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Genetic Diversity Based on SCoT and ISSR Markers in Agrobacterium isolated from Egyptian soil.

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ABSTRACT

Nineteen local *Agrobacterium* isolates from Egyptian soil were selected after 48 h at 30°C on selective MG medium and tests for biochemical characterization and one identified *Agrobacterium tumefaciens* (*Ag.t*) strain was used as standard. The production of alpha amylase was determined for all nineteen isolates and *Ag. tumefaciens.* Two molecular marker systems, eight SCoT primers (Start codon targeted polymorphism) and five ISSR primers (inter-simple sequence repeat) were employed for identification and genetic relationship between nineteen indigenous *Agrobacterium* species from Egyptian soil and one identified strain *Ag. tumefaciens*. A total of 398 and 328 amplicons were detected by eight SCoT and five ISSR primer combinations, among which 98.8% and 97.5% bands were polymorphic respectively. The genetic similarity among the species ranged from 0.348 to 0.036 using SCoT and 0.457 to 0.032 using ISSR marker systems. Based on the marker analysis all the nineteen isolates and identified *Ag. tumefaciens* were clustered into three major groups for ISSR primer and two for SCoT primers. Cluster analysis also reveals high genetic variation among the genotypes. Present investigation suggests the effectiveness of these two marker systems could be useful for identification and genetic diversity analysis of indigenous species and provide an important input into conservation biology.

Keywords: *Agrobacterium*,α-amylase, SCoT, ISSR marker

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Introduction

Agrobacterium sp. is Gram negative rod-shaped bacteria belonging to the α-Proteobacteria. Crown gall, caused by bacteria belonging to different species of the genus *Agrobacterium*, is one of the most serious diseases affecting nursery production of fruit trees and nuts. From a practical point of view, determination if the soil of fields designated for nursery plantations is free from tumor-inducing agrobacteria is very important [1].The latest taxonomic proposal, although far from unanimous acceptation, rejected the genus *Agrobacterium* and incorporated its members into the genus *Rhizobium* [2]. At present, tumorigenic bacteria belong to the species complex *A. tumefaciens* (*A. radiobacter* biovar 1) which consist of 10 genomovars (G1 to G7, G9, G13 and G14) and the six species *A. vitis* (biovar 3)*, A. rubi, A. larrymoorei, A. rhizogenes* (biovar 2)*,* the recently described *A. fabrum* [3] and *Rhizobium skierniewicense* [4].

In recent years, many new marker techniques have been developed in line with the rapid growth of genomic research [5]. Due to the tremendous growth in public biological databases, the development of functional markers that are located in or near the candidate genes have become considerably easy [6]. Initiating a trend away from random DNA markers towards gene-targeted markers, a novel marker system called Start Codon Targeted (SCoT) Polymorphism [7] was developed based on the short conserved region flanking the ATG start codon in plant genes. SCoT markers are generally reproducible, and it is suggested that primer length and annealing temperature are not the sole factors determining reproducibility. These are dominant markers like random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) and could be used for genetic analysis, quantitative trait loci (QTL) mapping and bulk segregation analysis [7]. In principle, SCoT is similar to RAPD and ISSR because the same single primer is used as the forward and reverse primer [7-5]. The use of these markers in diversity analysis and diagnostic fingerprinting has been successfully demonstrated in peanut, potato and grape [8-9-10]. Here, the use of SCoT polymorphism markers technique for studying genetic diversity was reported for the first time in *Agrobacterium*. Genetic variations based on Molecular markers for between and within different species of *Agrobacterium* were reported by many researchers [11-12]. Objectives of the present study are as follows: to identify the relationships between different *Agrobacterium* isolates using SCoT and ISSR molecular markers.

MATERIALS AND METHODS

Bacterial strains and culture conditions

All local bacterial strains used in this study were isolated from two different areas from Egyptian soils and the one identified strain *A. tumefaciens* obtained from Botany department, Al-Azhar University, Cairo, Egypt. The *Agrobacterium*, strains were grown and isolated on King's B medium and Nutrient agar medium [13].

Isolation of bacteria

selective agar media (based on the ability to use specific compounds Agrobacteria can be isolated directly from their ecological niches in plants or soil using selective media

combined with several purification steps method [14**]**. Samples of bulk soil, in 1 ml of sterile distilled water for at least 30 min. The suspension can be directly streaked or dilution can be plated onto appropriate as carbon and nitrogen sources and to resist toxic compounds). Inoculated agar media are incubated 2-3 days at 28°C. After isolation, typical *Agrobacterium* colonies (*e.g*. black colonies due to incorporation of tellurite in the colony) must be purified at least twice. To this aim, a single typical colony is suspended in sterile distilled water (at least 100 µl) and kept at 28°C overnight with shaking. The suspension is then spread on appropriate agar media without tellurite. Then one typical colony from the general agar plate is purified again (water plus streaking on selective media). These two purification steps are determinant to ascertain a pure culture of an Agrobacterial strain. Several selective media are useful to isolate and to identify agrobacteria. Agrobacteria are able to reduce tellurite (K_2TeO_3) and concentrations of 200 mg/l of this toxic metal allow selective growth of almost all agrobacteria (*A. tumefaciens* species complex, biovar 2, *A. rubi*, *A. vitis* and *A.larrymoorei*), while inhibiting most of the saprophytes [15]. Thus it is recommended to supplement selective agrobacteria media with tellurite. On media amended with tellurite, colonies are black (Fig. 1).

Inoculum preparation

Bacterial inoculum was used in the present studies. Fifty millilitre of inoculum medium containing nutrient broth 8.0 g/l, pH 7.0 was transferred to a 250 ml conical flask and cotton plugged. It was sterilized in an autoclave at 15 lbs/in2 pressure (121°C) for 15 min. After cooling to room temperature, a loopful of bacteria was a sceptically transferred to it. The flask was rotated at 200 rpm (37°C) in a rotary shaking incubator for 24 h.

Shake flask fermentation

The optimization of alpha amylase fermentation was carried out using submerged technique in 250 ml Erlenmeyer flasks. Fifty millilitre of the fermentation medium was transferred to the individual flasks and cotton plugged. The flasks were sterilized in an autoclave at 121°C for 15 min and cooled to room temperature. Each flask was inoculated with 2.0 ml of the bacterial inoculum. The flasks were placed in the rotary shaking incubator (200 rpm) at 37°C for 48-96 h. After the incubation, the fermented broth was centrifuged in a centrifuge at 5.631×g for 15 min.

Biochemical and physiological tests

The biochemical and physiological of isolates was conducted [16]. For each strain of *Agrobacterium* the following tests including Gram staining motility, catalase, urease, citrate utilization production, nitrate reduction, aerobic growth, and gelatin and hydrogen sulfide production. Bacterial strains were tested for their ability to oxidize carbon substrates including Sucrose, D-mannitol, D-sorbitol, Glucose, starch hydrolysis. All the isolates with a positive reaction are considered as *Agrobacterium* [17]. Reduction of tellurite and the tolerance to 2% NaCl were also tested. The growth was evaluated after 48h at 28°C.

Enzyme assay

Alpha amylase estimated technique was used [18]. One milliliter of enzyme extract was added to a test tube containing 1.0 ml of 1.0 % soluble starch solution, pH 7.0. The mixture was incubated at 60ºC for 10 min. After the incubation, 1.0 ml of DNS reagent was added to each of the tubes. The tubes were placed in boiling water for 5 min and cooled to room temperature. The contents of tubes were diluted up to 10 ml with distilled water. The optical density (OD) of reaction mixture was determined at 546 nm using a spectrophotometer. One unit of activity is equivalent to that amount of enzyme, which in 10 min librates reducing group from 1.0 % Lintner's soluble starch corresponding to 1.0 mg maltose hydrate.

DNA Extraction

DNA was extracted from the twenty *Agrobacterium* isolates using DNeasy Tissue Mini Kit (QIAGEN). The concentration of DNA was then determined based on a comparison of the DNA samples with standard lambda DNA on 1% (w/v) agarose gel, after which it was adjusted to 5 ng/µl.

ISSR-PCR Amplification

Five ISSR primers were used in this study. PCR amplification was done using a Perkin Elmer (GeneAmp PCR system 9600 –USA). Amplification was performed for 35 cycles with denaturation at 94^{o}C for 1 min, annealing at 45^{o}C for 1 min. and extension at 72^{o}C for 2 minutes. Initial denaturation was done at 94^{o}C for 5 min and a final extension step of 5 min. at 72^{o}C was also included. The reaction mixture (25 µl) contained 20 ng of DNA template, 10 mM Tris-HCl pH 9.0; 50 mM KCl; 0.1% Triton X-100; 1.5 mM $MgCl_2$; 0.1 mM dNTP; 2 mM primer; 0.5 unit of Taq DNA polymerase. Master mixes of each reaction were overlaid with 10 µl of mineral oil to prevent evaporation. Amplification products were separated on 1.2% agarose gel in 1X TAE (Tris base, acetic acid and EDTA) buffer.

SCoT-PCR Amplification and Detection

Eight SCoT primers were used in this study. SCoT-PCR reaction volume was 25 µL, containing 1.5 µL of template DNA (25 ng/µL), 1.0 µL primer at 10 µM, 2.0 µL dNTPs at 10 µM, 0.125 µL Taq DNA polymerase at 5 U/µL, 2.5 µL 10X PCR buffer, and 17.875 µL ddH_2O. SCoT-PCR was performed on an Eppendorf Perkin Elmer (GeneAmp PCR system 9600 –USA). Initial denaturation was carried out at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and final extension at 72°C for 5 min. The amplification products were separated in 1.2% agarose gels containing 0.5 µg/mL of ethidium bromide through electrophoresis in 1X TBE buffer solution at 5 V/cm and photographed by gel documentation system, Bio-Rad - Gel Doc.XR+ with Image lab Software.

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Data Analysis

SCOT and ISSR bands were manually scored as present (1) or absent (0) for estimation the similarity among all tested samples. The matrix of similarity (Jaccard) was obtained by clustering according to the Unweighted Pair-Group Method using Gene Tools-gel analysis software of SPSS (ver. 18). Polymorphism percentage was estimated by dividing the number of polymorphic bands over the total number of bands.

RESULTS AND DISCUSSION

Isolation methods of bacteria and α-amylase production

Table 1: Alpha amylase production U/ml/min of local Agrobacterium isolates and *Ag. tumefaciens*.

Sources	Isolates Number	Alpha amylase production U/ml/min
El-Monofia	1	105
	2	327
	3	38
	4	14
	5	63
	6	221
	7	172
	8	100
	9	65
	10	37
El-Bhera	11	114
	12	63
	13	73
	14	44
	15	55
	16	78
	17	25
	18	360
	19	77
Agrobacterium tumefaciens (*A.t*)	20	50

The bacterial strains isolated from rhizosphere were screened for α-amylase activity and *Agrobacterium tumefaciens* (*A.t*) (Table 1). After 3 days at 30°C on production media the isolate no.18 was selected to give the maximum amount of α-amylase activity (360 U/ml/min), while the identified strain (no.20) give the lowest amount of α-amylase activity (50 U/ml/min) this result is similar to the obtained when comparing the isolates with the original strain, and gave a noticeable increase in amylase production [18]. The nineteen isolate was gram Negative, motile, cream-white, smooth, convex, glistening, and circular with entire edges and mucoid, the strain exhibits moderate growth at NaCl concentrations 2% and temperature 30°C to 42°C. It was reduction of tellurite positive, catalase positive and having distinct fermentation profiles of different carbon sources. The strains possessed

the ability to hydrolyze starch, gelatine and casein positive, Urea test and reduction of nitrate to nitrite gave a positive results. On the basis of the observed phenotypic characteristics the above cited isolate was grouped into genus *Agrobactrium*. Such tests have already been performed and their obtained results are in agreement with our results [14–19].

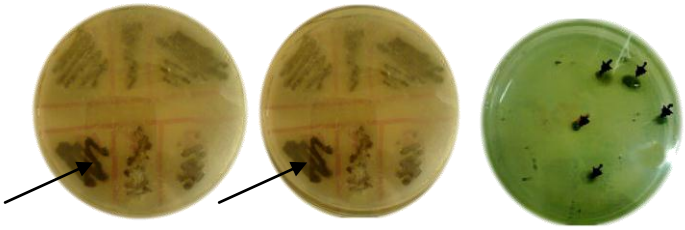

Figure 1: Plating of a 1/10 dilution soil suspension on medium MG amended with 60 ppm of K_2TeO_3. Arrows point to Agrobacterial colonies.

ISSR polymorphism and species identification

Gene-targeted markers are preferable for numerous applications in plant molecular genetics especially QTL mapping since recombination levels between marker and gene/QTL are generally lower compared with 'indirect random markers' such as RAPDs, ISSRs, or SSRs [6]. The SCoT technique is based on the single primer amplified region principle since it uses a single primer as a forward and reverse primer, like the RAPD or ISSR technique. However, PCR amplification using SCoT primers targets gene regions surrounding the ATG initiation codon on both DNA strands. It is possible that some SCoT markers would be codominant due to insertion–deletion mutations; these would be the minority like codominant RAPDs [20].

ISSR analysis is based on inter tandem repeats of short DNA sequences proven to be highly polymorphic even among closely related genotypes due to the lack of functional genetic constraints in these non-coding DNA regions14). Out of ten ISSR primers initially screened, five primers were found to be polymorphic and produced clear and re-producible amplification pattern. Analysis of the five primers could produce 328 distinct reproducible amplicons across the nineteen *Agrobacterium* isolates and *A. tumefaciens* with a mean of 65.6 bands per primer. The primer ISSR-3(UBC-834) produced the largest number of monomorphic bands (5 in total) (Fig.2A and Table 2). The size of the amplified products ranged from 110 bp to 2359 bp. (for the primer UBC-442) with the total 328 amplified bands, 323 were polymorphic, with an average of 64.6 polymorphic fragments per primer. All the ISSR primers except UBC-834 showed 100% polymorphism. The primer UBC-834 showed 87.5% of polymorphism. The average polymorphism percentage recorded with ISSR primers is 97.5%. Table (2) showed that five ISSR primers generated 219 unique fragments in all isolates and *Ag. tumefaciens.* The maximum numbers of unique fragments were generated by primer UBC-442 (90 band) and the least number of unique fragments by primer UBC-834 (19 band). The genetic similarity derived from the data of the ISSR marker analysis varied from 0.032 between isolate *Agrobacterium.*no12 and isolates *Agrobacterium* no.4 to 0.457 between isolate *Agrobacterium* no.1 and isolates *Agrobacterium* no,2. Among the Egyptian *Agrobacterium* isolates, isolate *Agrobacterium* no.1 showed maximum genetic similarity with isolate *Agrobacterium* 2, based on the ISSR marker analysis. The dendrogram

based on ISSR data was constructed by UPGMA analysis and grouped the nineteen *Agrobacterium* isolates and identified strain into three major clusters (Fig.3A). In cluster one 11 isolates were grouping together in two subgroup whereas in cluster 2 and 3 six and three isolates were grouped, respectively. *Ag. tumefaciens* strain no.20 in the cluster 3 is closely related with isolate no.19 with similarity 0. 90% also isolate no.17 is grouped in the same cluster. This result is in parallel with the data in Table (1) which showed that the two *Agrobacterium* isolate; 14, 15 and willd type produced equal approximately efficiency of alpha amylase 44 U/ml/min, 55 U/ml/min and 50 U/ml/min ,respectively.

Also, in this study, we describe a novel marker system called start codon targeted polymorphism that is based on the short conserved region in plant genes surrounding the ATG translation start (or initiation) codon that has been well characterized in previous studies [21-22]. DNA markers are produced by polymerase chain reaction (PCR) using single primers that are designed from the short conserved region flanking the ATG start codon that is conserved for all genes. Therefore, in principle, this technique is similar to RAPD or ISSR or single primer amplification reaction because a single primer is used as the forward and the reverse primer [23- 24]. Markers are visualized by standard gel electrophoresis with agarose gels and staining making this technique suitable for the vast majority of research labs with standard equipment.

Due to the basis of SCoT primer design, we expect SCoT markers to be distributed within gene regions that contain genes on both plus and minus DNA strands. It is also possible that pseudogenes and (genes within) transposable elements may be used as primer binding sites by SCoT polymorphism technique. An important factor is the distance in base pairs between primer binding sites of the template. Therefore, a relatively long extension time of the thermal cycle is important and we recommend at least 2 min.

SCoT primers in this study were designed based on a consensus sequence for the flanking region around the ATG start codon derived from the previous studies [21-22]. Specific nucleotides in the primer sequence were fixed: the ATG codon (at positions +1, +2, and +3), G at position +4, C at position +5, and A, C, and C, at positions +7, +8, and +9, respectively. Most primers differed from each other by at least one nucleotide with an emphasis on variations at the 3′ end, which has been shown too critical for primer-template specificity [25-26]. Nonconserved nucleotide positions (*i.e.,*N) were exploited, by designing primers such that these nonconserved nucleotides typically occurred within the last three or four nucleotides at the 3′ end. The eight SCoT primers generate DNA amplicons with the size range between 188–3318 bp. with an average of 49.75% per primer (Fig.2B and Table 2).

The results also indicated the effect of altering a single nucleotide within the last three nucleotides at the 3′ end (Fig.2B). For example, SCoT primers (29), (30), and (31) differ only in the last nucleotide at the 3′ end yet produced different profiles. SCoT primers 18 and 20 differed only in the second last nucleotide and SCoT primers 12 and 13 differed only at the third last nucleotide yet also produced very different DNA marker profiles (Fig.2B). SCoT primers were used to fingerprint a small diverse set of *Agrobacterium* strains, the number of bands generated ranged from 38 for SCoT-30 to 80 for SCoT-31 with a mean of 49.7 bands. The primers SCoT-31 and SCoT-29 produced the lowest number of monomorphic bands (1-2 in total) with a mean of 0.5 (Table 2). The primer (SCoT -30) generated a lowest total of 33

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polymorphic SCoT markers whereas; the primer 31 scored the highest total amplicons (80) with a polymorphism percentage 100% Table (2). These are similar obtained by comparable to the number of markers typically generated by RAPD and ISSR techniques in rice [17-27-28].

Cluster analysis with SPSS: Hierarchical Cluster Analysis

Cluster analysis works upwards to place every variable (strain) into a single cluster. The main part of the output from SPSS is the Dendrogram that represents the phylogenetic relationships among the variable (strains) under study. The Dendrogram for the ISSR and SCoT primers patterns of the nineteen *Agrobacterium* strains and *Ag*. *tumefaciens* using average linkage (between groups) is presented in Fig.2(A and B). The horizontal dotted line of the Dendrogram indicates the rescaled distance, in which the clusters are formed.

Table 2: Statistics of ISSR and SCOT primers used to detect polymorphism, in *grobactrium* isolates and identified strain.

rimers Name	Primer sequence 5'-3'	Total No. Of Bands	No. Of Mono-morphic Bands	Molecular weight bp		No. Of Unique Bands	No. Of Poly-morphic bands	Polymorphism(P) %	Mean of Band frequency
				Low M.W	High M.W				
ISSR Primers									
UBC 825-1	$(AG)_8YC$*	58	0	1583	177	39	19	100	0.17
UBC 826-2	$(Ac)_8YG$*	63	0	1511	168	42	21	100	0.162
UBC 834-3	$(AC)_8YT$*	40	5	2009	255	19	16	87.5	0.321
UBC 442-4	$(AG)_8YG$*	117	0	2359	110	90	27	100	0.143
UBC 881-5	$(GT)_8YG$*	50	0	1249	139	29	21	100	0.162
Total		328	5			219	104		0.195
average		65.6	1			43.8	20.8	97.5	0.039

SCOT Primers

Primer Name	Primer sequence 5'-3'	Total no. of bands	No.of polymorphic bands	No. of monomorphic bands	% polymorphism	Molecular weight bp	Mean of Band frequency
SCoT 11	5'AAGCAATGGCTACCACCA-3'	38	37	1	97	1703-	0.151
SCoT 12	5'-ACGACATGGCGACCAACG-3'	57	57	0	100	2195-	0.097
SCoT 13	5'-ACGACATGGCGACCATCG-3'	39	37	2	94	1984-	0.221
SCoT 18	5'- ACCATGGCTACCACCGCC -3'	41	41	0	100	1862-	0.102
SCoT 20	5'- ACCATGGCTACCACCGCG -3'	45	45	0	100	2177-	0.090
SCoT 29	5'- CCATGGCTACCACCGGCC -3'	65	64	1	98	2238-	0.111
SCoT 30	5'- CCATGGCTACCACCGGCG -3'	33	33	0	100	3318-	0.121
SCoT 31	5'- CCATGGCTACCACCGCCT -3'	80	80	0	100	2133-	0.083
Total		398	394	4			0.122
Average		49.75	49.25	0.5	98.8		0.015

* Y----C/T

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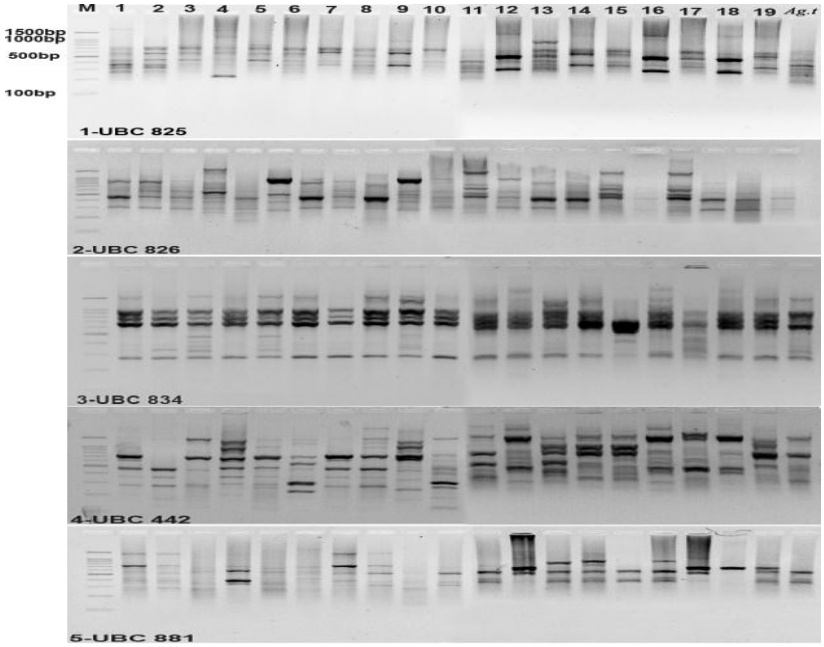
ISSR (A)



SCoT (B)

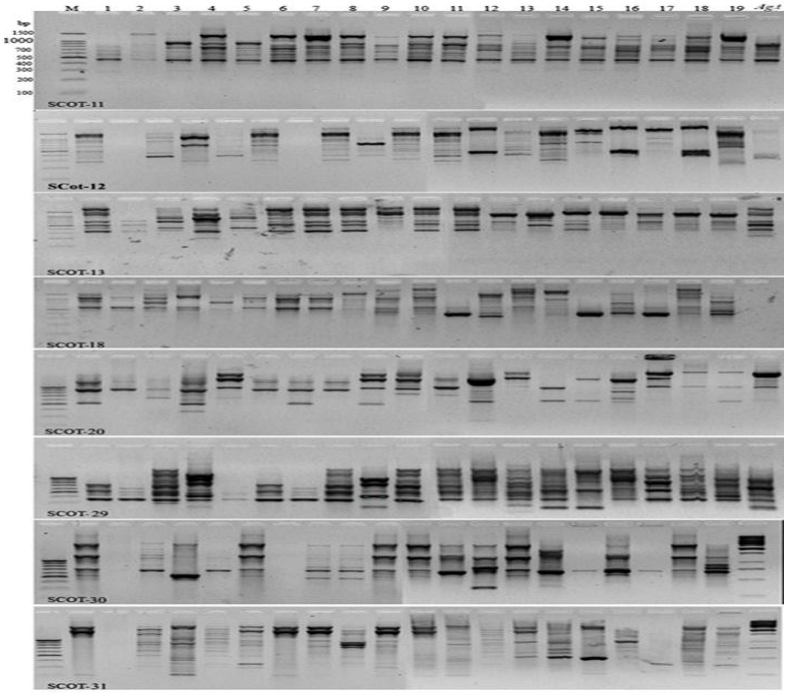


Figure 2: ISSR (A) and SCoT (B) marker profiles for the nineteen *Agrobacterium* and identified strains (Lane 2-20), M 100 bp DNA ladder (Lane 1).

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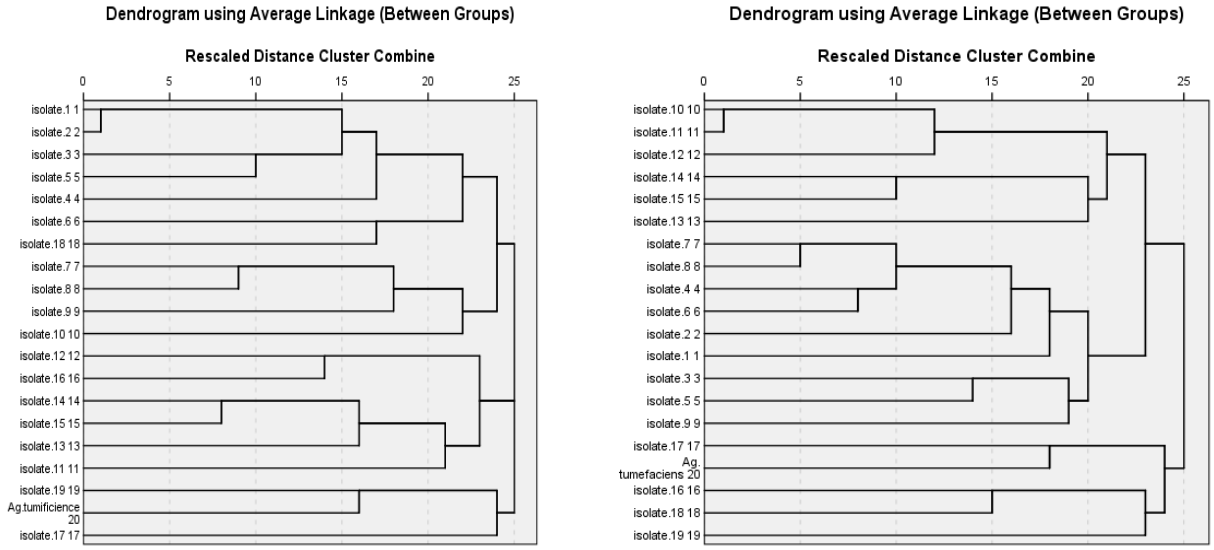

A -ISSR **B -SCoT**

Figure 3: Dendrogram generated using Un-weighted Pair Group Method with arithmetic average (UPGMA) analysis showing relationships among different *Agrobactrium* isolates from Egyptian soils, using ISSR (A), SCOT (B).

CONCLUSION

The present study showed existence of high genetic diversity in *Agrobacterium* isolated from Egyptian soils compared with *Agrobacterium tumefaciens* as identified strain. The magnitude and pattern of genetic variation detected in this study can be useful for more systematic characterization management and utilization in identification programs The percentage of polymorphic bands detected by ISSR primer (97.5%) was lower compared to SCoT (98.8%) although the number of polymorphic bands detected by ISSR primer (20.8) was higher than both SCoT primers (0.5) [29]. *Ag. tumefaciens* strain no.20 is a closely related with isolate no.19 with similarity matrix 0. 90%, with ISSR primer and closely related with isolate no.17 with SCoT primer.

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