doi.org/10.36721/PJPS.2019.32.5.SP.2509-2526.1

Antifungal and anti-aflatoxin efficacy of biogenic silver nanoparticles produced by *Aspergillus* species: Molecular study

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Abstract: Date palm (*Phoenix dactylifera* L.) is considered the main crop in deserts and arid areas such as Saudi Arabia. Fifteen species belonging to 7 fungal genera were isolated from date palm rhizosphere soil at Riyadh, Saudi Arabia. Twenty-one isolates of *Aspergillus* spp. were used in producing silver nanoparticles (SNPs), five of *A. flavus*, nine of *A. niger* and seven of *A. terreus*. Synthesis of SNPs by these fungi is emerging as an important branch of nanotechnology due to it'secofriendly, safe and cost-effective nature. SNPs have been characterized by UV-Visible Spectrophotometer and Transmission Electron Microscope (TEM). In order to increase the yield of biosynthesized SNPs of desired shape and size, it is necessary to control the cultural and physical parameters during the synthesis. We reported the optimum synthesis of SNPs on a liquid medium at 1.5mM of silver nitrate, pH 9 and 26°C after 96 hours. Antifungal activity of SNPs colloids has indicated that the highest inhibition zone was detected with SNPs. In the case of SNPs synthesized by *A. terreus* PNU37, the highest Inhibition percentage (IP %) 67.6% at the concentration 150 ppm of SNPs. Results have also indicated that the SNPs synthesized by *A. flavus* PNU05 at a concentration of 150 ppm/100 ml culture medium gave the highest reduction of B1 determined by HPLC, where the percentages of reduction (PR%) was 56.45%.ISSR analysis revealed a high level of genetic diversity in the *Aspergillus* spp. population and useful for genetic characterization. ISSR markers were not suitable to discriminate between producing and non-producing SNPs isolates. There was no clear-cut relationship between the ISSR markers (genotype of isolates), antifungal and anti-aflatoxigenic properties.

Keywords: Green synthesis, *Aspergillus* spp., silver nanoparticles, ISSR markers, optimization, antibacterial activity, anti-aflatoxigenic

INTRODUCTION

Nanotechnology, an emerging field of nanoscience deals with the synthesis and applications of nanoscale materials in diverse interdisciplinary fields like physics, chemistry, biology, medicine and agriculture (Albrecht *et al*., 2006). Recently, biosynthetic methods employing either biological microorganisms such as bacteria (Das *et al*., 2014) and fungus (Abd El-Aziz, 2014) or plants extract (Shaik *et al*., 2018). These biogenic processes are of low cost and high yield, safe, and eco-friendly in comparison with the physical and chemical synthetic procedures (Emeka *et al*., 2014). Many fungi are used for the synthesis of silver nanoparticles (SNPs) like *Aspergillus terreus* **(**Abd El-Aziz *et al*., 2013) and *Penicillium expansum* (Ammar HA and El-Desouky, 2016) *Trichoderma Longibrachiatum* (Elamawi *et al*., 2018), *Fusarium oxysporum* (Magdi *et al*., 2018) *Alternaria Solani* (Abdel-Hafez *et al*., 2016).

The mycosynthesis of nanoparticles depends on two factors, including (a) fungal isolate; isolates have secreted fairly large amount of proteins and secondary metabolites extracellularly and hence, leading to the rapid formation of nanoparticles (b) physicocultural conditions (b) physioculture conditions such as pH, incubation temperature, substrate concentration and incubation time (Saxena *et al*., 2016) Mycosynthesis methods seem to provide controlled particle size and shape, which is an important factor for various applications (Gurunathan *et al*., 2014). The other advantages of biological methods are the availability of a vast array of biological resources, a decreased time requirement, high density, stability, and the ready solubility of prepared nanoparticles in water (Thakkar *et al*., 2010).

Nanotechnology has led to the development of new concepts for agricultural applications especially at plant hormone delivery, seed germination, water management, transfer of target genes, nanobarcoding, nanosensors, and plant disease management (Sinha *et al*., 2014; Balaure *et al*., 2017). As agricultural nanotechnology develops, the potential to provide a new generation of green fungicides and other activities for plant disease management will greatly increase because of many advantages over conventional chemical fungicides (Hayles *et al*., 2017; Khandelwal *et al*., 2016). The nanofungicide provide us with a new advanced nano-based formulations that remain stable and active in the target environment (i.e., not heavily affected by sun, heat and rain), penetrate the target organism (fungi), resist defense of the fungi, remain benign to plants and mammals, be cost-effective to

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formulate and manufacture, and preferably possess a new mode of action (Benelli, 2016). The size, shape (spherical, rods, tubes, irregular), surface-to-volume ratio, crystal phase (crystalline, amorphous), and chemical composition (e.g., metallic, carbon, inorganic, organic, polymeric) are key parameters which define many outstanding properties of nanomaterials relevant for their use in green nanofungicide application, including toxicity (Athanassiou *et al*., 2018).

The most important mycotoxins for human and animal health are aflatoxins (AF) (B1, B2, G1, G2), ochratoxin A (OTA), AF and OTA are known to be hepatocarcinogenic, mutagenic, teratogenic and immunosuppressive in both animals and human (IARC, 2002). Several strategies were developed to either prevent toxin production either pre- or post-harvest. The recent advancements in agricultural nanotechnology have made SNPs to be powerful anti-a flatoxigenic activity just few articles have been published on their activity against aflatoxin B1 (Al-Othman *et al*., 2014; Zhao *et al*., 2017; Deabes *et al*., 2018).

A variety of techniques have been used to examine the population diversity of *Aspergillus* spp. present in various crops and geographical locations. Here we focused on *Aspergillus flavus*, *A. niger,* and *A. terreus*. To date, Random amplified polymorphic DNA (RAPD) (Kermani *et al*., 2016) amplified fragment length polymorphism (AFLP) (Kathuria *et al*., 2015) microsatellite (Tran-Dinh *et al*., 2018), restriction fragment length polymorphism (RFLP) (Majid *et al*., 2016), (inter-simple sequence repeats (ISSR) (Zhang *et al*., 2013; Neal *et al*., 2011) molecular marker technologies are used most extensively to study the genetic diversity among the species of *Aspergillu*s spp.

The present study deals with the extracellular synthesis of SNPs using three fungi, *Aspergillus flavus*, *A. niger* and *A. terreus* followed by its characterization and optimization for rapid SNPs synthesis. Furthermore, evaluate their antifungal and anti-aflatoxigenic effectiveness against a highly toxigenic strain of *A. flavus* studies have also been carried out. Finally, genetic characterization of these *Aspergillus* spp. producing and non-producing SNPs using ISSR markers and evaluate the association between antifungal and anti-aflatoxigenic properties and genotype in this collection of isolates.

MATERIALS AND METHODS

Collection of samples

Twenty four date palm soil samples collected from different localities of Riyadh, in the Kingdom of Saudi Arabia, at a depth of 10-15cm. Soil samples were collected over the year 2017, samples were stored in polythene bags at 5°C in the dark and were processed within 1-2 weeks after collection.

Isolation of fungi from soil samples

One gram of soil was suspended in 9mL of sterilized distilled water which gave a dilution of 1:10, from which the dilutions of 1:100, 1:1000 and 1:10000 were made. One mL aliquot of each dilution 1:1000 and 1:10000 was poured in sterilized Petri plates containing Potato Dextrose Agar medium (PDA) supplemented with penicillin (100,000 units) and streptomycin sulphate (0.2 g) to inhibit bacterial growth. Three replicates for each dilution were made. The plates were incubated at 28 ± 2°C for one week. Fungal colonies growing on plates were counted and identified. The number of colonies of each fungus was multiplied by the dilution factor which gave a total number of propagules/g of soil (Waksman *et al*., 1992).

Identification of fungi

General and specific taxonomic literatures were used for the identification of fungal species: (Domsch *et al.,* 1993; Klich, 2002; Nelson *et al*., 1983; Sutton, 1980; Tousson and Nelson 1976).

Genomic DNA extraction

Fungal isolates were cultured on double layer media in 50 mm Petri dishes, one solid (potato dextrose agar as a film) and the other liquid (peptone yeast glucose (PYG, 1200µl). DNA extraction was completed according to the protocol of (Amer *et al*., 2011).

PCR amplification and sequencing of internal transcribed spacer regions and 5.8S rRNA

Aspergillus spp. isolates were molecularly identified on the basis of their internal transcribed spacer regions (ITS1-ITS2) and 5.8S rRNA gene in a similar manner to the study performed by (Gehlot, 2011). PCR amplification by primer ITS-1 (5′-TCC GTAGGTGAACCTGCGTG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′). The amplified PCR product was sequenced using ITS-4 primer and using an automated ABI-Prism 377 DNA Sequencer (Applied Biosystems Inc., CA, USA) at the DNA Sequencing Facility, King Abdulaziz City for Science and Technology (KACST), Riyadh, Saudi Arabia.

Multiple sequence alignments and phylogenetic analysis

Nucleotide sequences were compared with those maintained in the GenBank Database through NCBI Blast (http://www.ncbi.nlm.nih.gov). Alignment of nucleotide sequences was done using a cluster method W of the MEGA6 software program (Tamura *et al*., 2013). A phylogenetic tree was generated based on the percentage difference between the sequences using the neighbor-joining method with the same program main point of reaction and occurred with changing the color of solution.

Fungal biomass preparation for SNPs biosynthesis

To prepare biomass for biosynthesis studies, the fungus was grown in 250ml Erlenmeyer flasks aerobically containing 100 ml liquid medium containing (g/l) KH_2PO_4

7.0 g; K_2HPO_4 2.0 g; $MgSO_47H_2O$ 0.1 g; $(NH_4)_2SO_4$ 1.0 g; yeast extract 1.0 g and glucose 15.0 g. Inoculated media were incubated at 28±2°C and proceeding of a method carried out as described (Abd El-Aziz, 2014). Control included the deionized water and silver nitrate 10-3 M and the sample replacement deionized water with cell-free filtrate.

Ultraviolet-Visible Spectrophotometer

UV–Vis is a widespread method of detection of SNP (Chan and Don, 2013). When bioreduction of SNPs in $AgNO_3$ solution, this is the main point of reaction and occurred with changing the color of solution. Color change in the reaction mixture (Cell-free filtrate and silver nitrate) was the initial indicator of the formation of SNPs. The solution was monitored by using double beam UV-Vis spectrophotometer, Cintra10e GBC (Victoria, Australia). The absorption spectra of the supernatants were taken between 300 and 700 nm. Scanning was performed after reaction times ranging from 6 hours to 96 hours.

Transmission electron microscopy (TEM)

TEM was performed on JEOL model JEM-1010, (Tokyo, Japan) within accelerating voltage of 80 kV after drying of a drop of aqueous SNPs on the carbon coated copper. TEM grid samples were dried and kept under vacuum in desiccators before loading on a specimen holder determined by TEM as described by Singhal *et al*. (2011).

Optimization of SNPs biosynthesis

Four main parameters were selected for the optimization of SNPs biosynthesis: substrate concentration, reaction pH, reaction temperature, and reaction time. Each variable was optimized by varying only a single parameter at a time (table 1). The absorbance of resultant samples was measured at 420 nm.

Minimum inhibitory concentration (MIC) assay

The goal of this assay was to determine the lowest concentration of NPs, which inhibit the growth of the mycotoxigenic fungi. Three toxigenic fungi such as *A. flavus*, *A. parasiticus* and *F. verticillioides*were utilized in the assay. The antifungal activity method was performed according to (Savi *et al*., 2013). Different concentrations (50, 100 and 150 ppm) of silver nanoparticle were added to autoclaved potato dextrose agar (PDA), keeping one as Control (PDA without Silver nanoparticle), the method completing according to (Savi *et al*., 2013).

The inhibition rate (%) was calculated using the following formula:

$$\text{Inhibition \%} = \frac{R-r}{R} \times 100$$

Where R is radial growth of fungi in the control plate and r is the radial growth of fungi in silver nanoparticle-treated plates.

Effect of SNPs on fungal production of total aflatoxins

Aflatoxin B1produced by three isolates fungi of *A. flavus*. The same quantity (pervious experimental) was add to 250 flasks containing SKMY medium liquid (autoclaved) with (10, 20, 30, 40, and 50µg) AgNPs colloids then incubated at 27°C for 21 days. After the end of incubation period, B1 was estimated in the liquid culture medium.

Extraction and measurement of aflatoxins by HPLC

The cultures obtained after incubation were filtered through Whatman number 1 filter paper. The culture filtrates were extracted in the presence of chloroform (1:2 v/v) and procedure apply as (Gertz, 1990). The concentrations of total B1 was quantitatively measured using an HPLC (Perkin Elmer model series 200 UV/VIS) with a C18 column (300 mm x 3.9 mm, 4 µm). The HPLC system was equipped with a UV detector and fluorescence with 365 nm excitation and 430 emission wavelengths. The mobile phase consisted of methanol: acetic acid: water (20:20:60, v/v/v). The total run time for the separation was approximately 25 min at a flow rate of 1 mL/min (Gertz, 1990).

Conditions for ISSR-PCR

Genomic DNA extraction was carried out using the method described in an earlier section. Twenty-eight of primers were employed to specifically amplify *A. flavus*, *A. niger* and *A. terreus* genomic DNA (table 2). PCR was carried out as the method described by (Zhang *et al*., 2013) for *A. flavus*, *A. niger* and (Neal *et al*., 2011) for *A. terreus*. PCR products were detected with 2% agarose ethidium bromide gels in TAE 1x buffer (40mM Tris-acetate and 1.0mM EDTA). A 100-bp DNA ladder (Intron Biotechnology, South Korea) was used as the molecular marker.

Data analysis

The DNA profiles were scored visually from gel photographs. The clear and reproducible amplified bands were chosen for the analyses. Polymorphism information content (PIC) value was estimated using the following

Table 1: Four parameters were used for optimization of SNP biosynthesis

Parameters	Conditions
Substrate concentration ($AgNO_3$)	1mM, 2mM, 3mM, 4mM
Reaction pH	3.0, 5.0, 7.0, 9.0, 11.0
Reaction temperature	22,24,26,28,30 ºC
Reaction time	24, 48, 72, 96, 120 hr

Table 2: ISSR primers used in the present study

No.	Primer code	Sequence (5'–3')	Length (bp)	GC content (%)	MW	Optimum AT (°C)
A. flavus and *A. niger*						
1	*UBC809	AGAGAGAGAGAGAGAGG	17	52.9	5487.18	55.0
2	UBC811	GAGAGAGAGGAGAGAC	16	56.3	5133.90	45.9
3	UBC817	CACACACACACACACAA	17	47.1	5150.94	44.6
4	UBC825	ACACACACACACACACT	17	47.1	5141.92	46.8
5	UBC834	**AGAGAGAGAGAGAGAGYT	18	44.4	5751.37	48.0
6	UBC841	GAGAGAGAGAGA GAGAYC	18	50	5736.36	50.3
7	UBC856	ACACACACACACACACYA	18	44.4	5440.15	48.0
8	UBC864	ATGATGATGATGATGATG	18	33.3	5698.30	48.0
9	UBC881	GGGTGG GGTGGGGTG	15	80	4881.63	52.9
10	UBC895	AGAGTTGGTAGCTCTTGATC	20	45	6243.68	51.3
A. terreus						
1	ISSR_7	***DDB$(AGG)_5$	18	66.7	4876.69	47.2
2	ISSR_8	DDB$(CAG)_5$	18	66.7	4676.54	47.4
3	ISSR_9	DDB$(GAG)_5$	18	66.1	4516.69	48.0
4	ISSR_10	DDB$(CTC)_5$	18	65.4	4431.29	47.5
5	ISSR_11	DDB$(GTG)_5$	18	62.8	4831.59	48.5
6	ISSR_12	DDB$(AACG)_4$	19	50	4997.81	50.5
7	ISSR_13	DDB$(CGCA)_4$	19	75	4901.69	58.4
8	ISSR_14	DDB$(GCCA)_4$	19	75	4901.69	58.4

MW = molecular weight, AT = annealing temperature *ISSR primers sequences published by University of British Columbia (http://www.michaelsmith.ubc.ca/services/NAPS/ Primers sets/primers-Oct2006.pdf) **Y=(C, T) ***Capital letters in ISSR primer sequences denote degenerate sites: B denotes nucleotides C, G, or t; D denotes A, G, or T.

Table 3: Isolation frequencies of fungi recovered from rhizosphere soil of date palm at dilutions of $10^3$and 10^4 of Riyadh, Saudi Arabia.

No.	fungi	Treatment		Difference[a]
		10^3	10^4	
1	*Alternaria alternata*	2.95	1.26	1.54NS
2	*Alternaria* sp.	1.30	0.95	1.09NS
3	*Aspergillus flavus*	4.89	2.41	2.96NS
4	*Aspergillus niger*	9.61	6.85	7.51**
5	*Aspergillus terreus*	16.73	14.19	11.76**
6	*Aspergillus* sp.	5.85	4.27	4.37NS
7	*Cunninghamella echinulata*	3.79	2.21	2.66NS
8	*Fusarium moniliforme*	4.83	3.14	3.85NS
9	*Fusarium oxysporum*	2.54	1.59	1.94NS
10	*Fusarium solani*	11.33	9.63	10.59**
11	*Fusarium* sp.	3.86	1.42	1.62NS
12	*Penicillium chrysogenum*	1.57	0.83	1.18NS
13	*Penicillium digitatum*	1.71	0.96	1.42NS
14	*Mucor* sp.	1.59	0.91	1.20NS
15	*Rhizoups* sp.	2.38	1.21	1.53NS

[a] The difference was significant at *P≤0.05, **P≤0.01, or not significant (NS)

equation described by (Anderson *et al*., 1993): PICj = 1-ΣPlj2 where Plj is the frequency if the l allele for locus j is summed over its L alleles. Markers were classified as informative when PIC was ≥0.5.

STATISTICAL ANALYSIS

The isolation frequency (Fq) of genera was calculated according to the method described by (Marassas *et al*., 1988). A randomized complete block design was used in

Table 4: Analysis of variance of effect of sterilization on frequency of fungi isolated from rhizosphere soil of date palm.

Source of variation	D.F	M.S	F. value	P>F
Replication[a]	23	1.357	0.0086	
Treatment (T)	1	1.149	0.0079	
Fungi (F)	14	2915.218	16.2417	0.000
T X F	14	2151.867	11.5013	0.000
Error	1409	193.491		

[a] Samples were used as replicates

Table 5: Identification of *Aspergillus* species isolated from rhizosphere soil by sequencing of ITS1 and ITS 2 and region of 5.8S rRNA gene compared with sequences listed in the Gen Bank.

No.	*Aspergillus* species soil fungi			Fungi of gene bank		Identity (%)
	Isolate code	Fungi	Size (bp) of ITS1-5.8S-ITS2 region	Gen Bank accession number	Fungi	
1	PNU01	*A. flavus*	584	KY488467	*A. flavus*	99%
2	PNU02	*A. flavus*	584	KY488467	*A. flavus*	99%
3	PNU03	*A. flavus*	584	KY488467	*A. flavus*	99%
4	PNU04	*A. flavus*	584	KY488467	*A. flavus*	99%
5	PNU05	*A. flavus*	584	KY488467	*A. flavus*	99%
6	PNU21	*A. niger*	539	MF436175	*A. niger*	99%
7	PNU22	*A. niger*	539	MF436175	*A. niger*	99%
8	PNU23	*A. niger*	539	MF436175	*A. niger*	99%
9	PNU24	*A. niger*	539	MF436175	*A. niger*	99%
10	PNU25	*A. niger*	539	MF436175	*A. niger*	99%
11	PNU26	*A. niger*	539	MF436175	*A. niger*	99%
12	PNU27	*A. niger*	539	MF436175	*A. niger*	99%
13	PNU28	*A. niger*	539	MF436175	*A. niger*	99%
14	PNU29	*A. niger*	539	MF436175	*A. niger*	99%
15	PNU31	*A. terreus*	777	KJ528526	*A. terreus*	98%
16	PNU32	*A. terreus*	777	KJ528526	*A. terreus*	98%
17	PNU33	*A. terreus*	777	KJ528526	*A. terreus*	98%
18	PNU34	*A. terreus*	777	KJ528526	*A. terreus*	98%
19	PNU35	*A. terreus*	777	KJ528526	*A. terreus*	98%
20	PNU36	*A. terreus*	777	KJ528526	*A. terreus*	98%
21	PNU37	*A. terreus*	777	KJ528526	*A. terreus*	98%

the present study. Analysis of variance (ANOVA) of the fungal isolation frequency was performed with the MSTAT-C statistical package, Michigan State Univ., USA used to compare fungi means. Cluster analysis by the unweighted pair-group method based on the arithmetic mean (UPGMA) was performed using SPSS6.0 software package.

RESULTS

Isolation frequencies of fungi recovered from date palm rhizosphere soil

Fifteen species belonging to 7 fungal genera were obtained from the test samples (table 3). Of these, *Aspergillus terreus, Fusarium solani* and *Aspergillus niger* were the dominant fungi isolated from rhizosphere soil, with isolation frequencies of 16.73, 11.33 and 9.61%, respectively at 10-3 dilutions. Isolation frequencies of *A. terreus* (14.19%), *F. solani* (9.63%) and A. niger (6.85%) were the dominant fungi isolated from rhizosphere soil at 10-4 dilutions. These results indicate that *A. terreus, F. solani* and *A. niger* may colonize the rhizosphere soil. The other fungi isolated occurred in frequencies ranging from 1.57 to 5.85%. Analysis of variance of the fungal isolation frequency (table 4) revealed that fungus and fungus×treatment interaction were all highly significant sources of variation in frequencies of fungi isolated from rhizosphere soil (table 4 & fig. 1). The fungus was the

Table 6: Screening of *Aspergillus* species isolated from rhizosphere soil for the biogenic synthesis of silver nanoparticles.

No.	Isolate code	Name of isolate	silver nanoparticles synthesis
1	PNU01	*A. flavus*	-
2	PNU02	*A. flavus*	-
3	PNU03	*A. flavus*	-
4	PNU04	*A. flavus*	+
5	PNU05	*A. flavus*	+
6	PNU21	*A. niger*	-
7	PNU22	*A. niger*	+
8	PNU23	*A. niger*	-
9	PNU24	*A. niger*	-
10	PNU25	*A. niger*	-
11	PNU26	*A. niger*	+
12	PNU27	*A. niger*	+
13	PNU28	*A. niger*	-
14	PNU29	*A. niger*	+
15	PNU31	*A. terreus*	+
16	PNU32	*A. terreus*	+
17	PNU33	*A. terreus*	-
18	PNU34	*A. terreus*	+
19	PNU35	*A. terreus*	-
20	PNU36	*A. terreus*	-
21	PNU37	*A. terreus*	+

Table 7: Analysis of variance of the effect of different concentration of SNPs synthesized by *A. flavus* PNU05 and *A. terreus* PNU27on fungal growth

Source of variation	DF	MS	F. value	P>F
50 ppm				
Nano (N)	1	4.275	6.176	
Fungi (F)	2	12.976	18.225	0.000
N X F	2	0.181	0.293	0.826
Error	14	0.835		
100 ppm				
Nano (N)	1	20.366	11.498	
Fungi (F)	2	6.402	3.369	0.000
N X F	2	0.587	0.172	0.000
Error	14			
150 ppm				
Nano (N)	1	74.586	17.114	
Fungi (F)	2	11.317	12.516	0.000
N X F	2	9.665	10.759	0.000
Error	14			

most important as a source of variation in isolation frequency, while fungus × treatment interaction was of secondary importance (fig. 1). Fungi isolated from rhizosphere soil of date palm formed several distinct groups based on their distribution patterns over the samples (fig. 2).

Identification of Aspergillus spp

The morphological analysis that was initially used to identify isolates revealed that all of the isolates analyzed were including A. flavus, A. niger and A. terreus. The isolates were further confirmed by analyzing the ITS1-

Table 8: Analysis of variance of the effect of the type and concentration of AgNPs on the amount of B1 produced in SKMY media

Source of variation	DF	MS	F. value	P>F
50 ppm				
Nano	2	5.248	4.982	
Fungi	1	10.476	15.481	0.000
NXF	2	0.247	0.314	0.152
Error	12	0.543		
100 ppm				
Nano	2	17.582	10.961	
Fungi	1	4.512	2.467	0.000
NXF	2	0.857	0.153	0.000
Error	12			
150 ppm				
Nano	2	33.691	9.235	
Fungi	1	5.148	7.274	0.000
NXF	2	4.875	5.931	0.000
Error	12			

df, degree of freedom; MS, mean square; P, probability at confidence 0.95.

5.8S-ITS2 regions from these isolates. The amplified Aspergillus spp. sequences were ranged from 584 to 777 bp. Aspergillus spp. isolated from rhizosphere soil compared with closely related Aspergillus spp. in the GenBank (table 5).

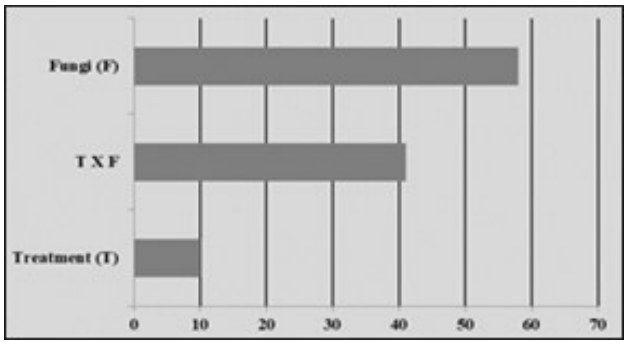

Fig. 1: Relative contribution of treatment (T), fungi (F) and their interaction (T x F) to variation in frequencies of fungi isolated from rhizosphere soil of date palm.

Biosynthesis of SNPs using Aspergillus spp.

The biosynthesis of SNPs using *Aspergillus* spp. was investigated. The reaction of silver nitrate with the filtered cell-free culture, the color of the mixture was gradually changed from light yellow to brown; this color change confirmed the formation of nanoparticles. Fig. 3 shows a photograph of conical flasks containing the filtrate of the *A. flavus* PNU05 biomass in an aqueous solution of 10-3 M AgNO3 at the beginning of the reaction (a) and after 3 days of reaction (b). Table 6 shows two isolates of *A. flavus* were capable of producing SNPs although three isolates failed to produce SNPs. The percentage of isolates produced was SNPs 40%. Three of the nine *A. niger* isolates were SNPs producers with 33%. Four of the seven *A. terreus* isolates were SNPs producers with 57%. In general, ten isolates of *Aspergillus* spp. (47%) were able to produce SNPs while eleven isolates (53%) failed to produce SNPs.

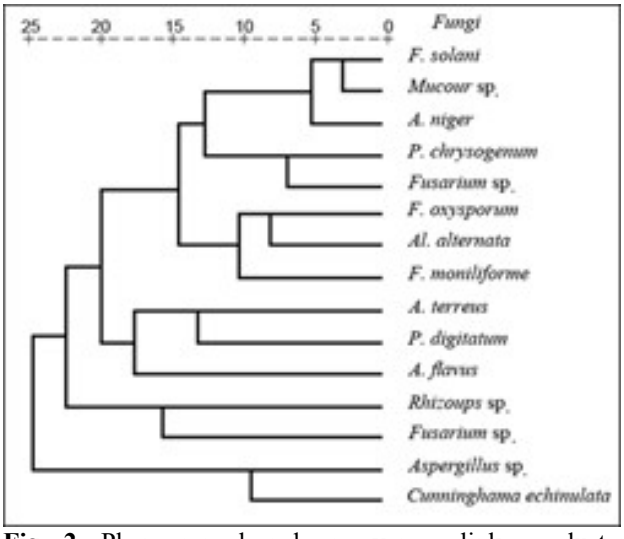

Fig. 2: Phenogram based on average linkage cluster analysis of frequency of 15 fungi isolated from 24 samples of rhizosphere soil of date palm.

Transmission electron microscopy (TEM)

TEM measurements were used to determine the morphology and shape of nanoparticles (fig. 4). The TEM

image showed variable, but predominantly spherical nanoparticles spherical in shape and are uniformly distributed (monodispersed) without significant agglomeration shape, spherical or nearly spherical and without significant agglomeration.

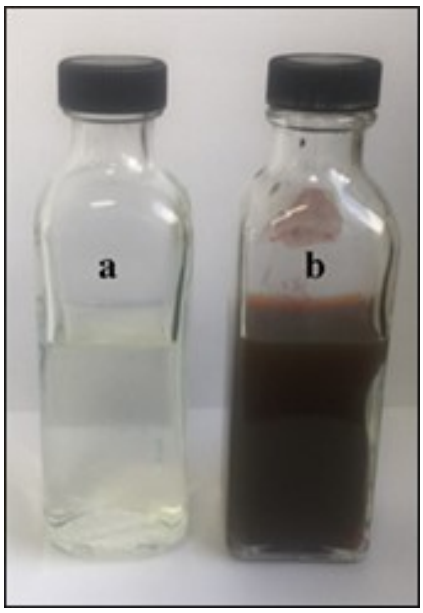

Fig. 3: photograph of two bottles containing the aqueous solution of 10^{-3} M $AgNO_3$ at the beginning of the reaction (a) and after 3 days of reaction (b)

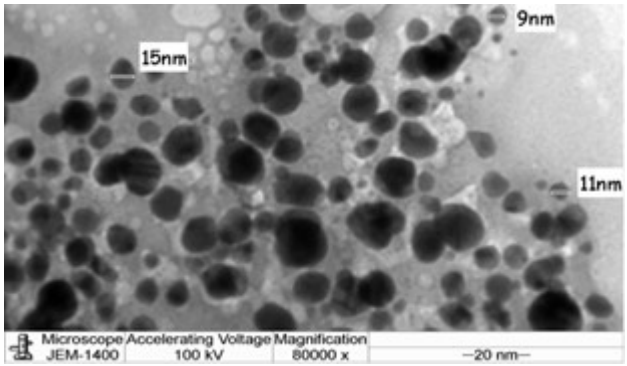

Fig. 4: Transmission Electron Microscopy (TEM) images of synthesized silver nanoparticles by *A. flavus* PNU05

Ultraviolet-Visible Spectrophotometer

UV-Visible spectrum was performed to examine the optical absorbance of SNPs when using *A. flavus* PNU05 (fig. 5). The reaction had run for 6 to 96 hours, showed a strong broad peak at 420 nm (surface plasmon resonances band), which corresponds to indicate the formation of SNPs

Energy Dispersive X-ray (EDX) Spectroscopy

Reflecting the crystalline of the SNPs (fig. 6), intense XRD peaks were observed corresponding to the (111), (200), (220) and (311) planes at 2θ angles of 37.69, 42.21°, 64.38° and 73.52°, respectively.

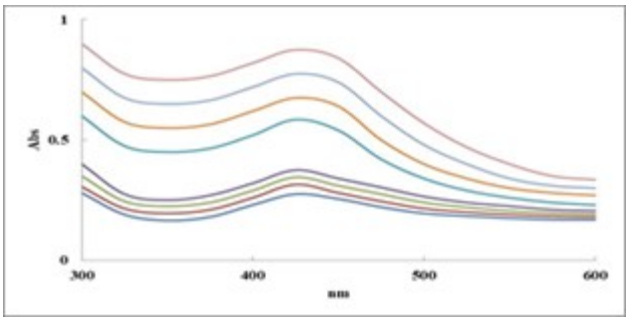

Fig. 5: UV–visible absorption spectrum of SNPs biosynthesized by *A. flavus* PNU05 with different time intervals. 6 h 8 h 10 h 12 h 24 h 48h 72h 96 h

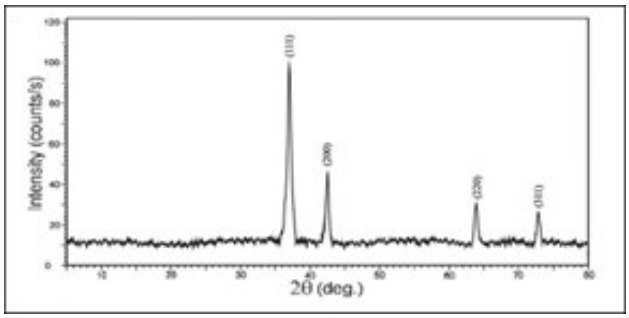

Fig. 6 Representative X-ray diffraction patterns of SNPs synthesized by *A. flavus* PNU05

Optimization conditions for biosynthesis of SNPs

Effect of substrate concentrations of $AgNO_3$

Fig. 7a shows that different substrate concentrations of $AgNO_3$ in the reaction mixture had an obvious influence on the biosynthesis of SNPs, and 1.5mM was the optimum concentration. When the substrate concentration reached 2mM, the biosynthesis of SNPs was both reduced and unstable, with aggregates and precipitation present at the bottom of the reaction container. Surface plasmon absorbance increased with increased salt concentration until the optimum concentration of $AgNO_3$ (1.5mM) after that a decline in the absorbance and synthesis of SNPs was observed.

Effect of pH

Five pHs 3, 5, 7, 9 and 11 were selected to experiment which is the best. fig. 7b shows pH 9 as the best for SNPs biosynthesis, meaning the acidic condition supports reaction and is more suitable for SNPs synthesis. Hydroxide ions (OH-) are nucleophiles which play pivotal role in maintaining the stability of SNPs by adsorbing on it and in the synthesis of smaller size SNPs by providing electrons for silver ions reduction.

The result of nine *A. niger* displayed that a total of 61 reproducible ISSR amplicons were generated from 10 primers out of the 20 ISSRs screened table 10. Total PB was 51, and ranged from 3 for UBC956 to 8 amplicons for UBC834; this accounted for a high PPB (82.9%),

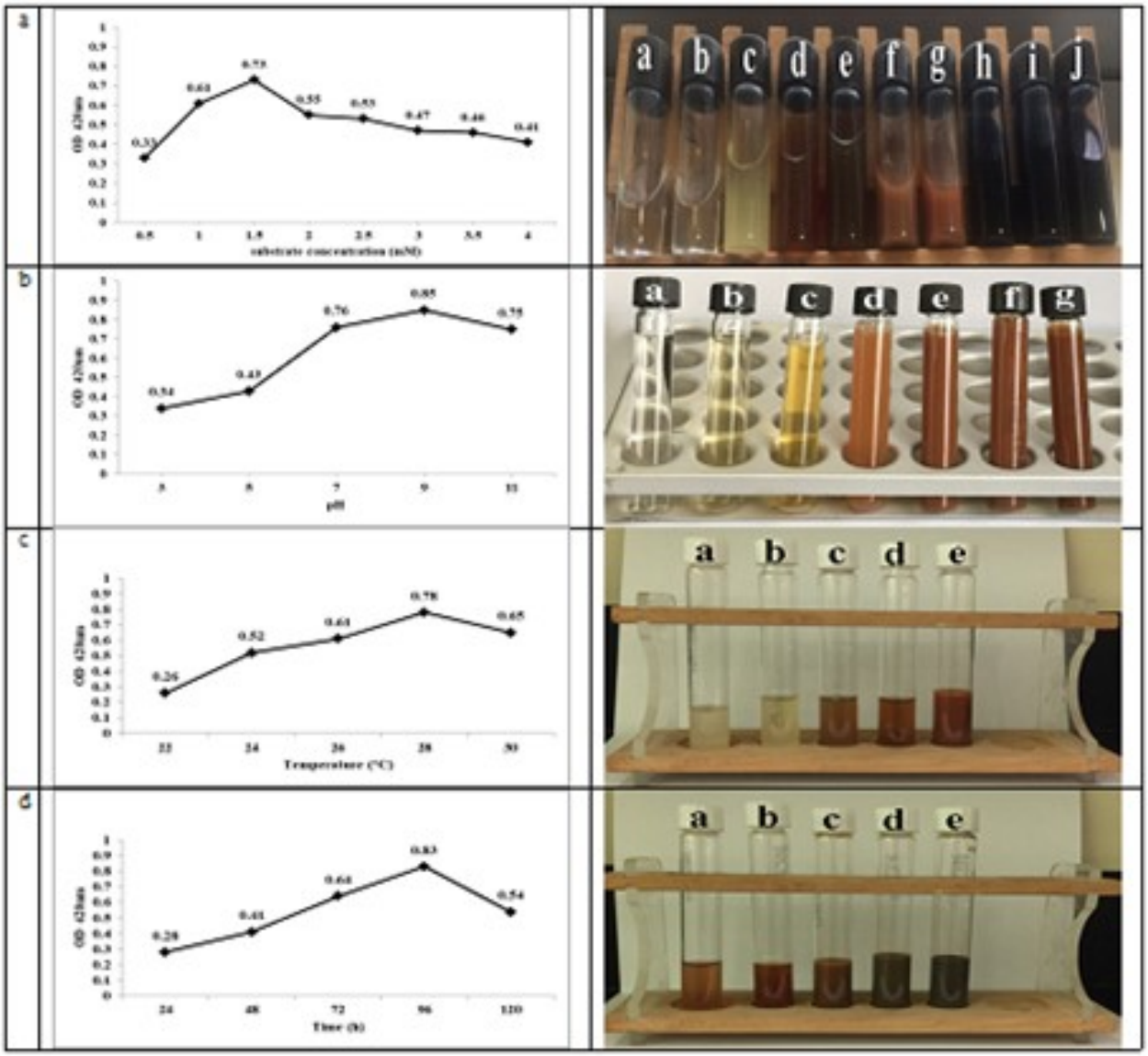

Fig. 7: Optimization of reaction conditions of biosynthesis of SNPs. Effect of (a) substrate concentration, (b) pH, (c) temperature and (d) time on SNPs biosynthesis by the reduction of $AgNO_3$ solution with the cell filtrate of *A. flavus* PNU05. Abbreviations: OD, optical density.

ranging from 71.4% for UBC908 to 100% for UBC841. Amplicon band size had various sizes ranging from 100 to 1700 bp. The PIC ranged from 64 for primer UBC809 to 90 for UBC956, with an average of 82.4%. UBC956 had the highest DP with a value of 16% and UBC908 had the lowest (5%) value. All the primers showed an average DP of 12.1%

Effect of temperature

Optimal temperature conditions for SNPs biosynthesis have varied by microbial isolate; our experiment hastemperatures ranging from 22 to 30°C during the study. The UV–visible absorption spectroscopy of the *A. flavus* incubated at 28°C showed a maximum absorbance. Our data suggest that 28°C was optimal and suitable for SNP biosynthesis (fig. 7c).The rate of synthesis was found to increase with an increase in reaction temperature up to 28°C, which showed maximum synthesis after which a decline in the synthesis was observed and at a lower temperature, reduction reaction could not finish completely within short time so that less SNPs were formed.

Effect of time

The optimal time for SNPs biosynthesis was 96 h, with reducing production at increasing time such as 120 h, as demonstrated by absorbance values had increased with time, up to 96 hours, but did not increase after that (fig.

Table 9: Marker parameters calculated for each ISSR primer used with *Aspergillus* species.

No.	Primer code	TB	PB	PPB (%)	Amplicon band size (bp)	PIC	DP
	A. flavus						
1	UBC809	8	7	87.5	200 –1000	80	10
2	UBC811	6	5	83.3	200–1000	86	12
3	UBC817	8	6	75	200 – 1100	66	6
4	UBC825	8	5	62.5	100 – 1100	75	4
5	UBC834	10	9	90	100 – 1500	89	14
6	UBC841	6	6	100	150 – 900	86	12
7	UBC856	4	3	75	300 – 1200	92	15
8	UBC864	5	5	100	200 – 1800	80	10
9	UBC881	7	6	85.7	100 –1700	87	13
10	UBC895	7	7	100	100 –1700	84	11
Total		69	59	–	–	–	–
Mean		6.9	5.9	85.9	–	82.3	10.7
Min		4	3	62.5	100	66	4
Max		10	9	100	1800	92	15
	A. niger						
1	UBC809	7	5	71.4	100 – 1000	64	5
2	UBC811	6	5	83.3	100 – 1000	85	14
3	UBC817	5	4	80.0	100 –1100	83	12
4	UBC825	8	7	87.5	150 –1150	81	11
5	UBC834	9	8	88.8	100 – 1400	84	13
6	UBC841	6	6	100	100 – 1000	89	15
7	UBC856	4	3	75.0	150 – 1100	90	16
8	UBC864	5	4	80	150 – 1700	83	12
9	UBC881	6	5	83.3	100 –1600	83	12
10	UBC895	5	4	80.0	100 – 1600	82	11
Total		61	51	–	–	–	–
Mean		6.1	5.1	82.9	–	82.4	12.1
Min		4	3	71.4	100	64	5
Max		9	8	100	1700	90	16
	A. terreus						
1	ISSR_7	9	8	88.9	100 – 1200	81	12
2	ISSR_8	7	5	71.4	200 – 1300	91	16
3	ISSR_9	8	7	87.5	100 – 1100	85	14
4	ISSR_10	7	6	85.7	100 – 1200	84	13
5	ISSR_11	8	6	75	200 – 1400	89	15
6	ISSR_12	7	6	85.7	100 – 1100	88	15
7	ISSR_13	7	7	100	100 – 1100	77	8
8	ISSR_14	8	8	100	100 – 1200	69	6
Total		61	53	–	–	–	–
Mean		7.6	6.6	86.8	–	83	12.4
Min		7	5	71.4	100	69	6
Max		9	8	100	1400	91	16

7d). The intensity of the absorbance peak increases with the increase in reaction time, which indicates the continued reduction of the silver ions. The increase of the absorbance with the reaction time indicates that the SNPs concentration increases.

Inhibitory effect of SNPs against mycelial growth of some mycotoxigenic fungi

Data presented in fig. 8a, b show the Inhibition percentage (IP%) of fungal growth after treatment with SNP colloid at the concentration 50, 100 and 150 ppm. In all studied mycotoxigenic fungi, medium to high IP% was observed. fig. 7a shows a low level of IP% (17.7) was detected at 50 ppm concentration SNPs synthesized by *A. flavus* PNU05 against *A. parasiticus*Where the highest IP% (56.6) at the concentrations of 150 ppm against *A. flavus*. In the case of SNPs synthesized by *A. terreus* PNU37, the highest IP% (67.6%) at the concentration of 150 ppm of SNPs was detected in *A. flavus* fig. 7b.

Moreover, the minimum IP% (16.6) was shown with the isolate. The inhibitory effect of SNPs against mycelial growth of some mycotoxigenic fungi was analyzed statistically as shown in table 7.

Effect of SNPs on the amount of B1 produced by three toxigenic A. flavus

The results displayed in fig. 8 a,b show the percentages of reduction (PR%) of B1 reduction produced by three toxigenic *A. flavus* in SKMY medium, which was treated with different source and concentrations of SNPs. fig. 9a shows the SNPs at the concentration of 150 ppm/100 ml culture medium gave the highest reduction of B1 determined by HPLC (fig. 10), where the percentages of reduction were 49.83% for SNPs synthesized by *A. flavus* PNU05 against *A. flavus* 3. fig. 8b indicated the same result with a higher PR% (56.45%). We have found highly efficient SNPs at three different concentrations on B1 production with three aflatoxigenic isolate of *A. flavus* in SMKY medium. The inhibitory effect of SNPs against of B1 produced by three toxigenic *A. flavus* was analyzed statistically as shown in table 8.

Molecular analysis

Out of the 60 primers used, 28 primers that produced unambiguous fragments with repeatable patterns when tested with three *Aspergillus* spp. were considered reproducible amplicons and were used in the analysis (table 9). Date of five *A. flavus* isolates showed a total of 69 amplicons (loci) were obtained, out of which 59 amplicons were polymorphic; the average number of polymorphic amplicons obtained per primer was 5.9 and the range was from 3 (for UCB956) to 9 (for UBC934). The PPB ranged from 62.5 to 100% with an average of 85.9%. Amplicon band size had different sizes ranging from 100 to 1800 bp. across isolates. The PIC ranged from 66 for primer UBC817 to 92 for UBC856, with an average of 82.3%. UBC856 had the highest DP with a value of 15% and UBC had the lowest (4%) value. All the primers showed an average DP of 10.7%.

The result of nine *A. niger* displayed that a total of 61 reproducible ISSR amplicons were generated from 10 primers out of the 20 ISSRs screened table 10. Total PB was 51, and ranged from 3 for UBC956 to 8 amplicons for UBC834; this accounted for a high PPB (82.9%), ranging from 71.4% for UBC908 to 100% for UBC841. Amplicon band size had various sizes ranging from 100 to 1700 bp. The PIC ranged from 64 for primer UBC809 to 90 for UBC956, with an average of 82.4%. UBC956 had the highest DP with a value of 16% and UBC908 had the lowest (5%) value. All the primers showed an average DP of 12.1%.

of 91 for primer ISSR_8 and low PIC value of 69 for primer ISSR_14, with an average value of PIC per primer 83 was obtained. The highest value DP 16 was observed with the primer ISSR_8 and the lowest was observed with the primer ISSR_14 with an average DP of 12.4 per primer.

Based on the Jaccard's similarity coefficient, a genetic similarity matrix was constructed using the ISSR data to assess the genetic relatedness among the *Aspergillus* spp. (fig. 11). The ISSR data of *A. flavus* isolates showed a similarity coefficient (SC) ranging from 0.60 to 0.79 by using primer UBC811 (fig. 11a). Cutting the dendrogram at this similarity value resulted in aggregating two isolates, PNU02 and PMU05, with a medium similarity value 80, whereas PNU04 failed to form a cluster and were individually separated. Cluster I has 1 isolate and Cluster II has 4 isolates with three subcluster (A, B and C). The cluster II contained four isolates, three PNU02, PNU01 and PNU03 (non-producing SNPs) and PNU05 (producing SNPs), which showed 87.2% SC.

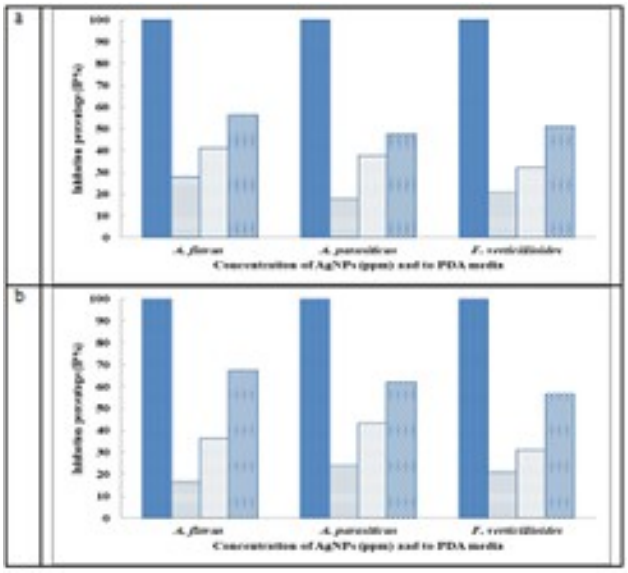

Fig. 8: Inhibition percentage (IP%) of SNPs synthesized by (a) *A. flavus* PNU05 and (b) *A. terreus* PNU37 against mycelial growth of *A. flavus*, *A. parasiticus* and *F. verticillioides* which treated by 50, 100 and 150 ppm of SNP colloids, control.

UPGMA dendrogram based on the similarity matrix for *A. niger* isolates with UBC811 displayed the SCs ranged from 0.62% to 0.98 between the isolates (fig. 11b). Cutting the dendrogram at this similarity value resulted in aggregating two isolates, namely PNU25 and PNU21 with a high similarity coefficient value 0.98, 0.96 for three isolates PNU25, PNU21 and PNU26. Cluster I has 2 isolates and Cluster II has 7 isolates with two subcluster (A and B). The cluster I contained 2 isolates, one PNU29 (producing SNPs) and PNU23 (non-producing SNPs), which showed 0.64% SC. The cluster II contained two subclusters, A and B. A subcluster contained five isolates, two PNU22, 26 (producing SNPs) and PNU28, 21 and 25 (non-producing SNPs) which showed 0.75% SC. B

subcluster contained two isolates, one PNU24 (non-producing SNPs) and PNU27 (producing SNPs), which showed High SC (0.90%).

A cluster analysis of *A. terreus* isolates (fig. 11c) was performed based on the similarity matrix with primer ISSR_9, which showed that the SC ranged from 0.87% to 0.95 (fig. 10c). High similarity value 0.95 between two isolates PNU34 and PNU31. Cluster I has 2 isolates and Cluster II has 5 isolates with two subcluster (A and B). The cluster I contained 2 isolates, one PNU37 (producing SNPs) and PNU33 (non-producing SNPs), which showed 0.78% SC. The cluster II contained mixing of isolates producing SNPs (3) and non-producing SNPs (2), which showed 0.82% SC.

DISCUSSION

Recovery of diverse fungal species isolated from Riyadh rhizosphere soil of date palm in the present study could be attributed to characteristics of the soil, fertilization, pH, root secretions, humidity percentage, microbial competition, and climatic conditions as well as date palm cultivars, tree age, and plant density per unit area. These variations were in agreement with the findings of (Abdullah *et al*., 2010; Ndubuisi-Nnaji *et al*., 2011). The most common fungal isolates were *A. flavus*, *A. niger*, *A. terreus*, *F. solani* and *F. oxysporum*. Our results were consistent with those reported by Ahmed *et al*., 2016; Manzelat, 2017). Fungi isolated from rhizosphere soil formed several distinct groups based on their distribution patterns over the samples. Within each group, fungi were strongly associated and positively in their distribution patterns over samples, whereas between groups, fungi were weakly associated and negative in their distribution patterns over samples (Aly *et al*., 2004).This phenogram implies that the potentia existence of a sample (environment) relate to groups of fungi.

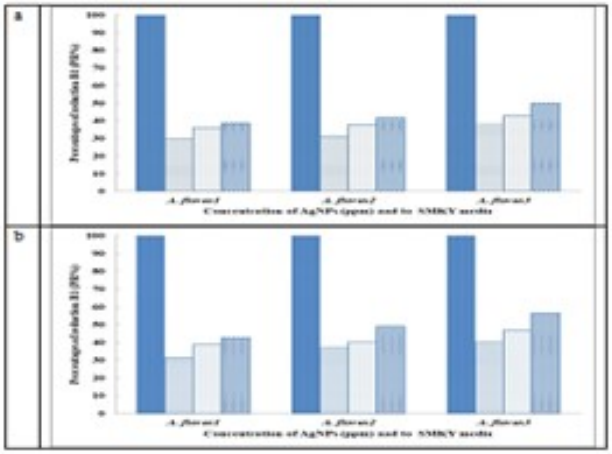

Fig. 9: The percentages of reduction (PR%) of B1 produced by three A. flavus in SKMY treated by 50, 100 and 150 ppm of SNP colloids, control of SNPs solutions synthesized by (a)A. flavus PNU05 and (b) A. terreus PNU37.

Most of *Aspergillus* spp. we isolated had 98–99% similarity with the related *Aspergillus* spp. listed in the GenBank. Comparison of the ITS1-5.8S-ITS2 regions sequences of these isolates using the BLAST algorithm revealed that all of the isolates belonged to *A. flavus*, *A. niger* and *A. terreus*. The sequence of the ITS1-5.8S-ITS2 region appears as the reliable molecular approach for fungal identification and fungal discrimination (Florez *et al*., 2007). Unlike the broader classification of *Aspergillus* spp. depending on the sequence analysis of more conserved rDNA region (5.8S rRNA), ITS1-ITS2 regions shows a plausible accuracy on molecular identification as ensured by (Henry *et al*., 2000; De-Aguirre *et al*., 2004).

A number of mechanisms have been proposed 1) first mechanism proposed protein involved with NADH-dependent reductase was responsible for the reduction of Ag ions and the subsequent formation of SNPs (Ahmad *et al*., 2003). 2) The second mechanism called the hypothetical mechanism where the cofactor NADH and nitrate reductase enzyme were responsible for the synthesis of SNPs. They also confirmed the presence of nitrate reductase in the fungal cell filtrate (Ingle *et al*., 2009). 3) Third mechanism bioreduction of metal nanoparticles was brought about by a protein containing amino acid with -SH bonds (cysteine) undergoes dehydrogenation on reaction with silver nitrate to produce silver nanoparticles. While the free amino acid groups possibly serve as capping for silver nanoparticles (Mukherjee *et al*., 2008) 4) fourth mechanism called hypothetical mechanism included two-step, in the first step, reduction of bulk silver ions (Ag+) to AgNPs takes place by a protein (32 kDa) which might be a reductase secreted by *A. flavus* NJP08, in the second step, SNPs were capped by a protein that binds with the SNPs and confers stability (Jain *et al*., 2011). 5) the fifth mechanism depends on aqueous extracts of most fungi containing proteins, alkaloids, tannins, steroids, phenols, saponins, and flavonoids, which could induce the formation of nanoparticles by serving as reducing agents (Mohamed *et al*., 2013).

The same type of nanoparticles having variable shapes and sizes were observed in common biological systems in the range of 5–50 nm, when synthesized by *A. terreus* (Abd El-Aziz *et al*., 2013).

These results agreement with the unit cell of the face-centered cubic (fcc) structure (JCPDS File No 03-0921) reported by (Abd El-Aziz, 2014).

Our result is in agreement with many researches (Abd El-Aziz *et al*., 2013; Magdi *et al*., 2018). This can be explained on the basis of enzyme-substrate kinetics; i.e. the active site in the key biomolecule responsible for bioreduction is already saturated with the silver ions, and no site is available for excess ions to get reduced, hence

there is no further increase in synthesis of SNPs despite the addition of more salt (Singh *et al*., 2013). Maximum biosynthesis takes place at an optimum concentration of silver nitrate due to increase in biosynthesis SNPs, after this stage we observedgreat difference in surface plasmon absorbance meaning the higher concentration of silver nitrate was not suitable for maximum synthesis (Birla *et al*., 2013).

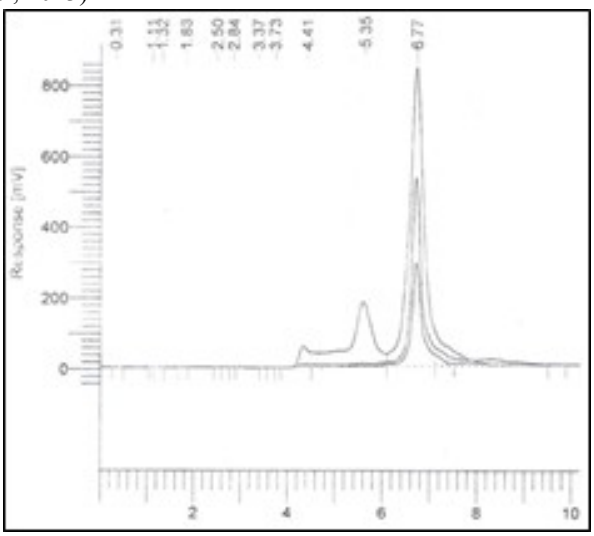

Fig. 10: HPLC chromatograms of B1 determined in liquid medium treated with 55, 10 and 150 ppm of SNPs solution.

OH− ions which help in preventing the aggregates are formed and maintain the smaller size of SNPs (Gurunathan *et al*., 2009) Many reports suggested that a variety of biomolecules are involved in biological nanoparticle synthesis, such biomolecules (polysaccharides and proteins) are likely to be inactivated under the extremely acidic conditions (pH 3.0) and start to work effectively in neutral and slightly alkaline conditions. Our results also differ from those of some researchers who stated that when the condition of the SNPs mycofabrication is alkaline, the synthesis will be faster than in acidic conditions. The activated proteins are involved in the synthesis, also, we know that the biological synthesis of SNPs enzymes catalyzes the reaction (Deepak *et al*., 2011).

At an elevated temperature, increased polydispersity and formation of nanoparticles at a faster rate are inferred from the UV-VIS absorbance intensity (Birla *et al*., 2013). The increasing of temperature due to the increasing kinetic energy of the synthesis SNPs also SNPs formation increases and these lead to the higher rate of agglomeration (Birla *et al*., 2013). Higher temperature leads to denaturation of some proteins, which leads to loosing its functions in reduction of silver ions to SNPs formation. SNPs synthesis process gets fast athigher temperature and slowed at lower temperature. Consequently, it can be concluded that the rate of particles formation and the size of particles can be controlled by temperature (Lee *et al*., 2010)

After 45 hours there is no further increase in the absorbance, indicating the complete reduction of the silver ions (Birla *et al*., 2013). The enhanced rate of SNPs synthesis at optimized conditions might be depending on the effect of substrate concentration ($AgNO_3$), pH, temperature and time on an important factor such as key biomolecule responsible for the bioreduction at aqueous filtrate of fungi.

NPs had a significant effect on plant pathogenic fungi, *Aspergillus terreus* (Abd El-Aziz *et al*., 2013) *A. alternata (*Abdel-Hadi *et al*., 2014*)*, *A. flavus* (Al-Othman *et al*., 2014*)*, *A. parasiticu* (Mousaviet *et al*., 2015), *F. oxysporum* (Abkhoo *et al*., 2017*)*, *F. solani* and *R. solani*, (Khatami *et al*., 2018). A decrease in the size of the SNPs from 29 nm to 20 nm, their antimicrobial activities increased considerably. When the particles are smaller in size, the microbial cells penetration will be faster. The antimicrobial properties are related to the total surface area of the nanoparticles (Mart'ınez-Castano'n *et al*, 2008; Mart'ınez-Gutierrez *et al*., 2010). SNPs attaches to the cell membrane and penetrated it in the fungal cells, then produced pores causing the leakage of ions and other materials with little molecular weight. Then, SNPs attached to the respiratory chain and following cell division stopped, leading to cell death (Hassan *et al*., 2013).

Similar results were discussed by (Al-Othman *et al*, 2014; Pietrzak *et al*., 2015). SNPs are able to inhibit the aflatoxin B1 production by *A. flavus* up to 86% at 150 ppm [21]. SNPs can change the metabolism and toxicity of moulds. SNPs decreases the mycotoxin production of *Aspergillus* sp. (81–96%) and reduces mould cytotoxicity (50–75%) (Pietrzak *et al*., 2015). SNPs were synthesized by *A. terreus* and *P. expansum* at a concentration of 220 lg/100 ml media giving the highest reduction of ochratoxin A, where the percentages of reduction were more than 50% (Ammar and El-Desouky, 2016).

To our knowledge, there is no clear and accurate information in the literature concerning the effect of SNPs on aflatoxin B1 production. Three genes *pksA*, *ver-1* and *omt-A* encode enzyme proteins were involved in the AF biosynthetic pathway. *A. flavus* ATCC 28542 treated by three kinds of SNPs, only two (*Aspergillus terreus* and Egyptian honey) have very low expression gene using quantitative real-time RT-PCR of aflatoxin B1 gene than control sample, with highly significant at ($P<0.01$) (Deabes *et al*., 2018). SNPs could stimulate the fungal mycelia to release O_2-rapidly; O_{2^-} is playing a key role in the quick reduction of intracellular Reactive oxygen species (ROS) level. The low intracellular oxidative level effect on the signaling pathway to the aflatoxin

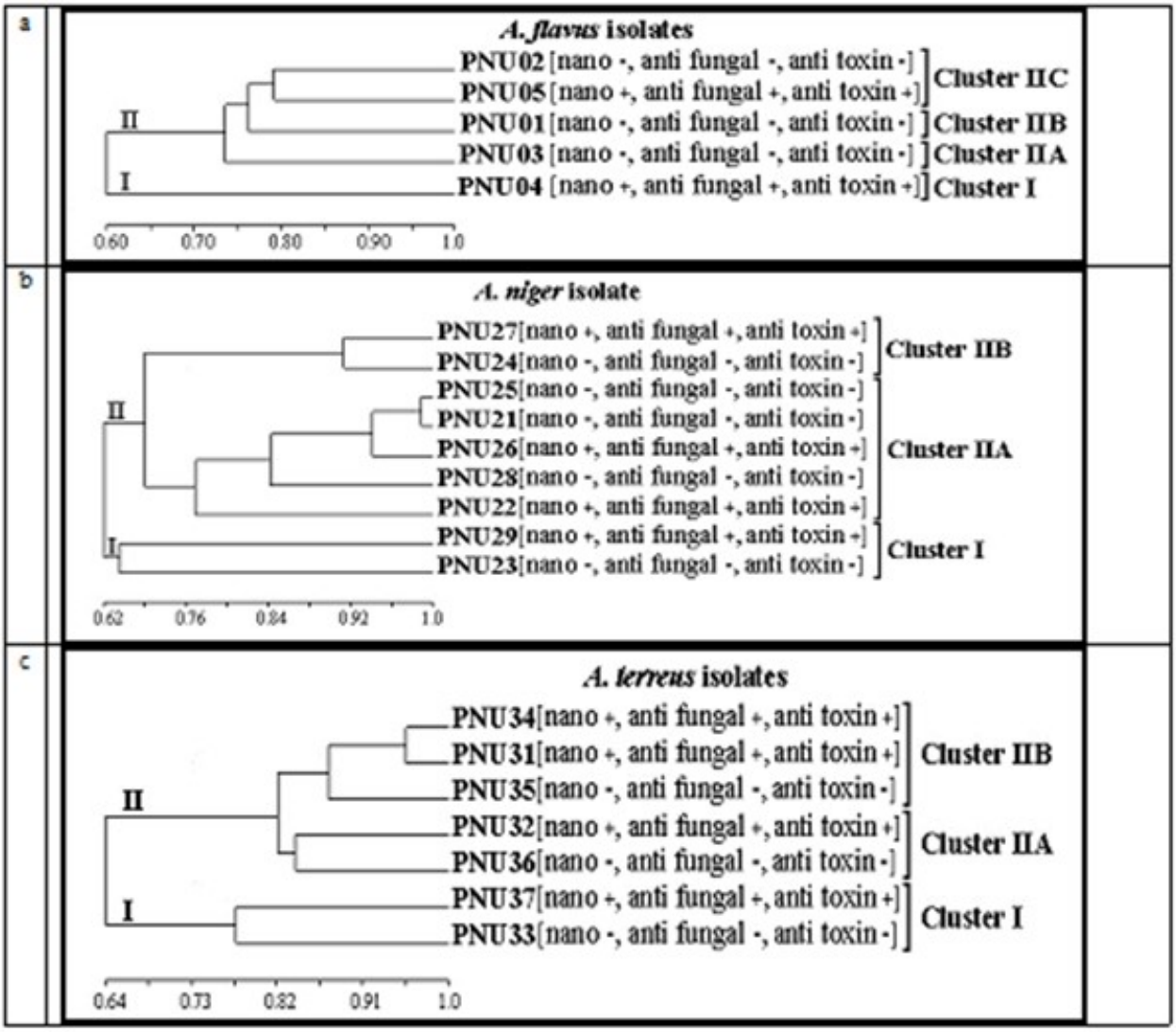

Fig. 11: UPGMA dendrogram based on Jaccard's coefficient illustrating the genetic similarities among *Aspergillus* spp. based on ISSR data) A. five isolates of *A. flavus* with primer UBC811, nine isolates of *A. niger* with primer UBC811, seven isolates of *A. terreus* with primer ISSR_9

biosynthesis. Finally, the aflatoxins production by *A. flavus* is significantly suppressed (Al-Othman *et al*., 2014).

ISSR markers were used with the aim of genetically characterizing isolates of *A. flavus* and *A. niger* and discriminate between producing and non-producing SNPs isolates. ISSR analysis revealed a high level of genetic diversity in the *A. flavus* and *A. niger* population useful for genetic characterization. ISSR markers were not suitable to discriminate between producing and non-producing SNPs isolates (Mahmoud *et al*., 2015).

The same results appeared when using ISSR markers with A. parasiticus and A. terreus isolates producing and non-producing SNPs (Abd El-Aziz *et al*., 2015). Fifty-two isolates of A. flavus were collected from three crops (peanut, maize, and pistachio) from different geographical regions of Iran. ISSR technology was used to determine genetic relatedness and polymorphism between isolates. A. flavus isolates exhibited high intraspecific variability and a significant number of polymorphisms. The varying similarity ranging within isolates of A. flavus could also be a result of isolates that share a host range and/or ecological niche (Houshyarfard *et al*., 2015).

Three ISSR primers were used to evaluate the genetic variability of 15 *A. flavus* isolates from maize in Saudi Arabia. ISSR analysis revealed a high level of genetic diversity in the *A. flavus* population. The ISSR dendrograms obtained were unrelated to geographic origin or aflatoxin production (Mahmoud *et al*., 2014).

Twenty-two primers succeeded in generating polymorphic bands for each primer. The study showed that ISSR technology is an effective molecular approach for studying the diversity of *A. flavus* from the soil of peanut crops in China (Zhang *et al*., 2013).

CONCLUSIONS

In conclusions, *Aspergillus* Spp. was used as green reducing agent for the biosynthesis of silver nanoparticles. Such type of synthesis may be considered as eco-friendly as it is free from any toxic chemicals or organic solvents during the biosynthesis process. The silver nanoparticles were characterized by using UV-Vis spectral analysis and TEM. The antifungal activity of silver nanoparticles was evaluated in vitro and the results showed that SNPs had the greater growth inhibition of the mycotoxigenic fungi (*A. flavus*, *A. parasiticus*and *F. verticillioides*) and control B1 amounts produced by *A. flavus*. This investigation can be applied in the treatment of feeds and foods as a means to reduce the hazards of mycotoxicosis in humans and animals, so more research work, especially on experimental animals, needs to be done. ISSR PCR analysis revealed a high level of genetic diversity in the *Aspergillus* spp. population and useful for genetic characterization. ISSR markers were not suitable to discriminate between producing and non-producing SNPs isolates. There was no clear-cut relationship between the ISSR markers (genotype of isolates) and antifungal and anti-aflatoxigenic properties.

ACKNOWLEDGEMENT

This research project was funded by the Deanship of Scientific Research, Princess Nourah Bint Abdulrahman University, through the Research Group Funding Program, grant No. 29 – ص – 235.

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