

Molecular diagnosis and intraspecific genetic variability of root pathogens of arid legumes in Western Rajasthan, India

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Abstract: The productivity of arid legumes, such as Clusterbean (*Cyamopsis tetragonoloba*), Cowpea (*Vigna unguiculata*), Moth bean (*Vigna aconitifolia*) and Horse gram (*Macrotyloma uniflorum*), may remain stagnant over decades because of their high susceptibility to root diseases. Besides, there is a limitation on the information about molecular diagnosis and intraspecific genetic variability of root pathogens in arid legumes. To contribute in this field, we assessed a total of 52 isolates from 88 root samples that were found infected with fungal pathogens in Jodhpur, Jaipur and Bikaner Districts of Rajasthan. Diseased roots samples were analyzed following standard microbiological methods for fungus extraction and purification, and for genetic studies. Irrespective of the geographical location from where the diseased samples were collected, all pathogen isolates were clustered in RAPD dendrograms as per their respective genera. Phylogram, based on multiple sequence alignment, revealed that different genera (i.e. *Fusarium*, *Neocosmospora* and *Syncephalastrum*), separated from each other, and species within the same genera, clustered together with their reference sequences with appreciable bootstrap values. Out of 20 representative isolates representing each cluster and all outgroups sequenced, eight were molecularly identified as *Neocosmospora vasinfecta*, five as *Fusarium solani*, two as *Neocosmospora striata*, two as *Fusarium acutatum*, one as *Syncephalastrum monosporum*, one as *Fusarium oxysporum* and one as *Fusarium* species. The root pathogens of the arid legumes were found neither restricted to a geographical location nor were host specific in nature. *Fusarium solani* wilt in cowpea and seedling rot in moth bean, *F. oxysporum* wilt in moth bean, *F. acutatum* damping off in cowpea and Clusterbean, *Fusarium* sp. seedling rot in Clusterbean, *Neocosmospora striata* root rot in cowpea and wilt in Clusterbean and *Syncephalastrum monosporum* root rot in Clusterbean were molecularly identified as new fungal records as pathogens causing root diseases in arid legumes. Rev. Biol. Trop. 64 (4): 1505-1518. Epub 2016 December 01.

Key words: RAPD, ITS, *Fusarium*, *Neocosmospora*, *Syncephalastrum*.

In India, arid legumes are grown in about 5 million hectares area with approximately 0.2 million tons production of grains annually. The major arid legumes are Clusterbean (*Cyamopsis tetragonoloba*), Cowpea (*Vigna unguiculata*), Moth bean (*Vigna aconitifolia*) and Horse gram (*Macrotyloma uniflorum*) which are predominantly grown in arid and semiarid tracks of Indian subcontinents. Due to inherent drought hardy characteristics arid legumes are mostly grown where land conditions are not

suitable for cultivation of cereals mostly under rain fed conditions. During rainy season, high temperature coupled with moisture stress put them into abiotic and biotic stresses. Besides, blights, leaf spots, powdery mildews and viral diseases, these arid legumes are attacked by serious soil-borne pathogens with a very wide host range causing substantial damages and yield reduction in arid legumes. The information on molecular diagnosis and intraspecific genetic variability of root pathogens

of arid legumes of the region is limited (Gautam, Singh, & Sharma, 2013).

The most common diseases caused by these pathogens are charcoal rot (damping off), dry root rot, wilt, leaf blight and ashy stem blight. Efforts have been made to characterize the fungus populations in different parts of the world. This is based on its pathogenic variability, the morphological characteristics, as well as the molecular characteristics (Jana, Sharma, Prasad, & Arora, 2003). Recently Random Amplified Polymorphic DNA (RAPD) and nuclear rDNA Internal Transcribed Spacer (ITS) polymorphism within *Macrophomina phaseolina* isolated from arid legumes of Western Rajasthan revealed genetic diversity among 33 isolates (Gautam et al., 2013). Species of *Fusarium* causes both major and minor diseases in these legumes (Aigbe & Fawole, 2010).

The productivity of these arid legumes remains virtually stagnant over decades because of their susceptibility to root diseases. Most of the root pathogens are soil or seed borne, and colonize the xylem vessels by clogging and blocking completely to effect wilting. Accurate and rapid identification of pathogens is necessary for appropriate management of plant diseases (Narayananasamy, 2001).

The classical biological pathotyping techniques alone are not enough for a reliable identification and characterization of fungal pathotypes and population (Jamil et al., 2000). Therefore, present study was carried out to identify the cause of the disease (Koch's postulates), molecularly identify and characterize root pathogens of arid legumes using RAPD and ITS rDNA polymorphism to reveal inter and intra specific genetic relationships among and within root pathogens, to facilitate effective management of major diseases.

MATERIALS AND METHODS

Isolation of pathogens: Surveys were conducted to collect diseased plants of Clusterbean (*Cyamopsis tetragonoloba*), Cowpea (*Vigna unguiculata*), Moth bean (*Vigna aconitifolia*) and Horse gram (*Macrotyloma uniflorum*)

from Jodhpur, Jaipur and Bikaner Districts of Rajasthan during the months of August and September, 2011. A total of 88 infected plants i.e., Clusterbean (38), Cowpea (16), Moth bean (24) and Horse gram (10) were collected, but this report only assessed those infections different from *Macropomina phaseolina*. To obtain pure cultures of root pathogens, these infected roots were cut into small pieces and surface sterilized using 5 % sodium hypochlorite for three minutes, washed thrice in sterilized distilled water and air dried. Two or three infected root bits from each infected root sample were then aseptically transferred onto Petri plates containing PDA (39 g PDA, HiMedia Company) culture medium, containing 150 mg/litre of streptomycin sulphate (to avoid bacterial contamination) and incubated in an incubator (Jindal, S.M. Scientific Instruments (P) Ltd, New Delhi, India) at $25 \pm 2^\circ\text{C}$ for five days. To raise pure cultures, the mycelia from the growing margins of each culture along with a piece of media from Petri plates were aseptically transferred into test tubes containing PDA, and incubated at $25 \pm 2^\circ\text{C}$ for a week. The 52 pure cultures obtained were stored at 4°C in a refrigerator until use.

Pathogenicity tests: Sandy loam soil was sterilized by autoclaving it (Apex, Conica Enterprises, New Delhi, India), at 121°C for one hour. PVC plastic pots were filled with two kg of sterilized soil, and a pathogenicity test was set in a complete randomized block design, considering legumes origin and all 20 isolates: JD-HG10, JAI-CP11, JAI-CP16, JD-CB7, JD-CB9, BK-CB15, BK-CB17, BK-CB20, BK-CB26, JAI-CB37, JAI-MB22, JAI-MB24, BK-CB18, JD-CB10, BK-CB30, BK-CB22, JAI-CB34, JAI-CP13, JAI-CB36 and JAI-MB21. Each isolated pathogen was raised in conical flasks on liquid Malt Extract Dextrose Broth culture medium (Malt Extract-10 g; Dextrose-5 g with antibacterial agent streptomycin 150 mg; 1 litre of sterilized distilled water) for 10 days. Conidial suspension was obtained by filtering mycelial mats and blending it with sterilized distilled water and again filtering it

through two layers of sterile cheesecloth. Two week old plants of arid legumes i.e., clusterbean, cowpea, moth bean and horse gram, were then inoculated with conidial suspension of specified isolate (from which it was originally isolated) at the base by disturbing the soil. The inoculated plants were incubated in green house under high temperature (28-30°C) and moisture stress conditions (100 mL water every 4th day and humidity 60-70 %) to facilitate disease development. A total of five pots were inoculated with each isolate. Plant symptoms were recorded and the pathogen reisolated. Besides, upon microscopic examination, the pathogenicity of each isolated pathogen was confirmed. Identifications to genus level were carried following Barnett and Hunter (1990).

DNA isolation: For DNA isolation, a small piece of growing mycelia from each of the 52 isolated cultures was aseptically transferred to separate Malt Extract Dextrose Broth culture medium and incubated in a BOD incubator at 25 ± 2 °C for seven days. The fungal mycelial net from each raised pure culture was filtered using filter paper through funnel. The genomic DNA was extracted from approximately 100 mg of fresh mycelium by crushing it in conical micro centrifuge tubes using micropestles in liquid nitrogen. The HiPura kit of HiMedia Company and protocols suggested by Birren and Lai (1993), Sambrook, Fritsch and Maniatis (1989) were followed for genomic DNA isolation. Finally, the DNA was eluted in 200µL of Tris EDTA buffer (TE buffer).

RAPD analysis: Eighteen decamer random primers of OPA, OPB and OPP series (Operon Technologies, USA: <http://www.operon.com>) were used for initial screening of all the isolates. Finally, the data of ten RAPD primers exhibiting consistent reproducible amplified products on gel electrophoresis were used for DNA fingerprinting. Each PCR was performed in a total reaction mixture of 25 µL following Gautam et al. (2013). Amplicons were separated on 1.5 % agarose gel (Sigma) pre-stained with ethidium bromide solution

using 1 × TAE (Tris Acetic acid EDTA, Himedia) buffer. The gel was run for 3 h at 50V and the RAPD amplicons profiles were recorded using Syngene Gel Documentation System with Genesnap software. The size of amplified fragments was determined using 100 bp plus ladder (MBI Fermentas). All RAPD reactions were performed twice to test the reproducibility of the amplicons profile.

Internal transcribed spacer (ITS) amplification: The universal primers ITS-1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC 3') were used for the amplification of ITS-1-5.8S-ITS-4 region. Each PCR amplification was performed in a total volume of 50 µL following Gautam et al. (2013). Amplified ITS regions were sequenced employing ABI Prism DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) using ITS-1 and ITS-4 primers separately for DNA labeling by the BigDye terminator method (Applied Biosystems, Foster City, CA, USA). Molecular identification of each fungal isolate was done on the basis of similarity with the best aligned sequence of BLAST search and accordingly species designated.

Molecular data analysis: The RAPD amplification products were scored as present (1) and absent (0) of scorable loci for each primer isolate combinations. Molecular data were entered into a binomial matrix and were used to determine Jaccard's similarity coefficient with NTSYS-pc software (Rohlf, 1997).

To perform analysis of molecular variance (AMOVA), the 52 isolates of root pathogens from arid legumes were divided into four populations based on the arid legume crop from which they were isolated i.e., population one (clusterbean), population two (horse gram), population three (cowpea) and population four (moth bean). Principal Component Analysis (PCA) via covariance matrix was calculated using GenALEX 6 software. Whereas, diversity in the frequency of fragment size of RAPD patterns was apportioned within and among root pathogen isolates using Shannon's information

index (*i*) and gene diversity index (*h*) following using PopGen 32 programme.

The multiple sequence alignment of ITS region (ITS-1, 5.8S r-RNA gene and ITS-2) of all the 20 representative root pathogen isolates was performed using ClustalX 2.0.11 software. The phylogenetic relationships of root rot pathogens were established by multiple alignment of sequences and generating phylogram depicting bootstrap values using NJ plot software based on Single Nucleotide Polymorphisms (SNPs), insertions/deletions (INDELS), and or length diversity in the ITS and 5.8S nuclear rDNA regions. To assess the robustness of phylogenetic relationships, the best aligned reference sequence representing of each species from GenBank database was downloaded in fasta format. A composite phylogenetic tree with bootstrap values showing grouping of 20 isolates sequenced with 11 reference sequences was generated to measure phylogenetic accuracy.

RESULTS

Isolation tests results: All the four steps of Koch's postulates were successfully completed for each test pathogen in a replicated trial. The details of fungal isolates, host, district and colony characteristics are shown at table 1. A total of 52 root samples were found infected with fungal pathogens belonging to genus *Fusarium*, *Neocosmospora* and *Syncephalastrum*. In all, four types of colony and growth patterns i.e. feathery, dense, fluffy and restricted, were recorded from different isolates. Similarly disease symptoms from seedling wilt (damping off) to seedling rot, wilt and root rot were recorded. Light pink to dark red pigmentations were also recorded in different cultures. Based on microscopic examinations, genus level identifications revealed that from the total of 52 diseased root samples considered in this study, 26 samples were identified as *Neocosmospora*, 25 as *Fusarium* and 1 as *Syncephalastrum*.

RAPD analysis: Out of 18 decamer random primers, 10 primers detected intraspecific variations generating scorable amplicons and reproducible patterns that have generated 147 marker bands in the range of 250 bp to 5 000 bp. Of which, 146 marker bands were polymorphic amounting to 99.41 % polymorphism ranging from 94.11 % to 100 %. The number of PCR amplified products ranged from 11 (OPP-16) to 18 (OPB-04) with an average of about 14.7 bands/primer (Table 2). The PIC values varied from 84 % to 92 % with an average of 90 %. The primer OPB-06 and OPB-14 exhibited the maximum PIC value of 92 % which was closely followed by OPA-09, OPA-13 and OPB-05 with 91 %. A representative RAPD profile generated using OPB-06 exhibiting the maximum PIC value is shown vide figure. 1A, figure 1B and figure 1C.

A cumulative RAPD dendrogram delineated all the 52 isolates into sub-clusters ranging from 32 to 95 % similarity. In all, the isolates can be seen in eight distinct clusters and seven isolates as out groups to these clusters (Fig. 2). Cluster one included two, cluster two contained nine, cluster three compiled 10, cluster four comprised seven, cluster five included six, cluster six and seven comprised four isolates each, and cluster eight compiled of three isolates.

Irrespective of the geographical location from where the diseased samples were collected, all isolates belonging to the genus *Neocosmospora*, aligned from cluster one to cluster five including all outgroups between these clusters. Whereas, all isolates of genus *Fusarium* converged into cluster six to eight and flanking outgroups including one isolate of *Syncephalastrum*.

ITS amplification: Based on the clustering patterns of different isolates of root pathogens of arid legumes, 20 representative isolates from all RAPD clusters and distinct isolates were selected for nuclear rDNA ITS region sequencing. All the isolates generated

TABLE 1
Morphological characterization of fungal pathogens causing root diseases in arid legumes

S. No.	Isolate	Host	District	Colony Growth Pattern	Colony Colour	Pigmentation in medium	Genus	Disease Symptom
1.	JD-CB7	Clusterbean	Jodhpur	Feathery	Off White	Light Pink	<i>Neocosmospora</i>	Wilt NV
2.	JD-CB8	Clusterbean	Jodhpur	Feathery	Off White	Light Pink	<i>Neocosmospora</i>	Wilt
3.	JD-CB9	Clusterbean	Jodhpur	Restricted	Light Purple	Dark Pink	<i>Neocosmospora</i>	Wilt NS
4.	JD-CB10	Clusterbean	Jodhpur	Restricted	Off White	Light Pink	<i>Neocosmospora</i>	Wilt NV
5.	JD-CB11	Clusterbean	Jodhpur	Restricted	Off White	Light Pink	<i>Neocosmospora</i>	Damping off
6.	JD-CB12	Clusterbean	Jodhpur	Dense	Off White	Light Pink	<i>Neocosmospora</i>	Wilt
7.	JD-CB13	Clusterbean	Jodhpur	Fluffy	Off White	Light Pink	<i>Neocosmospora</i>	Wilt
8.	JD-CB14	Clusterbean	Jodhpur	Dense	Off White	Light Pink	<i>Neocosmospora</i>	Wilt
9.	JD-HG9	Horse gram	Jodhpur	Dense	Off White	Light Pink	<i>Neocosmospora</i>	Wilt
10.	JD-HG10	Horse gram	Jodhpur	Restricted	Off White	Light Pink	<i>Neocosmospora</i>	Wilt NV
11.	JD-CP9	Cowpea	Jodhpur	Dense	Light Pink	Dark Pink	<i>Neocosmospora</i>	Wilt
12.	JD-MB14	Moth bean	Jodhpur	Feathery	Dark Pink	Dark Pink	<i>Neocosmospora</i>	Wilt
13.	JD-MB15	Moth bean	Jodhpur	Feathery	Off White	Light Pink	<i>Neocosmospora</i>	Wilt
14.	BK-CP10	Cowpea	Bikaner	Feathery	Off White	Dark Pink	<i>Neocosmospora</i>	Seedling rot
15.	BK-MB16	Moth bean	Bikaner	Restricted	Dark Pink	Dark Pink	<i>Neocosmospora</i>	Wilt
16.	BK-MB17	Moth bean	Bikaner	Fluffy	Dark Pink	Light Pink	<i>Neocosmospora</i>	Wilt
17.	BK-MB18	Moth bean	Bikaner	Fluffy	Off White	Light Pink	<i>Neocosmospora</i>	Seedling rot
18.	BK-CB15	Clusterbean	Bikaner	Dense	Off White	Dark Pink	<i>Syncephalastrum</i>	Root rot SM
19.	BK-CB16	Clusterbean	Bikaner	Restricted	Off White	Light Pink	<i>Neocosmospora</i>	Wilt
20.	BK-CB17	Clusterbean	Bikaner	Restricted	Light Pink	Light Pink	<i>Neocosmospora</i>	Wilt NV
21.	BK-CB18	Clusterbean	Bikaner	Feathery	Off White	Dark Red	<i>Neocosmospora</i>	Wilt NV
22.	BK-CB19	Clusterbean	Bikaner	Fluffy	Off White	Light Red	<i>Neocosmospora</i>	Wilt
23.	BK-CB20	Clusterbean	Bikaner	Restricted	Off White	Light Pink	<i>Fusarium</i>	Wilt FS
24.	BK-CB21	Clusterbean	Bikaner	Fluffy	Pink	Light Red	<i>Neocosmospora</i>	Root rot
25.	BK-CB22	Clusterbean	Bikaner	Restricted	Off White	Light Red	<i>Neocosmospora</i>	Root rot
26.	BK-CB23	Clusterbean	Bikaner	Restricted	Pink	Light Red	<i>Fusarium</i>	Wilt NV
27.	BK-CB24	Clusterbean	Bikaner	Dense	Off White	Light Red	<i>Fusarium</i>	Wilt
28.	BK-CB25	Clusterbean	Bikaner	Dense	Off White	Light Red	<i>Fusarium</i>	Wilt
29.	BK-CB26	Clusterbean	Bikaner	Restricted	Light Pink	Light Red	<i>Fusarium</i>	Damping off FA
30.	BK-CB27	Clusterbean	Bikaner	Restricted	Off White	Dark Pink	<i>Fusarium</i>	Damping off
31.	BK-CB28	Clusterbean	Bikaner	Restricted	Off White	Dark Pink	<i>Fusarium</i>	Damping off
32.	BK-CB29	Clusterbean	Bikaner	Restricted	Off White	Dark Pink	<i>Fusarium</i>	Root rot
33.	BK-CB30	Clusterbean	Bikaner	Dense	Off White	Dark Pink	<i>Neocosmospora</i>	Root rot NV
34.	JAI-CP11	Cowpea	Jaipur	Restricted	Light Pink	Dark Pink	<i>Neocosmospora</i>	Root rot
35.	JAI-CP12	Cowpea	Jaipur	Feathery	Off White	Light Pink	<i>Fusarium</i>	Wilt
36.	JAI-CP13	Cowpea	Jaipur	Feathery	Off White	Light Pink	<i>Fusarium</i>	Wilt FS
37.	JAI-CP14	Cowpea	Jaipur	Feathery	Off White	Light Pink	<i>Fusarium</i>	Wilt
38.	JAI-CP15	Cowpea	Jaipur	Feathery	Off White	Light Pink	<i>Fusarium</i>	Wilt
39.	JAI-CP16	Cowpea	Jaipur	Dense	Off White	Light Red	<i>Fusarium</i>	Damping off FA
40.	JAI-MB19	Moth bean	Jaipur	Feathery	Pink	Dark Red	<i>Fusarium</i>	Wilt
41.	JAI-MB20	Moth bean	Jaipur	Fluffy	Off White	Dark Red	<i>Fusarium</i>	Wilt
42.	JAI-MB21	Moth bean	Jaipur	Fluffy	Dark pink	Light Pink	<i>Fusarium</i>	Seedling rot FS
43.	JAI-MB22	Moth bean	Jaipur	Dense	Purple	Dark Red	<i>Fusarium</i>	Wilt F OX
44.	JAI-MB24	Moth bean	Jaipur	Dense	Pink	Light Red	<i>Fusarium</i>	Wilt
45.	JAI-CB31	Clusterbean	Jaipur	Dense	Off White	Light Red	<i>Fusarium</i>	Wilt
46.	JAI-CB32	Clusterbean	Jaipur	Dense	Off White	Light Red	<i>Fusarium</i>	Wilt
47.	JAI-CB33	Clusterbean	Jaipur	Restricted	Pink	Light Red	<i>Fusarium</i>	Wilt
48.	JAI-CB34	Clusterbean	Jaipur	Restricted	Off White	Light Pink	<i>Neocosmospora</i>	Seedling rot NV
49.	JAI-CB35	Clusterbean	Jaipur	Restricted	Off White	Dark Red	<i>Fusarium</i>	Wilt
50.	JAI-CB36	Clusterbean	Jaipur	Dense	Off White	Dark Red	<i>Fusarium</i>	Wilt FS
51.	JAI-CB37	Clusterbean	Jaipur	Dense	Off White	Light Pink	<i>Fusarium</i>	Seedling rot F Sp
52.	JAI-CB38	Clusterbean	Jaipur	Dense	Off White	Dark Red	<i>Fusarium</i>	Wilt

JD, Jodhpur, BK, Bikaner, JAI, Jaipur, CB, clusterbean, CP, cowpea, MB, moth bean, HG, Horse gram, NV, *Neocosmospora vasinfecta*, FS, *Fusarium solani*, FOX, *Fusarium oxysporum*, FA, *Fusarium acutatum*, SM, *Syncephalastrum monosporum*, NS, *Neocosmospora striata*.

TABLE 2
 Details of primer code, GC content, per cent polymorphism and PIC values of RAPD primers

S.N.	Primer Code	Primer Sequence	GC (%)	No. of Bands	No. of Polymorphic bands	Polymorphism (%)	PIC Values
1.	OPA-02	TGC CGA GCT G	70	13	13	100	90 %
2.	OPA-09	GGG TAA CGC C	70	15	15	100	91 %
3.	OPA-13	CAG CAC CCA C	70	13	13	100	91 %
4.	OPB-04	GGA CTG GAG T	60	18	18	100	90 %
5.	OPB-05	TGC GCC CTT C	70	12	12	100	91 %
6.	OPB-06	TGC TCT GCC C	70	17	16	94.11	92 %
7.	OPB-10	CTG CTG GGA C	70	16	16	100	90 %
8.	OPB-13	TTC CCC CGC T	70	14	14	100	89 %
9.	OPB-14	TCC GCT CTG G	70	18	18	100	92 %
10.	OPP-16	CCA AGC TGC C	70	11	11	100	84 %
	Total			147	146		
	Average					99.41	90.00

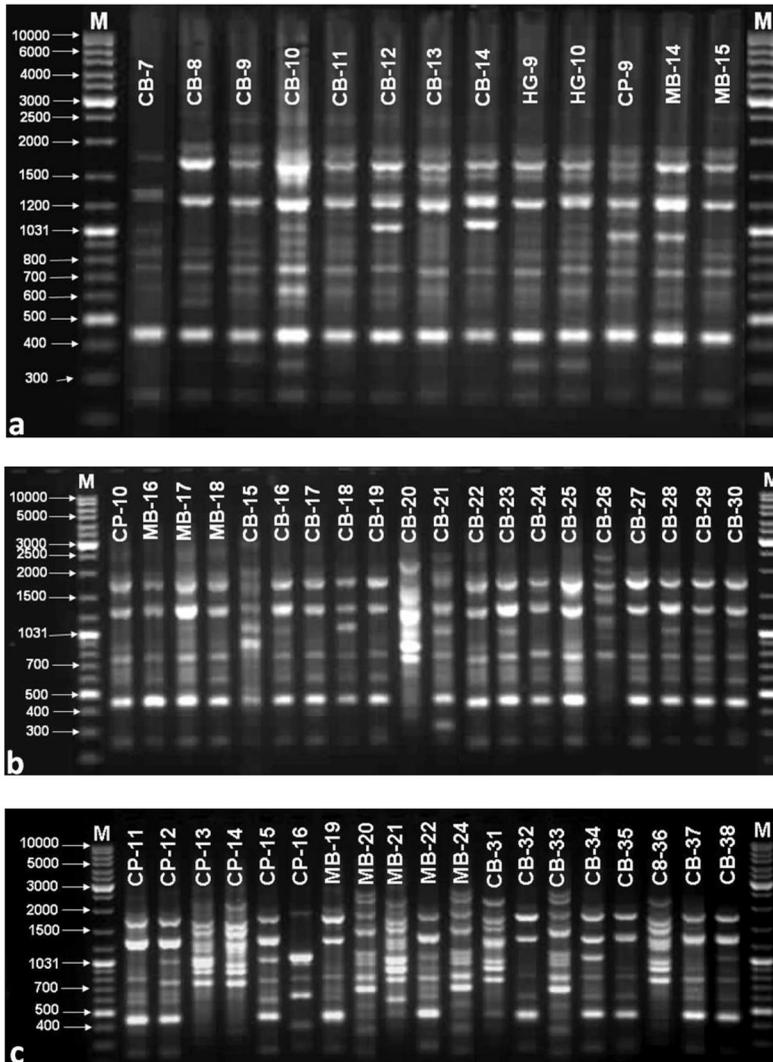


Fig. 1(A,B,C). RAPD profiles of root pathogens amplified by OPB-06 primer (a) Jodhpur, (b) Bikaner and (c) Jaipur.

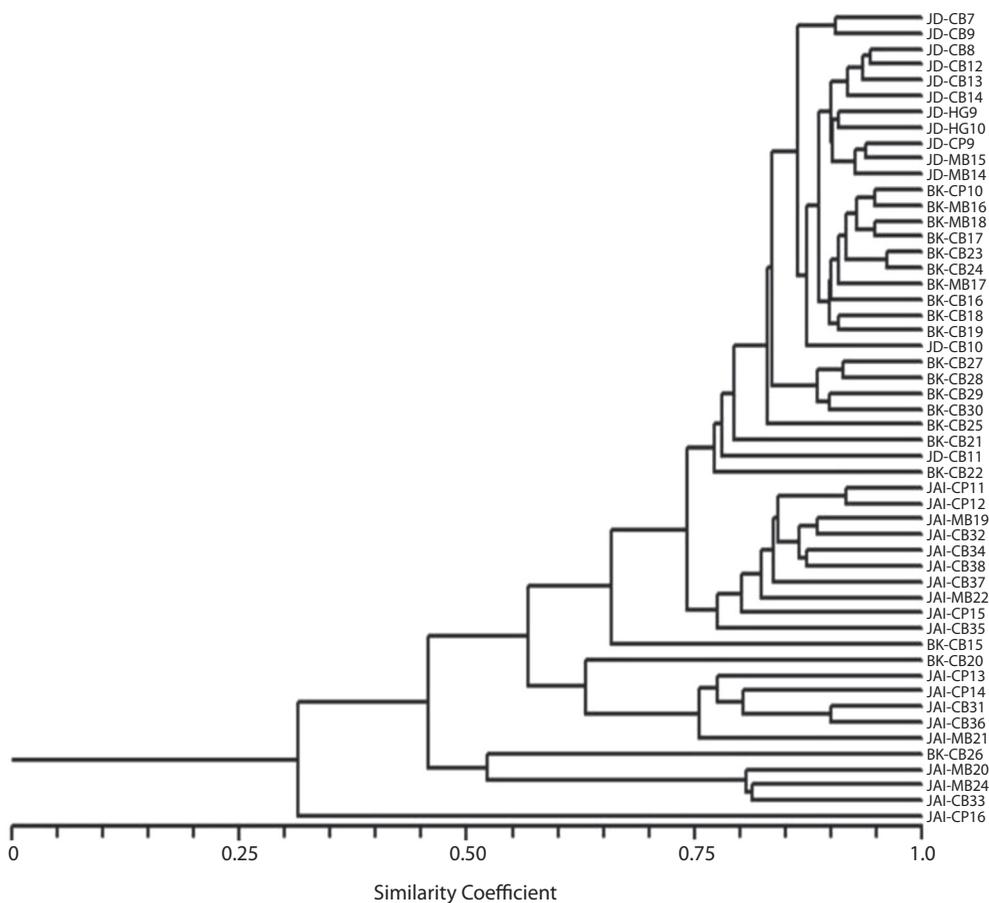


Fig. 2. Dendrogram of 52 isolates of root pathogens based on 10 RAPD informative primers.

a single prominent band on agarose gel which included partial sequence of 18S gene, complete sequence of ITS-1, 5.8S gene, ITS-2 and partial sequence of 28S gene. The nucleotide sequences were subjected to BLAST search using NCBI, USA databases. The species level designation were assigned to each isolate considering the maximum identities with that of available reference sequences of NCBI databases (Table 3).

Molecular data analysis: The novel gene sequences were submitted to NCBI and gene bank accession numbers obtained. In order to detect SNP's, insertions and deletions

(INDELS) and total length polymorphism of rDNA region, all the isolates were subjected to multiple sequence alignment using clustal X software programme. A phylogram was generated using tree view software and boot strap values determined using NJ plot programme (Fig. 3) to validate the species designation and to reveal the nucleotide polymorphism vis-à-vis reference sequence available in NCBI databases. The best aligned reference sequences were also downloaded and subjected to multiple sequence alignment to establish lineages. It is clear from the phylogram that different genera (i.e. *Fusarium*, *Neocosmospora* and *Syncephalastrum*) separated from each other and species

TABLE 3
Nucleotide base pair lengths of nuclear ribosomal RNA gene of representative genotypes
of RAPD sub-clusters Red Culture

S. N.	Isolate	Molecular identification	Gen accession number	ITS-1 (bp)	5.8 S (bp)	ITS-2 (bp)	Total (bp)
1.	JD-HG10	<i>Neocosmospora vasinfecta</i>	JQ954881	149	167	156	472
2.	JAI-CP11	<i>Neocosmospora striata</i>	JQ954882	148	156	171	475
3.	JAI-CP16	<i>Fusarium acutatum</i>	JQ954883	148	156	167	471
4.	JD-CB7	<i>Neocosmospora vasinfecta</i>	JQ954884	150	166	158	474
5.	JD-CB9	<i>Neocosmospora striata</i>	JQ954885	149	158	165	472
6.	BK-CB15	<i>Syncephalastrum monosporum</i>	JQ954886	192	150	200	542
7.	BK-CB17	<i>Neocosmospora vasinfecta</i>	JQ954887	148	156	171	475
8.	BK-CB20	<i>Fusarium solani</i>	JQ954888	148	157	173	478
9.	BK-CB26	<i>Fusarium acutatum</i>	JQ954889	148	158	166	472
10.	JAI-CB37	<i>Fusarium sp.</i>	JQ954890	112	158	170	440
11.	JAI-MB22	<i>Fusarium solani</i>	JQ954891	148	157	173	478
12.	JAI-MB24	<i>Fusarium oxysporum</i>	JQ954892	132	162	156	450
13.	BK-CB18	<i>Neocosmospora vasinfecta</i>	JX517195	150	158	165	473
14.	JD-CB10	<i>Neocosmospora vasinfecta</i>	JX517196	149	158	165	472
15.	BK-CB30	<i>Neocosmospora vasinfecta</i>	JX517197	149	158	165	472
16.	BK-CB22	<i>Neocosmospora vasinfecta</i>	JX517198	150	158	165	473
17.	JAI-CB34	<i>Neocosmospora vasinfecta</i>	JX517199	149	158	165	472
18.	JAI-CP13	<i>Fusarium solani</i>	JX517200	150	158	172	480
19.	JAI-CB36	<i>Fusarium solani</i>	JX517201	150	158	172	480
20.	JAI-MB21	<i>Fusarium solani</i>	JX517202	150	158	172	480

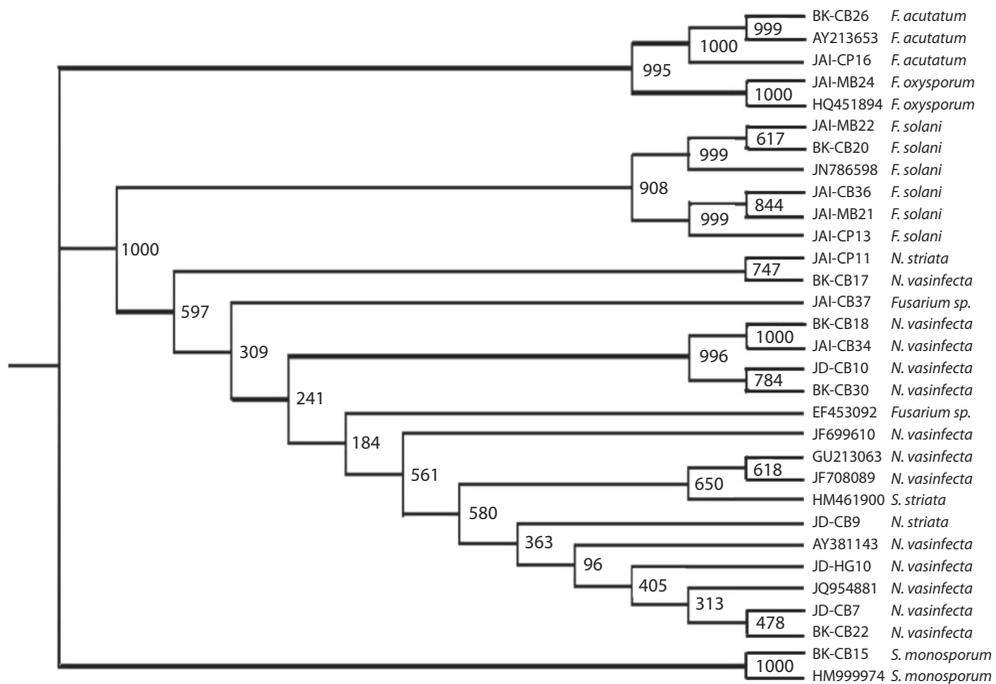


Fig. 3. Phylogram generated using Tree View of multiple sequences aligned rDNA region of 20 root pathogen isolates along with reference sequences.

TABLE 4
Summary of genetic variation statistics for all loci

Locus (mean)	Sample Size	<i>na</i>	<i>ne</i>	<i>h</i>	<i>i</i>
Pop 1	32	1.9660	1.5273	0.3066	0.4627
Pop 2	2	1.0952	1.0673	0.0394	0.0576
Pop 3	8	1.8299	1.5045	0.2930	0.4378
Pop 4	10	1.8299	1.5543	0.3087	0.4539
Mean of all loci	52	2.0000	1.5427	0.3129	0.4712

na, observed number of alleles, *ne*, effective number of alleles, *h*, Nei's gene diversity, *i* Shannon information index.

TABLE 5
Matrix of unbiased genetic identity and genetic distance according to Nei (1973)
among 4 populations of root pathogens based on RAPD markers

Population	Pop 1	Pop 2	Pop 3	Pop 4
Pop1	****	0.8860	0.9840	0.9972
Pop2	0.1210	****	0.8273	0.8766
Pop3	0.0161	0.1896	****	0.9796
Pop4	0.0029	0.1317	0.0206	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

within the genera converged together and grouped with respective reference sequences with appreciable bootstrap values.

Out of 20 isolates, eight were molecularly identified as *Neocosmospora vasinfecta*, five as *Fusarium solani*, two as *Neocosmospora striata*, two as *Fusarium acutatum*, one as *Syncephalastrum monosporum*, one as *Fusarium oxysporum* and one as *Fusarium* species. AMOVA revealed 100 % variation within population of root rot pathogens isolated from arid legumes. All the four populations of arid legumes, root pathogens were subjected to PCA using GenALX. The first three principle coordinates accounted for 44.74, 17.78 and 15.03 respective amounting to a total of 77.55 % of total variance. The eigen vector analysis indicated that the contribution of the first three factors were 38.2, 15.2 and 12.8 respectively explaining a total of 66.2 % of total variability. The analysis of RAPD binary data using Popgene revealed that the coefficient of gene differentiation between populations (*Gst*) was 2.0170 among all the populations. The genetic analysis of RAPD data of all the 52 isolates

of root pathogens isolated were performed by dividing the isolates on the basis of host from which they were initially isolated. For example, population one (32 isolates of Clusterbean), population two (two isolates of horse gram), population three (eight isolates of cow pea), and population four (10 isolates of moth bean).

The summary of genetic variation statistics of all loci is presented in table 4. The mean values of all the four populations together for Nei's genetic diversity (*h*) was 0.3129 and Shannon information index (*i*) was 0.4712. The data revealed that the genetic diversity of root pathogen isolates of population four (moth bean) was the richest among all the four populations. The matrix of unbiased genetic identity and distance among four populations of root pathogens based on RAPD markers is presented in table 5. The Nei's genetic distance ranged from 0.0029 to 0.1896 and the genetic identity ranged from 0.8273 to 0.9972. The largest distance was recorded between population two (horse gram) and three (cowpea) and the least between the population one (Clusterbean) and population four (moth bean).

DISCUSSION

Besides dry root rot caused by *Macrophomina phaseolina* (Gautam et al., 2013), 52 root samples were found infected with fungal pathogens. Irrespective of the geographical location from where the diseased samples were collected, all pathogen isolates clustered in RAPD dendrogram as per their respective genera. An insight of morphological characters and RAPD dendrogram revealed no consistency in grouping root pathogen isolates as morphologically similar isolates were genetically cataloged into different RAPD clusters. For instance, *N. vasinfecta* isolates with identical morphological characters such as restricted colony growth pattern, off white colony color and light pink pigmentation in the culture medium were genetically cataloged into distinct RAPD clusters i.e. JD-HG10 (cluster two), JAI-CB34 (cluster five) and JD-CB10 as out group between clusters three and four. Similarly, the phylogenetic clustering of isolates was also not in accordance with the host from which they were isolated. The isolates belonging to different hosts were well distributed among different RAPD phylogenetic clusters. For example, isolates from Bikaner aligned in cluster three, four and between cluster four and five, six and seven and seven and eight clusters as out groups.

The results indicated that the measures of relative genetic distances among populations did not completely correlate the geographical distances of places of their isolations and or hosts suggesting that the root pathogens of the arid legumes are neither confined to a geographical location and or are host specific in nature. Present results are in agreement with earlier molecular studies suggesting that grouping of *Macrophomina phaseolina* isolates independent of host and geography (Gautam et al., 2013) and DNA polymorphism in *Fusarium* spp. failed to show a positive correlation with geographical origin (Gargouri, Bernier, Hajlaoui, & Marrakchi, 2003) performed genotyping with RAPD markers resolves pathotype diversity in the ascochyta blight and *Fusarium* wilt pathogens of chickpea in Pakistan. By

contrast, they reported different levels of virulence among *F. oxysporum* f. sp. *ciceri* collected from different geographical locations.

RAPD technique has been used for the characterization of the microbes and detection of microbial diversity (Gautam et al., 2013; Sharma, Verma, & Sharma, 2013). Under present study, RAPD revealed high levels of polymorphism among root pathogens coupled with higher PIC percentage due to discretionary ability of selective primers in evaluating genetic diversity of root pathogens in arid legumes. Further genus wise RAPD clustering and alignment of isolates belonging to the genus *Neocosmospora* from cluster 1 to 5 including all outgroups between these clusters and *Fusarium* isolates aggregating from cluster 6 to 8 and flanking outgroups including one isolate of *Syncephalastrum* revealed both inter and intraspecific genetic diversity among root pathogen isolates.

Fusarium taxonomy and species designation based on morphological characterization is most frustrating (Brayford, 1989). More than 120 different formae speciales of *F. oxysporum* have been identified based on specificity to host species belonging to a wide range of plant families some formae speciales are not primarily vascular pathogens but cause foot and root rot or bulb rot (Pietro, Madrid, Caracuel, Jarana, & Roncero, 2003).

Riveros, Munoz, Gonzalez, Rojas and Hinrichsen (2001) compared RAPD-PCR with classical taxonomy, morphology, pathogenicity of *Fusarium* strains and found that the obtained results were inconsistent. Different workers (Jana et al., 2003; Ibrahim & Nirenberg, 2000) have grouped *Fusarium* spp. population from different plant host by using RAPD analysis and suggested that RAPD markers can be a quick and reliable alternative for differentiating isolates of *Fusarium* spp.

A high degree of nucleotide variations in the nuclear rDNA ITS region allowed separation of 20 representative isolates of all RAPD clusters and distinct isolates and of which eight were molecularly identified as *N. vasinfecta*, five as *F. solani*, two as *N. striata*,

two as *F. acutatum*, one as *Syncephalastrum monosporum*, one as *F. oxysporum* and one as *Fusarium* species. Due to SNP's, insertions and deletions (INDELS) and total length polymorphism of rDNA region, upon multiple sequence alignment different genera (i.e. *Fusarium*, *Neocosmospora* and *Syncephalastrum*) separated from each other and species within the genera were clustered together and with that of reference sequences with appreciable bootstrap values that validate reliable grouping. Bootstrap values are dependable measures of phylogenetic accuracy and higher values are likely to indicate reliable groups (Hillis & Bull, 1993). A broad pathogenic and genetic diversity was detected among isolates of *Fusarium oxysporum* f. sp. *cepae* restriction digestions of IGS regions of rDNA classified the isolates from Turkey and Colorado into 13 groups (Bayraktar, Türkkan, & Dolar, 2010; Mess et al., 1999) suggested that *F. oxysporum* f. sp. *lycopersici* is a polyphyletic taxon. Stewart, Kim, James, Dumroese and Klopfenstein (2006) carried out molecular characterization of morphologically undistinguished isolates of *Fusarium* sp., *F. oxysporum* and *F. commune* isolates from a conifer nursery using AFLP and ITS rDNA sequencing, mitochondrial rDNA and nuclear translation elongation factor 1- α . Each isolates had a unique RFLP phenotype.

Out of various regions of rDNA, the ITS and intergenic spacer (IGS) of the nuclear rDNA repeat units have been reported to be evolved fast and may vary among species within a genus or among populations and hence can be used for phylogenetic studies at various taxonomic levels. Molecular phylogenetic analysis has helped to clarify ubiquitous in traditional classification systems of *Fusarium* species by ITS marker Datta, Choudhary, Shamim, Singh and Dhar (2011) performed molecular diversity analysis in Indian isolates of *Fusarium oxysporum* f. sp. *lentis* insighting wilt disease in lentil and reported 77.14 % polymorphism using RAPD. Three molecular markers revealed varying degree of genetic diversity in the selected isolates ranging from 54 % in case of RAPD and up to 35 % with ITS markers.

Llorens et al. (2006) characterized *Fusarium* species isolates by PCR-RFLP analysis of the intergenic spacer region of rRNA gene (rDNA). They reported that the haplotypes obtained with six restriction enzymes permitted to discern the six assayed *Fusarium* species and claimed it as a rapid and suitable methodology that allows closely related strains and to estimate the genetic relationship between the groups Chehri, Salleh, Yli-Mattila, Reddy and Abbasi (2011) molecularly characterized pathogenic *Fusarium* species of cucurbits. They reported that PCR-ITS-RFLP method is simple and rapid procedure for the differentiation of *Fusarium* strains at species level.

Bayraktar (2010) studied the genetic diversity in *F. oxysporum* f. sp. *cepae* causing basal plate rot on onion, using RAPD markers. The cluster analysis revealed distinct lineages at 65 % similarity. The Nei's genetic distances supported populations grouping according to the geographic regions and 11.4 % of total genetic diversity (GST) attributed to differentiation among the geographical locations. AMOVA confirmed that there were low genetic differences among populations. Their results suggest that RAPD-PCR is a useful method for analyzing genetic variation within and between populations of *Fusarium oxysporum* f. sp. *cepae*.

The AMOVA revealed 100 % variation within population indicating that the total proportion of variability exists within host as compared to different hosts signifying that root pathogens of arid legumes are not host specific in nature. The estimates of Nei's genetic distance and identity between the populations provided a better understanding of genetic relationships. The low GST of 2.0170 among all the populations revealed little evidence for geographical subdivision among four populations. The results of molecular analyses clearly indicates that the out of the four populations of arid legumes, the genetic diversity of root pathogen isolates of population four (moth bean) was the richest. Similarly, low levels of genetic differentiation among geographic populations and high genetic variability have been reported

on asexually reproducing fungi such as *F. oxysporum* f. sp. *vasinfectum* (Wang et al., 2006).

The diversity analysis based on DNA sequence polymorphisms existing within highly conserved regions of the nuclear ribosomal DNA, such as the internal transcribed spacer or the intergenic spacer region (Edel, Steinberg, Gautheron, Recorbet, & Alabouvette, 2000). Zaccardelli, Vitale, Luongo, Merighi, & Corazza (2008) performed molecular and morphological characterization of *Fusarium solani* isolates based on host range tests. *F. solani* were subdivided into different formae speciales and varieties while DNA sequence of 28S rDNA, ITS-rDNA and elongation factor (EF-1 α) distinguished *F. solani* complex in 50 subspecific lineages.

The documented 'Fungi of India' records of fungi are mostly reported with identifications based on microscopic characteristics following traditional and obsolete keys of classification. Fungi reported herein were identified on the basis of both morphological and nuclear rDNA analysis. The available 'Fungi of India' records and internet searches revealed that *Fusarium solani* in India as pathogen on leguminous crop was first reported causing root rot of pea (Sen, Lal, & Majumdar, 1970) and on guar (Satyaprasad & Ramarao, 1981).

Neocosmospora vasinfecta, from rhizosphere of peanut (*Arachis hypogaea* L.), as a pathogen of new wild disease of horse gram in India, was first reported by Mishra (1988). *N. vasinfecta* has a wide distribution in tropical and temperate regions and is commonly associated with roots of leguminous crops and has been isolated from soil. *N. vasinfecta* an incident of wilt of Clusterbean have been reported by Patel, Patel, Desai and Khandar (1998). *N. vasinfecta* associated with the root rot complexes of peanuts has been reported in Vietnam (Dau et al., 2010) and China (Huang, Chen, & Chung, 1992). Cornely et al. (2001) reported *N. vasinfecta* as human pathogenic fungi in a patient with acute non lymphocytic leukemia on the basis of 5.8S nuclear rDNA region. They reported SNP's at two nucleotide positions and on insertion of a single base pair.

The root pathogens of the arid legumes were found neither restricted to a geographical location and nor were host specific in nature. A high degree of nucleotide variations in the nuclear rDNA ITS region allowed separation of different genera and species within the genera clustered together and with that of reference sequences validating reliable grouping. We report association of several fungi for the first time as pathogens causing root diseases in arid legumes. Precisely, *F. solani* wilt in cowpea and seedling rot in moth bean, *F. oxysporum* wilt in moth bean, *F. acutatum* damping off in cowpea and Clusterbean, *Fusarium* sp. seedling rot in clusterbean, *Neocosmospora striata* root rot in cowpea and wilt in Clusterbean and *S. monosporum* root rot in Clusterbean. The present findings reveal a complete spectrum of major root pathogens associated with the arid legume and warrant concerted research towards effective management of these pathogens.

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RESUMEN

Diagnóstico molecular y la variabilidad genética intraespecífica de patógenos de raíces en leguminosas resistentes a sequías del oeste de Rajasthan, India. La producción de leguminosas resistentes a sequías como *Cyamopsis tetragonoloba*, *Vigna unguiculata*, *Vigna acutifolia* y *Macrotyloma uniflorum*, puede permanecer inactiva durante décadas debido a su alta susceptibilidad a enfermedades en las raíces. Además, hay información limitada relacionada con el diagnóstico molecular y la variabilidad genética intraespecífica de patógenos de raíces en estas leguminosas resistentes a sequías. Para contribuir en esta área, evaluamos un total de 52 extractos de 88 raíces infectadas con patógenos fúngicos en los distritos de Jodhpur, Jaipur y Bikaner de Rajasthan. Las muestras de raíces infectadas se analizaron siguiendo los métodos estándar de microbiología para extracción y purificación

de hongos y para estudios genéticos. Independientemente del sitio donde se recolectaron las muestras contaminadas, todos los extractos patógenicos se agruparon en dendrogramas RAPD en cada uno de sus respectivos géneros. El filograma, basado en alineamiento de secuencias múltiples reveló que distintos géneros (*Fusarium*, *Neocosmospora* y *Syncephalastrum*) separados entre ellos y especies del mismo género se agrupan con sus secuencias de referencia con valores de bootstrap significativos. De cada 20 extractos representantes de cada agrupamiento y todos los grupos externos secuenciados, ocho fueron identificados molecularmente como *Neocosmospora vasinfecta*, dos como *Fusarium acutatum*, una como *Syncephalastrum monosporum*, una como *Fusarium oxysporum* y una como *Fusarium*. Los patógenos de estas leguminosas resistentes a sequías no están restringidos por la localidad ni por un hospedero específico. *Fusarium solani* que marchita el frijol de vaca y pudre la semilla de *Vigna aconitifolia*, *F. oxysporum* que marchita a *Vigna aconitifolia*, *F. acutatum* que marchita a *Vigna unguiculata* y *Cyamopsis tetragonoloba*, *Fusarium* sp. que pudre la semilla de *Cyamopsis tetragonoloba*, *Neocosmospora striata* que pudre la raíz de *Vigna unguiculata* y marchita a *Cyamopsis tetragonoloba* y, *Syncephalastrum monosporum* que pudre la raíz en *Cyamopsis tetragonoloba*, fueron identificados molecularmente como nuevos registros de patógenos fúngicos que causan daños en las raíces de leguminosas resistentes a sequías.

Palabras clave: RAPD, ITS, *Fusarium*, *Neocosmospora*, *Syncephalastrum*.

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