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Determination of Genetic Divergence Based on DNA Markers Amongst Monosporidial Strains Derived from Fungal Isolates of Karnal Bunt of Wheat

J. M. Seneviratne[1], Atul K. Gupta[1], Dinesh Pandey[1], Indu Sharma[2] and Anil Kumar[1]*

[1]*Department of Molecular Biology and Genetic Engineering, G.B. Pant University of Agriculture and Technology, Pantnagar 263 145, India*

[2]*Department of Genetics and Plant Breeding, Punjab Agricultural University, Ludhiana, India*

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Genetic variation among the base isolates and monosporidial strains derived from these isolates of *Tilletia indica*- the causal agent of Karnal bunt (KB) in wheat, was analyzed by morphological, growth behaviors and RAPD-ISSR based molecular polymorphism. Genetic make up of fungal cultures vary among each other. The magnitude of variation in KBPN group is less (narrow genetic base) when compared to the other groups KB3, KB9 and JK (broad genetic base) reflecting that variability is a genetically governed process. The generation of new variation with different growth characteristics is not a generalized feature and is totally dependant on the original genetic make-up of the base isolate generating new monosporidial strains. Thus, it can be concluded that monosporidial strains derived from mono-teliosporic isolate, consists of genetically heterogeneous population. The morphological and genetic variability further suggests that the variation in *T. indica* strains is predominantly derived through the genetic rearrangements through para sexual means.

Keywords **:** DNA polymorphism, fungal variability, genetic divergence, ISSR, RAPD

The pathogen, *Tilletia indica*, Syn. *Neovosia indica* causes the disease Karnal bunt in wheat, durum wheat and triticale which is sometimes called partial bunt because only part of the kernel is usually affected. Unlike the cereal rusts, the disease never caused severe production losses but it always existed, causing concerns intermittently. But this cosmetic disease of wheat has become a quarantine concern interfering with free and fair grain trade as it causes reduction in grain quality and market acceptability of the produce. Management of the disease has become very much crucial due to unavailability of tolerant wheat cultivars and the mode of dispersal of the pathogen, survival of teliospores in soil for a long period. Wheat shows considerable variation in degree of susceptibility among cultivars and in turn *T. indica* exhibits high level of genetic variability among the isolates (Mishra et al., 2001) exhibiting varying degree of virulence. The development of resistant wheat varieties with a wide genetic base claims thorough knowledge of molecular mechanism in the development of variability in *T. indica*. The variability in *T. indica* has been documented on the basis of pathogen morphology, cultural characteristics and temperature response and pathogenicity tests (Gill et al., 1993).

T. indica has emerged as a useful model organism for studying the role of development in pathogenesis. In the life cycle of *T. indica* distinct developmental phases i.e. sporidial phase, vegetative mycelia phase and teliospore phase have been described to play different roles. The direct role of mating in pathogenesis of the smut fungi comes from the fact that fusion of haploid cells of opposite mating types is generally a pre-requisite that proliferates within host tissues. In addition, mating function may also be required at subsequent stages when karyogamy takes place within dikaryotic mycelium, when sporulation occurs to yield diploid teliospores which increase the chances of variation. Studying mechanisms of genetic variation in *T. indica* is therefore likely to be critical for the future control of the pathogen.

Understanding the genetic variability in pathogen is extensively achieved through the use of genetic markers. DNA polymorphism generated through very popular genetic markers viz. random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) have been proven to be important in detecting genetic and phylogenetic relationship in a wide range of organisms (Mishra et al., 2001; Majer et al., 1996; Milbourne et al., 1997; Powel et al., 1996). However, RAPD and SSRs generally require less development efforts than RFLP and AFLP and usually yield many more bands and therefore represent many more loci (Milbourne et al., 1997).

The ISSR or random amplified microsatellites (RAMS) technique, originally described by Zietkiewicz et al. (1994) to analyze genetic diversity in plants and animals, has also

*Corresponding author.
Phone) +91-5944-233898, FAX) +91-5944-233473
E-mail) ak_gupta2k@rediffmail.com

proven to be a powerful tool for unraveling the diversity existed in fungi (Hantula et al., 1996). This technique combines the simplicity of the RAPD approach, the reliability of bands derived from known heritable domains of the genome, and the potential to differentiate populations or recently diverged species (Zhou et al., 2001). In the ISSR method, the DNA between the distal ends of two closely located microsatellites is amplified in multiple loci throughout the genome, using primers containing microsatellite sequences with or without degenerate anchors at the 5' ends. Each amplified band corresponds to a unique DNA sequence delimited by two inverted microsatellites. This method produces polymorphic patterns between different individuals and was found useful in describing genetic diversity in several groups of fungi, such as: *Phytophthora cactorum* (Hantula et al. 1997), *Claviceps* spp. (Tooley et al., 2000), *Botryosphaeria* spp. (Zhou et al., 2001), *Phaeoisariopsis griseola* (Mahuku et al., 2002), *P. citrophthora* (Cohen et al., 2003), *Fusarium culmorum* (Mishra et al., 2003), *Rhizoctonia solani* (Elbakali et al., 2003), *Colletotrichum lindemuthianum* (Mahuku and Riascos 2004), *Gremmeniella* spp. and *Phomopsis* spp. (Borja et al., 2006).

The main objectives of the present work were: (1) to test the utility of the RAPD and ISSR method as tools to study genetic diversity amongst Karnal bunt isolates and monosporidial strains (2) to study the relationship of genetic variation with morphological development and growth behaviors.

Materials and Methods

Collection of KB isolates and preparation of monosporidial strains. Three mono-teliosporic cultures *T. indica* isolates KB 3, KB 9 and JK prepared as described earlier (Geeta et al., 2000), collected from Delhi, Punjab, Jammu & Kashmir provinces were received from Indian Agricultural Research Institute, New Delhi, India and one monoteliosporic culture of KBPN isolate was collected from Uttarakhand province and obtained from Wheat Pathology Lab, Department of Plant Pathology, College of Agriculture, G. B. Pant University of Agriculture and Technology, Pantnagar, India. Fungal isolates showing differential pathogenicity patterns on host differentials were cultured at Molecular Markers Laboratory, Department of Molecular Biology and Genetic Engineering, G. B. Pant University, Pantnagar, India on modified potato dextrose agar (PDA) and potato dextrose broth (PDB) medium in Petri plates and flasks respectively. The cultures were incubated in BOD incubator at 22±1 °C under light and dark conditions.

After 21 days of growth of fungus in PDA, sporidia were collected separately by decanting and germinated on the same media. Single germinating sporidia were randomly picked microscopically from two petriplates of same culture grown on PDA and cultured as individual mono sporidial cultures. Two mono sporidial strains from KB 3 base isolate (KB3msa, KB3msc), seven mono sporidial strains from KB 9 base isolate (KB9ms1-KB9ms7), six mono sporidial strains from JK base isolate (JKmsa-JKmsf) and five mono sporidial strains from KBPN base isolate (KBpn1, KBpn3, KBpn5, KBpn6 and KBpn8) were prepared and used in this study.

Harvesting of mycelium. The growing liquid cultures of *T. indica* were harvested at desired time intervals. The media containing the mycelial mat of *T. indica* was filtered through a folded muslin cloth and washed several times in PBS (0.05 M, pH 7.2) and it was followed by washing with sterilized distilled water. The wet weight of the mycelial mass was taken and the wet masses were lyophilized for 5 hours to obtain the dry weight. Dried mycelial masses were stored at −20 °C until use.

Harvesting of sporidia for detection of developmental stage dependent variation. Developmental stage dependent variations were detected by isolation and counting of sporidia by haemocytometer at 21st days of growth. The sporidia were harvested from the 21 days old fungal cultures by decanting method. 10 ml of sterilized distilled water was added to the plates and gently moved back and forth and water containing sporidia was decanted to sterilized Oakridge tubes and centrifuged at 4000 rpm for 10 min to get sporidial pellet. The pellets were re-suspended in minimal amount of sterilized distilled water and stored at −20 °C.

Determination of growth kinetics and microscopic examination. All 24 fungal cultures were cultured on solid as well as liquid media. Radial diameters of mycelia were measured at 3 days intervals. In order to study the fungal morphology through microscopic examination, following method was used. Some mycelia were picked from the cultures at desired 7 days and 21 days interval and placed on the glass slides. A drop of cotton blue was added over the fungal materials and teased to get an even and thin spread over the slide. Then, a drop of Lactophenol was added to fix the slide and cover slip was placed to prepare temporal mounts for microscopic examinations.

DNA extraction and quantification. In order to isolate DNA from the fungus *T. indica*, a modified procedure based on cetyltrimethyl ammonium bromide (CTAB) method was used (Murray and Thompson, 1980). The DNA was purified and quantified by the method as described by Sambrook et al. (1989).

Primers used. A set of 30 decanucleotide RAPD and 19 ISSR primers synthesized and purchased from Life Technology were employed for PCR amplification. The sequence and the details of primers are given in the Table 1 and 2.

PCR amplification through RAPD and ISSR Marker. PCR was carried out in 25 µl reaction mixture containing 20 ng of genomic DNA, 0.3U Taq DNA polymerase enzyme, 200 µM each dNTPs, 10 mM Tris-HCl, 1.5 mM Mg Cl_2 and 0.2 µM primer, 30 ng from a single primer and 50 ng from each template DNA was added in each tube. The PCR amplification was carried out three times in a Biometra T-gradient DNA thermocycler. For RAPD amplification, PCR cycle was programmed as follows: initial denaturation of 5 min at 94°C; 37 cycles of denaturation at 94°C for 1 min, annealing at 41°C for 1 min and extension at 72°C for 2 min; followed by a final extension at 72°C for 7 min. In order to perform ISSR amplification, initial denaturation of 5 min at 94°C; 45 cycles of denaturation at 94°C for 1 min, annealing at 41°C for 1 min and extension at 72°C for 2 min; followed by a final extension 72°C for 10 min were utilized. The amplified products were stored at –20°C until they were subjected to electrophoresis. PCR products were electrophoresed in triplicate in 1.6% agarose gel in 0.5X TAE buffer along with 100 bp DNA marker at the concentration of 250 ng/ml to identify respective bands. The only reproducible bands were scored manually.

Dendrogram, UPGMA and Principle coordinate analysis.

Table 1. Details of RAPD primers used for the molecular characterization of *T. indica*

S. No.	Primer code	Primer Sequence	Amplified product (bp)	Total bands	Mono bands	Poly bands	% Poly.	Unique Band (bp)
1	R1	GAA ACG GGT G	100-1350	15	–	15	100	–
2	R 3	GGG TAA CGC C	275-1300	16	2	14	87.5	1(KB3msc)
3	R 4	GTG ATC GCA G	150-1350	17	–	17	100	–
4	R 5	TGC CGA GCT G	225-1300	16	–	16	100	–
5	R 6	AGT CAG CCA G	200-1300	14	1	13	92.85	–
6	R 7	AAT CGG GCT G	300-1250	15	–	15	100	–
7	R 8	CAA TCG CCG T	200-1400	17	2	15	88.2	2(KB3msc,KB9)
8	R 9	CAG CAC CCA C	200-1500	16	–	16	100	1(KB9)
9	R 10	TTC CGA ACC C	400-1350	14	–	14	100	2(KB3msc)
10	R 15	AGT CAG CCA C	250-1500	15	–	15	100	–
11	R 16	GTG AGG CGT C	250-1600	18	–	18	100	–
12	R 17	TGG ACC GGT G	250-1500	10	–	10	100	3(KB9)
13	R 18	GAC GGA TCA G	200-1325	16	–	16	100	3(KBPN,KB3msc)
14	R 22	TTG GCA CGG G	250-1500	18	–	18	100	2(KB9)
15	R 27	AGG GCG TAA G	200-1600	16	–	16	100	2(KB9ms1)
16	R 28	GAG AGC CAA C	200-1600	18	–	18	100	–
17	R2	GTG ACG TAG G	300-1250	8	8	–	0	–
18	R11	AGG TGA CCT G	300-1250	8	8	–	0	–
19	R12	GTT GCG ATC C	200-1500	6	6	–	0	–
20	R13	AGG TGA CCG T	400-1350	10	10	–	0	–
21	R14	CAG GCC CTT C	300-1250	6	6	–	0	–
22	R19	GGA CCC AAC C	400-1350	6	6	–	0	–
23	R20	GTC GCC GTC A	300-1250	8	8	–	0	–
24	R21	TGA GAG GAC A	200-1500	8	8	–	0	–
25	R23	GTG TGC CCC A	300-1250	6	6	–	0	–
26	R24	AGC GCC ATT G	200-1500	6	6	–	0	–
27	R25	GGG GTG ACG A	0	–	–	–	–	–
28	R26	GGA CTG CAG A	0	–	–	–	–	–
29	R29	GAT CCT GGA G	0	–	–	–	–	–
30	R30	GGC CCG ATG G	0	–	–	–	–	–
TOTAL			-	306	77	249	–	–
AVERAGE			228-1420	11.7	6.41	15.56	60.32	–

Table 2. Detail of ISSR primers used for the molecular characterization of inter microsatellite region of *T.indica*

S. No.	Primer code	Primer Sequence	Amplified product (bp)	Total bands	Mono bands	Poly bands	% Poly.	Unique Band
1	I-4	((AG)(8) T)	300-1350	13	–	13	100	1(KB3)
2	I-6	((GA)(8) A)	350-1350	15	–	15	100	1(KB9)
3	I-9	((AC)(8) T)	500-1600	9	–	9	100	–
4	I-10	((GA)(8)Y T)	300-1600	17	–	17	100	–
5	I-11	((AC)(8) YG)	500-1600	11	–	11	100	–
6	I-15	(HVH (TG) 7)	200-1400	16	4	12	75.0	–
7	I-18	((CTC)(8))	500-1300	6	–	6	100	1(KB9ms6)
8	I- 20	((GGGTG)(3))	400-1400	14	–	14	100	2(KB9ms6,KB3mscC)
9	I-13	(BHB (GA)(7))	200-1400	15	–	15	100	1(KB9)
10	I-1	(HVH(TG)(7))	200-1400	12	12	–	0	
11	I-2	(GA(8)T)	350-1350	10	10	–	0	
12	I-3	((GA)(8)G)	200-1400	11	11	–	0	
13	I-5	((AG)8G)	200-1400	13	13	–	0	
14	I-7	((CT)8G)	350-1350	12	12	–	0	
15	I-8	((TC)8G)	200-1400	10	10	–	0	
16	I-12	(HBH(AG)7)	350-1350	8	8	–	0	
17	I-14	(VHV(GT)7)	–	–	–	–	–	–
18	I-16	((CT)8G)	–	–	–	–	–	–
19	I-20	((GGGTG)3)	–	–	–	–	–	–
TOTAL				192	80	112	54.68	–
AVERAGE			416-1450	12.0	0.44	12.44	–	–

Fragments amplified by RAPD & ISSR were visually scored as present (1) or absent (0), fragments with the same size were considered equal. The similarity indices were generated after multivarient analysis using Nei and Li's Coefficient (Nei and Li, 1979). The similarity matrix was used to construct dendrograms with the help of the Unweighted Pair Grouping by mathematical averaging (UPGMA) method using NTSYS-pc version 2.1 software. Cophenetic values and Principle Coordinates Analysis were performed. The analysis work was based on Jacquard's similarity coefficient given below.

$$\text{Similarity coefficient} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}}$$

Results and Discussion

Fungi cause the most serious diseases of plants and display greater complexity and diversity of form than other microbial pathogens. This diversity is highlighted by the extraordinary host range observed by some pathogens and the extreme pathogenic specialization of others. Variability in the pathogen *T. indica* has been established by using single teliospore cultures but the stability of such pathogens to be proved as pathogen is heterothallic organism, primary or secondary sporidia or hyphae as compatible mating types must fuse to form dikaryon which readily increases the chances of variation due to heterozygosity. The greater frequency of out-crossing in *T. indica* suggests that intraspecific and inter-specific hybridization in *T. indica* presumably facilitates sexual recombination and greater genetic diversity. Knowledge of diversity of the Karnal Bunt pathogen in the form of distinct monoteliosporic and monosporidial cultures are considered essential before effective measures for its management are developed and understood. In the present investigation, attempts have been made to study the morphological and genetic changes of isolates and monosporidial strains of *T. indica* grown in culture medium. More emphasis has been categorically given to detect the variation using RAPD-ISSR based molecular tools among the monosporidial strains in order to understand the mechanism of genetic polymorphism in *T. indica.*

Determination of growth kinetics. As indicated in Fig. 1 (A) and (B), drastic variations in total biomass production as well as radial diameter were observed in all base isolates and monosporidial strains. JK, KB3 and KB9 group of strains showed much variation in their growth patterns; however KBPN group indicated very less variation amongst their monosporidial strains. It is quite interesting to note that some slow growing monosporidial strains (like KB9ms5) were isolated from fast growing isolate (KB9). KB3 iso-

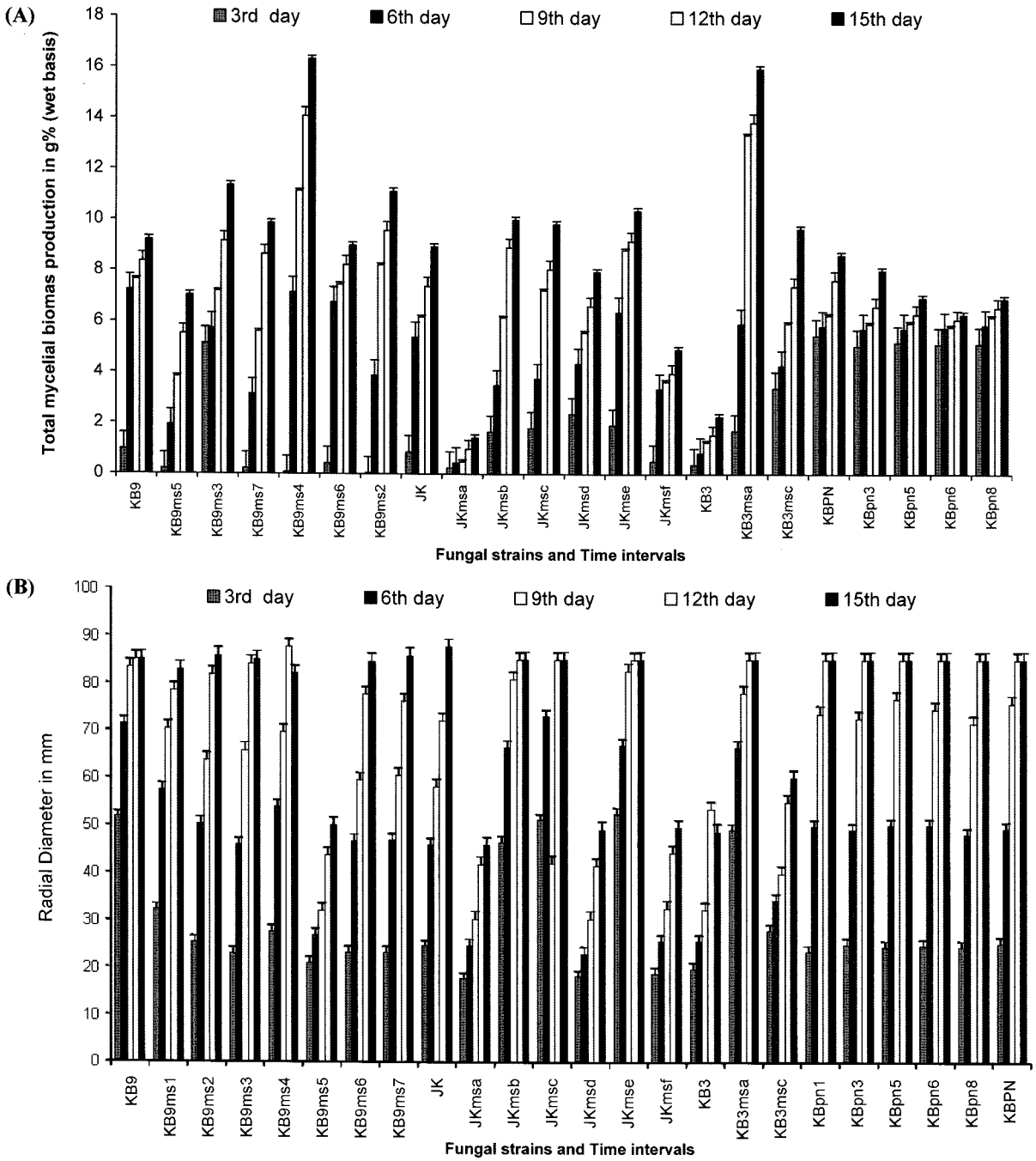

Fig. 1. (A) Total biomass production in g% on wet basis and (B) Radial diameters in mm of 4 isolates and 20 monosporidial strains of *T. indica* at different time intervals of their growth.

late showed slow growth, however monosporidial strains (KB3msa and KB3msc) generated from this showed significantly fast growth. Statistical analysis performed on each time interval indicated that the variation is highly significant ($P>0.05$) at five time intervals studied. Based on the total biomass and radial diameter at sixth day of the growth, the total 4 base isolates and 20 monosporidial strains were grouped as follows.

Fast growing (>4* and >50**): KB9, KB9 ms1, KB9 ms4, KB9ms6, KB9ms3, JK, JKmse, KB3msa, KBPN, KBpn1, KBpn3, KBpn5, KBpn6, KBpn8.

Moderate (2-4* and 25-50**): KB9ms7, KB9ms2, JKmsb, KB3msc

Slow growing (<2* and <25**): KB9ms5, JKmsc, JKmsd, JKmsf, KB3

*Total biomass production at 6th day (g % mycelial wt on

wet basis)
**Radial diameter at 6th day (mm)

Most of the fungal cultures used in the present study follow a linear pattern in time course increment in both total biomass production and radial diameter. It is pertinent to note that KBPN isolate and all monosporidial strains in KBPN group follow a pattern of fast growth behavior. At the sixth day of their growth, these strains showed fast growth but there after sudden retardation in total biomass and radial diameter increment was observed with the time course. This observation was made from an *in vitro* study

Table 3. Morphological variation of fungal isolates and monosporidial strains under microscopic observation

S. No.	Isolates/ Monosporidial strains	Sporidial count (Number×10^6)	Morphological Characteristics	
			7 days after inoculation	21 days after inoculation
1.	KB3	3.2	Extremely thin and some thick mycelium, moderate allantoid sporidium production	Thin mycelium with heavy allantoid sporidium production
2.	KB3msa	52.7	Thin and thick mycelium few allantoid sporidium production	Thin mycelium few sporidium production
3.	KB3msc	1.8	Thin mycelium with heavy filiform sporidium production	Thin and thick mycelium with heavy filliform and some allantoid sporidium
4.	KB9	37.0	Thin and thick mycelium few allantoid sporidium	Thin mycelium few allantoid sporidium
5.	KB9ms1	5.4	95% filiform sporidium production and few allantoid sporidium thin mycelium	Thin and thick mycelium, all filiform sporidium.
6.	KB9ms2	3.3	Thin and thick mycelium few allantoid sporidium production	Thin and thick mycelium few allantoid sporidium production
7.	KB9ms3	2.3	Thin and thick mycelium	Thin and thick mycelium few allantoid sporidium production
8.	KB9ms4	1344.0	Thin and thick mycelium	Thin mycelium and chlamydospore formation, moderate allantoid production
9.	KB9ms5	672.0	Thin and mycelium	Thin mycelium, moderate allantoid sporidium production
10.	KB9ms6	918.0	Thin and thick mycelium few allantoid sporidium production	Thin and thick mycelium moderate allantoid sporidium production
11.	KB9ms7	201.0	Thin mycelium moderate allantoid sporidium production	Thin and thick mycelium few allantoid sporidium production
12.	JK	87.0	Thin and thick mycelium, Chlamydospores formation	Mycelium thickening and moderate allantoid sporidium production
13.	JKmsa	3.7	Thin mycelium heavy allantoid sporidium production	Thin mycelium heavy allantoid sporidium production
14.	JKmsb	1630.0	Thin and thick mycelium few allantoid sporidium production	Thin mycelium, Chlamydospores formation and few allantoid sporidium formation
15.	JKmsc	217.9	Thin and thick mycelium, moderate allantoid and few filiform sporidium production	Mycelium thickening and few allantoid sporidium production
16.	JKmsd	37.5	Thin and thick mycelium heavy allantoid sporidium formation	Thin mycelium moderate allantoid sporidium production
17.	JKmse	8.6	Thin mycelium few allantoid formation	Thin mycelium heavy allantoid formation
18.	JKmsf	17.5	Thin mycelium heavy allantoid formation	Thin mycelium moderate allantoid formation
19.	KBpn1	3.8	Thin and thickened mycelium no sporidium formation	Mycelium thickening and few allantoid sporidium formation
20.	KBpn3	387.0	Thin mycelium moderate allantoid formation	Thin mycelium moderate allantoid formation
21.	KBPN5	432.0	Thin mycelium no sporidium formation	Mycelium thickening, chlamydospore formation and few allantoid sporidium formation
22.	KBpn8	328.0	Thin and thickened mycelium clamidospore formation	Mycelium thickening and few allantoid
23.	KBpn6	341.0	Thin mycelium	Mycelial thickening moderate allantoid
24.	KBPN	289.0	Mycelium thickening, Chlamydospores formation and few allantoid sporidium formation	Mycelial thickening moderate allantoid

carried out without replenishment of nutrients. High demand for the nutrients by a fast growing organism can be thought to explain the retardation of fungal growth when the culture media is not replenished. Further, formation of different monosporidial strains having differential growth behaviors is indicative of heterogeneous population derived from single isolate. It is interesting that the magnitude of variation in growth behaviors of mono-sporidial strains in KBPN group is less pronounced as compared to other groups of mono-sporidial strains (KB3, KB9 and JK). It can be concluded that the generation of new strains with different growth characteristics is not a generalized feature but totally dependant on the original genetic make up of the base isolate, which generated new types of monosporidial strains during fungal development.

Microscopic examination and enumeration of sporidia and mycelial growth variation. As indicated in Table 3, a significant variation was observed in both number and the form of the sporidium. Most of the cultures produced only one type of sporidia at 21 days except KB9ms6, KB9ms7, JK, JKmsb and KB3msa which produced both types (allantoid and filiform) of sporidia and also generated high variation in sporidial counts. Morphological variations of all fungal cultures were monitored at two different time intervals (7^{th} day and 21^{st} day). A clear variation was observed between the base isolates as well as among the monosporidial strains irrespective of their origin. All three fungal groups (JK, KB3 and KB9) showed a large variation in sporidial count as well as morphological features; however KBPN group showed uniformity in terms of morphology and sporidial count amongst themselves. Some fungal strains (JKmse) showed the formation of chlamydospores indicating transition in the developmental stage towards sporulation after sensing the starvation of nutrients.

Molecular Characterization of variation amongst isolates and monosporidial strains. *T. indica* exhibits high level of genetic variability among the isolates (Mishra et al., 2001) exhibiting varying degree of virulence. Understanding the mystery of genetic variability in pathogen is extensively achieved through the use of genetic markers. Such type of variability studies amongst fungal isolates with respect to virulence are very important for disease management and resistance breeding programmes. DNA markers such as RAPD & ISSR have been proven to be useful for the detection, differentiation and determination of phylogenetic relationship between isolates of a morphological species of several pathogens (Narayanasamy, 2001). The RAPD technique was applied to distinguish the pathogenic and nonpathogenic isolates of *Fusarium oxysporum* f.sp. *dianthi* causing wilt disease of carnation (Hernandez et al., 1999). In a later investigation, the analysis of genetic distance data showed that the pathogenic isolates tend to cluster together in one group, whereas the non-pathogenic isolates constituted another group (Zanotti et al., 2006). The genetic diversity among isolates of a worldwide collection of *Fusarium culmorum* and *F. graminearum* was assessed by RAPD fingerprinting (Schilling et al., 1994). The RAPD technique was applied to assess the genetic diversity among rice blast pathogens. The analysis of RAPD polymorphism on *M. grisea* isolates exhibited high level of polymorphism indicating a wide and diverse genetic base. A repeat sequence designated MGR 586 present in the genomic DNA of rice infecting strains of *M. grisea* and another retrotransposon named as *fosbury* have been used for differentiation of isolates of *M. grisea* (Schull and Hamer, 1994). The phylogenetic grouping based on the RAPD data did not seem to be congruent with geographical locations. A high genetic diversity was evident among the isolates due to spontaneous mutation and stress induced transposition which may be the main reason for genetic diversity within isolates of *M grisea* from Andhra Pradesh, Maharashtra and Karnataka states in India and could be due to different crop seasons (Chadha and Gopalakrishna, 2005). The results indicate that populations of *M. grisea* in India are heterogenous genetically and the RAPD technique can be effectively used to assess the interrelationship among the isolates.

RAPD

Twenty six of these primers out of 30 primers used in the present study showed a total of 306 reproducible bands. Each of the primer varied greatly in their ability to resolve variability among the genotypes. The individual primer produced bands in a range of 06 (R12, R14, R19, R23 and R24) to 18 (R28, R22 and R16) bands with an average of 16 bands per primer. Out of the 306 bands, 77 bands were monomorphic i.e. they were present in all fungal cultures (Table 1) showing polymorphism percentage of 60.32%.

The amplified fragment ranged from 100bp (primer R1) to 1600bp (primers R27, R16 and R28). Unique bands identified by primers R3 and R10 (for KB3msc), R8 (for KB3msc, KB9), R9, R17 and R 22 (for KB9), R18 (for KBPN and KB3msc) and R27 (for KB9ms1) were exclusive to these cultures which made it distinct from other fungal cultures. Presence of higher numbers of polymorphic bands (16) indicate that the fungal genome contains hot spots of variability in these regions defined by the used primers and the presence of monomorphic bands (10) indicate that the fungal genome is quite consistent in the regions defined by used primers and the primers which

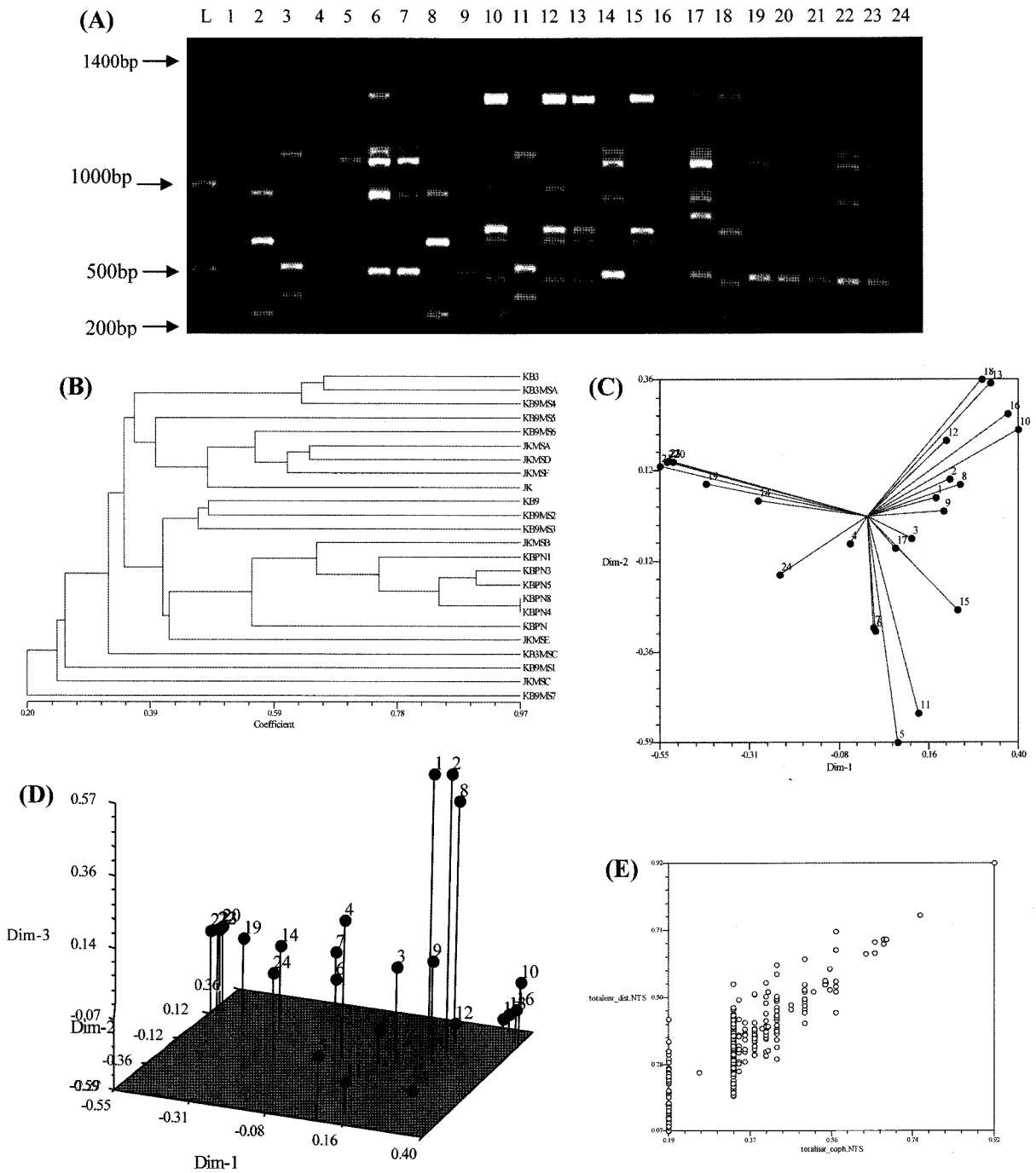

Fig. 2. (A) RAPD marker analysis through PCR amplification of fungal genomic DNA with R 03 primer, (B) Dendrogram constructed by using UPGMA based on Jacquard's Coefficient, (C) 2 D (D) 3 D (E) Matrix comparison with score plots Depiction of RAPD profiles of *T. indica* isolates and monosporidial strains.
L- 100bp Ladder, 1- KB3, 2- KB3msa, 3- KB3msc, 4- KB9, 5- KB9ms1, 6- KBms2, 7- KB9ms3, 8- KB9ms4, 9- KB9ms5, 10- KB9ms6, 11- KB9ms7, 12-JK, 13- JKmsa, 14- JKmsb, 15- JKmsc, 16- JKmsd, 17- JKmse, 18- JKmdf, 19- KBpn1, 20- KBpn3, 21- KBpn5, 22- KBpn8, 23- KBpn6, 24- KBPN

produced unique bands during amplification were considered as highly informative markers for identification of fungal cultures. The banding profile of primer R-03 is depicted in (Fig. 2A).

Similarity coefficient, Cluster analysis, Matrix Comparison and Mantel Test. A dendrogram was constructed based on the PCR amplification results. The UPGMA cluster analysis method was followed for the construction

of the phylogenetic tree (Fig. 2B). Principal coordinate analysis was performed in order to highlight the resolving power of the coordinates. Two and three dimensional views are depicted in (Fig. 2C & 2D), respectively. In the situation of total RAPD analysis Jaccard's similarity values between pairs of accessions ranged from 0.13 for KB9ms7 and KB3msa to 0.96 for KBpn4 and KBpn8. KB9ms7 monosporidial strain from KB9 isolate branched out from the base of the dendrogram leaving the rest fungal strains on multi storey clusters (Fig. 2B). JKmsc, KB9ms1and KB3msc branched out from the dendogram in stepwise manner leaving all the rest 20 strains on two major clusters. The dendogram readily grouped the other 20 strains into two main clusters and the cluster I was further divided in to two sub clusters. Sub cluster I had a group of three strains, KB3, KB3msa and KB9ms4 which branched out as a solitary strain. The second group of sub cluster I was further divided into several mini clusters showing varied degrees of similarities. Sub cluster II comprised of eleven strains which were further clustered in to several mini clusters with varying degrees of similarities. Isolates and monosporidial strains were grouped in to same clusters in most of the cases and in rare situation some monosporidial strains (JKmsb, JKmse, KB9ms4, KBms5 and KB9ms6) were clustered in to different clusters other than their origin.

The cophenetic value matrix generated from SAHN's cluster analysis was used for the 2-way Mantel test (z-statistic). The estimated cophenetic correlation (r) was 0.96179 indicating very good fit of the cluster analysis (Fig. 2E). The approximate Mantel t-test revealed that t value equal to 8.8314 and the probable random Z was less than observed Z exhibited significant correlation between similarity matrix and cluster analysis.

Molecular characterization of monosporidial strains through RAPD indicates very high variation within and between the groups. It is quite interesting to notice the generation of new fast growing strains from slow growing base isolates as well as onset of slow growing strains from fast growing base isolates. KBPN is profoundly important as it shows unique features in genetic modulation. All strains of KBPN group show very low genetic variation between each other indicating very low genetic rearrangements taking place in this genome. It can be hypothesized that this phenomenon can be due to the presence of low levels of recombination pockets in the fungal genome compared to all the other monosporidial groups (KB9, KB3 and JK) which showed comparatively very high levels of genetic rearrangements. In general, phenotypic characters are well modulated by the environmental effects. But the phenotypic features detected in terms of growth kinetic studies are in well accordance with this molecular characterization features. Total biomass as well as radial diameter of fungal strains is highly fluctuated in all monosporidial groups except KBPN monosporidial group indicating that the low genetic variability observed here is well confirmed through those morphological features.

ISSR

A total of 192 bands were detected using 19 ISSR primers out of which 112 bands were polymorphic, 80 were monomorphic (Table 2). The number of amplified bands varied from 6 bands in primer ISSR 18 to a maximum of 17 bands in primer ISSR 10, with an average of 12 bands per primer while the amplified fragment ranged from 200bp (primers I 13, I 15 and I 6) to 1600 bp (primers I 10, I 11 and I 9) with an average of 416bp to 1450bp. A total six unique bands were obtained with primers I 4 (for KB3), I 6 (for KB9), I 18(for KB9 ms6), I 20 (for KB9ms6 and KB3msc) and I 13(for KB9). Out of nineteen primers tested seven primers amplified only monomorphic bands indicating the presence of stagnant regions of variability. The 9 primers amplified polymorphic bands indicate the presence of hot regions of variability in fungal genome. The banding profile of primer I-15 is depicted in (Fig. 3A).

Similarity coefficient and cluster analysis. Jaccard's similarity coefficients, estimated using nine ISSR primers ranged from 0.07% between pairs KB9 and KB 3 to the maximum of 92% between pairs KBpn8 and KBpn4. Thus the result revealed closeness between KBpn8 and KBpn4 and diversity between KB9 and KB9ms7 isolates.

Phylogenetic tree generated using SAHN cluster analysis and UPGMA method is shown in (Fig. 3B). All the 24 strains were clearly separated by dendrogram. Two and three dimensional views are depicted in (Fig. 3C & 3D), respectively. Clustering feature in total ISSR dendogram is much more pronounced compared to the total RAPD dendogram and at the very first level two major clusters are formed showing 0.19 similarity coefficient. First main cluster remains as it is through out the dendogram while the second main cluster is grouped in to two another main sub clusters.

Clustering feature in total ISSR dendogram is much more pronounced compared to the total RAPD dendogram and at the very first level two major clusters are formed showing 0.19 similarity coefficient. The first main cluster is not sub divided while the second main cluster is grouped in to two another main sub clusters in the dendrogram. In the situation of total RAPD analysis, Jacquard's similarity values between pairs of accessions ranged from 0.07 for KB9ms 7 and KB3msa to 0.92 for KBpn4 and KBpn8. In most of the situations main isolates and monosporidial strains originated from the same are clustered together (KB3, KB3msa,

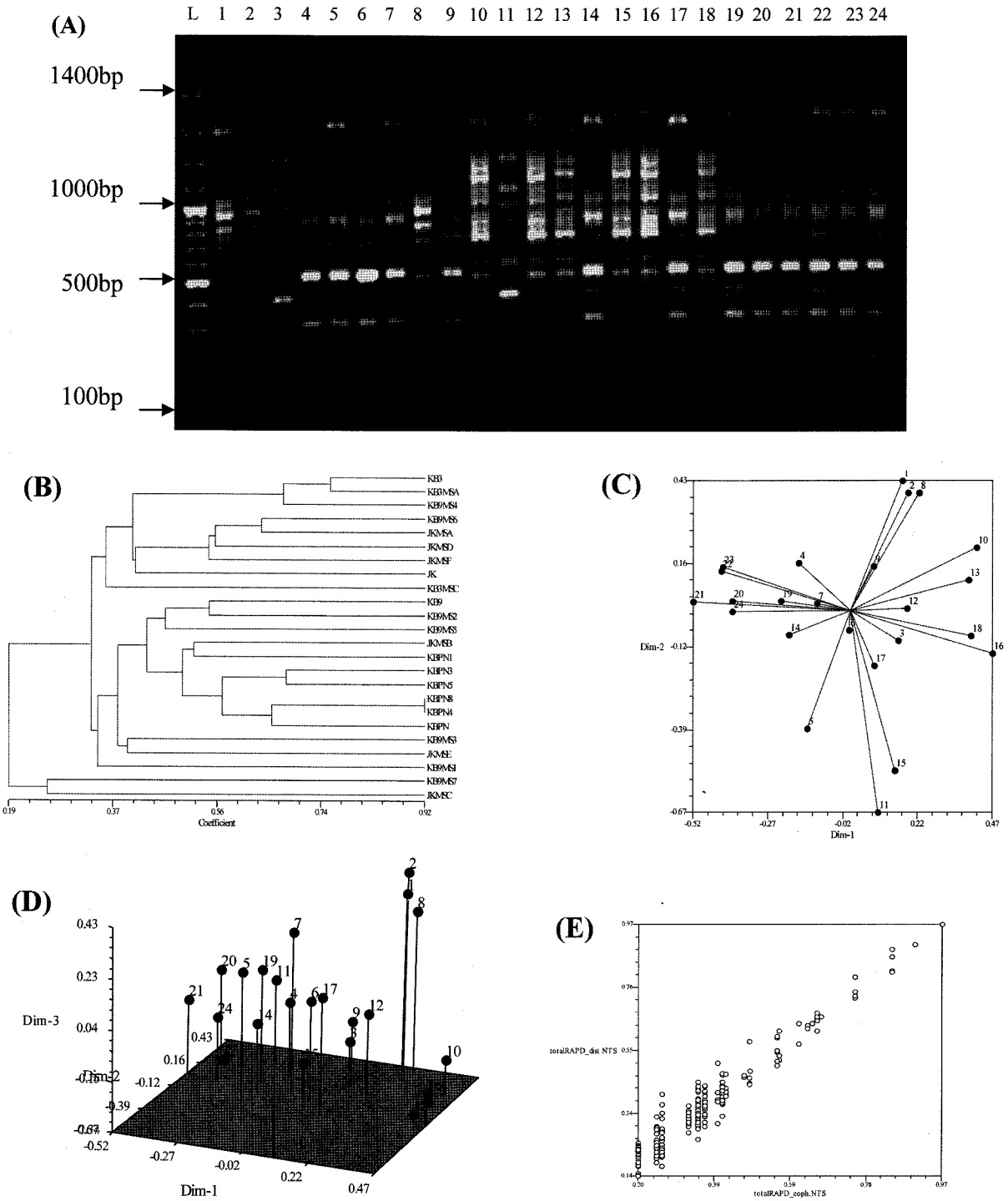

Fig. 3. (A) ISSR marker analysis through PCR amplification of fungal genomic DNA with R 03 primer, (B) Dendrogram constructed by using UPGMA based on Jacquard's Coefficient, (C) 2 D (D) 3 D (E) Matrix comparison with score plots Depiction of ISSR profiles of *T. indica* isolates and monosporidial strains.
L- 100bp Ladder, 1- KB3, 2- KB3msa, 3- KB3msc, 4- KB9, 5- KB9ms1, 6- KBms2, 7- KB9ms3, 8- KB9ms4, 9- KB9ms5, 10- KB9ms6, 11- KB9ms7, 12- JK, 13- JKmsa, 14- JKmsb, 15- JKmsc, 16- JKmsd, 17- JKmse, 18- JKmdf, 19- KBpn1, 20- KBpn3, 21- KBpn5, 22- KBpn8, 23- KBpn6, 24- KBPN

JK, JKmsa, JKmsd, JKmsf, KB9, KB9ms2, KB9ms5) and some monosporidial strains deviate from this rule showing very high genetic variation from the origin (KB9ms3, JKmse, KB9ms1,KB9ms7 and JKmsc).

In general ISSR marker analysis indicated more or less similar features to that of RAPD analysis. The analysis

revealed that RAPD analysis in molecular characterization in *T. indica* is much more informative as it indicated 60% polymorphism while ISSR analysis indicated only 54%. This may be due to the reason that ISSR markers generated polymorphism, only from the intervening regions of introns while RAPD can cover both exon and intron regions. The estimated cophenetic correlation (r) was 0.85358 and showed a very good fit for cluster analysis. The approximate mantel t-test recorded t equal to 7.7685 and probable random Z was less than observed Z indicated good representation between similarity matrix and cluster analysis (Fig. 3E).

Molecular characterization with two marker systems indicated that RAPD is more informative over ISSR as it can resolve the genetic variability of *T. indica* strains more effectively. Genetic make up of isolates and monosporidial strains significantly vary among each other and members of same monosporidial group vary among each other in their genetic makeup. This variability is not a generalized phenomenon as some monosporidial groups exhibited nearly zero variability (KBPN). Hence it can be concluded that variability is a genetically governed process. This genetic variability clearly reflected in terms of morphological variability suggests that the variations in *T. indica* strains are predominantly derived through the genetic rearrangements thus altering the growth behavior.

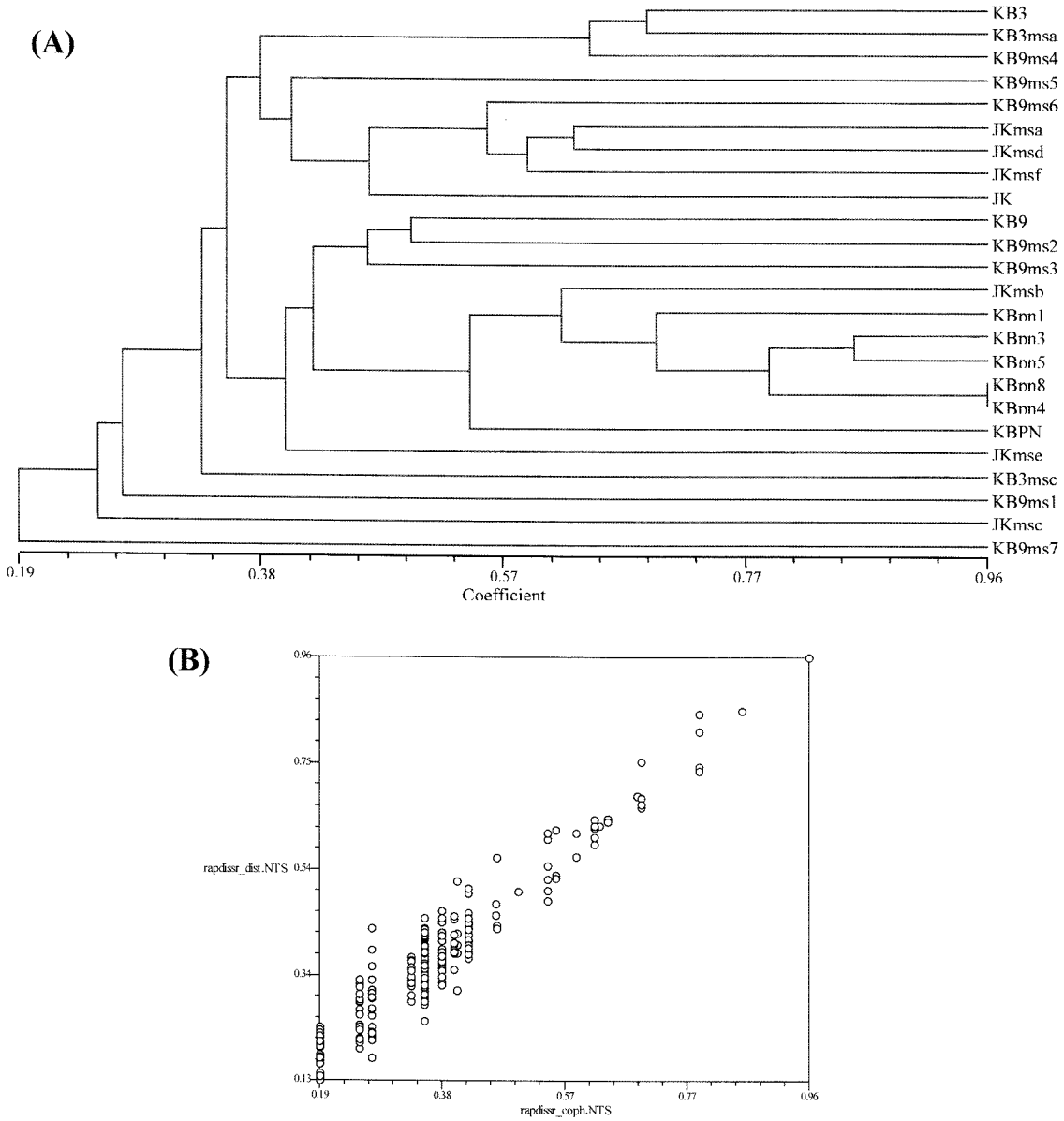

Fig. 4. Total RAPD-ISSR profiles of *T. indica* isolates and monosporidial strains. (A) Dendrogram constructed by using UPGMA based on Jacquard's Coefficient (B) d Matrix comparison with score plots.

Combination of RAPD and ISSR markers. Features of total RAPD-ISSR similarity matrix and dendrogram (Fig. 4A) are quite similar to the total RAPD analysis, indicating the RAPD analysis is dominant over the ISSR analysis. The combined allelic diversity dataset using two different PCR based molecular markers generated bands from 25 primers with an average of 12.88 bands per primer. The lowest number of six bands per primer was obtained from I 18 ISSR marker. The highest number of bands (18) was recorded for three RAPD primers (R 16, R22 and R28). Out of 25 primers used in the study, twelve primers resulted in unique bands (seven in RAPD and five in ISSR) on amplifying single strain.

Similarity coefficient, Cluster analysis, Matrix Comparison and Mantel Test. The Jaccard's similarity coefficient was estimated using pooled marker diversity data. The range of the coefficient varied from 0.13 (KB3msa, KB9ms7 and KB9, KB9ms7) to a maximum of 0.85 between KBpn5 and KBpn3 followed by KBpn8 and KBpn5 with the same similarity. The phylogenetic tree constructed through SAHN cluster analysis using UPGMA method revealed that pooled cluster analysis is much similar to the cluster analysis of RAPD markers, indicating that RAPD markers are contributing more as compared to the ISSR markers. The pooled allelic diversity data revealed clear and distinct grouping of all the isolates.

Matrix comparison was performed using MXCOMP programme of NT SYS PC 2.11v to calculate the cophenetic correlation between the similarity matrix and the cophenetic value matrix. The degree of relationship between two matrices measured for all the genetic diversity data generated through different markers are represented in Figure 6B. The cophenetic correlation (r) was also used to measure the goodness of fit for different cluster analysis. The cophenetic correlation, r=0.95323 for combined molecular markers (RAPD and ISSR) indicated very good fit of the cluster generated using SAHN UPGMA method with the similarity matrix. The two way Mantel test resulted t equal to 8.6356 and probable Z less than observed Z which showed the cluster was well represented by the similarity matrix value. Matrix comparison in the score plots depicted in (Figure 4B) indicate that the total RAPD score plot show much more linear relation compared to the total ISSR and RAPD combined depiction, indicating RAPD is a good marker for *Tilletia indica* genetic evaluation.

Principle Co-ordinate Analysis (PCoA). The patterns of cluster analysis was further confirmed by Principal coordinate analysis (PCoA) for molecular markers. The results for different markers are presented in two and three dimensional score plots. The PCoA for RAPD, ISSR and combined markers also resulted in similar relationship between the score plots and the pattern of genetic diversity estimated by the UPGMA cluster analysis. Maximum of 53.49% of the total variation recorded for SSR markers using principal coordinate analysis was recorded. It was indicated that maximum number of distinct clusters were generated using all the markers with cumulative variance for first three components (31.75). From the score plots generated through PCoA analysis and the dendrogram constructed through UPGMA method for all the marker systems were in close affirmation (Fig. 6A & 6B) as studied earlier by Jain et al. (2004) and Semgan et al. (2006). The clusters generated through the combination of two molecular markers (RAPD, ISSR) were compared with the score plots generated by PCoA analysis. The total variation of first five principal components for RAPD, ISSR and combined was estimated as 47.02, 45.8 and 46.2%, respectively. The first five components resulted in a cumulative variance of 46.2% which showed relatively similar result with individual molecular markers. The different groups resulted from the score plots (PCoA) and the clusters generated from the dendrogram (UPGMA cluster analysis) were found to be similar.

Estimation of relative efficiencies of different markers. The relative efficiency of a marker was assessed by correlating the genetic similarity measures with different markers. The similarity coefficients of 24 fungal cultures measured through two different molecular markers (RAPD, ISSR) were subjected to calculate the Pearson's correlation coefficient. Among the molecular markers highest Pearson correlation value (r=0.9925) was recorded in RAPD×Combined markers which suggested that these two markers are concordant to each other in terms of the genetic similarity produced. The Combined and ISSR markers produced the second highest correlation value (r=0.9087). It was observed that the involvement of RAPD markers increases the Pearson correlation coefficient compared to ISSR markers. The result suggested that both molecular markers have produced somewhat similar genetic similarity but RAPD markers were the most influential type of molecular marker while used in combination to study the genetic diversity. The results indicated differences among various marker systems in terms of identifying degrees of genetic diversity present in a particular set of isolates.

Based on the data set generated through combined molecular markers (RAPD and ISSR) isolates were distributed in 20 clusters. The clustering pattern obtained through combined molecular markers finds its place in between ISSR and RAPD markers. The result revealed that RAPD markers came out with best discrimination among all the isolates and mono-sporidial strains studied for genetic

diversity analysis hence it may be considered as a more efficient marker technique for determination of genetic divergence amongst *Tilletia indica* pathogen.

Since the pathogen is heterothallic, filamentous and multinucleate in nature only compatible monosporidial cultures are able to produce infection. Till now cultures from single teliospores are being utilized for variability studies and testing for compatible monosporidial lines for variability is laborious and difficult by conventional plant pathological methods. This is the first report demonstrating the application of molecular markers to determine the vast genetic diversity within monosporidial strains derived from different mono-teliosporic isolates. Such diversity is possible through recombination and mating between compatible mating types and karyogamous changes (Geeta et al., 2000) as well as recombination originated through anastomosis and subsequent para sexual recombination (mitotic crossing over) or simply by the mutations (Nicholas, 1998). It has been demonstrated that heterothallism and pathogenicity are controlled by multiple alleles of one locus on a homologous chromosome and there exist two mating types (Dural and Cromarty, 1977). Royer and Ryter (1985) and Aujla and Sharma (1990) also indicated presence of at least three mating types which is suggestive of the probability of tetrapolar incompatibility system in *T. indica*. There is a great deal of genetic variability within the species but researchers do not agree on the designation of specific races. The molecular characterization approaches used in the present study may also help in the identification of the possible mating types generated during fungal life cycle and elucidation of the molecular mechanisms underlying the sexual development and pathogenicity.

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