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Research Article
Evaluation of Resistance of Cotton Genotypes to Fusarium Wilt Disease by Using Inter Simple Sequence Repeats (ISSR) and Start Codon Targeted (SCoT) Molecular Techniques

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Abstract

Background and Objective: In Egypt, cotton plant (*Gossypium barbadense* L.) is considered the first important economic crop. Fusarium wilt of cotton is a serious fungal disease responsible for significant losses throughout the world. The objectives of this study were to evaluate 10 cotton genotypes according to their resistance and susceptibility to Fusarium wilt and to explore the possibility of using Inter Simple Sequence Repeat (ISSR), Start Codon Targeted (SCoT) technique and their combined analysis to differentiate between reactions of cotton genotypes to Fusarium wilt disease. **Materials and Methods:** The genotypes divided into two distinct groups. The first group included the susceptible genotypes 10/2017, 13/2017, 29/2017, 31/2017 and 46/2017 where the disease incidence ranged from 83.33-93.33%, while the second group included the resistant genotypes 130/2017, 143/2017, 152/2017, 155/2017 and 163/2017 where the disease incidence ranged from 0-13.33%. DNA was extracted from genotypes leaves and subjected to ISSR by using five primers and SCoT by using five primers. **Results:** Five primers of ISSR pooled together. The resulting cluster was unable to differentiate between resistant and susceptible genotypes, while the pooled results of the five primers used in SCoT analysis were able to differentiate between genotypes based on their reactions to the disease. The combined analysis using ISSR and SCoT markers succeeded in placing the resistant cotton genotypes in a distinct group unrelated to the group of susceptible genotypes. **Conclusion:** It can be concluded that high level of polymorphism for ISSR and SCoT techniques suggesting their effectiveness for assessing genetic diversity, characterization and identification of reactions to cotton Fusarium wilt disease.

Key words: Cotton, Fusarium wilt, molecular markers, Inter Simple Sequence Repeat, Start Codon Targeted

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cotton (*Gossypium* spp.) is one of the most important commercial fiber and oil yielding crops playing a key role in economic. Because of its worldwide economic importance, new cultivars are constantly being released in the world[1]. Significant losses throughout the world by Fusarium wilt disease. The causal organism *Fusarium oxysporum* Schlecht. f. sp. *vasinfectum* (Atk). Snyd. and Hans., (FOV) invades the host through the tap roots behind the root tip[2]. The pathogen can infect cotton at all stages of growth and produces symptoms, which include seedling death, wilting, vascular discoloration and plant death[3]. The pathogen can survive for several decades in soil and cannot be eradicated from infested fields. The most successful strategy to manage Fusarium wilt is the use of resistant cultivars[4]. Ulloa *et al*.[5] reported that foliar damage and vascular discoloration caused by FOV were negatively correlated with node number and plant height. Wu *et al*.[6] found that incidence level of Fusarium wilt was negatively correlated with fiber strength, micronaire value and the fiber span length. Currently, up to eight races of FOV, most of which are geographically separated are recognized worldwide[7]. The Egyptian race (race 3) of FOV has long been known in the Nile valley, where it remains as one of the most damaging pathogen on Egyptian cotton (*Gossypium barbadense* L.). Increasing the probability that new races other than race 3 or new biotypes of this race may arise to cofound cotton breeders because FOV is still well established in the Egyptian soil[8].

Molecular markers has provided an ideal means for identifying cultivars, estimation of relatedness between different accessions and following inheritance of economically important characters[9]. Molecular markers and comparative mapping of various species have been very helpful in enhancing our understanding of genome structure and function. A molecular marker is defined as a particular segment of DNA that is representative of the differences at the genome level. An ideal marker should be polymorphic, independent and reliable, providing sufficient resolution relatively easily, quickly and with fairly low costs[10]. It was shown that marker data about genetic relationships and diversity are very important for any breeding program to select promising cultivars[11].

In recent years, many new alternative and promising marker techniques have been developed[12]. DNA markers as Inter Simple Sequence Repeats (ISSR) and Start Codon Targeted (SCoT) are used efficiently for studying genetic diversity of plants[13-15]. The ISSR markers are simple, randomly distributed in the genome, exhibit mostly dominant inheritance pattern and require low quantity of DNA[9]. They are employed in species and plant varietal identification, taxonomic and genetic diversity studies, gene mapping and clonal fidelity testing of *in vitro* derived plants[16]. This marker is a PCR-based technique, which involves amplification of DNA segment between adjacent and inversely oriented microsatellites[17].

A novel marker system developed based on the short conserved regions flanking the ATG Start Codon Targeted (SCoT) in plant[14]. SCoT involved the use of 18-mer primers with annealing temperature at 50°C. Single primer is used in PCR which means the same primer is utilized as forward and reverse primers as in ISSR markers. Yan *et al*.[18] used SCoT, successfully to evaluate genetic diversity and population structure and have assisted in selection for crop production. There have been very few reports utilizing ISSR or SCoT to investigate the level of genetic variation in cotton cultivars and genotypes according to their resistance or susceptibility to Fusarium wilt. The objectives of this study were to evaluate 10 cotton genotypes according to their resistance and susceptibility to Fusarium wilt and to explore the possibility of using ISSR, SCoT and their combined analysis to differentiate between reactions of cotton genotypes to Fusarium wilt disease.

MATERIALS AND METHODS

Cotton genotypes: The genotypes used in the present study were selected from the collection of germplasm available at the Department of Cotton Breeding, Cotton Research Institute, Agriculture Research Center (ARC), Giza, Egypt (Table 1).

Preparation of Fusarium isolates inocula: The fungal inoculum used in the greenhouse test was a mixture of equal parts (w/w) of 50 isolates of FOV race 3 which obtained from the Fungal Collection of Cotton and Fiber Crops Diseases, Dept. Plant Path. Inst. ARC. Giza, Egypt. The isolates

Table 1: Reaction of selected cotton genotypes to Fusarium wilt under greenhouse conditions

Cotton genotypes	Fusarium wilt incidence (%)
10/2017	86.67[a]
13/2017	93.33
29/2017	90.00
31/2017	83.33
46/2017	90.00
130/2017	0.00
143/2017	0.00
152/2017	0.00
155/2017	13.33
163/2017	6.67

LSD ($p \leq 0.05$): 19.15, [a]Mean of three replicates

were originally isolated from almost all cotton growing areas in Egypt. Autoclaved clay loam soil was infested with the mixture of isolates at rate of 10 g kg^{-1} soil. Substrate for growth of each selected isolate was prepared in 500 mL glass bottles where each bottle contained 50 g of sorghum grains and 40 mL of tap water. The inocula were taken from one-week old culture on potato dextrose agar (PDA) medium and allowed to colonize sorghum for 3 weeks. Infested soil was dispended in 10 cm diameter clay pots, which were planted with 10 seeds per pot. The greenhouse was equipped with a heating system. Temperature ranged from 28-35°C.

Evaluation of cotton genotypes against Fusarium wilt race 3 under greenhouse conditions: Evaluation of cotton genotypes was conducted in greenhouse of cotton and fiber crops diseases Research Dept, Plant Pathology Research Institute (PPRI), ARC trough February-May, 2018. The experimental design used in this study was randomized complete block with three replicates (pots) for each genotype.

Percentages of infected seedling were recorded 45 days from planting date. The infected seedling included the dead and the surviving seedling, which showed external or internal symptoms. The external symptoms usually began at the margin of cotyledons as yellowing along the veins (vein clearing), eventually, the entire cotyledons turned yellow and dropped from the seedlings. Seedling that remained apparently healthy 45 days after planting were cut diagonally across the root and stem to examine the internal symptoms. If discoloration of xylem vessels was observed, they were considered infected. If seedlings were free of such a discoloration, they were considered healthy. Thus, the seedlings of each genotype were placed in two distinct classes: healthy if they were free of any external or internal symptoms or infected if the seedlings died or survived showing any external or internal symptoms[19,20].

Molecular analysis

DNA isolation: Leaves of the 10 selected cotton genotypes were collected and soaked in liquid nitrogen for DNA extraction. DNA was extracted by Cetyl Trimethyl-Ammonium Bromide (CTAB) method[21].

Polymerase chain reaction (PCR) procedure: ISSR analyses were performed as described by Adawy *et al.*[22] and Hussein *et al.*[23]. The sequences of the five ISSR primers were used in this investigation showed in Table 2. SCoT amplification was performed as described by Collard and Mackill[14] and Xiong *et al.*[24]. The sequences of the five SCoT primers were used in this investigation showed in Table 3.

The PCR amplification was performed in a 25 µL reaction volume containing the following: 2.0 µL of dNTPs (2.5 mM), 1.5 µL of Mg Cl_2 (25 mM), 2.5 µL of 10x buffer, 2.0 µL of primer (2.5 µM), 2.0 µL of template DNA (50 ng μL^{-1}), 0.3 µL of *Taq* polymerase (5 U μL^{-1}) and 14.7 µL of sterile ddH_2O. The reaction mixtures were overlaid with a drop of light mineral oil per sample. Amplification was carried out in Perkin Elmer GeneAmp PCR System 2400. The reaction was subjected to one cycle at 95°C for 5 min followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, then a final cycle of 72°C for 5 min. PCR products were run at 100 V for 1 h on 1.2% agarose gels which mixed with I×TBE buffer and ethidium bromide was added to the melted gel after the temperature became 55°C. Gels were photographed and scanned with Bio-Rad video densitometer model 620 at a wavelength of 577.

Table 2: Code and sequences of 5 ISSR primers used in molecular characterization of identifying 10 cotton genotypes

PN	S 5′→3′	TB	MB	PB	UB	P (%)
14A	CTC TCT CTC TCT CTC TTG	5	1	4	0	80.00
44B	CTC TCT CTC TCT CTC TGC	15	1	14	8	93.33
HB9	GTG TGT GTG TGT GG	15	0	15	10	100.00
HB12	CAC CAC CAC GC	14	1	13	3	92.80
HB15	GTG GTG GTG GC	7	2	5	2	71.00
Total		56	5	51	23	91.07

PN: Primer name, S: Sequence, TB: Total bands, MB: Monomorphic band, PB: Polymorphic band, UB: Unique band and P (%): Polymorphism percentage

Table 3: Code and sequence of 5 SCoT primers used in molecular characterization of identifying 10 cotton genotypes

PN	S 5′→3′	TB	MB	PB	UB	P (%)
SCoT 1	ACG ACA TGG CGA CCA CGC	13	2	11	2	84.60
SCoT 2	ACC ATG GCT ACC ACC GGC	16	2	14	7	87.50
SCoT 3	ACG ACA TGG CGA CCC ACA	7	3	4	1	57.14
SCoT 8	ACA ATG GCT ACC ACT GAG	10	1	9	1	90.00
SCoT 9	ACA ATG GCT ACC ACT GCC	12	2	10	2	83.33
Total		58	10	48	13	82.75

PN: Primer name, S: Sequence, TB: Total bands, MB: Monomorphic band, PB: Polymorphic band, UB: Unique band and P (%): Polymorphism percentage

Statistical analysis: Data were subjected to analysis of variance and least significant difference (LSD) was one way which calculated by using MSTAT-C statistical package. The similarity matrices were done using Gel works ID advanced software UVP-England Program. The relationships among genotypes as revealed by dendrograms were done using PASST program. DICE computer package was used to calculate the pairwise difference matrix and plot the phenogram among genotypes[25].

RESULTS AND DISCUSSION

Fusarium wilt incidence: Cotton genotypes can be divided into two groups (Table 1). The first group included highly susceptible genotypes (10/2017, 13/2017, 29/2017, 31/2017 and 46/2017) where disease incidence ranged from 83.33-93.33%. The second group included the highly resistant genotypes (130/2017, 143/2017, 152/2017, 155/2017 and 163/2017) where the Fusarium wilt incidence ranged from 0-13.33%. The differences between any two genotypes from the two groups were always significant, while the differences between genotypes within each group were non-significant. Wang *et al.*[26] reported that FOV is a soil borne highly virulent pathogenic fungus of cotton and the pathogen invades the plant through the roots and subsequently infects the vascular system, rustling in wilt symptoms. Fusarium wilt has become a major factor affecting cotton production and planting resistant varieties is the most economical and effective way to solve this problem[26].

Molecular characterization using ISSR markers: The amplified fragments of ISSR which generated as well as the monomorphic bands, polymorphic bands, primer sequences, unique bands and the percentage of polymorphism in ten cotton genotypes are presented in Table 2 and Fig. 1. A maximum of 56 DNA bands were scored in ISSR profiles

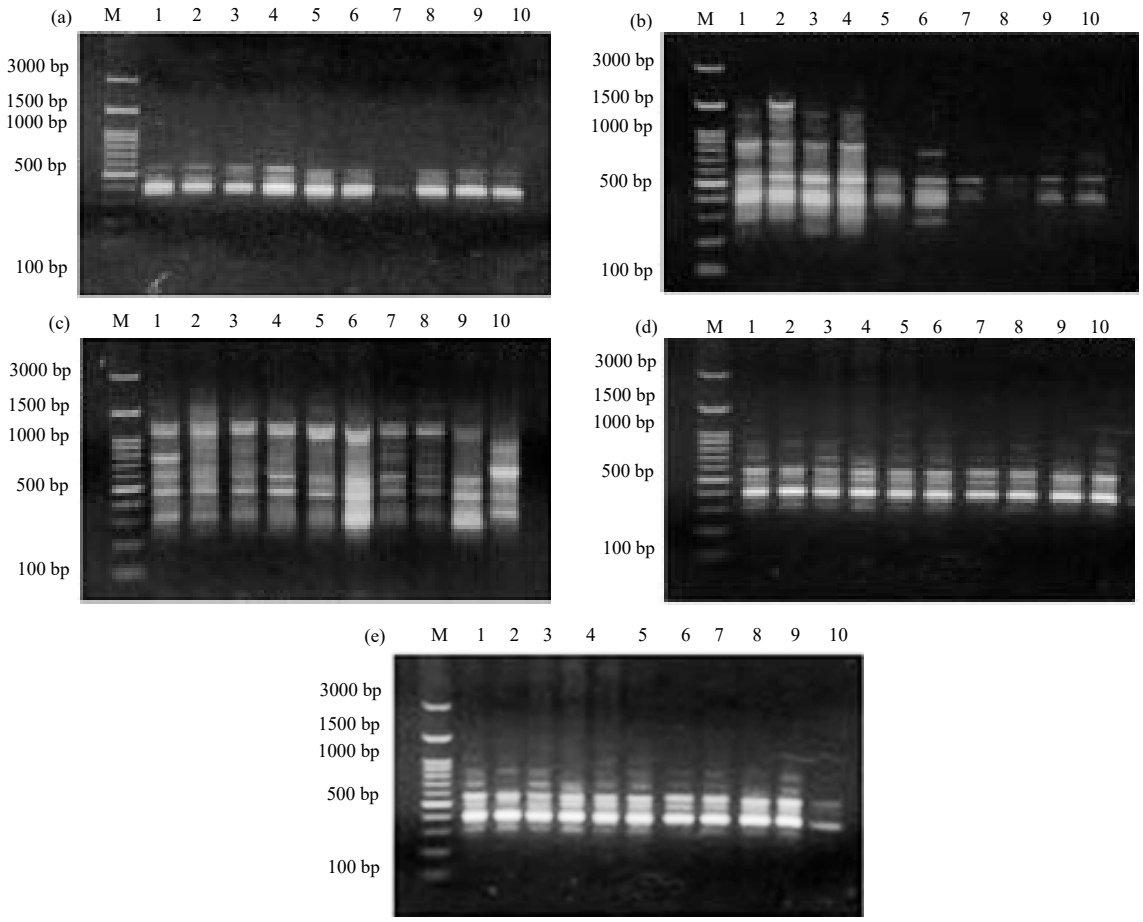

Fig. 1(a-e): Separation pattern of the ISSR products generated by five primers, (a) 14A, (b) 44B, (c) HB-9, (d) HB-12 and (e) HB-15
M: DNA marker, 1-10: Ten genotypes

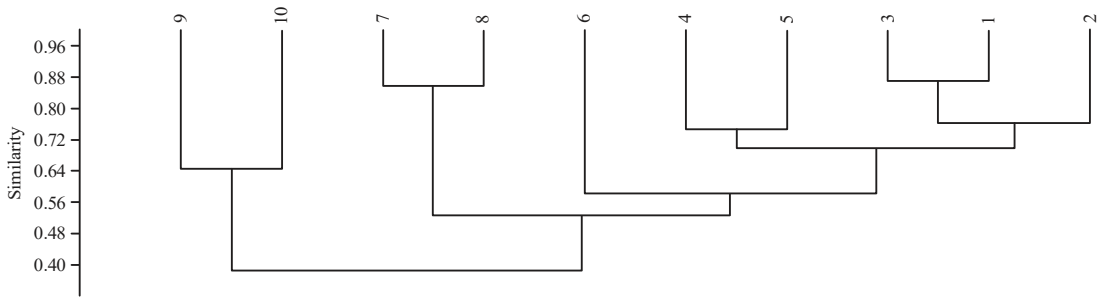

Fig. 2: Dendrogram illustrating genetic distance based on the data generated by the 5 ISSR primers between 10 genotypes of cotton (1-10)

generated by 5 ISSR primers. The size of the amplified bands ranged from about 260-1800 bp. These bands were identified as 51 polymorphic fragments and 5 monomorphic (91.07% polymorphism). Twenty three unique bands were identified in the resulted ISSR profile generated by all primers except 14A.

The primer 14A generated one monomorphic fragment and 4 polymorphic fragments, the polymorphism percentage was 80%. While the polymorphic percentage was 93.33% with primer 44B and four unique fragments were only generated with susceptible genotype no. 2 at molecular sizes 1800, 1550, 680 and 280 bp, so these were considered as positive markers for genotype no. 2. Furthermore, two unique fragments were appeared with the susceptible genotype no. 3 with molecular sizes 1430 and 320 bp, so these were considered as positive markers for genotype no. 3. Likewise one positive marker was exhibited with both the resistant genotype no. 6 and the susceptible genotype no. 1 at molecular sizes 770 and 730 bp, respectively. The fragment with molecular size 400 bp was only absent with the resistant genotype no. 8. Therefore, it considered as a negative marker for genotype no. 8. No monomorphic band was detected by primer HB-9 (100% polymorphism). Three unique fragments were appeared with the resistant genotype no.10 at molecular sizes 850, 650 and 330 bp, so, these were considered as positive markers for genotype no. 10. Likewise, two unique fragments were exhibited with the resistant genotype no. 9 at molecular sizes 580 and 280 bp. Therefore, these were considered as positive markers for genotype no. 9. As well as two unique fragments were detected with the resistant genotype no. 8 with molecular sizes 700 and 320 bp. Therefore, these were considered as positive markers for genotype no. 8. Furthermore, one unique fragment was appeared with susceptible genotypes no. 2, 3 and 4 at molecular sizes 1650, 1400 and 880 bp, respectively, so it was considered as positive markers for genotypes no. 2, 3 and 4, respectively.

A total of 13 polymorphic bands were detected by the primer HB-12 and one band was monomorphic, the polymorphism percentage was 92.8%. Two unique fragments were appeared with the resistant genotype no. 10 at molecular sizes 750 and 630 bp, so these were considered as a positive markers for genotype 10. Likewise, one unique fragment was exhibited with the susceptible genotype no. 4 at molecular sizes 660 bp. Therefore, it was considered as a positive marker for genotype no. 4. The band at molecular size 310 bp was detected in all genotypes except the resistant genotype no. 7, so it considered as a negative marker for genotype no. 7. Primer HB-15 has detected two monomorphic fragments and five polymorphic fragments, the polymorphism percentage was 71%. Two unique fragments were observed with the susceptible genotypes no. 4 and 1 at molecular sizes 790 and 500 bp, respectively; so, these were considered as positive markers for genotypes no. 4 and 1 respectively. As well as the band with molecular size 330 bp was only appeared with all genotypes except the resistant genotype no. 7, so it was considered as a negative marker for genotype no. 7.

The results of ISSR analysis were pooled together to generate the dendrogram (Fig. 2). The ten genotypes were divided into two main groups. The first one comprises the resistant genotypes no. 9 and 10, while, the second group comprises all other genotypes. The second group was divided into two sub-groups the first one included the resistant genotypes no. 7 and 8 and the second sub-group was divided into two classes, the first class included only the resistant genotype no. 6 and the second class was divided into two subclass; the first subclass included the susceptible genotypes no. 4 and 5. The second sub-class divided into two clusters, the first one included susceptible genotypes no. 1 and 3, while the second cluster included only the susceptible genotype no. 2.

Similar results were obtained by many authors, Noormohammadi *et al.*[27] examined agronomic characteristics and molecular genetic diversity in the Opal cotton (*Gossypium hirsutum*) cultivar and in F_2 progenies by

using 10 ISSR primers produced 206 reproducible bands, with 49.4% polymorphism and found that some ISSR bands occurred only in the parental genotype, while others were only present in the hybrid genotypes. Abdein *et al.*[28] mentioned that ISSR markers are considered very useful in studies of genetic diversity, phylogeny, genomics and evolutionary biology. Genetic variability and relationships among 15 cotton genotypes were investigated using 9 ISSR primers. A total of 86 bands were detected out of 54 which polymorphism[29] 62.79%. Genetic diversity for 15 cotton cultivars were assessed by 9 ISSR primes gave 85 bands of which 81 were polymorphic with 95.29% polymorphism[30]. Our results showed that ISSR markers were unable to differentiate between resistant and susceptible cotton genotypes as it placed the resistant genotypes no. 6, 7 and 8 in the same group with the susceptible genotypes. At the same time, it had distinguished 23 unique bands can be easily characterizing the genotypes at the individual level. Among the molecular markers, ISSR revealed more genetic variation among cotton genotypes[27].

Molecular characterization using SCoT markers: The amplified fragments of SCoT which generated as well as primer sequences, the monomorphic bands, polymorphic bands, unique bands and the percentage of polymorphism in 10 cotton genotypes are presented in Table 3 and Fig. 3. A maximum of 58 DNA bands was exhibited with SCoT profiles generated by five primers. The size of the amplified bands ranged from about 230-2300 bp. These bands were identified as 48 polymorphic fragments and 10 monomorphic ones (82.75% polymorphism). Thirteen unique bands were identified in the resulted of SCoT profile.

Primer SCoT1 exhibited two monomorphic fragments and 11 polymorphic. The polymorphism percentage was 84.6%. Two unique bands were appeared at molecular sizes 750 and 700 bp with the resistant genotype no. 10 and the susceptible genotype no. 2 respectively. So, these were considered as positive markers for genotypes no. 10 and 2 respectively. Seven unique bands were detected by SCoT2 primer, the first two with the susceptible genotype no. 1 at molecular sizes 2300 and 650 bp, so these were considered as

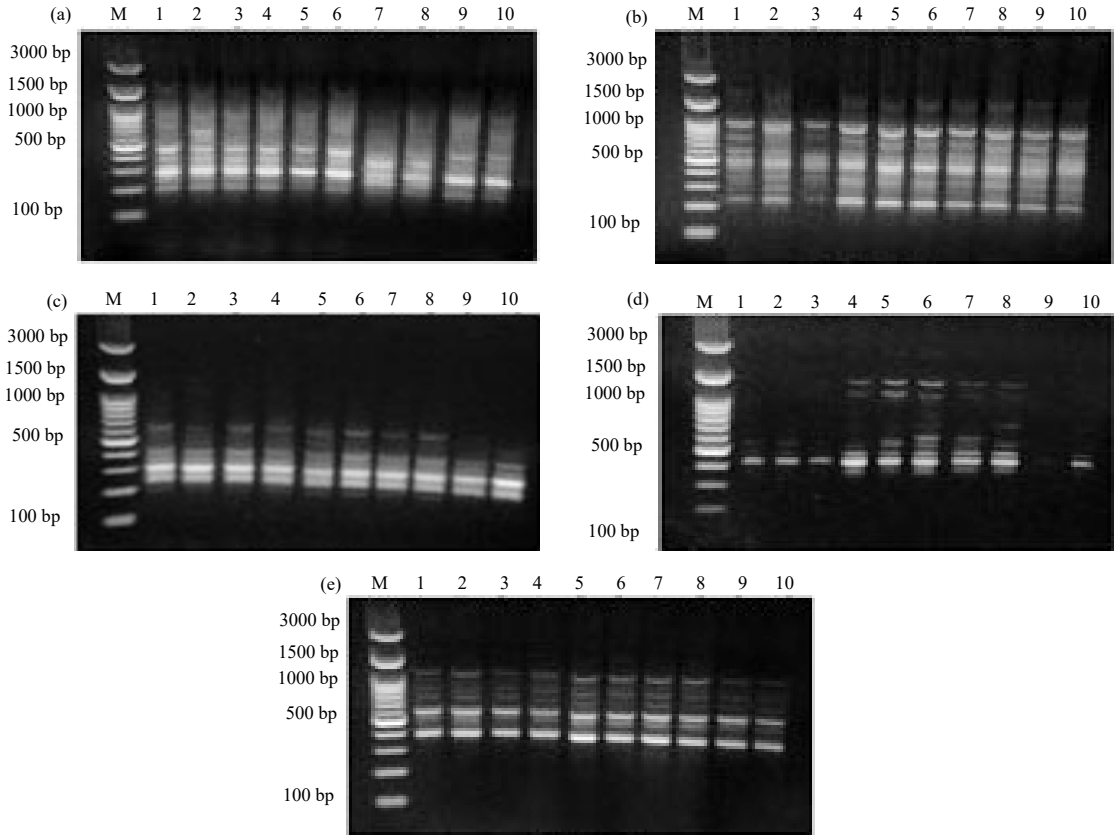

Fig. 3(a-e): Separation pattern of the SCoT products generated by five primers, (a) SCoT 1, (b) SCoT 2, (c) SCoT 3, (d) SCoT 8 and (e) SCoT 9

M: DNA marker, 1-10: Ten genotypes

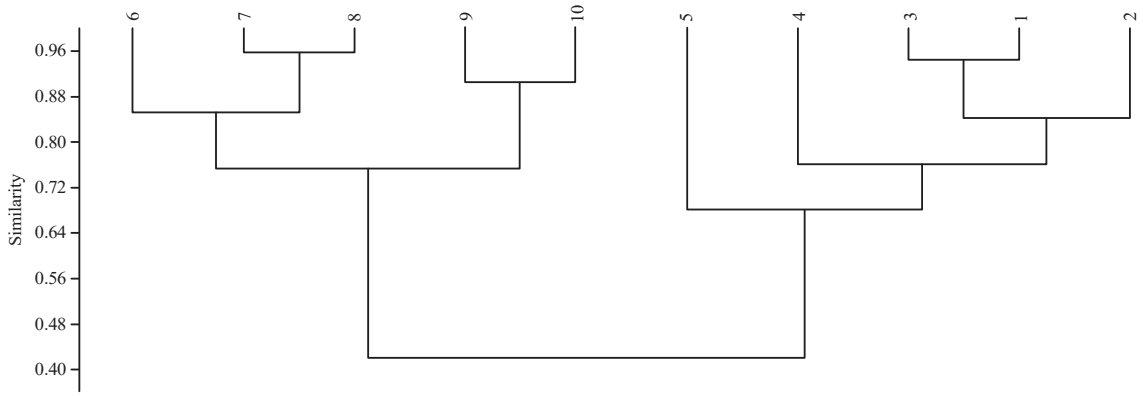

Fig. 4: Dendrogram illustrating genetic distance based on the data generated by the 5 SCoT primers between 10 genotypes of cotton (1-10)

positive markers for genotype no. 1. Three positive markers had scored with susceptible genotype no. 2 at molecular sizes 1800, 350 and 300 bp. The last two positive markers were distinguished with susceptible genotype no. 4 at molecular sizes 1300 and 850 bp. Two monomorphic fragments and 14 polymorphic fragments were generated, the polymorphism percentage was 87.5%. One positive marker was generated by primer SCoT3 at molecular size 500 bp with susceptible genotype no. 5. Three monomorphic fragments were appeared and four fragments were polymorphic, the polymorphism percentage of this primer was 57.14%. One monomorphic fragment was generated with primer SCoT8 and 9 were polymorphic, the polymorphism percentage was 90%. One positive marker was detected at molecular size 620 bp with susceptible genotype no. 5. The fragment at molecular size 500 bp was only absent with the resistant genotype no. 9, so it considered as a negative marker for this genotype. Primer SCoT9 has detected two monomorphic fragments and 10 polymorphic, the polymorphism percentage was 83.33%. Susceptible genotype no. 4 has two unique fragments at molecular sizes 800 and 580 bp, so, these were considered as positive markers for genotype no. 4.

The results of SCoT analysis were pooled together to generate the dendrogram (Fig. 4). The ten genotypes were divided into two main groups. The first one comprises the Fusarium wilt susceptible genotypes no. 1, 2, 3, 4 and 5. This group divided into two sub-groups the first one included only the genotype no. 5 and the second sub group divided into two classes, the first included only genotype no. 4 and the second class divided into two sub-class, the first subclass included only genotype no. 2 and the second subclass involved genotypes no. 1 and 3. The second group comprises the Fusarium wilt resistant genotypes no. 6, 7, 8, 9 and 10. This group divided into two sub-groups the first one included genotypes no. 9 and 10 and the second sub-group divided into two classes, the first class included only genotype no. 6 and the second class included genotypes no. 7 and 8.

Similar results were obtained by many authors, Heikrujam *et al.*[31] assessed 15 SCoT primers to detected genetic polymorphism among 39 Jojoba genotypes. Mahjbi *et al.*[32] used 12 SCoT primers for their ability to reveal polymorphism of the targeted codon of initiation at the interspecies level in Citrus genus. A total of 132 fragments were generated and 93.9% of them were polymorphic. Miro *et al.*[33] described 12 SCoT primers with 14 genotype Hungarian and international grape varieties and found one primer producing 17 polymorphic bands after data normalization, which was sufficient to separate the varieties. In both rice and Chinese grape varieties, 36 primers were tested[14], while in peanut, mango and ramie, 18, 33 and 20 primers were evaluated, respectively[24,34,35]. The use of SCoT markers succeeded in differentiation between Fusarium wilt resistant and susceptible cotton genotypes. The SCoT markers could classify cotton genotypes based on their susceptibility and resistance to Fusarium wilt disease into two separate groups. Xiong[24] and Abdel-Lateif and Hewedy[36] reported that the SCoT marker method was utilized for its simplicity, its ability to target gene sequences and for being a dominant marker system.

Combined analysis using ISSR and SCoT markers: The results of ISSR and SCoT markers were pooled together to generate the dendrogram (Fig. 5). The ten genotypes were divided into two main groups. The first one comprised all the resistant genotypes (no. 6, 7, 8, 9 and 10). This group was divided into two sub-groups, the first one included genotypes no. 9 and 10 and the second sub-group divided into two classes. The first class included only genotype no. 6 and the second class

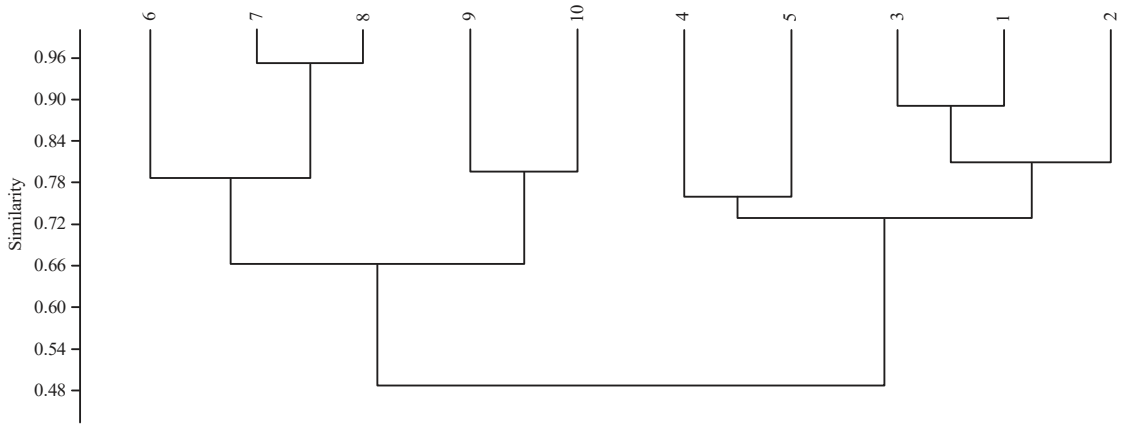

Fig. 5: Dendrogram illustrating genetic distance based on the data generated by the 5 ISSR primers and the 5 SCoT primers between 10 genotypes of cotton (1-10)

included genotype no. 7 and 8. The second group comprised all the susceptible genotypes (no. 1, 2, 3, 4 and 5). This group was divided into two sub-groups, the first one included genotypes no. 4 and 5 and the second sub-group divided into two classes, the first one included genotypes no. 1 and 3 and the second class included only genotype no. 2.

The combination of the analysis of the two DNA molecular markers (ISSR and SCoT) was successful in differentiation between cotton genotypes according to their resistance or susceptibility to Fusarium wilt. Zhang *et al.*[37] mentioned that in recent years, with the application of molecular biology techniques in cotton production, the creation of molecular genetics maps for purpose of tagging character marker gene, a great deal of progress has been made. It was shown that marker data about genetic relationships and diversity are very important for any breeding program to select promising cultivars[11]. Noormohammadi *et al.*[27] found that cluster analysis based on three types of molecular markers (RAPD+ISSR+SSR) were differentiated cotton genotypes and their progenies. However, using all three types of molecular markers provided a better overall view of cotton genome polymorphism. Combined molecular data can provide more information and clear discrimination of genotypes. In addition, intraspecific hybridization showed more diversity and distinctness in comparison to parental genotypes giving great information to cotton breeding programs for achieving more genetic variations in cotton genotypes[27].

CONCLUSION

This study confirmed that Fusarium wilt of cotton is a serious fungal disease. The present study demonstrated the possibility of using ISSR and SCoT techniques in cotton breeding programs as a marker assisted selection for Fusarium wilt resistance.

SIGNIFICANCE STATEMENT

This study confirmed that Fusarium wilt of cotton is a serious fungal disease. The present study demonstrated the possibility of using ISSR and SCoT techniques in cotton breeding programs for Fusarium wilt resistance.

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