RESEARCH ARTICLE

GENETIC RELATEDNESS AMONG *FUSARIUM* POPULATIONS ASSOCIATED WITH SUGARCANE WILT IN INDIA: BRIDGING MOLECULAR VARIABILITY AND PHYLOGENETIC DIVERSITY

M. Poongothai, R. Viswanathan*, P. Malathi, A. Ramesh Sundar, C. Naveen Prasanth and C.G. Balaji

Abstract

Fifty *Fusarium* isolates associated with sugarcane wilt that widely differed in their cultural characters and geographical origin were subjected to molecular characterization using different molecular markers, viz. RAPD, rDNA-IGS-RFLP and ISSR. Among the markers, ISSR generated 98% polymorphism followed by RAPD and rDNA-IGS-RFLP with 97 and 92% polymorphism, respectively. The *Fusarium* population was divided into two groups, namely A and B by RAPD analysis and such grouping remained consistent with ISSR. ISSR and RAPD grouped 76 - 86% of isolates as *F. sacchari* in group A and the remaining isolates as other species isolates in a separate group B. The dendrogram of IGS-RFLP had many clusters in which the isolates that belonged to species other than *F. sacchari* separated first in many small clusters as in RAPD. The detailed phylogenetic analyses established that ISSR is more efficient than RAPD and rDNA-IGS-RFLP for grouping the isolates. The detailed investigation taken up to define genetic variability among the *Fusarium* population associated with sugarcane wilt in India correlates with earlier established taxonomy and pathogenicity data. Our investigation clearly indicates *F. sacchari* as the major causative organism of sugarcane wilt.

Key words: Sugarcane, wilt, *Fusarium sacchari*, genetic diversity, ISSR, RAPD, rDNA-ITS, rDNA-IGS-RFLP.

Introduction

The taxonomy of *Fusarium* species had always been a controversial issue. At one time, there were more than 1000 species, varieties and forms named on the basis of superficial observations, with little or no regard for the cultural characteristics of these specimens. The necessity for a precise and reliable system of classification became apparent when it was shown that *Fusarium* species caused serious diseases in many plants, animals and human beings. The identification and classification of *Fusarium* isolates based on morphological and physiological criteria could not resolve the existing variability (Nelson 1991). The study of *Fusarium* spp. variability has been greatly advanced by the adoption of molecular techniques including random amplified polymorphic DNA (RAPD) analysis (Kini et al. 2002; Prasad et al. 2007; Zamani et al. 2004), specific diagnostic PCR primers (Schilling et al. 1996), analysis of PCR products by either restriction fragment length polymorphism (Bateman et al. 1996; Edel et al. 1996; Nicholson et al. 1993) or DNA sequencing and amplified fragment length

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polymorphisms (Leissner et al. 1997). Ubiquitous distribution of microsatellites in many genomic loci enables species level recognition (Wei et al. 2005).

Wilt is a devastating disease of sugarcane reported in India as early as 1913 and it was responsible for failure of many elite varieties. Conflicting claims have been made regarding the causal organism as species of *Fusarium, Cephalosporium* and *Acremonium* by different workers (Viswanathan 2013). However, detailed studies by Viswanathan et al. (2006) failed to recover *Acremonium* from wilt infected canes of tropical and subtropical India and only *Fusarium* species could be recovered from both nodal and internodal tissues. Subsequently, cultural and morphological characteristics of majority of 263 isolates from different regions revealed that *F. sacchari* is the most commonly isolated wilt fungus in sugarcane (Poongothai et al. 2014a, b). Further, pathogenicity studies revealed that the isolates that belonged to species of *F. verticilloides, F. proliferatum, F. napiforme* or *F. subglutinans* were nonpathogenic or less virulent in sugarcane and only *F. sacchari* isolates were pathogenic (Viswanathan et al. 2011).

The objective of the present study was to examine the genetic variability among the *Fusarium* associated with sugarcane wilt and to clarify any correlation with important traits such as taxonomic status of the pathogen(s) causing sugarcane wilt, regional specificity and pathogenicity. To date, different molecular markers have been used to study the genetic variability of different *Fusarium* species and such information is lacking in the case of *Fusarium* associated with sugarcane wilt. This is the first detailed investigation taken up to define genetic variability among *Fusarium* populations isolated from sugarcane wilt in tropical and subtropical India using RAPD, ISSR and rDNA-IGS-RFLP markers.

Materials and methods

Fungal isolates

Two hundred and sixty three isolates obtained from wilt infected sugarcane stalks and rhizosphere soils were maintained at the Plant Pathology laboratory of ICAR-Sugarcane Breeding Institute, Coimbatore (Poongothai et al. 2014a). Fifty out of the 263 *Fusarium* isolates that widely differed in their cultural characters and geographical origin were used in this study for molecular characterization (Table 1). These isolates were isolated on oatmeal agar (OMA) or Coon's agar and were maintained on OMA slants at 4°C or in filter paper discs at -20°C.

Genomic DNA extraction and purification

The 50 *Fusarium* isolates were multiplied on 90 ml of potato dextrose broth in 500 ml Erlenmeyer flasks for 10 days. Two hundred µl of spore suspension prepared from actively growing cultures was used as a source of inoculum. The culture flasks were placed stationary and incubated at room temperature (~28°C) for 10 days. The fungal mat was filtered through cheese cloth, blotted dry with sterile filter paper towels and used immediately for DNA extraction. Template DNA for PCR was isolated as reported earlier by Du Teau and Leslie (1991). The mycelial mat (250 mg) was ground in liquid nitrogen to a fine powder, suspended with equal volume (1 ml) of SDS buffer and incubated in water bath at 65°C for 45 min. The tubes were homogenized with equal volume of phenol: chloroform: isoamyl alcohol in 25:24:1 ratio and centrifuged at 12,000 rpm and 25°C for 10 min. Aqueous phase was carefully removed after centrifugation without disturbing the interphase. To the aqueous phase, 0.8 volume isopropanol was added and left at room temperature for 45 min and centrifuged at 12,000 rpm and 4°C for 15 min. The pellet obtained after centrifugation was washed

Table 1. *Fusarium* isolates used in molecular studies

Isolate	Host cultivar	Place of collection	District	State
Fs 805 AP1L1	Co 7805	Chinnathadepallii	West Godavari	Andhra Pradesh
Fs 805 AP2L1	Co 7805	Chinnathadepalli	West Godavari	Andhra Pradesh
Fs 805 AP3L2	Co 7805	Vodlamurru	East Godavari	Andhra Pradesh
Fs 032 AP1L1	Co 86032	Mortha	West Godavari	Andhra Pradesh
Fs 032 AP2L2	Co 86032 (TR)	Rudrur	Nizamabad	Telangana
Fs 009 AP	Co 2002-09	Rudrur	Nizamabad	Telangana
FsV 048 AP1	81 V 48	Tirumali	East Godavari	Andhra Pradesh
FsV 048 AP2	81 V 48	Tirumali	East Godavari	Andhra Pradesh
FsV 048 AP3	81 V 48	Tirumali	East Godavari	Andhra Pradesh
Fs LC ArP1	Local cultivar	-	-	Arunachal Pradesh
Fs LC A1	Local cultivar	Nagaon	Nagaon	Assam
Fs LC A2	Local cultivar	Jorhat	Jorhat	Assam
FsBln 173 B1	CoBln 03173	Motipur	Muzaffarpur	Bihar
FsBln 173 B2	CoBln 03173	Motipur	Muzaffarpur	Bihar
FsBln 175 B1	CoBln 03175	Motipur	Muzaffarpur	Bihar
FsBln 175 B2	CoBln 03175	Motipur	Muzaffarpur	Bihar
FsBln 176 B1	CoBln 03176	Motipur	Muzaffarpur	Bihar
Fs 006 G1	Co 95006	Navsari	Navsari	Gujarat
Fs 006 G2	Co 95006	Navsari	Navsari	Gujarat
Fs 010 G	Co 98010	Chalthan	Surat	Gujarat
FsSi 071 G	CoSi 95071	Chalthan	Surat	Gujarat
FsV 102 G	CoV 94102	Chalthan	Surat	Gujarat
FsNG 159 K1	57 NG 159 yellow	Kannur	Kannur	Kerala
FsNG 159 K4	57 NG 159 yellow	Kannur	Kannur	Kerala
Fs BT K1	Black Tanna	Kannur	Kannur	Kerala
Fs BT K2	Black Tanna	Kannur	Kannur	Kerala
FsJn 964 MP1	CoJn 964	Powarkheda	Hosangabad	Madhya Pradesh
Fs 032 M1L1	Co 86032	Someshwar Nagar	Pune	Maharashtra
Fs 032 M2L2	Co 86032	Kopargaon Bolki	Ahmednagar	Maharashtra
Fs 012 M2	Co 94012	Pravaranagar	Ahmednagar	Maharashtra
FsA 085 O1	CoA 89085	Chikinia	Cuttack	Orissa
FsA 085 O2	CoA 89085	Chikinia	Cuttack	Orissa
FsA 085 O3	CoA 89085	Chikinia	Cuttack	Orissa
FsA 085 O6	CoA 89085	Chikinia	Cuttack	Orissa
Fs 003 P1L1	Co 89003	Taggadabadala	Hoshiarpur	Punjab
Fs 003 P5L1	Co 89003	Taggadabadala	Hoshiarpur	Punjab
Fs 003 P6L2	Co 89003	Mukerian	Gurdaspur	Punjab
Fs 120 P3	Co 0120	Panwan	Hoshiarpur	Punjab

Table 1 Continued

Isolate	Host cultivar	Place of collection	District	State
Fs 032 TN3L1	Co 86032	Polur	Tiruvannamalai	Tamil Nadu
Fs 032 TN4L2	Co 86032	Vedapatti	Coimbatore	Tamil Nadu
FsAVT 153 TN2	AVT 21153	Cuddalore	Cuddalore	Tamil Nadu
FsC 063 TN1	CoC 90063 (TR)	Vaidipakkam	Cuddalore	Tamil Nadu
FsV 101 TN2L2	CoV 94101	Karamani Kuppam	Cuddalore	Tamil Nadu
Fs 032 TN8L4	Co 86032	Sakthinagar	Erode	Tamil Nadu
Fs 003 TN	Co 94003	Palakudi	Thanjavur	Tamil Nadu
FsSi 071 TN1	CoSi 86071	Vayalur	Kancheepuram	Tamil Nadu
TN SF TN1	Sugarcane seed	Coimbatore	Coimbatore	Tamil Nadu
Fs 047 TN	2003-47	Vedapatti	Coimbatore	Tamil Nadu
Fs 121 UP	Co 0121	Shimbaoli	Ghaziabad	Uttar Pradesh
Fs 003 H1	Co 89003	Karnal	Karnal	Haryana

twice with 70% ethanol, air dried and suspended in sterile MilliQ water. To the suspension, RNase enzyme was added to a final concentration of 25µg/ml and incubated at room temperature for 1h. Later, equal volume of phenol: chloroform in 1:1 ratio was added and centrifuged at 12,000 rpm 25°C for 10 min. To the aqueous phase, 0.8 volume ice cold absolute ethanol was added, the mixture placed at -20°C for 30 min and centrifuged at 12,000 rpm and 4°C for 15 min. The pellet was washed twice with 70% ethanol and suspended in TE buffer. The isolated DNA (2 µl) was loaded on 1% agarose gel and electrophoresed at 80 V. The purity and concentration of DNA were checked by using standard markers of known concentration.

Random amplified polymorphic DNA assays (RAPD)

RAPD reactions were typically performed in a total volume of 25 µl, containing 1x PCR Buffer, 0.2mM of each dNTP, 3mM $MgCl_2$, 0.5µM of a single RAPD primer (Operon Technologies Ltd), 25ng of genomic DNA template, and 1 unit of *Taq* DNA polymerase. Initial screening of 50 random primers on 10 representative isolates showed 34 primers, viz. OPA03, OPA04, OPA06, OPA09, OPA10, OPA11, OPA12, OPA13, OPA17, OPB05, OPB06, OPB08, OPB11, OPB12, OPB14, OPB16, OPB17, OPB18, OPC01, OPC05, OPC09, OPC10, OPO12, OPP18, OPP19, OPR15, OPW14, OPW17, OPY01, OPY02, OPY03, OPY14, OPY19 and OPZ03 to be polymorphic (Table 2). These 34 decamers were used to screen all the 50 isolates selected for the study. Amplification was performed in a thermal cycler (Mastercycler gradient, Eppendorf, Germany) programmed for 40 cycles (1 min at 94ºC, 1 min at 36ºC, and 2 min at 72ºC) using the fastest available transitions between each temperature. Initial denaturation and final extension were at 94ºC and 72ºC, respectively for 5 min. Amplified products were analyzed by electrophoresis in 1.5% agarose gels.

PCR amplification of IGS region

Universal IGS region was amplified using genomic DNA of all the 50 isolates selected for molecular studies. The PCR reaction mixture (25µl) for the amplification of rDNA ITS region and rDNA IGS region included 1µl of template DNA, 2.5 units of *Taq* polymerase per reaction, 1x buffer supplied with

Table 2. Sequence of random primers used and number of fragments generated by RAPD in isolates of *Fusarium* spp.

S. No	Primer	Sequence 5'-3'	No. of amplified fragments*	No. of polymorphic fragments
1	OPA10	GTGATCGCAG	6	6
2	OPA12	TCGGCGATAG	4	4
3	OPA13	CAGCACCCAC	9	9
4	OPB05	TGCGCCCTTC	11	10
5	OPB06	TGCTCTGCCC	9	9
6	OPB08	GTCCACACGG	8	8
7	OPB14	TCCGCTCTGG	8	8
8	OPB16	TTTGCCCGGA	7	7
9	OPB17	AGGGAACGAG	4	4
10	OPB18	CCACAGCAGT	3	3
11	OPC01	TTCGAGCCAG	5	5
12	OPC05	GATGACCGCC	3	2
13	OPC10	TGTCTGGGTG	2	2
14	OPW14	CTGCTGAGCA	10	10
15	OPY01	GTGGCATCTC	6	6
16	OPY02	CATCGCCGCA	7	6
17	OPY03	ACAGCCTGCT	5	5
18	OPY14	GGTCGATCTG	6	6
19	OPY19	TGAGGGTCCC	4	4
	Total no. of bands		117	114

*bands <200bp were not included

the enzyme, 3 mM $MgCl_2$, 200 µM dNTPs and 1 µM of primers each. The thermocycler conditions include initial denaturation at 95°C for 2 min, followed by 30 cycles of 54°C for 30 sec (annealing), 72°C for 1 min (elongation), 94°C for 1 min (denaturation) and a final elongation was allowed for 10 min at 72°C to ensure a double-stranded amplification product.

IGS amplicon (1µg) of 2.6-2.8kb was restricted with the restriction enzymes *Alu*I, *Bam*HI, *Eco*RI, *Hind*III, *Hpa*II and *Taq*I (MBI Fermentas, USA) using the manufacturer's instructions. The restricted fragments were electrophoresed on 1.5% agarose gel. Reaction condition of the used restriction enzymes and the number of polymorphic bands generated by each enzyme are given in Table 3.

Table 3. Reaction conditions and number of RFLPs generated by restriction enzymes in the IGS region of *Fusarium* spp. isolates

S. No	Restriction enzyme	Recognition Sequence	Incubation		RFLPs
			Temp (ºC)	Time (h)	
1	*Eco*RI	G↓AATTC	37	1	6
2	*Alu*I	AG↓CT	37	1	>11
3	*Taq*I	T↓CGA	65	1	5
4	*Hae*III	GG↓CC	37	1	5
5	*Xho*I	C↓TCGAG	37	1	4

Analysis of inter specific spacer region (ISSR)

Nine pairs of simple sequence repeats (SSR) primers (1st Base, Malaysia) were used to amplify ISSR region from 50 isolates. Amplification reactions contained 1x PCR buffer, 1U *Taq* polymerase, 0.2mM each dNTP, 2mM $MgCl_2$, 0.5µM each primer and 25ng of genomic DNA per 25µl of reaction mixture. PCR was performed in a thermocycler (Mastercycler gradient, Eppendorf, Germany) at initial denaturation of 94ºC for 4 min, followed by 35 cycles of 94ºC for 30 sec, 57ºC for 1 min (primer annealing), and 72 ºC for 1 min (primer extension) with a final extension of 7 min at 72ºC. Annealing temperature of the primers varied as given in Table 4 and the amplified fragments were checked on 1.5% agarose gel.

Phylogenetic analysis

Fragments which migrated the same distance in agarose gel electrophoresis were considered to be fragments in common. A data matrix was constructed from the gel photographs of RAPD, ISSR and rDNA IGS-RFLP. The presence or absence of amplified or restricted bands was coded as 1 or 0, respectively and 9 when the band was not determined. Binary matrices were analyzed to obtain Jaccard's coefficients among the isolates using NTSYS-pc (version 2.0; Exeter Biological Software, Setauket, NY). Jaccard's coefficients were clustered to generate dendrograms using the SAHN clustering program, selecting the unweighted pair-group method with arithmetic average (UPGMA) algorithm in NTSYS-pc (Rohlf 2005).

Results

Random amplified polymorphic DNA (RAPD) assay

Of the 34 decamers screened, only 19 RAPD primers gave scorable RAPD bands from the 50 isolates subjected to molecular studies. A total of 117 bands with a size range of 200bp - 4 kb were observed in 19 RAPD reactions and 114 of them were polymorphic, resulting in 97% polymorphism among the isolates (Fig. 1). The number of polymorphic bands generated varied from primer to primer (Table 2). Also, 100% polymorphism was recorded in 16 out of the 19 primers tested. One band generated by RAPD was monomorphic in each of the three primers, viz. OPB5, OPC5 and OPY2 of size ~950 bp, 1200 bp and ~550 bp respectively. A high degree of polymorphism pointed out that there is a greater genetic variability among the isolates collected from different regions. Dendrogram generated from a binary matrix of RAPD profile clustered the 50 isolates into two groups at a similarity index of 0.57 (Fig. 2). Group

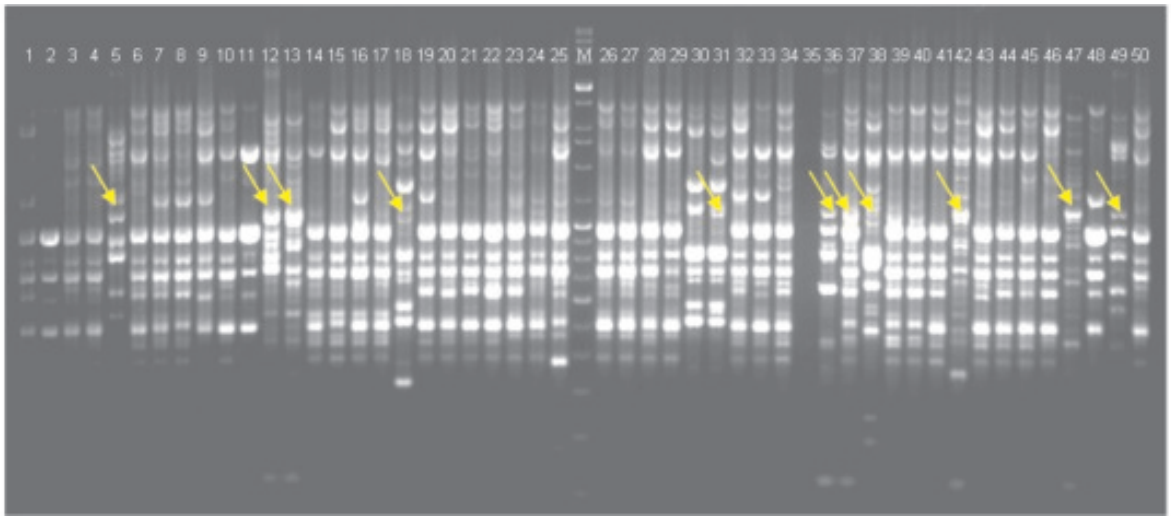


Fig. 1. Random amplified polymorphic DNA (RAPD) analysis of genomic DNA from 50 *Fusarium* isolates-(Primer: OPW14). Lanes 1-50 represent RAPD of *Fusarium isolates* 1- FsSi 071 G, 2- FsV 102 G, 3- Fs 010 G, 4- Fs 006 G1, 5- Fs 006 G2, 6- Fs 805 AP1L1, 7- Fs 805 AP2L1, 8- Fs 032 AP1L1, 9- Fs 805 AP3L2, 10- FsV 048 AP1, 11- FsV 048 AP2, 12- FsV 048 AP3, 13- Fs 032 AP2L2, 14- Fs 009 AP, 15- FsA 085 O1, 16- FsA 085 O2, 17- FsA 085 O3, 18- FsA 085 O6, 19- Fs 003 P1L1, 20- Fs 003 P5L1, 21- Fs 003 P6L2, 22- Fs 120 P3, 23- Fs 003 H1, 24- Fs 121 UP1, 25- FsJn 964 MP1, 26- Fs LC A1, 27- Fs LC A2, 28- Fs LC ArP1, 29- FsBln 173 B1, 30- FsBln 173 B2, 31- FsBln 175 B1, 32- FsBln 175 B2, 33- FsBln 176 B1, 34- Fs 032 M1L1, 35- Fs 032 M2 L2, 36- Fs 012 M1, 37- FsNG 159 K1, 38- FsNG 159 K4, 39- Fs BT K1, 40- Fs BT K2, 41- Fs 032 TN3L1, 42- Fs 032 TN4L2, 43- FsC 063 TN1, 44- FsAVT 153 TN2, 45- FsV 101 TN3L3, 46- Fs 032 TN8L4, 47- Fs 003 TN, 48- FsSi 071 TN1, 49- Fs SF TN1, 50- Fs 047 TN; M: Bangalore Genei high range DNA ladder; amplified products were separated on a 1.5% agarose gel. Yellow arrow indicates presence of 1.1 kb fragment

A comprised 39 *Fusarium* isolates and the 11 remaining isolates formed group B. Within group B, Fs 032 AP2L2 and FsBln 175 B1 out-grouped from the nine other isolates at a similarity value of 0.60. FsNG 159 K4 separated out from the cluster of eight remaining isolates at a similarity index of 0.62. Among the eight isolates, Fs 805 AP1L1, Fs 009 AP, Fs 003 P1L1 and FsBln 175 B2 formed a sub cluster and Fs 012 M2, FsS 268 TN2, Fs 003 TN and Fs SF TN1 formed another sub cluster in group B. Overall grouping by RAPD revealed that majority of the isolates clustered into group A.

rDNA-IGS-RFLP

The length of the amplified IGS region was ~2.8 kb in 49 isolates and ~2.6 kb in Fs 121 UP1. rDNA-IGS region was restricted with *Eco*RI, *Eco*RV, *Alu*I, *Hae*III, *Xho*I, *Hind*III, *Taq*I, *Kpn*I and *Hpa*II. Of them *Eco*RV, *Hind*III, *Kpn*I and *Hpa*II did not digest the amplified rDNA-IGS region. Restriction digestion of rDNA-IGS region by *Alu*I, *Eco*RI, *Xho*I, *Hae*III and *Taq*I gave 24 polymorphic bands (Fig. 3). The three isolates Fs 009 AP, Fs 012 M2, Fs 032 TN4L2 were grouped in a cluster with a similarity value of 0.58. Fs 032 TN3L1 outgrouped from the cluster of 46 remaining isolates at a similarity index of 0.64 (Fig. 4). At a similarity index of 0.68, Fs 003 TN and FsSi 071 TN1 separated in a cluster. Fs 805 AP1L1, Fs 032 AP2L2, Fs 003 P1L1, FsBln 175 B2, Fs 032 TN8L4 and Fs SF TN1 subdivided into a new group with the similarity value of 0.68 and the 36 remaining isolates formed a cluster which

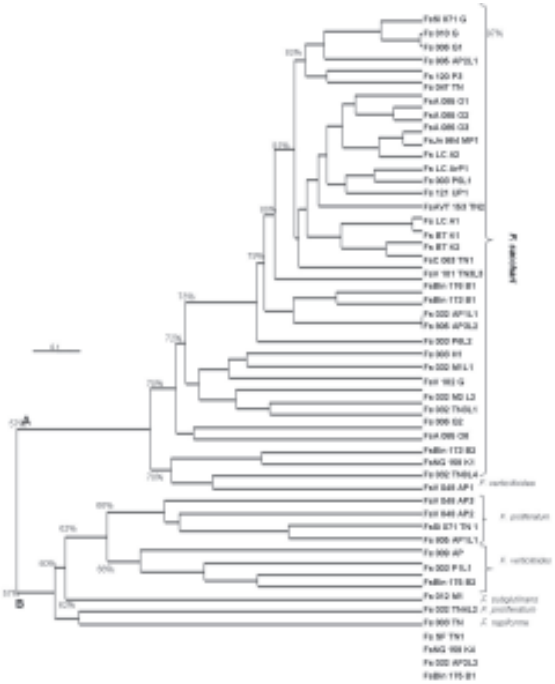


Fig. 2. RAPD dendrogram showing relationship among 50 *Fusarium* isolates generated by unweight pair group method of arithmetic means (UPGMA) Dendrogram was generated by converting the binary data to a distance matrix using Jaccard's co efficient by UPGMA method of clustering by NTSYS-pc 2.0. Bar represents number of substitutions per site. Similarity percentage of the isolates is indicated on the nodes. *F. sacchari* isolates clustered in group A and other *Fusarium* sp. clustered in group B.

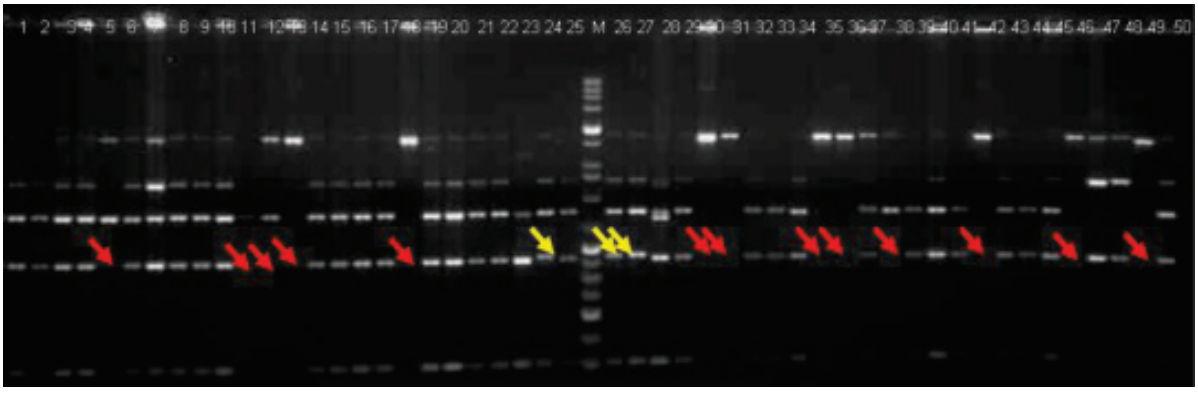


Fig. 3. rDNA inter genic spacer (IGS) amplification and *Xho*I restriction pattern of rDNA-IGS region of *Fusarium* spp. isolates. Yellow arrow indicates absence of 920 bp fragment and red arrow indicates absence of 900 bp fragment; Lanes 1-50 represent *Fusarium* isolates as listed in Fig. 1. M: Bangalore Genei high range DNA ladder; amplified products were separated on a 1.5% agarose gel.

further subdivided into smaller subgroups. The dendrogram of IGS-RFLP had many clusters in which the isolates that belong to species other than *F. sacchari* separated first in many small clusters as in RAPD. The isolates Fs 009 AP, Fs 032 M2L2 and Fs 032 TN4L2, which separated from the other

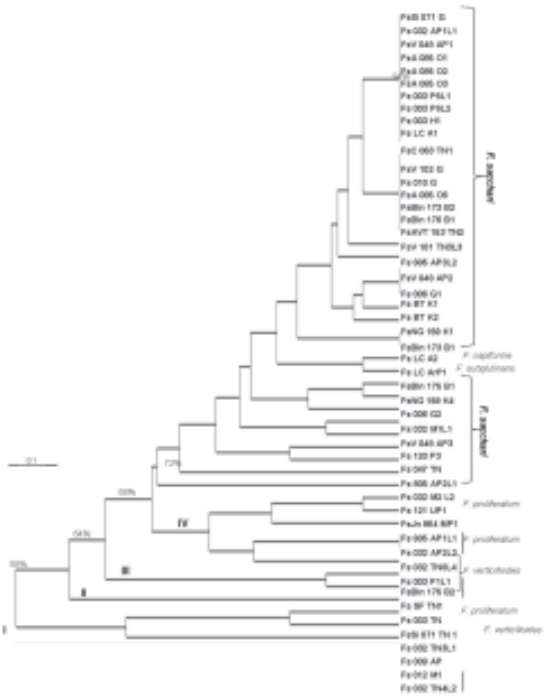
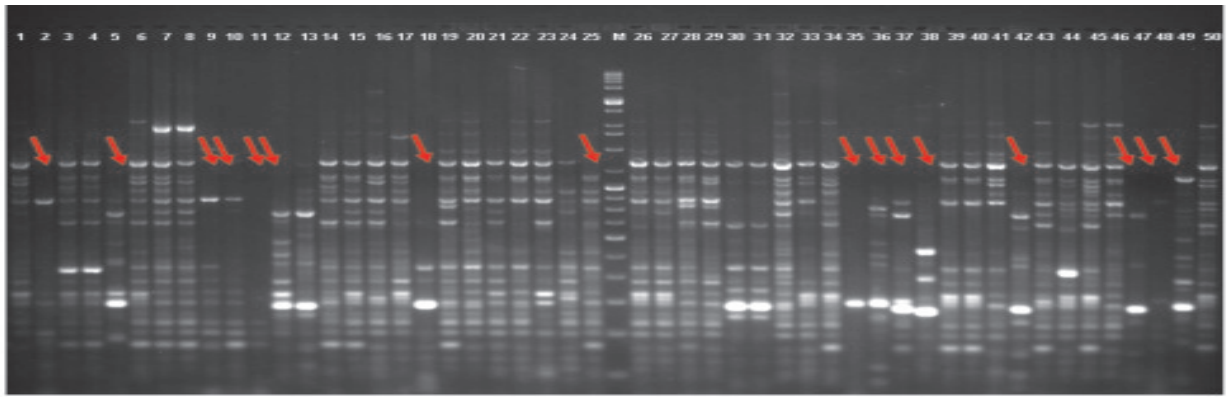


Fig. 4. UPGMA dendrogram showing relationship among 50 isolates of *Fusarium* by rDNA IGS restriction pattern of 5 different restriction enzymes. Dendrogram was generated from rDNA IGS RFLP analysis by converting the binary data of amplified products to a distance matrix using Jaccard co efficient and clustered by UPGMA method using NTSYS-pc 2.0. Bar represents distance of 0.1. Similarity percentage of the isolates is indicated on the nodes. *F. sacchari* isolates are not delineated in to separate clusters as in RAPD and ISSR dendrograms. *F. proliferatum* and *F. verticilloides* were separated from *F. sacchari* in groups I, II, III and IV but *F. napiforme* and *F. subglutinans* are not distinguished from *F. sacchari* isolates based on IGS RFLP restriction pattern

47 isolates in group I, had their morphological dissimilarity from *F. sacchari*; however, these isolates separated from other *F. proliferatum* and *F. verticilloides* isolates also. The reason for separation of Fs 032 TN4L2 from other Tamil Nadu isolates that produced chains of conidia is probably it had oblong microconidia. Similarly, Fs 032 TN3L1, which too had oblong microconidia, separated next in cluster II. The other Tamil Nadu isolates that had oval microconidia formed group III. Fs 003-P1L1, FsBln 175 B2, Fs 032 TN8L4, Fs SF TN1, Fs 805 AP1L1 and Fs 032 AP2L2 out-grouped from the remaining 40 isolates in group IV. The isolate Fs 032 TN8L4 that produced microconidia in false heads separated out in group IV as it produced oval microconidia similar to other isolates in group IV.

Analysis of inter specific spacer region

Nine ISSR primers were used with the initial set of 10 isolates. Seven of the nine ISSR primers screened, viz. ISSR1, ISSR2, ISSR3, ISSR5, ISSR6, ISSR7 and ISSR9 were found to give polymorphic profiles. ISSR analysis amplified 45 bands of 100bp - 3kb size (Fig. 5), of which 44 were polymorphic resulting in 98% polymorphism. The number of polymorphic bands generated by the seven ISSR primers is given in Table 4. The dendrogram based on ISSR primer amplification separated the 50



Red arrow indicates absence of 1.3 kb fragment

Fig. 5. Inter simple sequence repeat (ISSR) profile of 50 *Fusarium* isolates using primer ISSR 9. Lanes 1-50 represent *Fusarium* isolates as listed in Fig. 1. M: Bangalore Genei high range DNA ladder; amplified products were separated on a 1.5% agarose gel

Table 4. Sequence of primers used for amplification and polymorphic bands generated by ISSR in isolates of *Fusarium* spp.

S. No.	Primer	Sequence 5'-3'	No. of amplified fragments*	No. of polymorphic fragments
1	SSR1	TGCTGTGTATGGATGGATGG	6	6
2	SSR1R	CATGGTCGATAGCTTGTCTCAG		
3	SSR2	ACTTGGAGGAAATGGGCTTC	6	6
4	SSR2R	GGATGGCGTTTAATAAATCTGG		
5	SSR3	TGGCTGGGATACTGTGTAATTG	3	3
6	SSR3R	TTAGCTTCAGAGCCCTTTGG		
7	SSR5	GTGGACGAACACCTGCATC	9	9
8	SSR5R	AGATCCTCCACCTCCACCTC		
9	SSR6	GGAGGATGAGCTCGATGAAG	6	5
10	SSR6R	CTAAGCCTGCTACACCCTCG		
11	SSR7	CGTCTCTGAACCACCTTCATC	5	5
12	SSR7R	TTCCTCCGTCCATCCTGAC		
13	SSR9	GGTAGGAAATGACGAAGCTGAC	10	10
14	SSR9R	TGAGCACTCTAGCACTCCAAAC		
	Total number of bands		45	44

*bands <200bp were not included

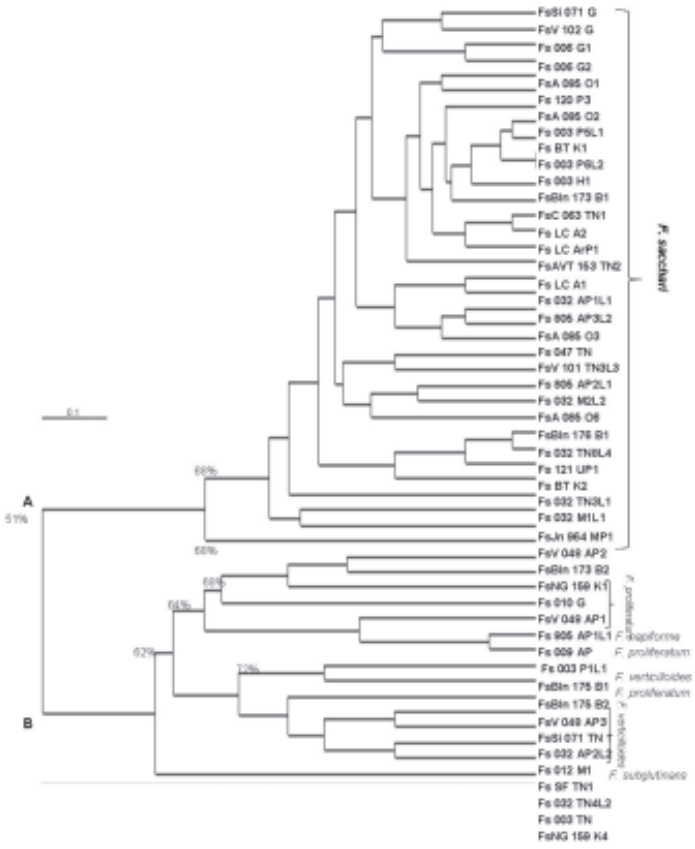


Fig. 6. Dendogram derived from ISSR fingerprints of 50 *Fusarium* isolates generated by UPGMA. Dendrogram produced by relationship between the *Fusarium* isolates based on ISSR amplification. The amplified products were converted to a distance matrix using Jaccard coefficient. The distance matrices were used to construct the dendrogram by UPGMA using NTSYS-pc 1.8 version. Values at the nodes represent similarity percentage among the *Fusarium* isolates. *F. sacchari* (35 isolates) formed group A and species other than *F. sacchari* (15 isolates) are grouped in group B

isolates into two groups (Fig. 6). Cluster A comprised 35 isolates and the remaining 15 isolates formed cluster B at a similarity value of 0.51. Of the 15 *Fusarium* isolates in cluster B, Fs 010 G, Fs 805 AP1L1, FsV 048 AP1, Fs 009 AP, Fs 003 P1L1, FsBln 175 B1 and FsBln 175 B2 formed a subgroup at a similarity value of 0.68. FsV 048 AP3, Fs 032 AP2L2, Fs 032 M2 L2, Fs 032 TN4L2, Fs 003 TN, FsSi 071 TN1 and Fs SF TN1 formed a separate sub cluster within cluster B at a similarity value of 0.72. FsNG 159 K4 out-grouped from both the sub clusters in cluster B, at a similarity value of 0.62.

Phenotype vs genotype relationship

Cultural and morphological characters of the *Fusarium* isolates were not sufficient to identify the species of the pathogen. Phenotypical characters of the 50 isolates resembled those of *F. sacchari, F. subglutinans, F. proliferatum* and *F. verticillioides. F. sacchari* and *F. subglutinans* resemble each other and is difficult to distinguish them based on cultural characters. Morphologically, formation of chains of microconidia by *F. verticillioides* and *F. proliferatum* delineate them

from *F. sacchari* and *F. subglutinans*. However, *F. proliferatum* also formed false heads and this could lead to wrong identification of *F. proliferatum* as *F. sacchari* or *F. subglutinans*. Four different molecular tools were used for the characterization of 50 *Fusarium* isolates. Fs 003 P1L1 not only formed chains of conidia inconsistently but also formed false heads (results not shown). On the other hand, Fs 805 AP1L1 and FsBln 175 B2 also formed false heads and these three isolates were separated from other isolates by rDNA ITS sequencing. rDNA ITS sequencing confirmed the identity of *F. proliferatum* by nucleotide insertions and resolved the confusion in understanding the basis of grouping the isolates in cluster B in the other three molecular tools used (results not shown). RAPD and ISSR were consistent in dividing the 50 isolates into two groups, and *F. verticillioides* and *F. proliferatum* were included in group B that is comprised by seven other isolates in RAPD dendrogram and 12 other isolates in dendrogram generated by ISSR. Majority of the isolates (70-80%) in ISSR and RAPD dendrogram formed group A. Nucleotide BLAST results of rDNA-ITS sequences confirmed that the other isolates in group B were similar to *F. napiforme, F. proliferatum* and *F. oxysporum* and the isolates in group A were similar to *F. sacchari.* However, rDNA-ITS sequencing failed to separate *F. verticillioides* and nucleotide BLAST results showed similarity of these isolates with *F. sacchari* and *F. subglutinans* (results not shown). Cultural characters together with the molecular data support the identification of the pathogen as *F. sacchari.* Of the 50 isolates, 37 isolates were distinguished conclusively as *F. sacchari*, six were confirmed as *F. proliferatum* and six were identified as *F. verticillioides* using the dendrogram generated from the three molecular tools used. FsBln 175 B1 that produced papillate macroconidia was clustered with *F. verticillioides* in RAPD and *F. proliferatum* in ISSR; however, BLAST of rDNA-ITS sequence exhibited greatest similarity of 99% with *F. napiforme*. This study very clearly revealed that majority of the 50 isolates belonged to *F. sacchari* and a few isolates belonged to *F. proliferatum* and *F. verticillioides*. Probably this is the first detailed study that established the ideality of sugarcane wilt pathogen at molecular level.

Discussion

Conflicting claims have been made regarding the true causal organism of sugarcane wilt as species of *Fusarium*, *Cephalosporium* and *Acremonium* by different authors. Even though the disease was recorded long back with substantial loss to sugarcane production, aspects of pathogen(s) involved and its variation were not investigated (Viswanathan 2013). The confusion was sorted out by Gams et al. (1971) and Nirenberg (1976) who established that *F. sacchari* causes sugarcane wilt. However, further studies were not carried out to establish the pathogen associated with the disease, pathogenicity and pathogenic variation. Species of *Acremonium* are common in substrates such as soil, plant debris and rotting mushrooms. But we failed to recover *Acremonium* from nodal tissues of various cane samples collected from tropical and subtropical regions and only *Fusarium* were recovered from both nodal and internodal tissues (Viswanathan et al. 2006, 2011). Further, cultural and morphological characteristics of majority of 263 isolates from different regions revealed that *F. sacchari* is the most commonly isolated wilt fungus in sugarcane (Poongothai et al. 2014 a,b).

In the present study, all the molecular markers separated six isolates that formed chains of conidia in group B. Although morphological characters support their presence in the group, the reason for the clustering of a few other isolates in group B is

not easy to explain with mere cultural and morphological data. In studies of molecular diversity of *F. verticilloides* isolated from different hosts (Kini et al. 2006), the profile varied not only among the isolates belonging to the same host but also between the isolates obtained from different hosts. We had similar observations of *F. sacchari* isolates and other *Fusarium* isolates like *F. verticilloides* isolated from wilt infected sugarcane stalks. This variation among the isolates belonging to the same species clustered the organisms further into many subgroups in the dendrogram. Chelkowski et al. (1999) in their RAPD studies showed species specific bands with toxigenic cereal pathogens *F. culmorum*, *F. graminearum*, *F. crookwellense* and *F. avenaceum*. Similarly our molecular profile also separated *F. sacchari* isolates from *F. verticilloides, F. proliferatum, F. oxysporum* and *F. subglutinans*. Prasad et al. (2007) grouped species and formae speciales of *F. oxysporum* f. sp. *carthami* belonging to eight different sections by RAPD. Similarly, we demarcated 50 isolates in to different species in separate clusters. Comparison of ITS sequences with database sequences clearly proved that 16 out of 26 isolates matched with *F. sacchari* and the remaining 10 isolates belonged to different species (R. Viswanathan, unpub. data). The isolates other than *F. sacchari* were established conclusively by the nucleotide BLAST results of rDNA-ITS sequence. However, rDNA-ITS alone cannot be used to demarcate the species as rDNA ITS sequence of *F. verticilloides* was similar to *F. sacchari*. However, rDNA-ITS sequencing is an easy way to identify the species and has been widely accepted to study phylogenetic relationships (Hibbett 1992). ITS sequences of four isolates were homologous and differed from others with nucleotide insertions of 9 bp and BLAST results proved that the isolates are similar to *F. proliferatum* (R. Viswanathan, unpub. data). This nucleotide variation was supported by Waalwijk et al. (1996) who studied variation in ITS region of *Fusarium* belonging to different sections which was observed to be discordant and they also grouped *F. proliferatum* in a separate group from *F. sacchari* and *F. subglutinans*. In rDNA-IGS analysis, we found three enzymes to be useful in studying the variation in IGS region of *F. sacchari* isolates. Similarly, this technique was used earlier in characterizing *F. oxysporum* f. sp. *ciceris, F. oxysporum* forma specialis and *F. culmorum* (Chakrabarti et al. 2001; Kim et al. 2001; Mishra et al. 2002). As in RAPD, the morphologically dissimilar isolates were separated out in IGS-RFLP, but with many clusters. Lesser polymorphism of IGS-RFLP than ISSR and RAPD supports the idea that more number of restriction enzymes would further group the isolates into reliable groups. Patino et al. (2006) also discriminated molecular variability in Fumonisin producing and non-producing strains of *F. verticillioides* using *Eco*RI digestion. Our IGS haplotypes correlated with morphology and geographical origin had no influence on the clustering as has been observed by Llorens et al. (2006). The IGS haplotypes obtained in their study with restriction enzymes *Cfo*I, *Alu*I, *Hap*II, *Xho*I, *Eco*RI and *Pst*I permitted to discern *F. oxysporum, F. graminearum, F. culmorum, F. cerealis, F. poae* and *G. fujikuroi* complex isolates but the restriction patterns of the IGS region did not show any relationship with the geographic origin of isolates.

Prasad et al. (2007) found the DNA banding pattern specific to formae speciales of *F. oxysporum* and it was different from other *Fusarium* species by RAPD markers and ISSR 10 primer. Similarly, RAPD markers and ISSR1, 5 and 9 banding patterns in our study demarcated species and intraspecific variation among *Fusarium* isolates. The dendrogram generated by ISSR amplification correlated with the RAPD in separating out morphologically dissimilar

isolates. Bayratkar et al. (2008) found that ISSR and RAPD data sets provided a substantially similar discrimination among Turkish isolates and divided *F. oxysporum* f. sp. *ciceris* isolates into three major groups as has been observed in our studies which divided the same set of *Fusarium* isolates into two groups in both RAPD and ISSR. However, ISSR has been found to be more reliable than RAPD and grouping of our isolates into clusters by ISSR has correspondence with morphological characters. Dinolfo et al. (2010) observed that ISSR showed high intraspecific variability within *F. poae* isolates but did not reveal a clear relationship between variability and the host/geographic origin. ISSR markers generated from the Alberta 1998 and Saskatchewan 2000 populations of *F. pseudograminearum* by Mishra et al. (2006) resulted in genetic differentiation and gene flow values similar to those obtained with the IGS data. In our studies too genetic differentiation of *Fusarium* isolates in ISSR was same as that of IGS.

In all the three different molecular tools used for characterization, morphologically distinct isolates formed separate clusters and isolates of *F. sacchari* grouped together in a cluster. Within this cluster, due to intraspecific variation, *F. sacchari* isolates were further grouped into many sub clusters. In all the three, the chain forming species separated in a cluster with minor variation. We could not bring out the basis of intraspecific variation within *F. sacchari* isolates. As many studies reported molecular characterization in relation to production of secondary metabolites such as gibberellins or toxins like fumonisin, trichothecene, zearlenone, etc. (Nelson et al. 1994; Mitter et al. 2002), the basis for intraspecific grouping in our *F. sacchari* isolates could also be chemotypic which needs further studies. The grouping of isolates in the dendrogram and percent of polymorphism observed implies that ISSR is a more reliable technique compared to RAPD and IGS-RFLP. Based on all these results, it is inferred that ITS sequencing combined with three ISSR primers will be highly helpful in delineating the phylogenetic relationship of *Fusarium* population infecting sugarcane. The reason for higher polymorphic pattern in the tools used may probably be the collection of different isolates across the country and wide range of sugarcane varieties used for isolation of the pathogen.

All the 50 *Fusarium* isolates used for molecular characterization were isolated from wilt-infected sugarcane stalk tissues. While sampling, we could not determine virulence or pathogenicity of the isolates since the infected stalks dried either partially or completely. These isolates represented different geographical regions in India and the host cultivars also varied from place to place. In our previous study, testing of a sub-set of isolates revealed pathogenicity of *F. sacchari* in sugarcane (Viswanathan et al. 2011). In that, *F. sacchari* isolates Fs 002 G1, Fs 006G1, Fs 032 G1, Fs032 G2 and Fs 032 TN4L2 were highly virulent on the host. Re-isolation of the pathogenic and non-pathogenic isolates from the artificially inoculated canes and molecular analyses with specific ISSR and RAPD markers confirmed that variation in molecular profile of the isolates correlated with pathogenicity and taxonomy. We also found that some of the *F. sacchari* isolates like Fs LCA1, Fs010 G, Fs C063 TN1 and Fs NG159 K1 exhibited non-pathogenic profile. Similarly, the isolates that belonged to species *F. verticilloides, F. proliferatum*, *F. napiforme* or *F. subglutinans* were less virulent or non-pathogenic on sugarcane in our earlier study (Viswanathan et al. 2011). Our detailed investigation taken up to define genetic variability among the *Fusarium* population associated with sugarcane wilt in India correlates with earlier established taxonomy and pathogenicity data and clearly indicated *F. sacchari* as the major

causative organism of sugarcane wilt. This is the first comprehensive work carried out on the molecular variability in *F. sacchari* associated with sugarcane wilt.

Acknowledgements

The financial support received from ICAR, New Delhi, as part of Network Project on Wilts of Crops is gratefully acknowledged. The authors are also thankful to Dr. N.V. Nair, former Director of the Institute for his encouragement and facilities.

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