.78±0.49
	1.5	4.23±0.39	5.50±0.36	7.06±0.71
	3	3.90±0.35	4.93±0.52	4.41±0.36
KKU-F17	0.75	4.26±0.26	5.20±0.83	6.10±0.78
	1.5	3.66±0.16	4.36±0.68	6.66±1.01
	3	3.33±0.66	4.03±0.43	3.68±0.25

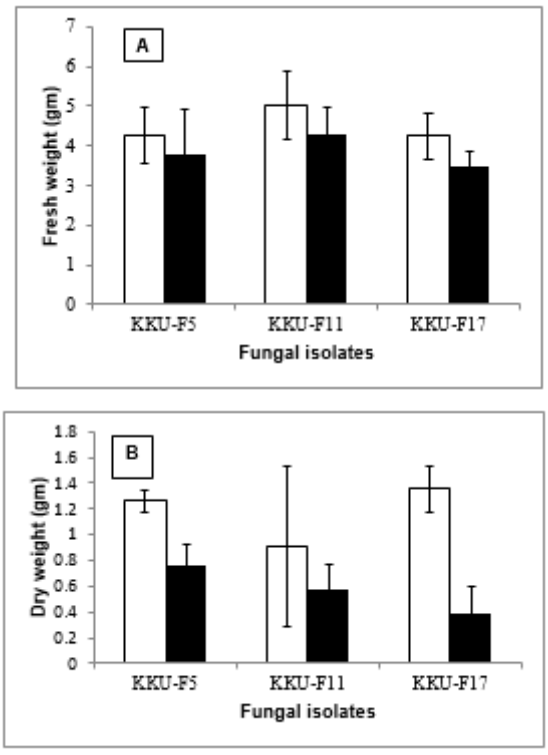

Figure 1. Fresh (a) and dry (b) weight of the fungal isolates KKU-F5, KKU-F11 and KKU-F17 cultured in PDB with 3 gm/L of the pesticide carbofuran 7 days after inoculation. (White columns indicate the control, and black columns indicate the carbofuran treatment.)

ITS1-5.8S rRNA-ITS2 region sequences and phylogenetic analysis

The alignment and comparisons of the ITS1-5.8S rRNA-ITS2 sequences of the isolates KKU-F 5, KKU-F11, and KKU-F17 to the published ITS1-5.8S rRNA-ITS2 sequences in the GenBank database by BLAST search were determined. The sequence results of the ITS1-5.8S rRNA-ITS2 region of the isolates KKU-F5, KKU-F11, and KKU-F17 were shown to be highly homologous to *Fusarium polyphialidicum*, *Fusarium oxysporum* and *Fusarium verticillioides*, respectively, with similarities ranging from 99 to 100%. To determine the position of each strain in a phylogeny, several sequences representative of *Fusarium* species were selected from the GenBank database to construct the phylogenetic trees.

To confirm the position of strain KKU-F5 in the phylogeny, several sequences representing *Fusarium* spp. were selected from the GenBank database to construct a phylogenetic tree. As shown in Figure 2, the phylogenetic tree indicated that strain KKU-F5 and *Fusarium polyphialidicum* shared one clade. Therefore, strain KKU- F5 was identified as *Fusarium polyphialidicum*.

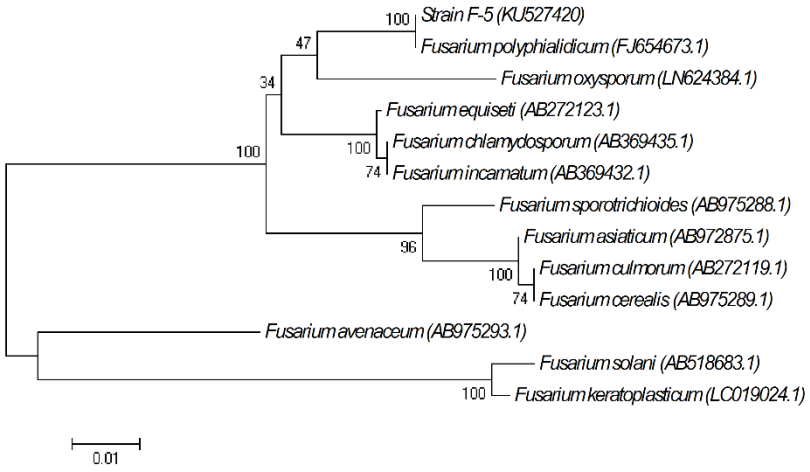

Figure 2. The phylogenetic relationship between isolate KKU-F5 and other ITS1- 5.8S rRNA- ITS2 region sequences of the isolates published. In the phylogenetic tree, KKU-F5 and *Fusarium polyphialidicum* clustered together as one clade.

For the fungal isolate KKU-F11 phylogeny, several sequences representing *Fusarium* spp. were selected from the GenBank database to construct a phylogenetic tree. As shown in Figure 3, the phylogenetic tree indicated that strain KKU-F11 and *Fusarium oxysporum* shared one clade. Therefore, strain KKU-F11 was identified as *Fusarium oxysporum*.

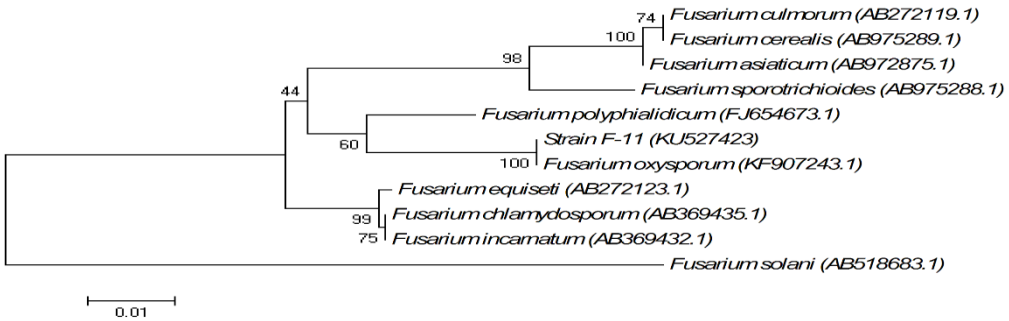

Figure 3. The phylogenetic relationship between the strain KKU-F11 and other ITS1- 5.8S rRNA- ITS2 region sequences of the isolates published. In the phylogenetic tree KKU-F11 and *Fusarium oxysporum* clustered together as one clade.

Finally, to confirm the position of strain KKU-F17 in phylogeny, several sequences representing various *Fusarium* spp. were selected from GenBank database to construct a phylogenetic tree. As shown in Figure 4, the phylogenetic tree indicated that strain KKU-F17 and *Fusarium verticillioides* shared one clade. Therefore, strain KKU-F17 was identified as *F. verticillioides*.

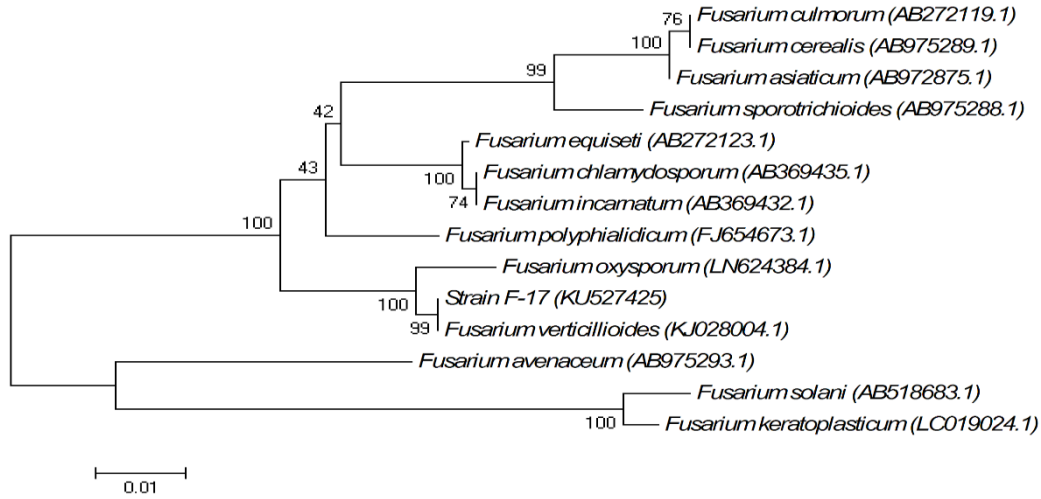

Figure 4. Phylogenetic relationship between the strain KKU-F17 and other ITS1- 5.8S rRNA- ITS2 region sequences of the isolates published. In the phylogenetic tree, KKU-F17 and *Fusarium* verticillioides clustered together as one clade.

Carbofuran degradation and HPLC analysis

HPLC analysis was used to determine the degradation rate, and the HPLC spectra are shown in Figure 5.

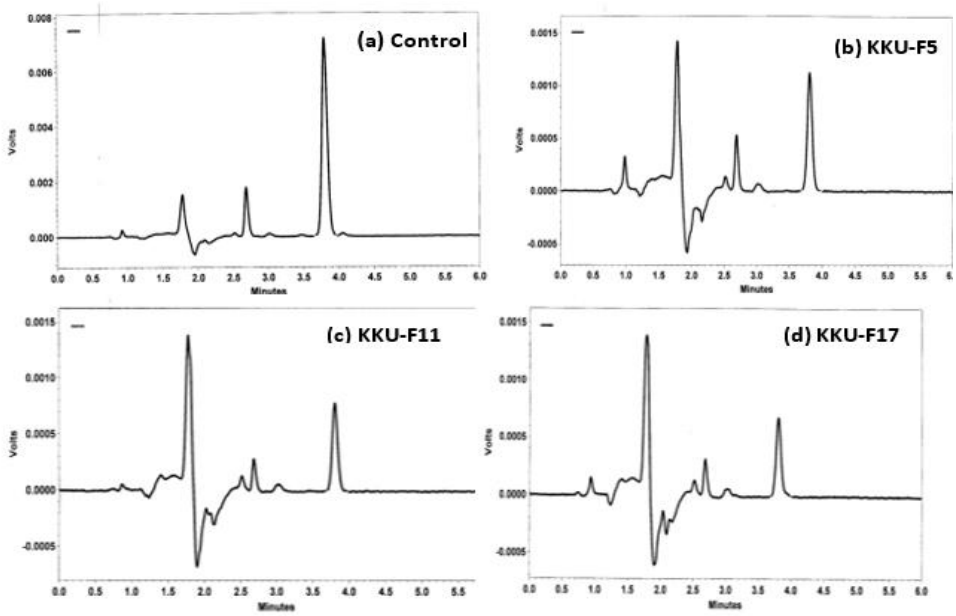

Figure 5. Spectra of the HPLC analysis of Carbofuran degradation (at a retention time of approx. 38 min.) by the fungal isolates (a) Control (Carbofuran), (b) Carbofuran with *Fusarium polyphialidicum* KKU-F5, (c) Carbofuran with *Fusarium oxysporum* KKU-F11 and (d) Carbofuran with *Fusarium verticillioides* KKU-F17.

The pesticide carbofuran was detected at a retention time of approximately 38 minutes. The peak height of carbofuran was reduced after incubation with the three fungal isolates. As shown in Figure 6, the percentage of carbofuran removal determined by HPLC analysis for the fungal isolates KKU-F5, KKU-F11 and KKU-F17 was 88%, 77% and 90.10%, respectively, within the period of analysis. The HPLC results indicated that *Fusarium polyphialidicum* KKU-F5, *Fusarium oxysporum* KKU-F11 and *Fusarium verticillioides* KKU-F17 almost completely removed the carbofuran after seven days of incubation.

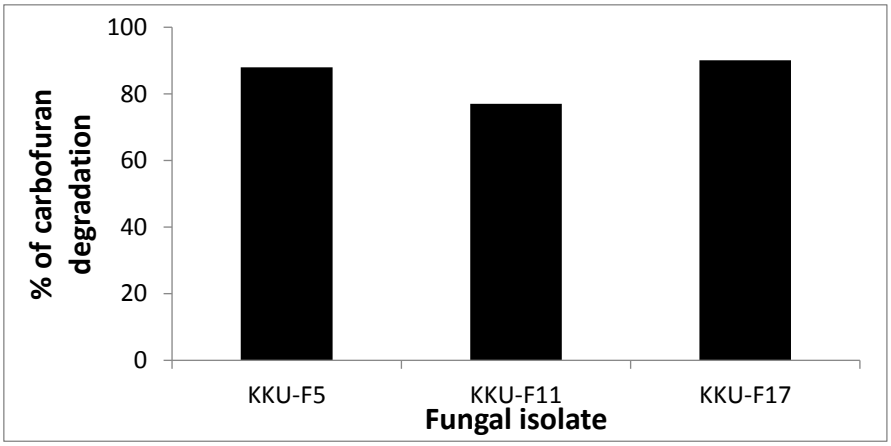

Figure 6. Biodegradation of carbofuran by the fungal isolates KKU-F5, KKU-F11 and KKU-F17.

RAPD analysis

RAPD-PCR fingerprinting was performed to determine the differentiation at the molecular level within the three selected *Fusarium* spp. using five random primers. All primers reacted with the selected strains except for OPA-15. The results revealed that the number of fragments amplified for each primer varied between 5 and 24 fragments (Table 2) and that their sizes ranged from 50 to 1600 bp based on Figure 7.

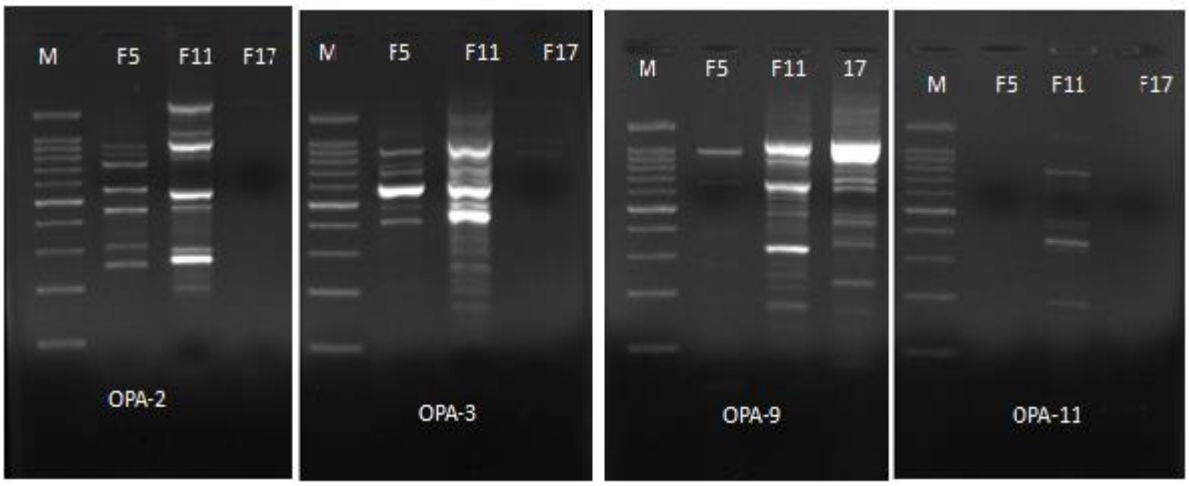

Figure 7. Agarose gel electrophoresis of RAPD products by five random ten-mer primers: OPA-2, OPA-3, OPA-9, OPA-11, and OPA-15. M-represents 100 pb DNA ladder.

Alternatively, Table 2 also illustrates that the total number of bands was 69, while the polymorphism percentage was 98.5%.

Based on the calculated genetic similarity shown in Table 3, an estimation of the relationship between different isolates could be deduced. Data also showed that the lowest genetic similarity was observed between isolates KKU-F11 and KKU-F17 (25.5%), while the highest value was observed when comparing isolate KKU-F5 to isolate KKU-F17 (0.51%).

Table 2. Polymorphism obtained by RAPD analysis among *Fusarium* isolates

RAPD Primers	Number of Bands (a)	Number of Monomorphic Bands (b)	Number of Polymorphic Bands (b)	Polymorphism -b/a * 100%
OPA-2	16	0	16	100
OPA-3	24	1	23	95.8
OPA-9	24	0	24	100
OPA-11	5	0	5	100
OPA-15	0	0	0	0
Total	69	0	68	98.5

Table 3. Genetic similarity values calculated from the DNA Fragments amplified from the three isolates of *Fusarium* spp. using RAPD primers

Fusarium isolates	KKU-F5	KKU-F11	KKU-F17
KKU-F5	1.0000000		
KKU-F11	0.2765957	1.0000000	
KKU-F17	0.5106383	0.2553191	1.0000000

DISCUSSION

Carbofuran is a widely used pesticide in global agriculture that acts as an insecticide, acaricide and nematicide (Chelinho et al., 2011). Its high toxicity to animals and humans, significant mobility in soils and poor management practices associated with overuse can cause potential risks to public health (Bonner et al., 2005). The degradation of pesticides using microorganisms has been one of the most eco-friendly, safe, and cost-effective methods to remove them (Adelowo et al., 2014). Since microorganisms exist in abundance in the soil environment; it is important to search for ones that are native to the contaminated environment and provide excellent degradation potential, considering their advantages when compared to exogenous microorganisms (Larik et al., 2015). In this study, among the 20 isolates, KKU-F5, KKU-F11 and KKU-F17 were determined to robustly grow in the PDB-carbofuran medium. Eman et al. (2013) reported that specific microorganisms use organic pollutants as nutrients and can increase their growth. Recently, some scientists have reported on the isolation of pesticide-utilising fungi from pesticide-contaminated environments (Pinto et al., 2012; Tamer and El-Naggar, 2013; Adelowo et al., 2014; Birolli et al., 2015).

The three fungal isolates selected were identified based on their ITS sequences that contain ITS1, 5.8 s, and ITS2 regions and a phylogenetic analysis as *Fusarium polyphialidicum* KKU-F5, *Fusarium oxysporum* KKU-F11 and *Fusarium verticillioides* KKU-F17. The nuclear internal transcribed spacer (ITS) regions have been used as molecular markers because of their relative variability and ease of PCR amplification (Nilsson et al., 2008). ITS genes are usually used to identify fungal isolates. Based on these facts, the recent identification of the fungal strains was conducted with the 5.8S gene and ITS1 and ITS2 regions (Nilsson et al., 2008; Khan et al., 2009; Sconrad et al., 2012; Hesham et al., 2017). In addition to being widely used for phylogenetic inference and in systematics, the ITS region is the formal fungal barcode and is the primary choice for the molecular identification of fungi from a number of sources (Schoch et al., 2012).

Our results indicated that the *Fusarium* isolates can degrade a high percentage of carbofuran, and this was confirmed by HPLC analysis. The enhanced degradation of pesticides can be caused by repeated use of these compounds over a number of seasons (De Wilde, 2009 ; Kulshrestha et al., 2000). There is some data that fungi

are very effective because they are robust organisms and may tolerate higher concentrations of pollutants than bacteria. Fungal degradation occurs extra-cellularly (Pinto et al., 2012).

RAPD analysis was performed to determine the variation at the molecular level among the three *Fusarium* strains. Our RAPD-PCR results exhibited different RAPD banding profiles indicating that the three *Fusarium* strains, *F. polyphialidicum* KKU-F5, *F. oxysporum* KKU-F11 and *F. verticillioides* KKU-F17, differed from each other and could be clearly distinguished by their amplification patterns. According to Mitrakul et al. (1999) and Martorella et al. (2006), OPA-2, OPA-3 and OPA-9 were the best tests to discriminate microbial strains. Although the isolates *F. polyphialidicum* KKU-F5, *F. oxysporum* KKU-F11 and *F. verticillioides* KKU-F17 belong to the same genus, the genetic similarity and dendrogram regrouped these isolates into two different clusters that were distinct from each other. These results indicated that the isolates KKU-F5 and KKU-F17 are closely related. Our RAPD marker results are similar to those previously reported (Gopal et al., 2008; EL-Fiky et al., 2012; Hesham et al., 2016). They showed the usefulness of the RAPD-PCR technique in fungal strain differentiation and identification.

CONCLUSION

The pesticide carbofuran is a common environmental pollutant associated with agricultural applications, which is known to pose health risks to humans. The three fungal isolates KKU-F5, KKU-F11 and KKU-F17 exhibited a high ability to degrade carbofuran. The three isolates were identified and phylogenetically analysed to be *F. polyphialidicum* KKU-F5, *F. oxysporum* KKU-F11, and *F. verticillioides* KKU-F17. HPLC analysis indicated that *F. verticillioides* KKU-F17 almost completely removed the carbofuran after one week. RAPD-PCR results exhibited different RAPD banding profiles indicating that the three *Fusarium* strains were different from each other and could be clearly distinguished by their amplification patterns.

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