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Morphological and molecular diversity assessment of *Fusarium oxysporum* f. sp. *cubense* isolates from District Thatta, Pakistan

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Key words: Panama disease, *Fusarium oxysporum*, Morphological study, Genetic study, ISSR.

Abstract

Banana fruit crop is one of the most valued commodities in the agriculture export industry. *Fusarium* wilt disease also well known as the Panama disease has been plaguing this industry since 1919. Its causal agent, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is a highly destructive and a variable pathogen for which no effective control measure exists. The durability of a sustainable management strategy depends on the pathogen population structure. In this study 55 *Foc* isolates from nine different banana farms of district Thatta of Pakistan were investigated for morphological and genetic diversity. The *Foc* isolates under study produced septate to unseptate microconidia, monophialides, sickle shaped 3-5 septate macroconidia with basal foot, and globose chlamydospores present singly, in pairs or in chains. Pigmentation in colonies varied from white, purple, salmon pink to peach. The optimum temperature for colony growth and sporulation was 25°C. The growth rate among the *Foc* isolates ranged from 6 to 8 days. *Foc* isolates mostly exhibited good to medium sporulation rate. No statistical correlation was observed between growth rate and sporulation potential. Out of six, only five ISSR primers amplified and generated 42 scorable bands ranging from 350bp to 2.5Kb in sizes and 40 were polymorphic (83.4% to 100%). Structure software revealed a genetic admixture population structure with low level of variation among the isolates. UPGMA dendrogram clustered *Foc* population into two major clades. The present study reports low levels of diversity in Pakistani *Foc* isolates at both morphological and genetic level.

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Introduction

Banana of genus *Musa*, is the world's main cash crop and a staple food for the poor. It is cultivated over 120 countries around the world, feeding around 400 million people (Molina and Valmayor, 1999). The sweet dessert banana dominates the global fruit trade and it is the most popular fruit in the world (Anania, 2006). Banana is an important fruit crop in Pakistan. Its cultivation spreads over 34,800 hectares with annual production averages of 154,800 tons (Junejo, 2014) and export contribution of around 45.0 thousand tons (FAO, 2018). Banana fruit is grown mainly in the tropics in areas between 20°N and 20°S latitudes (Robinson *et al.,* 1996). In Pakistan, Sindh province is the major banana production region of the country accounting for 84% of the produce while 16% is received from the rest of the country (Memon, 2015).

The major constraint plaguing the banana production since 1919, is the *Fusarium* wilt of banana or Panama wilt caused by *F. oxysporum* f. sp. *cubense* (*Foc*). It is a soil-borne pathogen that causes wilting in plants by infecting the host's vascular tissue entering via the host root system. It spreads through the soil, irrigation water, infected plant residues, and dirt on the shoes of the farmers working in the infested fields. It produces three types of asexual spores, microconidiaa, macroconidia, and chlamydospores. The dormant chlamydospores are one of the reasons for its persistence in the field (Stover, 1954; Stover, 1960; Stover, 1962; Su *et al.,* 1977).

This pathogen has several physiological races, of which Tropical race4 is the most deadly variant. All existing cultivars are susceptible to this race including the Cavendish sweet banana which is resistant to race1 of *Foc* (Ploetz, 2015). After the report of *Fusarium* wilt disease in 2012 in small farms of district Thatta, Sindh province (Syed *et al.,* 2015; Ordoñez *et al.,* 2015; Aish *et al.,* 2017), it has put the banana industry of Pakistan under serious threat. As Pakistan's banana industry heavily relies on dwarf Cavendish banana that is susceptible to *Foc* Tropical race4, there is a need to come up with an effective disease management strategy against this pathogen. It is a pre-requisite for the implementation of both effective long-term and short-term management strategies to have sufficient and thorough understanding of pathogen diversity and variability (Belabid *et al.,* 2004). Since the first incursion report of *Foc* Tropical race4 in banana farms of Pakistan, no such genetic and morphological studies have yet been carried out. Morphological and genetic studies have been successfully carried out in variability studies of *Fusarium oxysporum* species to discern characters associated with pathogenicity in regions long challenged with *Fusarium* wilt disease. Genetic variability studies in *Foc* has been successfully executed using ISSR and Random Amplified Polymorphic DNA (RAPD), Random Amplified Microsatellites (RAMS), Restriction Fragment Length Polymorphism of Intergenic Spacer (RFLP-IGS), and sequence analysis of TEF-1 gene (Bentley *et al.,* 1995; Bentley and Dale, 1995; Bentley *et al.,* 1998; O'Donnell *et al.,* 1998; Belabid *et al.,* 2004; Groenewald *et al.,* 2006; Fourie *et al.,* 2009; Kumar *et al.,* 2010; Katkar *et.al.,* 2015). Therefore, the present investigation is the first attempt aimed at morphological studies and genetic studies using ISSR markers to analyze the variability of *Fusarium oxysprum* f. sp. *cubense* isolates collected from nine different banana farms of district Thatta, Pakistan.

Material and methods

Isolates

The 55 isolates of Panama wilt pathogen, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) that formed the basis of this study, were collected from nine different banana farms of district Thatta. The cultures stored in 50% glycerol solution were re-cultured on potato dextrose agar medium (PDA) at 25°C.

Morphological characterization

Morphological characterization of isolates under study was assessed based on macroscopic (colonial morphology, color, texture, and pigmentation) and microscopic characteristics including conidia shape and conidiogenous cells, shape of fruiting bodies and formation of chlamydospores, by observing at the magnification of 40X, and 100X, as described by Booth (1971), Nelson *et al.* (1983), Gupta (1986), Watanabe (2002), Leslie and Summerell (2006).

In addition, growth studies of *Foc* isolates was carried out by monitoring its growth rate and sporulation rate on PDA medium. For growth rate assessment, 5mm disk punctured from 5 days old culture was transferred to a fresh PDA plate. The disk inoculated PDA plate was then incubated at 25°C. Colony diameter was measured for each isolate and days till full confluency attained in 9cm Petri dish was recorded.

Seven days old *Fusarium* cultures were flooded with 10ml distilled water and gently scraped with glass spreader to release the spores. The mycelial solution was filtered to collect the spores. The filtered spore solution collected was diluted up to 10^{-2} and 2ul from the final solution was pipetted on to microscope slide and the number of macroconidia and microconidia was counted in the field view of microscope at 40X magnification. Three replicate plates were counted per isolate, and an average was determined. The isolates were grouped according to the number of microconidia and macroconidia counted by using a scale from 0-3, where 0 = absent (0 spores), 1 = few (1-20 spores), 2 = regular (21-50 spores) and 3 = abundant (>50 spores).

Molecular characterization

DNA extraction of *Foc* isolates under study was achieved by culturing the fungal isolates in 50ml of potato dextrose broth (PDB) in 100-ml Erlenmeyer flasks at 25°C for 4 days without shaking. The resulting mycelial mat was filtered and used for DNA extraction. Total genomic DNA was extracted using GeneJet Plant Genomic DNA purification mini kit (Thermo Scientific, #K0791) according to the manufacturer's instructions.

Genomic DNA of *Foc* isolates were amplified using six ISSR primer sets supplied by Ligo, Macrgen (Table 2). Each 25µl reaction contained 2.5µl of 10x Taq buffer, 0.5µl dNTP mix, 0.5µl of ISSR primer used, 2.0µl of 25mm $MgCl_2$, 1.0µl of gDNA template, 0.25µl Taq polymerase. The programme carried out for the amplification was; 1 cycle at 94°C for 3mins, 40 cycles of 94°C for 1min, annealing at 48-52°C for 1min, 72°C for 2min, and 1 cycle of final extension for 8min at 72°C. The PCR product was visulaized under UV run in a 0.1% ethidium bromide amended 1.5% agarose gel at 80 volt for 45mins.

Table 2. Features of ISSR primers used in the study.

Sr. No.	Primer Sequence	Annealing Temperature (°C)	Amplified Bands		Polymorphism (%)
			Total no of bands generated	Polymorphic bands	
1	$(CAC)_3$ GC	50	6	6	100%
2	$(GTG)_3$ GC	48	7	7	100%
3	$(AG)_8$ C	52	12	10	83.4%
4	$(AG)_8$ G	52	9	9	100%
5	$(CA)_8$ A	52	8	8	100%
6	$(TAG)_4$	50	0	0	-
	Total	-	42	40	95.2%

During ISSR data analysis only the distinct and reproducible fragments with the size of more than 250bp were included in scoring of the bands. Each band was considered as single allele and scored present (1) or absent (0) on Excel worksheet for each of ISSR loci. To assess the genetic population pattern of the *Foc* population, Structure software 2.3.4 was used. Length of Burnin period and number of MCMC reps were set at 50,000 with k of 2 to 9. Percentage polymorphism was calculated and a dendrogram was generated by Dice coefficient distance matrix constructed by the unweighted pair group method with arithmetic mean (UPGMA) using Free Tree programme with boostrap of 2000.

Statistical analysis

Pearson's correlation was used to estimate the difference in growth rate among the isolates and between growth rate, sporulation and pigmentation. ANOVA CRD was used to calculate the difference in growth rates of the isolates.

Result

Based on the colony color, the 55 *Foc* isolates under study were divided into 4 morphotypes namely; white, whitish pink, whitish purple, and pink. The white colony color was the most dominant morphological type containing 32 isolates, while the next most dominant morphotype exhibited whitish pink color with 18 representative isolates. The least prominent colony color among the recovered isolates was pink and whitish purple. Pigmentation observed in isolates ranged from no color to light pink, peach to dark purple (Fig. 1).

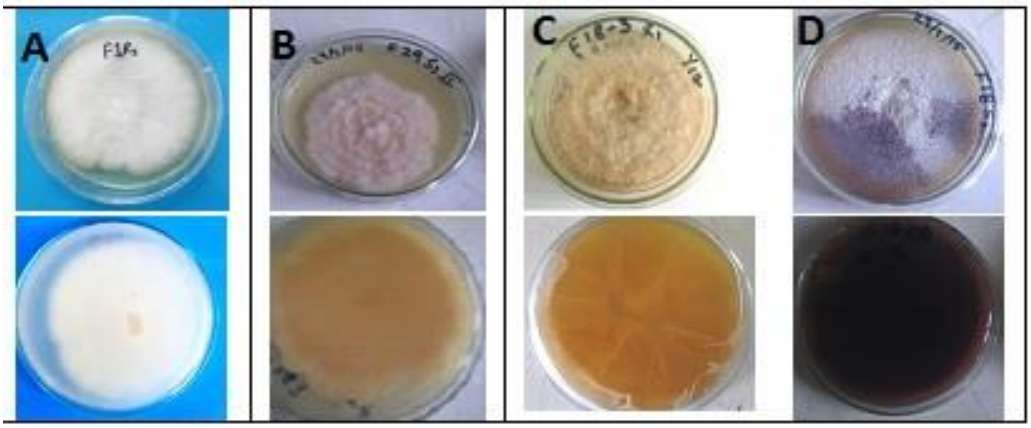

Fig. 1. Colony Morphology of field recovered isolates of *Fusarium oxysporum* f. sp. *cubense* on potato dextrose agar medium (PDA). The top panel in each pair is the upper surface and the lower panel is the under surface of respective plate. A: White colony color; B: Pink colony color; C: Whitish pink colony color, and D: Whitish purple colony color.

Microscopic observations revealed three types of conidia production in all the *Foc* isolates under study, namely microconidia, macroconidia and chlamydospores. The conidiogenous cells, the asexually spore producing structures, were observed to be short monophialides having flask-shaped projections. Microconidia were noted to be oval shaped, hyaline, and mostly single celled though bi-celled microconidia were also observed in some cultures while macroconidia were sickle shaped, hyaline and having variation in three to five septations with pointed apical tip and a pedicellate basal foot. Chlamydospores were observed to be present singly, in pairs, short chains or in combination, produced readily and profusely in some cultures, existing either terminally or/and intercalary (Fig. 2).

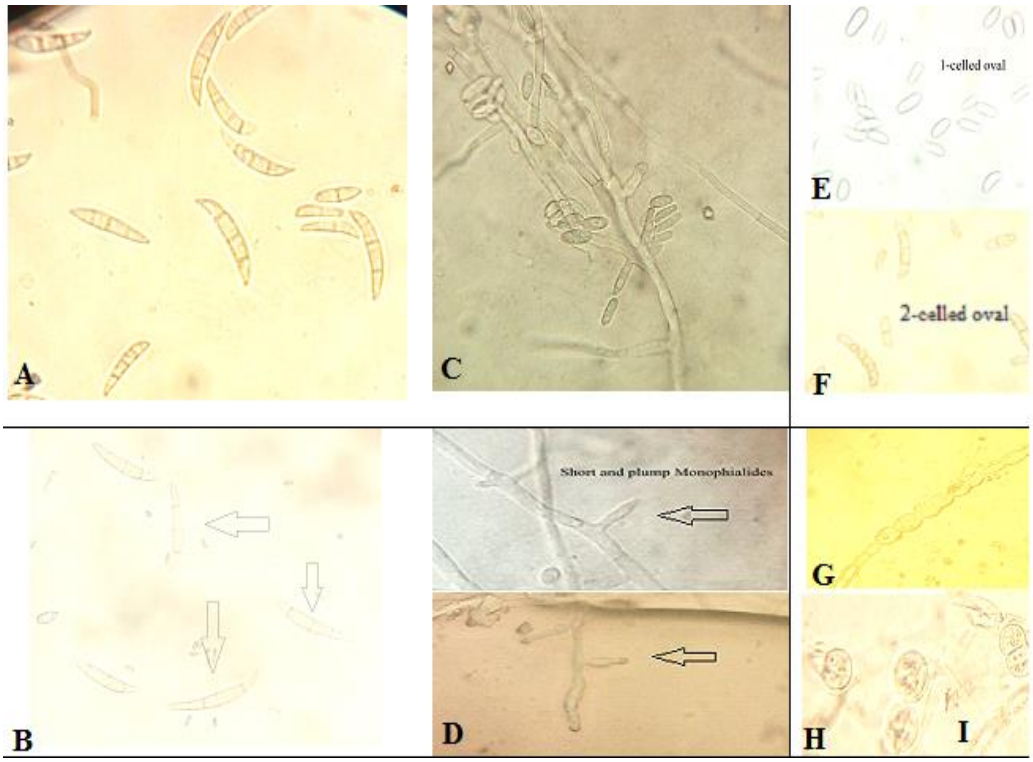

Fig. 2. Microscopic features of Fusarium oxysporum f. sp. cubense on PDA. (A): Macro-conidia; (B): Swollen intercalary cells in old conidia (C): Microconidia on false heads; (D): Conidiogenous cells; (E)-(F): Microconidia of single-celled and 2-celled oval shape; (G)–(I): Formation of Chlamydospores, G: Short chains, H: Singly, I: Pairs.

Variation in growth rate among the *Foc* isolates was observed at 25°C and it ranged from 6 to 8 days. The isolates were grouped into three categories; fast growth rate, medium growth rate, and slow growth rate on the basis of the colony growth observed. A statistically strong difference ($F=166.47$, $p<0.0001$) was found among the three growth rate groups in their mean colony diameter recorded at the 4th day of incubation at confidence interval of 95%. Twenty four isolates (A23, A37, A47, A54, A58, A59, A64, A12, A04, 08, A10, A11, A13, A14, A27, A33, A43, A44, A48, 49, A51, A52, A63, A42) attained 100% confluency in a 9 cm plate in 6 days and therefore proved to be the significantly fastest growing group, while ten isolates (A02, A46, A55, A03, A53, A56, A62, A06, A18, A31) exhibited the slowest growth rate of all isolates attaining 100% confluency in a 9cm dish in 8 days (Table 4.3). A statistically strong correlation ($p<0.0001$, $r= 0.890$) was found between the number of days taken by the isolate to attain 100% confluency in a 9cm Petri dish and the respective mean diameter on 4th day (Fig. 3).

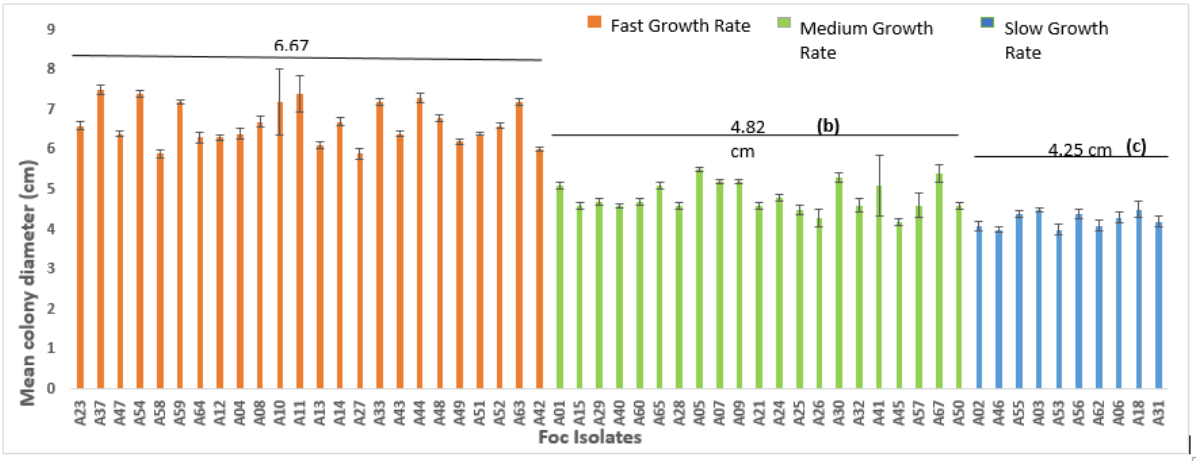

Fig. 3. Mycelial growth rate of *Foc* isolates from Pakistan recorded after 4 days of incubation at 25°C on potato dextrose agar medium. The isolates are color coded according to their respective growth rate groups (the horizontal black line above the columns further demarcates the respective groups). The average fungal colony diameter of each group is indicated above the line. Lower case letters assigned above the line shows significant difference at alpha P=0.05. Error bars on the columns indicate S.D (standard deviation) of the mean from three replicate readings.

Sporulation

Macroconidia was produced in variable amounts in all *Foc* isolates but absentee microconidia was observed only in three isolates; A58, A06, A18. These three isolates, with absentee microconidia though produced abundant macroconidia. Microconidia production was abundant in 10 isolates (A23, A47, A55, A04, A05, A11, A21, A41, A57, and A42), while sparse production was observed in 14 isolates (A02, A37, A59, A64, A28, A09, A10, A13, A14, A30, A44, A45, A51, A63). Abundant macroconidia production was noted in 14 isolates (A01, A02, A29, A37, A58, A64, A12, A13, A33, A45, A52, A67, A06, A18) and sparse production in 19 isolates (A15, A46, A54, A55, A60, A04, A07, A08, A10, A14, A24, A27, A41, A49, A51, A56, A62, A31, A42). Overall sporulation rate of isolates was determined by taking into account both micro and macroconidia production rate and ranked as poor, medium or good spore producing isolates. The highest sporulation rate was exhibited by 13 isolates (A01, A23, A29, A47, A12, A05, A11, A21, A26, A33, A52, A57, A67) and was ranked as "good", while poor sporulation rate was noted in only three isolates (A06, A18, A58). Most of the recovered isolates exhibited medium sporulation rate (39 isolates) (Table 1). No statistical correlation was found between growth rate and sporulation potential in the Pakistani *Foc* isolates investigated in this study.

Table 1. Morphological characterization of *Foc* isolates under study (*S=single, P=pair, C=chains).

Sr. No	Isolate Code	Popul-ation ID	Colony Color	Pigmentation	Days to 100% confluency	Septation in Macroconidia	Shape of Microconidia	Chlamydospore formation*	Sporulation Rate
1	A01	9	Whitish pink	Light peach	7	3-4	1celled oval	S, P	Good
2	A02	1	Whitish pink	Light pink	8	3	2 celled oval	S, P, C	Medium
3	A03	2	White	Light pink	8	3	1celled oval	S	Medium
4	A04	8	White	-	6	3	1 celled oval	S, P	Good
5	A05	9	White	Light pink	7	3	1 celled oval	S	Good
6	A06	5	Pink	Light pink	8	3-5	1 celled oval	S, P	Medium
7	A07	2	White	Light peach	7	3	1 celled oval	S	Medium
8	A08	9	White	-	6	3	1 celled oval	S, P	Medium
9	A09	2	White	-	7	3	1 celled oval	S, P, C	Good
10	A10	9	White	Peach	6	3	1 celled oval	S, P	Medium
11	A11	9	White	-	6	3	1 celled oval	S,P	Medium
12	A12	5	Light pink	Tan	6	3-5	1 celled oval	C	Poor
13	A13	6	White	Light pink	6	3	1 celled oval	S, P	Medium
14	A14	9	White	-	6	3	1 celled oval	S, P, C	Medium
15	A15	9	Whitish pink	Light pink	7	3-5	2 celled oval	S, P	Medium
16	A18	5	Pink	Light pink	8	3-5	1 celled oval	S	Medium
17	A21	6	White	Light pink	7	3	1 celled oval	P, C	Good
18	A23	9	Whitish pink	Light pink	6	3-5	1 celled oval	S, P	Medium
19	A24	3	White	-	7	3	1 celled oval	S, P	Medium
20	A25	2	White	Light purple	7	3	1 celled oval	S	Medium
21	A26	2	White	-	7	3	1 celled oval	S, P	Good
22	A27	6	White	-	6	3	1 celled oval	S, P	Medium
23	A28	9	Light pink	Light pink	7	3	2 celled oval	C	Medium
24	A29	9	Whitish pink	Light pink	7	3-5	1 celled oval	S, P	Medium
25	A30	9	White	Light peach	7	3	1 celled oval	S, C	Medium
26	A31	7	Pink	Light pink	7	3	1 celled oval	S, P	Good
27	A32	2	White	Light peach	7	3-5	2 celled oval	S, P, C	Medium
28	A33	6	White	-	6	3	1 celled oval	S, P, C	Medium
29	A37	2	Whitish pink	Light pink	6	3-5	1 celled oval	P, C	Good
30	A40	9	Whitish pink	Light pink	7	3	1 celled oval	S, C	Medium
31	A41	2	White	Light peach	8	3	1 celled oval	S	Medium
32	A42	9	Whitish purple	-	6	3	1 celled oval	S, P	Good
33	A43	6	White	Light pink	6	3	1 celled oval	S, P	Medium
34	A44	9	White	Light pink	6	3	1 celled oval	S, P	Medium
35	A45	3	White	-	7	3-5	1 celled oval	S	Medium
36	A46	1	Whitish pink	Light pink	7	3	1 celled oval	S	Good
37	A47	9	Whitish pink	Pink	6	3-5	1 celled oval	S, P	Medium
38	A48	9	White	-	6	3	1 celled oval	S, P, C	Medium
39	A49	9	White	Light pink	6	3	1 celled oval	S, P	Medium
40	A50	9	Whitish purple	Light peach	7	3	2 celled oval	S, P, C	Medium
41	A51	7	White	-	6	3	1 celled oval	S	Medium
42	A52	7	White	Light pink	6	3	1 celled oval	S, P	Medium
43	A53	4	White	-	6	3	1 celled oval	S, P	Medium
44	A54	2	Whitish pink	Light pink	6	3-5	1 celled oval	C	Good
45	A55	9	Whitish pink	Light pink	8	3	1 celled oval	S, P	Medium
46	A56	1	White	-	8	3	1 celled oval	S	Medium
47	A57	2	White	-	7	3	1 celled oval	S, P	Good

Sr. No	Isolate Code	Popul-ation ID	Colony Color	Pigmentation	Days to 100% confluency	Septation in Macroconidia	Shape of Microconidia	Chlamydospore formation*	Sporulation Rate
48	A58	2	Whitish pink	Light pink	6	3-5	1 celled oval	P, C	Medium
49	A59	2	Whitish pink	Tan	6	3-5	2 celled oval	S, C	Medium
50	A60	2	Whitish pink	Light pink	7	3	1 celled oval	P, C	Good
51	A62	2	White	-	8	3	1 celled oval	S, P	Poor
52	A63	8	White	-	6	3	1 celled oval	S, P	Poor
53	A64	9	Whitish pink	Tan	6	3	1 celled oval	S, P, C	Medium
54	A65	1	Whitish pink	-	7	3	1 celled oval	S	Medium
55	A67	9	White	Light peach	7	3	1 celled oval	S, P	Medium

The ISSR amplification of Pakistani *Foc* isolates with six primers generated a total of 42 bands out of which 40 bands were polymorphic. The size of fragments ranged from 350bp to 2.5Kb. The total scorable bands ranged from 62-254. *Foc* isolates amplified with all tested ISSR primers except for the primer $(TAG)_4$, which failed to produce any bands for the isolates. All the five working ISSR primers showed high level of polymorphism ranging from 83.4% to 100% (Table 2). Structure software analyzed the genetic pattern of Pakistani *Foc* population and revealed a mixed population structure with low level of variation among the isolates. The low level of genetic variation among *Foc* isolates recovered from different geographical location revealed shared genetic makeup making up a population with genetic admixture (Fig. 4).

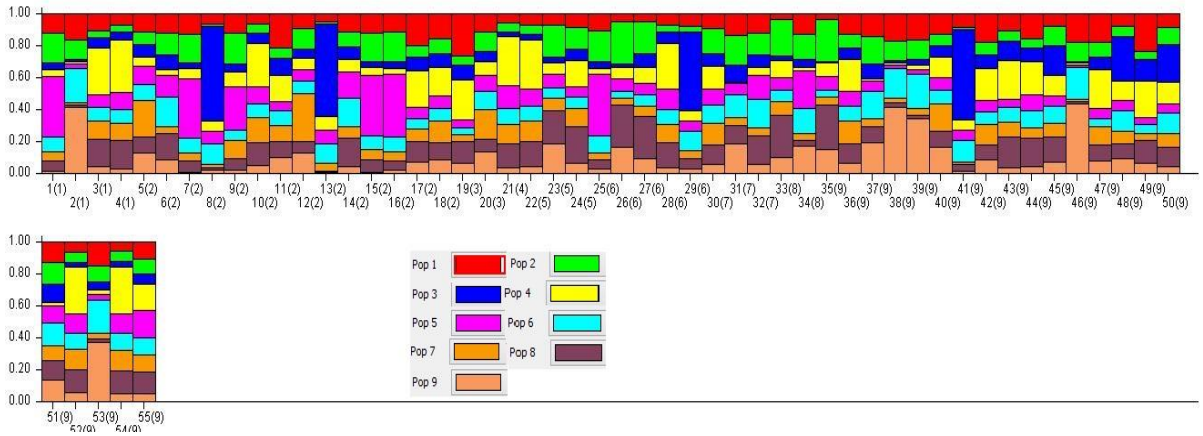

Fig. 4. Structure analysis represents the population structure for K9, coded in different colors in which each color shows the location of genotypes within Subgroups. Each individual is represented by a single vertical line on horizontal axis while vertical axis show the membership coefficient to sub populations. Pop1to Pop9 represents the different locations of banana farms of district Thatta from where the isolates were recovered.

The dendrogram constructed using the Free Tree programme clustered the *Foc* population into two main clades i.e. Clade I and Clade II. The Clade II clustered the populations 5 and 9 into one group with high bootstrap support (100%), while Clade I was further subdivided into two sub-groups. The major sub-group in Clade I harbored the most population showing close association with one another. Populations 4 and 6, and populations 3 and 1 were more closely related than others (bootstrap 17% and 100% respectively). The population 2 separated out into a single sub-group (91% bootstrap). This low variability in populations depicted in dendrogram reflected the results of low genetic variability projected by the Structure software among the *Foc* isolates (Fig. 5).

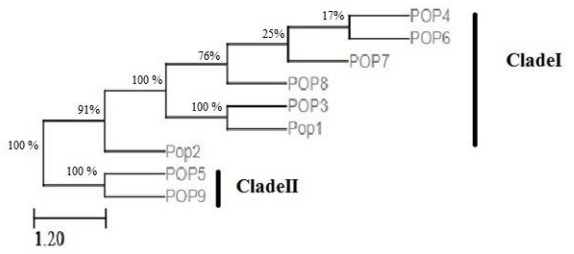

Fig. 5. Dendrogram generated by Free Tree programme clustered the population of Pakistani *Foc* isolates into two major clades.

Discussion

Macroscopic and microscopic observations of *Foc* isolates under study showed conformity in the morphological characters, typical of *Fusarium oxysporum*. For instance, the short monophialides bearing the microconidia on false heads, observed in all the recovered isolates of *Foc*, is the most distinguishing character of *Fusarium oxysporum* when compared to *Fusarium foetens, Fusariummonili forme* and *Fusarium solani* as outlined by Booth (1971), Nelson *et al.* (1983), Burgess *et al.* (1989), Seifert (1996), Watanabe (2002), Summerell *et al.* (2003), Leslie and Summerell (2006), and Mwaniki *et al.* (2011) that these isolates characteristically produce polyphialides and long monophialides, the features never observed in *Fusarium oxysporum*. Immense variation in the pigmentation in *Fusarium oxysporum* isolates have been known to exist on PDA. It is generally observed among the strains of *Fusarium oxysporum* that the aerial mycelium appears white in the initial stages of growth and then gradually attains a variety of pigmentation in the later stages though non-pigmentation is also observed in some strains of *Fusarium oxysporum* (Booth 1971, Seifert, 1996; Nelson, 1983; Watanabe, 2002; Leslie and Summerell, 2006; Groenewald *et al.*, 2006). Similar variation was also observed in the *Foc* isolates recovered in this study.

It is well documented in literature that the choice of medium greatly affects the rate of sporulation in *Fusarium* species (Linfield, 1986; Agrios, 2005; Sharma *et al.,* 2005; Kim *et al.,* 2005; Zhao *et al.,* 2010; Hussain *et al.,* 2012; Rahman *et al.,* 2012; Pradeep *et al.,* 2013; Djeugap *et al.,* 2017). As the recovered isolates under study were tested for their sporulation potential using the same procedure, it is likely that the variation observed is the true reflection of isolates behavior under artificial medium. It should be pointed out nonetheless, that the sporulation and growth rate potential of isolates observed under artificial medium did not correlate to each other. The reason for this non-correlation might be that pigmentation character does not provide any physiological advantage to the isolate rather it is the result of the richness of the medium in which the organism is studied that results in its characteristic colony color.

Genetic diversity analysis carried out using the ISSR markers revealed only 5 markers with high polymorphism while the one marker, $(TAG)_4$ failed to produce any bands in the Pakistani *Foc* isolates. Although in one of the studies the $(TAG)_4$ primer have been shown to produce good banding scores in the Indian isolates of *Fusarium oxysporum* f. sp. *cubense* (Das *et al.,* 2012).

The ISSR marker population analysis revealed very low genetic variability among the isolates indicating low rate of mutation. These results could probably be the result of a small number of individuals undergoing recent population expansion. Such inferences are assumed because the pathogen has been only recently detected in the banana farms of district Thatta (Syed *et al.,* 2015; Ordonez *et al.,* 2016; Aish *et al.,* 2017) and the pathogen is assumed to have been unintentionally introduced from across the borders probably during the exchange of plant material. So the low level of intra and inter-genetic variability results obtained, during population analysis, among the population of Pakistani *Foc* isolates support these assumptions.

Conclusion

The present study reports low variation in Pakistani *Foc* isolates at both morphological and genetic level. The ISSR markers used in the study showed high level of polymorphism among the isolates except for the

ISSR marker $(TAG)_4$. The low genetic variation in *Foc* isolates was observed at both intra and inter-population level indicating recent population expansion. The morphological and genetic characterization study have greatly enhanced the understanding of the variability within this pathogen which will ultimately facilitate in deciding the choice of appropriate management models. Nonetheless, it is recommended to carry out more detailed studies on genetic variation by including additional and more informative microsatellite markers to generate precise structure of the Pakistani *Foc* population.

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Conflict of interest statement

It is declared by the authors that the present work underwent without any potential conflict of interest.

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