MOLECULAR ANALYSIS OF ISOLATES OF *Fusarium udum* CAUSING PIGEONPEA WILT THROUGH RAPD AND ISSR MARKERS

THESIS

Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
in partial fulfilment of the requirements
for the Degree of

MASTER OF SCIENCE
IN
AGRICULTURE
(PLANT PATHOLOGY)

By

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DECLARATION OF STUDENT

I hereby declare that, the experimental work and its interpretation of the Thesis entitled **"MOLECULAR ANALYSIS OF ISOLATES OF *Fusarium udum* CAUSING PIGEONPEA WILT THROUGH RAPD AND ISSR MARKERS"** or part thereof has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or scientific organization. The source of materials used and all assistance received during the course of investigation have been duly acknowledged.

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CERTIFICATE

This is to certify that, the thesis entitled **"MOLECULAR ANALYSIS OF ISOLATES OF *Fusarium udum* CAUSING PIGEONPEA WILT THROUGH RAPD AND ISSR MARKERS."** submitted in partial fulfilment of the requirement for the degree of **"Master of Science in Agriculture (Plant Pathology)"** of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **MISS. DHAGE SHUBHANGI ASHOK** under my guidance and supervision.

The subject of thesis has been approved by the Student's Advisory Committee.

Place : Akola
Date : 11/6/2012

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Countersigned

Associate Dean
Post Graduate Institute
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THESIS APPROVED BY THE STUDENT'S ADVISORY COMMITTEE INCLUDING EXTERNAL EXAMINER (AFTER VIVA-VOCE)

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ACKNOWLEDGEMENTS

Traditional formal words of acknowledgement will not project the picture of feelings while expressing a deep sense of gratitude to my many known and unknown hands which helped and put me on right paths and enlightened with their experience, knowledge and wisdom. I shall ever remain grateful to them.

I consider myself fortunate and greatly priviledged in availing this golden opportunity to express my deepest sense of gratitude and humble indebtedness towards my guide and chairman Dr. V.R Gupta, Junior Plant Pathologist, AICRP on Pulses, Dr. P.D.K.V., Akola and Dr. S.S. Mane, Professor and Head, Department of Plant Pathology, Dr. P.D.K.V. Akola for their kind, generous and valuable guidance ,helpful suggestions and constructive critism right from the selection of this research topic from to its completion. The words all short to appropriately and sufficiently thanks them.

With infinite pleasure, I put on record indebtedness to member of my advisory committee, Dr. S. S. Mane Professor and Head, Department of Plant Pathology, Dr. G. K. Giri, Associate Professor of Plant Pathology and Dr. S. J. Gahukar, Chairman of Biotechnology Centre for their intellectual stimulation, kind suggestions and critical comments during investigation.

My inexplicable gratitude are also due to Prof. D. B. Tamgadge, Associate Dean, P.G.I. ,Dr. P.D.K.V. Akola for providing all necessary facilities during my course of study.

I also record my sincere thanks to Dr.R.M. Gade, Professor , Dr.R.W.Ingale, Associate Professor, Dr. Ashwini Charpe, Assistant Professor, Department of Plant Pathology, Dr. P.D.K.V., Akola for their valuable guidance and encouragement throughout the investigation.

I am much obliged to Dr. S.J.Gahukar, Chairman of Biotechnology Centre, Dr.P.R.Jadhav, Assistant Professor, Dr. M.P. Moharil Assistant Professor of Biotechnology Centre

Dr. P.D.K.V. Akola for providing every kind of technical help and guidance in dealing with my problems.

I also take this opportunity to express my sincere thanks to Shri R.S.Ghawade, Shri V.V.Deshmukh, Dr. Manjusha Gaikwad madam, Shri Milind Joshi and Shri K.K. Kakade sir, Department of Plant Pathology, P.G.I., Dr.P.D.K.V. Akola for providing all necessary facilities.

I also thanks to Shri. Pravin Deshmukh, Shri D.S. Barade, Shri G.P. Moharut kaka, Shri. Nilesh Belsare, Smt. N.W. Narkhede, Smt. K.S. Damodar and Smt. Rajkanya Bhaltilak for rendering necessary help during my research work.

I also thanks aspirants Shri. Sandesh Bangar, Shri. P.N. Rakhonde, Shri. D. L. Wasule, Shri. M.N.Ingole, Shri. Shivaji Wavare, Shri. Anil Gulhane, Shri Prashant Kale and my friends Prashant, Jayashri, Hemant sir for helping me during my Biotechnology research aspect.

The special thanks to my dear room partners Dipali and Harshada for giving me support at difficult time.

I have no words to express my regards and love to my friends. I wish to express my special thanks to my best friends Linus, Vrushali, Madhuri, Ashwini, Kalpana, Ranjitha Rani, Santoshi, Chaitanya, Namita, Priyanka, Karuna, Sarika, Sarala, Sonali, Harshal, Kunal, Ashish, Hrisikesh, Amol, Tushar, Milind, Aniket, Dipak and my senior Nivedita for their direct and indirect help and close co-operation throughout the course of study.

Words are inadequate to my feelings of love, affection and gratitude to my father Shri. Ashok and mother Sau. Manda whose kind blessing, constant encouragement, patience and unstinted support to me to complete thesis. This thesis is affectionately dedicated to my beloved parents as they are the architect of my past and presents.

Few words cannot thanks my brother Rahul and Suyog who stood by my side during my endeavour to pleasure they helped me always in the difficulties.

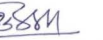
I am very thankful to all authors and researchers whose articles helped me in organizing my research on proper time.

At the end, I recall memory of my grandpa Shri. Bhaurao And Janabai Dhage who always cherished the dream of my higher education.

The words are inadequate to thank almighty "Saibaba" for his grace and blessing which enabled me to be happy and successful in every phase of my life.

I am thankful to Mr. Shailesh Kadam (Real Computers and Parvati Grafix, Akola) for computerizing photocopies and printing the transcript in time.

I was fortunate enough to receive the kind co-operation from almost everyone in one way or the other during my stay at this Institute. It is extremely difficult to thanks individually by name this shortcoming may please be pardoned.



Place-Akola

Date- 11/06/2012

Dhage Shubhangi Ashok.

Enrolment No.- II -1900

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D) Abbreviations

AFLP	-	Amplified Fragment Length Polymorphism
°C	-	Degree celcius
Cat	-	Catalase
CD	-	Critical Difference
cm	-	Centimetre
CTAB	-	Cetyl Trimethyl Ammonium Bromide
DAI	-	Days after incubation
dd H_2O	-	Double distilled water
Deptt.	-	Department
DNA	-	Deoxyribose Nucleic Acid
dNTP	-	2' – deosynucleoside – 5' – triphosphate
Dr. PDKV	-	Dr. Panjabrao Deshmukh Krishi Vidyapeeth
e.g.	-	*Exampli gratia* (For example)
EDTA	-	Ethylene Diamine Tetra Acetic Acid
et. al.	-	*Et alia* (and others)
etc.	-	*Et cetra*
TE buffer	-	Tris hydroxymethyl aminomethane ethylene
Fig.	-	Figure
F.U.	-	*Fusarium udum*
g	-	gram
HCl	-	Hydrochloric acid
HCN	-	Hydrocynic acid
i.e.	-	That is
ISSR	-	Inter Simple Sequence Repeat Marker
J.	-	Journal
kb	-	Kilobites
kbp	-	Kilo base pairs
M	-	Molar
Max	-	Maximum
mg	-	Milli gram(s)
min.	-	Minutes
ml	-	millilitre

mM	-	Millimolar
mm	-	Millimeter
nm	-	Nanometre
No.	-	Number
NS	-	Non significant
ŋg	-	Nano gram(s)
ŋm	-	Nano meter
PCR	-	Polymerase Chain Reaction
PDA	-	Potato dextrose agar
PDB	-	Potato dextrose broth
PO	-	Peroxidase
PPO	-	Polyphenol oxidase
RAPD	-	Random Amplified Polymorphic DNA
RFLP	-	Restriction Fragment Length Polymorphism
RNA	-	Ribonuclease acid
rpm	-	Revolution per minute
SE (M)	-	Standard error of means
sec	-	Seconds
Sig.	-	Significant
SSR	-	Simple Sequence Repeat
Taq polymerase	-	*Thermus Aquaticus* DNA polymerase Enzyme
TBE buffer	-	Tris hydroxymethyl aminomethane boric acid
Tris	-	Tris Hydroxymethyl amino methane
U	-	Unit
UPGMA	-	Unweighted Pair Group Method Analysis
UV	-	Ultra Violet
viz.	-	Videlicet (namely)
w/v	-	Weight by volume
µ	-	micron
µg	-	Microgram
µl	-	microlitre
%	-	Per cent
/	-	Per
@	-	At the rate of

Thesis Abstract

a) Title of the thesis	:	"MOLECULAR ANALYSIS OF ISOLATES OF *Fusarium udum* CAUSING PIGEONPEA WILT THROUGH RAPD AND ISSR MARKERS."
b) Full name of student	:	DHAGE SHUBHANGI ASHOK.
c) Name and address of Major Advisor	:	Dr. V.R.Gupta. Junior Plant Pathologist, AICRP on Pulses, Dr. PDKV, Akola (M.S.) - 444104.
d) Degree to be awarded	:	M.Sc. (Agriculture)
e) Year of award of Degree	:	2012
f) Major subject	:	Plant Pathology
g) Total number of pages in the thesis	:	56
h) Number of words in the abstract	:	357
i) Signature of the Student	:	
j) Signature, name and address of forwarding authority	:	

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ABSTRACT

Six isolates of *Fusarium udum* were collected from different districts of Vidarbha. It includes the isolates from Washim (FU-1), Yavatmal (FU-2), Buldhana (FU-3), Amaravati (FU-4),

Gadchiroli (FU-5) and Akola (FU-6).

The pathogenicity test of 6 isolates of *Fusarium udum* was done by water culture method using susceptible variety TAT-10. The isolates of Washim and Amaravati showed 100% wilting. Yavatmal, Buldhana and Gadchiroli isolates showed 66.66% wilting, and Akola showed 33.33% wilting.

The genetic variation was detected among six isolates of *Fusarium udum* using RAPD marker. Off 30 RAPD primers screened for amplification of DNA of six isolates of *Fusarium udum,* 18 produced reproducible and scorable bands with high percentage of polymorphism. The number of bands generated were primer and isolate specific ranged from 3 to 12. A total 154 amplicons were obtained with the 18 primers with an average fragment of 8.6 bands per primer. The level of polymorphism was 94.8 percent. The primer OPA-9, OPB-3 and OPB-14 amplified maximum fragments 12 and the least in OPB-20 (4).In similarity matrix showed that Buldhana isolate had higher value of similarity coefficient (0.6428) whereas Gadchiroli isolate had the lower value of similarity coefficient (0.448).The primer OPA-2, OPA-3, OPA-4,OPA-16 and OPB-17 showed the monomorphic band could be used for identification of *Fusarium udum.*

The genetic variation was detected among six isolates of *Fusarium udum* using 16 ISSR marker, among which 9 produced reproducible and scorable bands with high percentage of polymorphism. The number of bands generated were primer and isolate specific and ranged from 7 to 12. A total 80 amplicons were obtained with 9 ISSR primers. The average fragment of 8.9 bands per primer were obtained. All bands were polymorphic and the level of polymorphism was 100 percent. The primer $(GA)_9C$ amplified maximum fragments (12) and the least in $(ATG)_6$ and $(CA)_8RG$ found (7) fragments. In similarity matrix showed that the isolates of Gadchiroli was found to have higher value of similarity coefficient 0.6125 whereas Akola isolate had the lower value of similarity coefficient (0.40).

In similarity matrix of RAPD and ISSR the Akola isolate showed highest similarity coefficient (0.6239) whereas Amaravati isolate showed lowest value of similarity coefficient (0.4957)

CHAPTER I

INTRODUCTION

1.1 Background information

The pigeonpea (*Cajanus cajan*) is an important pulse crop in India. It is known as arhar, tur and red gram. It is most important pulse crop in tropics and subtropics. It is an often cross pollinated (20-70%) with diploid chromosome number 2n= 22 and genomic size 1C= 858Mbp (Annonymous, 2012).

The crop is grown in Asia, Africa, America etc. (Annonymous, 2012). It is the second major pulse crop in India accounting about 20% of the total production in world. In India, the area under this crop is 3.38 million hector with production of 2.27 million tones and average productivity 671 kg/ha (Annonymous, 2011).

India is the largest producer and consumer of pigeonpea in the world. It is nutritionally rich and contains 22% protein, 1.7% fat, 7.3 % calcium apart from Vit. A and B-complex in traces. It is consumed mainly in the form of split pulse as *dal.*

Being a legume crop it also improves the soil fertility. However, the crop is attacked by large number of fungal, bacterial and viral diseases *viz. Alternaria* leaf spot, Collar rot, Dry root rot, wilt, Powdery mildew, Sterility mosaic etc. Among these wilt caused by *Fusarium udum* is major limiting factor in production and on an average 10-100% losses occur due to wilt (Mesapogu *et al.,* 2012).

Fusarium is a heterogeneous and cosmopolitan organism. It's population varied greatly in respects of morphological, physiological and pathological characters as described by E.J. Butler in 1906. Later, he isolated and identified the causal organism as *Fusarium udum.* The fungus can survive on infected plant debris in soil for about 3 years (Mesapogu *et al.*, 2012). It enters through the rootlets as it possesses an ability to penetrate through the cell wall. It grows

through the vascular system of the plant and plug the xylem vessels. The pathogen is capable of causing infection to the plant at any stage of its growth but symptoms are more pronounced when the crop is in the flowering and podding stage.

1.2 IMPORTACE OF STUDY

Pigeonpea wilt is a major constraint to pigeonpea production, worldwide. The fungus can survive on infected plant debris in the soil for about three years and cause serious yield losses, sometimes 100% in susceptible cultivars. The total production loss due to this disease in India alone was estimated to be approximately 97000 tones per year (Mesapogu *et al.*, 2012).The disease occurs generally at seedling and flowering stage. Seeds harvested from wilted plants are lighter and dull than those from healthy plants.

Considering the nature of damage and survival ability of fungus use of resistant varieties is the only economical and practical solution. Research efforts has already been in progress in this direction at ICRISAT, Hyderabad and through All India Co-ordinated Research projects on pulses. But most of the resistant varieties have been found to be susceptible after some years because of breakdown in their resistance and co- evolution of pathogen.

The most practical and cost efficient method for management of this disease is through the use of resistant cultivars; however, pathogenic variation in *Fusarium udum* limits the effectiveness of this strategy (Sharma *et al.*,2009). *Fusarium udum* have extreme genotypic and phenotypic variability and can adopt to wide range of environmental conditions. Therefore accurate and rapid identification of pathogen is necessary for appropriate management of this disease.

In the recent years several types of molecular marker systems such as RAPD (Random Amplified Polymorphic DNA),ISSR (Inter Simple Sequence Repeat), AFLP(Amplified Fragment Length Polymorphism) etc. have been increasingly used to study the variability

in pathogenic populations of *Fusarium udum* (Sharma *et al.*,2009). The commonly used Polymerase Chain Reaction (PCR) based DNA marker systems *viz* Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and more recently Simple Sequence Repeats (SSRs) or Microsatellites are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology.

Considering this the present study was aimed to know the genetic and pathogenic variability amongst the isolates of *Fusarium udum* collected from Vidarbha regions of Maharashtra by RAPD and ISSR marker that will helpful in breeding the varieties resistant to pigeonpea wilt for Vidarbha region.

1.3 OBJECTIVES OF STUDY

The main objective of the studies was molecular analysis of isolates of *Fusarium udum* causing pigeonpea wilt through RAPD and ISSR markers.

1.4 HYPOTHESIS

Increased attention is being paid by the scientist to develop wilt resistant varieties for large scale cultivation of pigeonpea. In order to breed the wilt resistant varieties it is essential to know the genetic variability and pathotype variability in the region. Therefore accurate and rapid identification of pathogen is essential. Pathogenic types of isolates prevailing in pigeonpea growing areas in Vidarbha. Hence the present investigation was carried out to know the variability in *Fusarium udum* which in turn will be useful for its exploitation in wilt resistance breeding for development of area specific varieties.

1.5 SCOPE AND LIMITATIONS

Pigeonpea wilt has the potential to devastate the entire crop and can cause losses upto 94% (Mc Rae, 1923). Unfortunately the varieties which have been evolved through screening in wilt sick plots, showed varied types of reactions in the farmer's field and sick plots at different locations. It might be due to pathogenic variability in

pathogen. A variety having resistance in particular area may be susceptible in another area over the years resistance became of co-evolution of pathogen. Therefore, it is necessary to ascertain the genetic and pathogenic variability among the *Fusarium udum*. Hence present investigation was carried out on "Molecular analysis of isolates of *Fusarium udum* causing pigeonpea wilt by RAPD and ISSR markers." during 2011-12 at Department of Plant Pathology, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth Akola (M.S.) India.

CHAPTER II

REVIEW OF LITURATURE

The pigeonpea wilt (*Fusarium udum* Butler) is the most important disease of pigeonpea and was first described by E.J. Butler in 1906 from Bihar. The wilt disease of pigeonpea is responsible for 15-25% mortality of plants and the wilting may rise to more than 50% in epidemic years (Butler,1906).The grain losses are almost 100% when 100% wilt occurs at prior to early pod formation stage and its around 30% when pods are fully developed and the plants are close to harvest(Nene *et al.*,1979)

2.1. History

Butler (1910) identified and named the causal agent of wilt of pigeonpea for the first time in India as *Fusarium udum*.

Patil and Gokhale (1965) noticed the continuous cultivation of pigeonpea for 2-3 years in same field leads to more than 50% losses of plants due to wilt disease. They recorded that the wilt disease is more conspicuous in adult stage compared to seedling stage.

Kannaiyan and Nene(1981) reported that the losses are 100% when plants wilt at prepod stage but 67.1% and 29.5% at pod maturity and pre harvest stages respectively. The wilted plants produce over 70% normal seeds and in delayed wilting, seed are not affected adversely.

Patil *et al.* (1990) reported that the wilt disease of pigeonpea caused losses ranging from 89-100% when plants were in prepod and early pod stage. The rate incidence was maximum during flowering and pod formation stage.

Joshi (2000) studied pathogenic variability in pigeonpea wilt pathogen *Fusarium udum* Butler in Nepal. In Mid-Western terai region this disease is more severe killing 90% of plants in farmer's field.

2.2. Pathogenicity test

Wensly and Mckeen (1962) evolved a rapid test for testing the pathogenicity of *Fusarium oxysporum* f.sp. *melonis*. The symptoms developed within seven to eight days and reported several advantages.

Cases and Diaz (1985) studied the wilt complex in chickea. In the wilt complex individual leaves showed flaccidity followed by a dull green discolouration and dessication. Symptoms developed on all the foliage and plants died, necrotic leaflets remained attached to the petioles. Vascular and pith tissues were coloured brown. Wilt symptoms were occasionally observed on 20 days old plants but they were most conspicuous at the onset of flowering 50 days after planting.

Haware and Nene (1994) investigated a rapid method for inoculating pigeonpea seeding with *Fusarium udum* involving the root dips of test plants in an inoculums source with many advantages.

Rao and Krishnappa (1997) studied pathogenicity test of chickpea plants. He collected chickpea wilted plants from 15 districts of Karnataka. He used cultivar Annegiri-1 for pathogenicity test. Pot as well as water culture technique were used for pathogenicity. The observations on wilt incidence were recorded after 60 days after inoculation in pot culture.

Joshi *et al.* (2000) studied pathogenic variability in pigeonpea wilt pathogen in Nepal. Pathogenic variability of 2 isolates collected from different regions in Nepal was studied in using root dip inoculation and transplantation methods. 7-10 days old roots of 10-30 seedling immersed in spore suspension up to 30 minutes and then transplanted in sterilized sand and soil (1:1) mixture in plastic pots. Disease incidence and reactions were taken 11, 21 and 31 days after transplanting. In this pathogenicity test differential pigeonpea lines showed 4 types of reactions such as no apparent symptoms, chlorosis, chlorosis and early wilting (after 10-15 days) , chlorosis and late wilting (after 15-30days).

Ombe *et al.* (2008) conducted pathogenicity test of 52 isolates of *Fusarium solani* f.sp. *phaseoli.* He used cultivated susceptible bean variety Rosecoco for pathogenicity test. Firstly he prepared spore suspension and then these spore suspension was mixed with steam sterilized soil @ $3x10^5$ conidia/gm of soil. All 15 isolates were found to incite disease to varying levels of virulence. Symptoms observed on the infected plants were reddish streaks on the tap roots and hypocotyls. Symptoms included pale yellow leaves. The highest virulence was observed in 44 isolates i.e.84.6% and lowest was observed in 8 isolates i.e.15.4%.

Datta *et al.* (2011) carried the pathogenicity test of 15 isolates of *Fusarium oxysporum* f.sp. *lentil.* He collected 15 isolates of Lentil wilt from different agro climatic zones of India. Among them 5 isolates were highly pathogenic and 10 isolates were moderately pathogenic.

Mesapogu *et al.*(2012) noticed the typical symptoms of pigeonpea wilt such as epinasty, interveinal yellowing of lower leaves followed by drooping of leaves and discolouration of vascular tissues. 12-98% plants showed wilt symptoms with an average disease incidence of 56.31%. Necrosis generally began at 4-6 weeks of post inoculation and was near completion after 8 weeks.

3. Morphological variation

Gupta *et al.* (1986) observed variability among 6 isolates of *Fusarium oxysporum* f.sp.*ciceri* causing vascular wilt of chickpea. Morphological studies of 6 isolates of *Fusarium oxysporum* f.sp.*ciceri* revealed the variation in size of macro and micro conidia. He observed the variation in the growth parameters and cultural characters of 6 isolates differed in growth pattern, pigmentation and sporulation.

Reddy and Chaudhary (1985) collected 3 isolates of *Fusarium udum* from ICRISAT, Hyderabad, 1 from Jaipur and 2 from New Delhi and studied their morphological and cultural characters, radial growth, colony characters and size of macro and micro conidia

and chlamydospores. Cultural studies were recorded on Potato Dextrose Agar, Potato Sucrose Agar, Czapek's Dox Agar, Oat Meal Agar media. Best growth of *Fusarium udum* was observed on Potato Sucrose Agar media. The isolates based on colony characters on Potato Sucrose Agar showed that isolate 1, 2 were fluffy and forming concentrate rings, isolates 3 showed cottony forming sectors and isolate 4,5 and 6 showed fluffy and sticky growth. Macro conidia were found in isolate 2,3,4 and 6 only.

Raghuwanshi (1995) reported the cultural & physiological studies of *Fusarium oxysporum* f.sp.*sesami*. Cultural studies of *Fusarium oxysporum* f.sp.*sesami* were carried out on six different medium. The fungus showed luxuriant growth & maximum sporulation on Potato Dextrose Agar medium .The profuse growth & sporulation of *Fusarium oxysporum* f.sp.*sesami*. was recorded at 27^0C temperature. Further it was observed that the fungus growth & sporulation was maximum at pH range between 6.5-7.5.

Rao and Krishnappa (1997) isolated *Fusarium spp.* from wilted chickpea plants collected from different locations of Karnataka. These isolates differed in cultural characteristics and pathogenicity. Wilt inducing fungus *Fusarium oxysporum* f.sp.*ciceri* and seed rotting fungus *F.solani* were found associated with the infected plants, the former being predominant.

Groenewald *et al.* (2006) recorded physiological studies of *Fusarium oxysporum* f.sp.*cubense* in South African population. Twenty-six isolates of *Fusarium oxysporum* f.sp.*cubense* were selected for this study. Differences in growth rate, spore production and no. of spores was determined. Virulence of *Fusarium oxysporum* f.sp. *cubense* was determined by inoculating susceptible tissue culture banana plantlets. Differences in cultural characteristics and virulence among isolates were found. The south African isolates were divided into sporodochial, cottony and slimy pionnotal types. All isolates produced microconidia in abundance but production of macroconidia and sporodochia varied

Mahesh *et al.* (2010) showed existence of variation among 41 isolates of *Fusarium udum* from different parts of India with special references to culture on PDA media. All the 41 *F. udum* isolates showed wide variation with respective mycelial colour pigmentation, colony characters. Based on pigmentation 41 isolates were categorized into 5 groups. It include group 1- brown colour, group 2- dark yellow , group 3- light yellow, group 4- light yellow to brown colour and group 5- pink colour pigmentation. Based on mycelia colour the isolates were categorized into 3 groups *viz* fluffy, partially appressed and appressed growth.

Mesapogu *et al.*(2012)showed genetic diversity and pathogenic variability among *F.udum* collected from different geographical locations of India. All the isolates exhibited variable level of virulence against susceptible pigeonpea cultivarT-21. Majority of the isolates showed fluffy mycelia growth with yellow colour and golden brown to mulbur purple substrate pigmentation in the medium.

4. RAPD (Random Amplified Polymorphic DNA) and ISSR (Intersimple Sequence Repeat) markers

Belabid *et al.* (2003) studied pathogenic and genetic characterization of Algerian isolates of *Fusarium oxysporum* f. sp. *lentis* by RAPD and AFLP analysis. Thirty-two isolates of *Fusarium oxysporum* f. sp. *lentis* were collected from different lentil growing areas in north-west Algeria. A pathogenicity test was performed for all isolates. The amount of genetic variation was evaluated by polymerase chain reaction (PCR) amplification.

Sivaramkrishnan *et al.* (2002) found the genetic variability in 36 isolates of *Fusarium udum* [*Gibberella indica*] collected from 4 pigeonpea (*Cajanus cajan*) growing states in India (Andra Pradesh, Karnataka, Maharashtra and Uttar Pradesh) which was assessed using Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) techniques. Although the two molecular markers detected high levels of polymorphism among the

fungal isolates, the degree of polymorphism varied depending on the marker selected. Cluster analysis of the similarity index data from the two DNA markers classified the isolates into three major groups, suggesting the existence of a minimum of 3 specific races of the pathogen prevailing in the pigeonpea growing area of Maharashtra. All the five isolates from the pigeonpea fields of the International Crops Research Institute for the Semiarid Tropics in Patancheru, Andra-Pradesh were placed in the same group suggesting the prevalence of a single pathogenic race. AFLP proved to be better in assessing the genetic diversity among the fungal pathogen isolate than RAPDs.

Hou *et al.* (2005) studied genetic diversity in barley from west China based on RAPD and ISSR analysis. Two types of molecular markers random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) were assayed to determine the genetic diversity of 46 barley accessions including 27 landraces of *H. vulgare* sp. *vulgare* (HV), 6 accessions of *H. vulgare* sp. *spontaneum* (HS) and 13 accessions of *H. vulgare* sp. *agriocrithon* (HA) from west China. A high level of polymorphism was found with both RAPD and ISSR markers and the mean polymorphism information content (PIC) values were 0.574 and 0.631 for RAPD and ISSR markers respectively. In RAPD analyses 84 out of 109 bands (77.06%) were polymorphic. The number of alleles ranged from 2 to 8 per primer, In ISSR analyses, a total of 107 alleles were detected among which 105 alleles (98.13%) were polymorphic. The number of alleles per primer ranged from 2 to 10 with an average of 5.94 alleles per ISSR primer. Cluster analysis indicated that all 46 barley accessions could be distinguished by both RAPD and ISSR markers.

Sharma *et al.* (2005) studied genetic variability of pea wilt pathogen *Fusarium oxysporum* f. sp. *pisi* from three agronomically growing regions using cultural characteristics DNA RAPD and proteins (native- proteins and esterase isozymes) markers. Variability in growth pattern, colour of mycelium and pigments was observed. Based on cultural characters. Twenty-four isolates from three regions could be

assigned to six groups. Amplification of genomic DNA of 24 isolates of 10-mer primers generated 134 polymorphic markers whereas native proteins and esterase profile reveals 27 markers, based on NTSYS analysis of RAPD and protein data, the isolates were delineated into four region specific groups. Group PRI, PRIII and PRIV represented isolates from sub-tropical and sub-humid regions whereas group PRII consisted of isolates from dry-temperate region, indicating that pathogen population from sub-tropical and sub-humid regions evolved from three distinct lineages and those from temperate region from the fourth lineage.

Honnareddy *et al.* (2006) reported the genetic variability within 24 isolates representing seven races of *Fusarium oxysporum* f.sp. *ciceri* assessed by RAPD with a set of 40 primers. Out of 40 primers OPA-1, OPA-2, OPA-3, OPA-4, OPA-5, OPA-9, OPB-15, OPB-19 gave better polymorphic bands. UPGMA cluster analysis divided the isolates into seven distinct clusters at 0.55 genetic similarities. The most virulent isolate obtained from wilt sick field of IARI (MB-4C) New Delhi was found to be distinct from others.

Nagabhushana (2006) studied molecular basis interaction between *Fusarium udum* and *Heterodera cajani.* RAPD technique was used to demonstrate the polymorphism in 9 pigeonpea genotypes.Susceptible genotype GS-1 and resistant genotype C-11 had maximum similarity whereas Gulyal Red and GS-1 showed least similarity.

Bayraktar *et al.* (2007) analyzed the genetic variation among the isolates of *Fusarium oxysporum* f.sp. *ciceri* using molecular markers Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) polymorphism. Seventy-four isolates were assessed using 30 arbitrary decamers primers. Average cluster analysis of RAPD, ISSR and RAPD +ISSR database divided the isolates into three major groups. Groups 1,2,3 consisted of 41,18,15 isolates respectively. These method revealed a considerable genetic variation among Turkish isolates.

Prasad *et al.* (2007) studied the molecular variability among 45 isolates belonging to 8 sections of the genus *Fusarium* by using PCR based RAPD,ISSR and ITS-RFLP analysis using 12 random primers amplified 1145 loci of sizes ranging from 0.4kb to 5.5 kb and the cumulative analysis of similarity values placed different *Fusarium* spp. in 8 clusters. The clear grouping at spp. Level and more precise clustering at formae specialis level was resulted with DNA markers generated by RAPD primers.

Dubey *et al.*(2008) used RAPD, ISSR, SSR markers to assess the genetic diversity of *Fusarium oxysporum* f.sp.*ciceri* isolates . The cluster generated by RAPD grouped all isolates into 3 categories at 25% genetic similarity and into two major categories at 30% genetic similarity. Majority of these isolates were grouped in one category. While the isolates from all other state were grouped in other. Some of the RAPD (OPM-6, OPI-9,OPN-4, OPF-1, P-21and SC-1), ISSR (ISSR-7, ISSR-11, ISSR-12) and SSR(MB-17) markers clearly distinguished area specific isolates.

Bayraktar (2009) investigated the intra and inter specific polymorphism among fungal pathogens by using 30 RAPD and 20 ISSR primers. The no. of RAPD fragments produced per primer varied between 1-8 and ranged in size from 0.2-3.6 kb. The primer OPK-7, OPK-19 OPA-18 yielded polymorphic bands within and between fungal spp.

Prasanthi *et al.*(2009) studied molecular marker for screening *Fusarium* wilt resistance in pigeonpea (*Cajanus cajan*). *Fusarium* wilt is a serious disease of pigeonpea which causes severe yield losses. Screening of 88 lines along with ICPL 87119 and ICPL 8863 resistant checks for *Fusarium* wilt under field conditions and artificial inoculation identified 14 lines having 0-20% plant mortality grouped under 0-5 disease score. PCR reactions using different primers with genomic DNA of these lines resulted in identification of 6 resistant sources with specific amplification for resistance to wilt at 920bp with OPGO8 primer. Considering the wilt reaction and

resistance linked RAPD marker, it is possible to identify the new resistance sources in a short time and they can be utilized in breeding programme or for direct release.

Chakraborty *et al.* (2010) showed the identification patterns of *Trichoderma* isolates by using RAPD and ITS-PCR. Nineteen isolates of *Trichoderma viride* and *Trichoderma harzianum* obtained from rhizosphere soil of North Bengal region were studied using RAPD and ITS-PCR. The genetic relatedness among eleven isolates of *T. viride* and eight isolates of *T. harzianum were* analyzed with six random primers. RAPD profiles showed genetic diversity among the isolates with the formation of eight clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.67 to 0.95. ITS-PCR of rDNA region with ITS1 and ITS4 primers produced 600bp products in all isolates.

Datta *et al.* (2011) collected one hundred (100) isolates of *Fusarium oxysporum* f. sp. *lentis* causing vascular wilt in lentil from different agroclimatic region of India. Finally fifteen distinct *F. oxysporum* f. sp. *lentis* isolates selected for molecular characterization by three molecular markers. Twenty Randomly Amplified Polymorphic DNA (RAPD) primers produced a total of 105 reproducible bands, out of which 81 (77.14%) were polymorphic and 24 (22.85%) were monomorphic. Nine Simple Sequence Repeat (SSR) primer pairs amplified 21 alleles with 2.33 alleles per primer. Considerable length variations (561 to 668 bp) in rDNA regions were found as restrictions digestion of amplified rDNA region produced forty eight different DNA bands. Three molecular marker revealed varying degree of genetic diversity in selected isolates ranging from 54% in case of RAPD up to 35% with ITS markers. Based on the coefficient of similarity, the isolates grouped into two major clusters in the dendrogram. Isolates from North Indian regions grouped in same cluster, whereas isolates from north east regions and eastern region fell in another cluster.

Mesapogu *et al.* (2011) studied Genetic diversity and pathogenic variability among *Fusarium udum* isolates collected from

different geographical locations of India. All the isolates exhibited variable levels of virulence against a susceptible pigeonpea cultivar (T-21). The genetic diversity allelic variations among these isolates were estimated using RAPD molecular markers. All the thirteen RAPD primers were found to be highly reproducible and produced a total of 126 loci of which 69 loci were polymorphic. Primer OPB 17 amplified highest number of polymorphic bands with maximum polymorphic information content (PIC) of 4.00. Percentage of polymorphism revealed by individual primers varied from 33.3 to 76.9% with an average of 53.4%. Further, cluster analysis of OPB-17 provided a substantially discrimination of all the isolates.

Dhar *et al.* (2012) studied 32 pathogenic isolates of *Fusarium udum* from different pigeonpea growing areas in India for pathogenic and molecular variability. The amount of genetic variation was evolved by PCR amplification with 20 RAPD markers and 9 microsatellite markers. All amplification revealed scorable polymorphisms among the isolates and total 137 polymorphic fragments were scored for the RAPD markers and 16 alleles for the SSR markers. RAPD primers showed 86% polymorphism.

CHAPTER III

MATERIAL AND METHODS

The present investigation entitled "Molecular analysis of isolates of *Fusarium udum* causing pigeonpea wilt through RAPD and ISSR markers." was carried out in the laboratory of Department of Plant Pathology and Biotechnology Centre, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola (M.S.).

3.1 Materials:-

3.1.1 Glasswares:-

The glasswares used during the study were petridishes, reagent bottles, micropipettes, conical flasks of different capacity i.e. 2000 ml, 1000 ml, 500 ml, 250 ml and 100 ml, test tubes, pipette, beakers of borosil make.

3.1.2 Equipments:-

The laboratory equipments *viz.* autoclave, hot air oven, laminar air flow, electronic balance, BOD incubator, research microscope, centrifuge machines (Eppendorf 5418), PCR (Eppendorf), gel electrophoresis (Genexy), microwave oven, Gel Doc. Unit (Biorad), vortex etc. were used.

3.1.3 Seeds:-

Pigeonpea seeds of susceptible variety of TAT-10 were obtained from Pulse Research Unit Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

3.1.4 Miscellaneous material:-

Inoculation needle, distilled water, spirit lamp, spirit, scalpel, forcep, cork borer, slide, cover slip, haemocytometer, ependorff tube, PCR tubes and tips etc. were used.

3.2 Collection of wilt infected pigeonpea plants from pigeonpea growing region of Vidarbha:-

A total 6 isolates of *Fusarium udum* were obtained by isolation from wilted pigeonpea plants from six districts of Vidarbha *viz.* Washim, Yavatmal, Buldhana, Amaravati, Gadchiroli and Akola.

3.3 Sterilization of glassware, media, water, blotter paper and other material:-

The petriplates, test tubes, reagent bottles and conical flasks of different capacities i.e. 1000 ml, 500 ml, 250 ml etc. of 'Borosil' make were sterilized in hot air oven at 180^0 C for one hour. Whereas the media, distilled water and blotter paper were sterilized in autoclave at 15 psi for 15 minute. The material *viz.* needles, inoculating needle, forcep, scalpel were sterilized on flame by direct heating.

3.4 Precautions to eliminate contamination:-

All the isolation and inoculation work of microbial culture was carried out under aseptic condition in laminar airflow. The laminar airflow was sterilized by glowing ultraviolet light for half an hour prior to commencement of work. The working surface and side glasses of laminar airflow were sterilized with denatured spirit.

3.5. Preparation of media

The media with following composition was prepared for isolation of *F.udum* from infected plants

1. Peeled Potato 200 gm.
2. Dextrose 20 gm.
3.. Agar 20 gm.
4. Distilled water 1000 ml.

The potatoes were peeled, cleaned under running water and then sliced into pieces. Then 200 gm healthy peeled potato were taken and boiled in 500 ml sterilized distilled water in saucer pan for 30 min. until they get soft. In another 500 ml of water, 20 gm Agar and 20 gm Dextrose was added and dissolved. The potatoes were then strained through muslin cloth and the extract was collected. Both the parts were mixed and the volume was made to 1 lit. The media thus prepared was then dispensed in conical flask to half or $1/3^{rd}$ of its

capacity. Then the flasks were plugged with non-absorbent cotton. Similarly the potato dextrose broth was prepared without adding agar-agar. The flasks were sterilized in autoclave at 15 psi for 15 minutes.

3.6 Isolation of fungi *Fusarium udum* from infected plants :-

Material required:-

The infected plant part i.e roots of wilted pigeonpea plants, sterilized distilled water, sterile petriplates, sterile media, mercury chloride ($HgCl_2$) 0.1%, forceps, scalpel, spirit lamp / burner, were used for isolation.

Procedure:-

A) Before isolation:-

The working surface of laminar airflow was cleaned with denatured spirit. All the material *viz.* petriplate, blade, needle, forceps, denatured spirit were kept in laminar air flow and the ultraviolet lamp was blown for 30 min. before commencement of work.

B) Isolation:-

a) The sterilized molten media (Potato Dextrose Agar) was poured in pre sterilized petriplates and allowed to solidify.

b) The roots of wilted pigeonpea plants were cut into small pieces using sterile blade and surface sterilization of root bits was done by 0.1% $HgCl_2$.

c) The roots bits were washed successfully 3-4 times to remove the traces of $HgCl_2$.

d) Root bits were placed on sterile blotter paper for soaking of excess moisture. Then the root bits were placed on solidified PDA media and plates were incubated at 27 ± 1^0c for 7 days.

e) The mycelium coming out of roots bits was lifted with the help of sterilized inoculating needle and further purified by hyphal tip method.

f) In this way 6 isolates were obtained from different locations which is given as follows.

List of different isolates of *Fusarium udum* from Vidarbha region:-

Sr. No	ISOLATES	LOCATION
1	FU-1	Washim
2	FU-2	Yavatmal
3	FU-3	Buldhana
4	FU-4	Amaravti
5	FU-5	Gadchiroli
6	FU-6	Akola

3.6 Morphological study:-

The *Fusarium udum* isolates were confirmed by growing pure culture of these 6 isolates grown on PDA media. The fungal growth were examined on growth pattern and mycelium pigmentation.The identification of isolates of *Fusarium udum* was done as per Booth, C. 1977.

3.7 Pathogenicity test by water culture method

The water culture technique as given by Nene and Kannaiyan (1982) was adopted for testing virulence of *Fusarium udum* cultures. The spore suspension of $1.5x10^5$ spores /ml was prepared of each isolates. It was filled in bigger size test tube upto ¾ level. The 8 days old seedling of pigeonpea cultivar TAT-10 grown in sterilized sand, were transferred in each tube. The seedlings were held in straight position by cotton plugs. The sterilized distilled water was added to these tubes every 48 hrs. to make up the loss of water. One set of uninoculated seedling in sterilized water was kept as check. The observations on wilting were taken and wilting percentage was calculated.

3.8 RAPD and ISSR analysis of races of *Fusarium udum*

The total six isolates of *Fusarium udum* collected from the different district of Vidarbha were used for PCR-RAPD and ISSR analysis. The following reagents/chemicals and their composition were used.

A) Stock solutions:-

a) Tris HCl (100mM pH 8.0)

Tris HCl	1.576 g
Distilled water	100 ml

b) Tris HCl (1000mM pH 8.0)

Tris HCl	15.76 g
Distilled water	100 ml

c) EDTA (100 Mm)

EDTA	2.9225 g
Distilled water	100 ml

B) Extraction Buffer (150 ml)

a) CTAB 2%

CTAB	3 g

b) NaCl 1.4 m

NaCl	12.2724 g

c) EDTA 20 Mm

EDTA (100 Mm)	30 ml

d) Tris HCl 100mM

Tris HCl 1000 mM	45 ml

The Tris HCl and EDTA was taken in measuring cylinder from their respective stock solutions. In this solution NaCl and CTAB was added and then the volume was make up to 150 by addition of distilled water.

C) $T_{10}E_1$ buffer

Tris HCl (100Mm)	1 ml
EDTA (100Mm)	0.1 ml
Distilled water	8.9 ml

Tris HCl and EDTA was taken in measuring cylinder and then volume was make up to 10 ml by addition of distilled water

D) 70% Ethanol

Ethanol	70 ml
Distilled water	30 ml

E) RNAse (Diluted RNase)

RNase	43 ul
Distilled water	172 ul

F) 10X TBE (Tris- Borate EDTA)

Tris base	108 g/lit
Boric acid	55g/lit
0.5 M EDTA (pH-8.0)	40ml/lit
Distilled water	1lit

G) Liquid Nitrogen

H) Dicholoroform:Isoamylalcohol(24:1)

I) Isopropanol

J) Phenol: Chloroform

L) Absolute ethanol

M) $MgCl_2$ (25 mM)

N) Loading dye (6x)

O) Ethidium bromide (0.5µg/ml)

P) dNTPs (2 mM)

Q) Tag DNA polymerase (5 Units)

R) Primers (RAPD/ ISSR)

S) Nuclear free water

T) Genomic DNA

3.8.1. Selection of isolates for RAPD and ISSR:-

The total six isolates of *Fusarium udum* were used for PCR-RAPD and ISSR analysis as listed in table -1

3.8.2 Procedure for Genomic DNA Extraction:-

The DNA was extracted by the method given by Murray and Thompson (1980). The procedure of DNA extraction was carried

out in 2ml eppendorf tube. The 200 ml Pure culture of fungus was grown on Potato Dextrose Broth (PDB) in 1000 ml conical flask for 7 days at a temperature of 27 $\pm$ 2^{0}c in BOD incubator. The mycelial mat was harvested after 7 days. It was washed thoroughly and repeatedly and then dried using blotter paper and crushed to powder in pre-chilled pestle and mortar with liquid nitrogen. The powdered mass was mixed with pre-heated (65°C) 1 ml of CTAB extraction buffer (100 mM Tris HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl and 2% CTAB) per tube. The 2 µl Mercapthoethanol was added to it. The content was mixed gently by inversion and incubated at 65^{0}C for 1 hour in a water bath with occasional mixing by gently inverting the tube. The tubes were then centrifuged at 8000 rpm for 10 min. The supernatant was transferred to another tube and an equal volume of Chloroform:Isoamylalcohol (24:1) was added. The content was mixed gently for 5 min and centrifuged for 15 min at 8000 rpm. The aqueous phase was transferred to another tube .The nucleic acid was precipitated by adding 0.6 volume of Isopropanol (pre chilled) and tubes were centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the pellet was suspended in 70% ethanol and then centrifuged at 9000 rpm for 10 min and two ethanol wash were given and centrifuged for 10 min at 9000 rpm. The pellet thus obtained was air dried and sterile nuclease free water was added to dissolve the DNA as per the quantity of DNA and treated with RNAse (10.51µl for 200µl) and placed on thermocycler at 37^{0}c for 1 hr. The equal volume of Phenol: Chloroform was added to it after incubation. The content was mixed well for 5 min and centrifuged at 9000 for 15 min in micro centrifuge. The aqueous phase was transferred to another tube and equal volume of chloroform was added, mixed well and centrifuged. The aqueous phase was again transferred to another tube and DNA was precipitated by adding 2.5 volume of absolute ethanol. The DNA pellet was washed in 70% ethanol vacuum dried and redissolved in sterile water and stored at -20^{0}C for further study.

3.9 DNA quantification

The DNA obtained after extraction was confirmed by running it on 0.8% agarose gel containing ethidium bromide at 0.5 mg/ml in a horizontal gel electrophoresis system. The 2 µl of genomic DNA of each isolate + 3µl loading dye + 5µl sterile water was loaded in each well. After completion of 5 cm run, the gel was observed under UV light and the DNA yield and quality was confirmed.

3.10 Randomly Amplified Polymorphic DNA (RAPD)

The protocol given by Bayraktar and Dolar (2007) was used with slight modification in PCR thermal cycler programmer. The RAPD-PCR was performed in a total volume of 25 µl of reaction containing 10mM of Tris-HCl (pH8.8), 50mM of KCl, 1.5mM of $Mgcl_2$, 0.32 µl of primer, 0.125mM of dNTPs, and 1U of Taq polymerase. The thirty oligonucleotide were randomly selected from OPA, OPB, OPC and OPE primer series.

3.10.1 PCR mixture (25 ul) per reaction:-

1. Tris-HCl (pH-8.8)	10 mM
2. KCl	50 mM
3. $Mgcl_2$	1.5 mM
4 .Primer	0.32 µM
5. DNTPs	0.125 mM
6. Taq polymerase	1 U

The PCR tubes containing reaction mixture were placed in the Thermal cycler for 40 cycles with the following profile:-

Step 1:-	Initial denaturation	94^0C for 5 min
Step 2:-	Denaturation	94^0_{Cfor} 1 min
Step 3:-	Anneling	35^0c for 1 min
Step 4:-	Extension	72^0c for 2 min
	The step 2 to 4 were repeated for 40 cycles	
Step 5:-	Final elongation	75^0c for 5 min
Step 6:-	Retention	4^0C

3.11 Electrophoresis of RAPD-PCR :-

The RAPD analysis was carried out in horizontal gel electrophoresis. The PCR products were separated electrophoretically in 1.5% agarose gel using 1X TBE buffer. The gel was stained with ethidium bromide .

The cleaned and dried electrophoresis assembly was used for RAPD. The gel tray was wiped and cleaned with methanol. The agarose gel solution was prepared by mixing agarose in 1X TBE Buffer, this mixture was heated in microwave oven for 3 min. The ethidium bromide was added to the gel solution as staining agent. The gel solution was then poured in the gel casting tray and combs was placed in the gel and allowed to set. 1X TBE buffer was used as the tank buffer. After the casting of the gel the combs were removed and the gel was placed in electrophoresis assembly with 1X TBE buffer. The gel was pre-runned for 15 min. The care was taken while handling the gel as the ethidium bromide added is highly mutagenic. After the pre-run, the RAPD-PCR product was mixed with 5 ul 6X dye and the mixture was loaded in the wells, along with the 1 Kb DNA ladder in the first well. The gel was run for 2 hrs. at 80V. After the run, the gel was removed carefully from the unit and observed under Gel Doc instrument.

3.12 ISSR – PCR:

The protocol given by Bayraktar and Dolar (2007) was used with slight modification in PCR thermal cycler programmer for ISSR-PCR analysis. The sequences, annealing temperatures and G+C content (%) of ISSR primers screened against isolates of *Fusarium udum* is listed in Annexure-II.

The ISSR-PCR was performed in a total volume of 25 µl of reaction, containing 10 mM of Tris-HCl (pH- 8.8), 50 mM of KCl, 2.5 mM of $Mgcl_2$, 0.24 µM of primer, 0.2mM of dNTPs, and 1 U of Taq polymerase. The sixteen ISSR primers with di- or tri- nucleotide repeats were analyzed. The annealing temperatures were adjusted as per G+C content of the primers.

3.12.1 PCR mixture (25 µl) per reaction for ISSR

1.	Tris-HCl (pH-8.8)	10 mM
2.	KCl	50 mM
3.	$Mgcl_2$	2.5 mM
4.	Primer	0.24 µM
5.	dNTPs	0.2 mM
6.	Taq polymerase	1 U

The PCR tubes containing reaction mixture (25 µl per tube) were placed in the thermal cycler for 40 cycles with the following profile.

Step 1:-	Initial denaturation	94^0c for 5 min.
Step 2:-	Denaturation	94^0c for 1 min.
Step 3:-	Anneling	As per anneling temp. of primers
Step 4:-	Extension	72^0c for 2 min.
	The step 2 to 4 were repeated for 40 cycles	
Step 5:-	Final elongation	75^0c for 8 min.
Step 6:-	Retention	4^0C

3.12.2 Electrophoresis ISSR-PCR:-

The ISSR-PCR analysis was carried out in horizontal gel electrophoresis. The PCR products were separated electrophoretically in 1.4% agarose gel using 1X TBE buffer. The gel was stained with ethidium bromide.

The cleaned and dried electrophoresis assembly was used for ISSR. The gel tray was wiped and cleaned with methanol. The agarose gel solution was prepared by mixing agarose in 1X TBE Buffer, this mixture was heated in microwave oven for 3 min. The ethidium bromide was added to the gel solution as staining agent. The gel solution was then poured in the gel-casting tray and combs was placed in the gel and allowed to set. 1X TBE buffer was used as the tank buffer. After the casting of the gel the combs were removed and the gel was placed in electrophoresis assembly with 1x TBE buffer. The gel was pre-runned for 15 min. The care was taken while handling the gel as the ethidium bromide added is highly mutagenic. After the

pre-run, the ISSR-PCR product was mixed with 5 ul 6x dye and the mixture was loaded in the wells along with the 1 Kb DNA ladder in the first well. The gel was run for 2 hrs. at 80v. After the run, the gel was removed carefully from the unit and observed under Gel Doc. instrument.

3.13: Data analysis

The gel images were captured and visualizaed in gel documentation system (Biorad).The data was scored as the presence (1) or absence (0) of individual band for each isolates in RAPD-PCR and ISSR-PCR analysis of races of *Fusarium udum*. The percentage of polymorphism was calculated by following formula.

$$\text{Percentage of Polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}}$$

Chapter - IV

Results and Discussion

CHAPTER IV

RESULTS AND DISCUSSION

The wilt of pigeonpea caused by *Fusarium udum* Butler is one of the major constraints in pigeonpea production worldwide. The disease is widespread in the pigeonpea growing areas of the world and causing 10-100% losses annually (Mesapogu *et. al.*, 2012). Therefore an investigation was undertaken to study the pathogenic and genetic variability among the different isolates of *Fusarium udum* collected from Vidarbha region by RAPD and ISSR markers.

4.1 Collection, isolation, purification and identification of pathogen

4.1.1. Collection and Isolation

The wilted pigeonpea plants showing typical symptom of wilting were collected from different districts of Vidarbha *viz.* Washim, Yavatmal, Buldhana, Amaravati, Gadchiroli and Akola.

The tissue isolation technique was used to isolate the causal agent from infected roots. The Potato Dextrose Agar (PDA) was used for isolation.

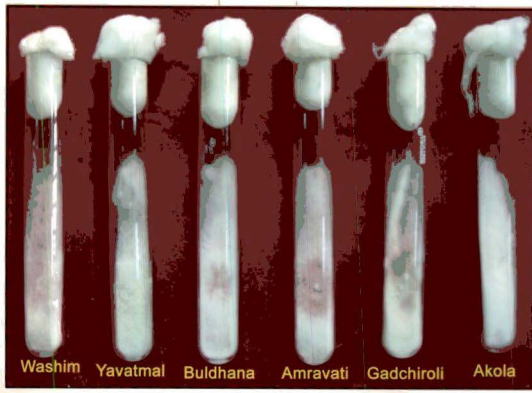
4.1.2. Purification and Identification

The cultures obtained after isolation were purified by hyphal tip method and identified as *Fusarium udum.* They were maintained on PDA slants for further studies.(Plate No.-1)

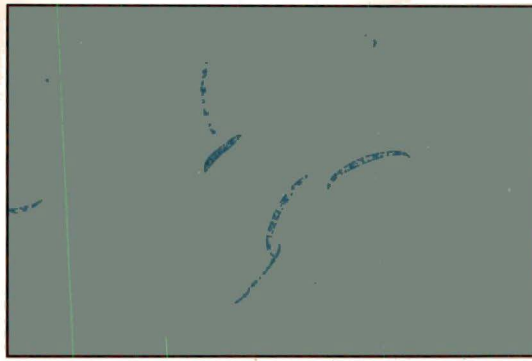
4.2 Pathogenicity and symptoms

4.2.1. Pathogenicity-

A total of six isolates of *Fusarium udum* were isolated and subjected to the pathogenicity test. The water culture method by Nene and Kannaiyan (1982) was used. The susceptible pigeonpea variety TAT-10 was used for pathogenicity test. Observations were recorded on percent wilted plants.

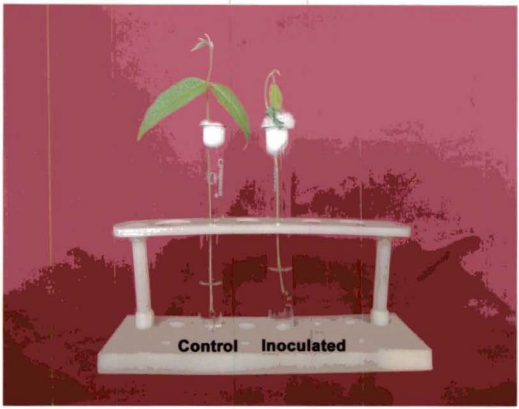


(A)



(B)

Plate 1 : (A) Isolates of *Fusarium udum*
(B) Macroconidia of *Fusarium udum*



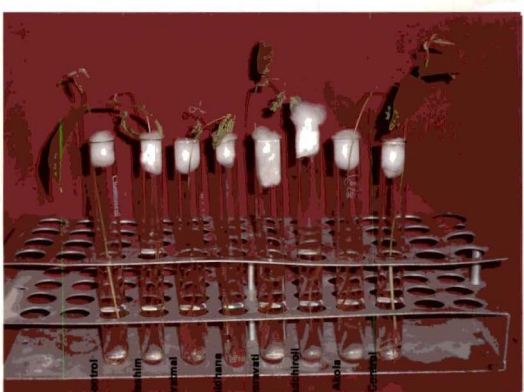


Plate 2 : Pathogenicity test of *Fusarium udum* against susceptible variety TAT-10 by water culture method

Table-1: Pathogenicity test of isolates of *Fusarium udum* against susceptible variety TAT-10 by water culture method.

Isolates	Total no. of plants placed in test tubes	Total no. of wilted plants	Percent-age of wilted plants	Wilting (Days After Inoculation)
FU-1 (Washim)	9	9	100	9
FU-2 (Yavatmal)	9	6	66.66	12
FU-3 (Buldhana)	9	6	66.66	15
FU-4 (Amaravati)	9	9	100	15
FU-5 (Gadchiroli)	9	6	66.66	18
FU-6 (Akola)	9	3	33.33	13

For the water culture test (Nene and Kannaiyan ,1982) the seedlings were initially grown in sterilized quartz sand. The eight days old seedling were uprooted carefully, the small portion of the root was cut from the base and seedlings were placed in the test tubes having spore suspension of *Fusarium udum* at a concentration of $1.5x10^5$ spore/ml (Plate-2). The three replications were made having 3 plants per replication. The results presented in table 1. revealed that there is variation in wilting percentage from 33.3% to 100% at concentration of $1.5x10^5$ spore/ml in the isolates collected from different vidarbha region. The early expression of symptoms was noticed 9 days after inoculation. Similar observations also reported by Wensly and Mckeen,1962.The isolates from Washim and Amaravati showed 100% wilting, whereas the isolates from Yavatmal, Buldhana,and Gadchiroli showed 66.66% wilting and isolates of Akola shows that 33.33% wilting.

4.2.2. Symptoms

The characteristic wilt symptoms such as dropping of upper leaves yellowing of the leaves from upper leaves which progress

downward. Wilting of the whole plant part were observed. Internal discoloration of the root vascular system was conspicuous in wilted plants. Similar observations were also reported by Cases and Diaz,1985.

4.3. *Fusarium udum* isolates selected for RAPD

The *Fusarium udum* isolates collected from different districts of from Vidarbha region namely Washim (FU-1), Yavatmal (FU-2), Buldhana (FU-3), Amaravati (FU-4), Gadchiroli (FU-5) and Akola (FU-6), Listed in Table 2 were selected for RAPD analysis.

Table 2 : List of isolates selected for RAPD analysis

Sr. no.	ISOLATES	LOCATION
1	FU-1	Washim
2	FU-2	Yavatmal
3	FU-3	Buldhana
4	FU-4	Amaravati
5	FU-5	Gadchiroli
6	FU-6	Akola

4.3.1. Primers used for RAPD analysis

In all 30 (10-mers) RAPD primers were selected for RAPD analysis of isolates of *Fusarium udum* consisting of 20 of OPA series, 8 of OPB series,1 of OPC series and one from OPE series from Genexy Pvt. Ltd.technologies. The PCR(Polymerase Chain Reaction) products amplified by each primer were resolved on 1.5 % agarose gel electrophoresis. The size of the amplified product was compared with 1 Kb DNA ladder from Fermentas India Ltd..

All 30 primers screened for RAPD analysis among them 18 primers OPA-1,OPA-2, OPA-3, OPA-4, OPA-7, OPA-10, OPA-12, OPA-13, OPA-14,OPA-16, OPA-18, OPB-3, OPB-7, OPB-10,OPB-11, OPB-14, OPB-15 and OPB-17 were polymorphic against six isolates of *Fusarium udum*.

The genetic variations were detected amongs six isolates of *Fusarium udum*. All 30 RAPD primers screened for analysis of DNA for six isolates of *Fusarium udum* among them 18 produced reproducible and scorable bands with high percentage of polymorphism. The number of bands generated were primer and isolate specific, ranged from 3 to 12. A total 154 amplicons were obtained with 18 primers with an average of 8.6 bands per primer. Out of 154 bands obtained 148 were polymorphic and the level of polymorphism was 94.8 percent. The primer OPA-9, OPB-3 and OPB-14 amplified maximum fragments 12 and the least in OPB-20 (4) respectively.

Table 3. The percent of polymorphism obtained in RAPD analysis of six isolates of *Fusarium udum*

Sr. No	Primer	Bands	Polymorphic bands	Percentage Polymorphism
1	OPA-1	7	7	100
2	OPA-2	7	6	85.71
3	OPA-3	8	5	62.5
4	OPA-4	9	7	77.77
5	OPA-7	9	9	100
6	OPA-10	7	7	100
7	OPA-12	9	9	100
8	OPA-13	10	10	100
9	OPA-14	10	9	90
10	OPA-16	10	10	100
11	OPA-18	11	11	100
12	OPB-3	12	12	100
13	OPB-7	9	9	100
14	OPB-11	8	8	100
15	OPB-14	12	12	100
16	OPB-15	7	7	100
17	OPB-17	6	5	83.33
18	OPB-20	3	3	100
	TOTAL	154	146	94.8

4.4. Primers selected for ISSR marker study

In all 16 primers were selected out of them 5 ISSR primers having same annealing temp. 50 °C, 4 (53 °C), 3 (54 °C) and 1 (55 °C) were used to evaluate the isolates of *Fusarium udum* for ISSR banding pattern. The PCR (Polymerase Chain Reaction) amplified products of each primer were resolved on 1.5 % agarose gel and size of the amplified product was compared with 1 Kb DNA ladder of Biotechnology Grade from Fermentas.

All 16 ISSR primers were screened, among them 9 primers $(GA)_8T$, $(GA)_9C$, $(AC)_8T$, $(AG)_8T$, $(ATG)_6$, $(CT)_8RG$, $(GA)_9RY$, $(GA)_8YC$,$(AG)_8YC$ were polymorphic amongst six isolates of *Fusarium udum*. The 9 primers produced reproducible and scorable bands with high percentage of polymorphism. The number of bands generated were primers and isolates specific ranged from 7 to 12. A total 80 amplicons were obtained with 9 primers with an average of 8.9 bands per primer. All 80 bands produced were found polymorphic and level of polymorphism was 100 percent. The primer $(GA)_9C$ amplified maximum fragments (12), $(ATG)_6$ and $(CA)_8RG$ produced the least fragments (7).

Table 4. The percent polymorphism observed in ISSR primers against *Fusarium udum*

Sr. No	Primer	Bands	Polymorphic bands	Percentage polymphism
1	$(GA)_8T$	8	8	100
2	$(GA)_9C$	12	12	100
3	$(AC)_8T$	9	9	100
4	$(AG)_8T$	10	10	100
5	$(ATG)_6$	7	7	100
6	$(CT)_8RG$	7	7	100
7	$(GA)_9RY$	8	8	100
8	$(GA)_8YC$	9	9	100
9	$(AG)_8YC$	10	10	100
	TOTAL	80	80	100

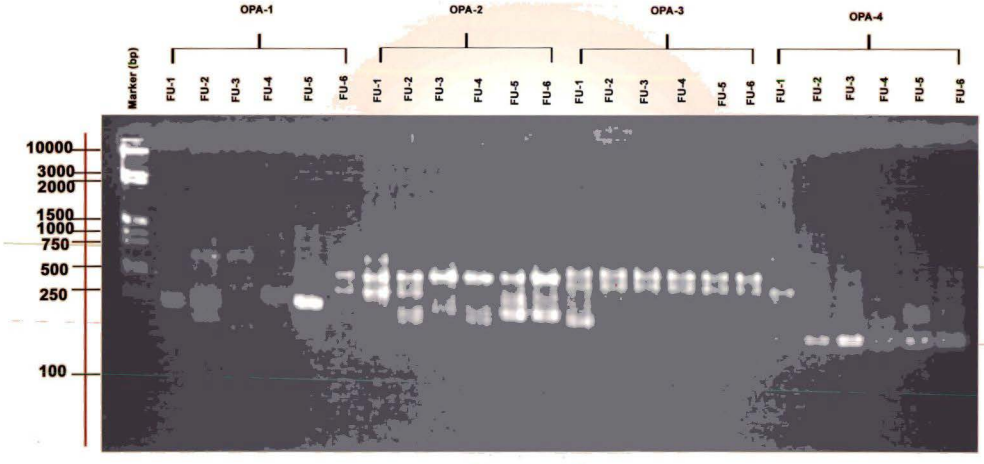


Plate 3 : RAPD banding pattern of primers OPA-1, OPA-2, OPA-3 and OPA-4

4.5. RAPD banding pattern

The banding pattern observed in 18 RAPD primers against *Fusarium udum* in each respective primer summarized below.

OPA-1

The primers amplified 7 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-1 ranged from 665bp-167bp.

The details of the 7 bands types of RAPD bands are as under-

Band type 1 (666 bp) - This type of band observed in FU-2, FU-3.
Band type 2 (602 bp) - This type of band observed in FU-5.
Band type 3(492 bp) - This type of band observed inFU-2, FU-3.
Band type 4 (346bp) - This type of band observed in FU-3, FU-6.
Band type 5 (290bp) - This type of band observed in FU-5.
Band type 6 (199 bp) - This type of band observed in FU-2.
Band type 7 (167 bp) - This type of band observed in FU-3.

OPA-2

The primers amplified 7 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-2 ranged from 649 bp-171 bp.

The details of the 7 bands types of RAPD bands are as under

Band type 1 (855 bp) - This type of band observed in FU-1.
Band type 2 (700. bp) - This type of band observed in FU-1, FU-2, FU- 3, FU-4, FU-5,FU-6.
Band type 3 (434 bp) - This type of band observed in FU-1,FU-2,FU-5, FU-6.
Band type 4 (337 bp) - This type of band observed in FU-1, FU-2, FU-3.
Band type 5 (237bp) - This type of band observed in FU-2, FU-5, FU-6.
Band type 6 (167 bp) - This type of band observed in FU-4.
Band type 7 (117bp) - This type of band observed in FU-2.

OPA-3

The primers amplified 8 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-3 ranged from 855 bp-117bp.

The details of the 8bands types of RAPD bands are as under

Band type 1 (855bp) - This type of band observed in FU-6.

Band type 2 (700bp) - This type of band observed in FU-5,FU-6.

Band type3(434bp)- This type of band observed in FU-1, FU-2, FU- 3, FU-4,FU-5,FU-6.

Band type 4 (337bp) - This type of band observed in FU-1, FU-2, FU-3, FU-4, FU-5,FU-6.

Band type 5 (237bp) - This type of band observed in FU-1, FU-2, FU- 3, FU-4, FU-5, FU-6.

Band type 6 (167bp) - This type of band observed in FU-5.

Band type 7 (117bp) - This type of band observed in FU-5.

Band type 8 (194bp) - This type of band observed in FU-1.

OPA-4

The primers amplified 9 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-4 ranged from 793 bp-140bp.

The details of the 9 bands types of RAPD bands are as under

Band type 1 (793bp) - This type of band observed in FU-1.

Band type 2 (617bp) - This type of band observed in FU-2, FU-3, FU-5,FU-6.

Band type 3(468bp) - This type of band observed in FU-3, FU-5, FU-6.

Band type 4 (355bp) - This type of band observed in FU-1, FU-5, FU-6.

Band type 5 (337bP) - This type of band observed inFU-2.

Band type 6 (305.69bp) - This type of band observed in FU-1, FU-6.

Band type 7 (243bp) - This type of band observed in FU-1, FU-2, FU-3, FU-4, FU-5, FU-6.

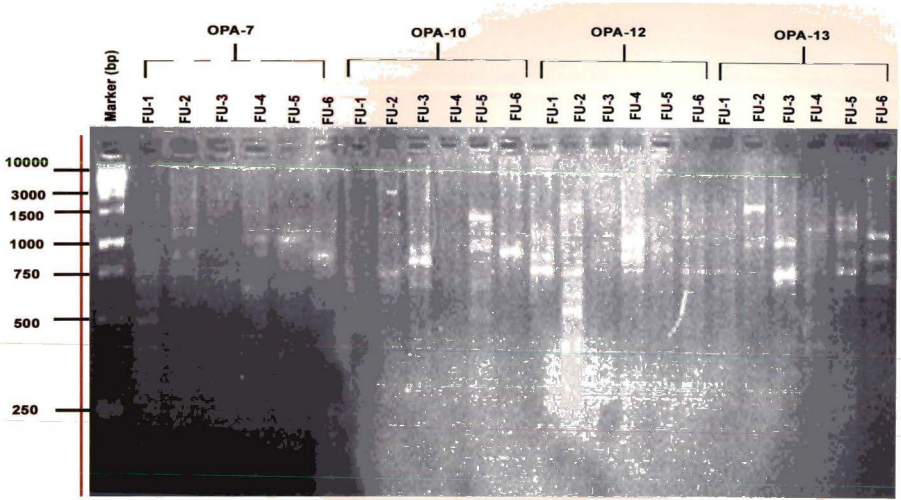


Plate 4 : RAPD banding pattern of primers OPA-7, OPA-10, OPA-12, OPA13

Band type 8 (199bp) - This type of band observed in FU-1 FU-2, FU-3, FU-4, FU-5,FU-6.

Band type 9 (140 bp) -This type of band observed in FU-1.

OPA-7

The primers amplified 9 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-9 ranged from 1771 bp-398 bp.

The details of the 9 bands types of RAPD bands are as under

Band type 1 (1771bp) - This type of band observed in FU-2.

Band type 2 (1633bp) - This type of band observed in FU-4, FU-5.

Band type 3(1335bp) - This type of band observed in FU-1, FU-3, FU-6.

Band type 4 (1027bp) - This type of band observed in FU-2.

Band type 5 (822 bP) - This type of band observed inFU-4, FU-5.

Band type 6 (714 bp) - This type of band observed in FU-1.

Band type 7 (527 bp) - This type of band observed in FU-1.

Band type 8 (449bp) - This type of band observed inFU-4.

Band type 9 (398bp) - This type of band observed in FU-1.

OPA-10

The primers amplified 7 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-10 ranged from 2302 bp-607bp.

The details of the 7 bands types of RAPD bands are as under

Band type 1 (2302bp) - This type of band observed in FU-5, FU-6.

Band type 2 (1507bp) - This type of band observed in FU-5.

Band type 3(1390bp) - This type of band observed in FU-3, FU-6.

Band type 4 (1183bp) - This type of band observed in FU-3.

Band type 5 (1027bp) - This type of band observed in FU-2.

Band type 6 (892bp) - This type of band observed in FU-3.

Band type 7 (607bp) - This type of band observed in FU-5.

OPA-12

The primers amplified 9 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-12 ranged from 2651bp-382 bp.

The details of the 9 bands types of RAPD bands are as under

Band type 1 (2651bp) - This type of band observed in FU-2, FU-4.

Band type 2 (2167bp) - This type of band observed in FU-2, FU-4, FU-6.

Band type 3(1601bp) - This type of band observed in FU-1, FU-4,FU-6.

Band type 4 (1335bp) - This type of band observed in FU-1, FU-2, FU-4, FU-5.

Band type 5 (1091.42 bp) - This type of band observed in FU-1, FU-2, FU-3, FU-4,FU-6.

Band type 6 (1048bp) - This type of band observed in FU-1, FU-2.

Band type 7 (790bp) - This type of band observed in FU-2.

Band type 8 (607 bp) - This type of band observed in FU-2.

Band type 9 (382bp) - This type of band observed in FU-2.

OPA-13

The primers amplified 10 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-13 ranged from 2495 bp-382bp.

The details of the 9 bands types of RAPD bands are as under

Band type 1 (2495bp) - This type of band observed in FU-2.

Band type 2 (2211bp) - This type of band observed in FU-5.

Band type 3(192bp) - This type of band observed in FU-4.

Band type 4 (1735bp) - This type of band observed in FU- 6.

Band type 5 (1601 bp) -This type of band observed in FU-2, FU-3.

Band type 6 (1256bp) - This type of band observed in FU-5, FU-6.

Band type 7 (1048bp) - This type of band observed in FU-1, FU-2, FU-3, FU-6.

Band type 8 (947 bp) - This type of band observed in FU-6.

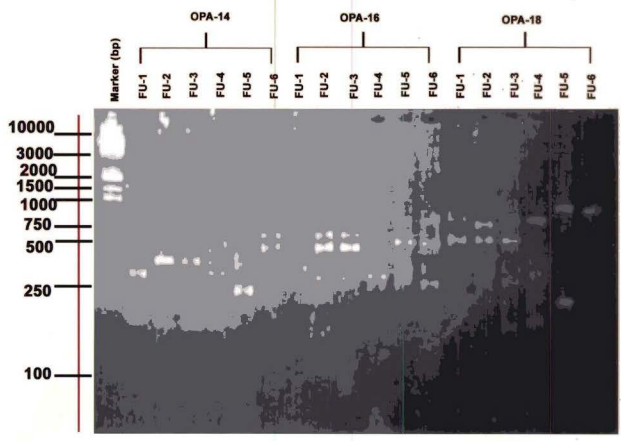


Plate 5 : RAPD banding pattern of primers OPA-14, OPA-16 and OPA-18

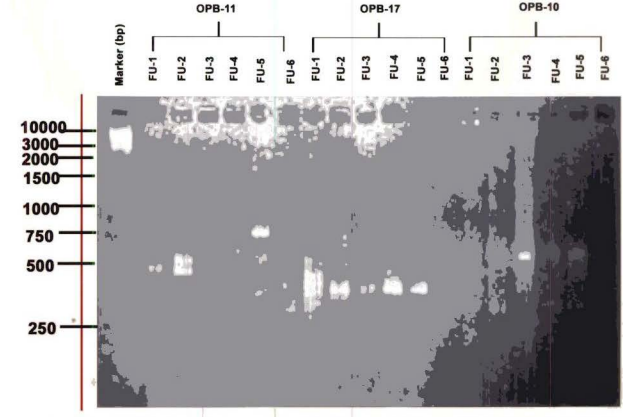


Plate 6 : RAPD banding pattern of primers OPB-11, OPB-17 and OPB-10

Band type 9(549bp) - This type of band observed in FU-3.

Band type 10 (382 bp) - This type of band observed in FU-2, FU-3.

OPA-14

The primers amplified 10 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-14 ranged from 805bp-156bp.

The details of the 10 bands types of RAPD bands are as under

Band type 1 (805bp) - This type of band observed in FU-3, FU-5.

Band type 2 (717bp) - This type of band observed in FU-1, FU-5.

Band type 3(570bp) - This type of band observed in FU-6.

Band type 4 (508bp) - This type of band observed in FU-6.

Band type 5 (485 bp) -This type of band observed inFU-2, FU-3, FU-5.

Band type 6 (376bp) - This type of band observed in FU-3, FU-6.

Band type 7 (359bp) - This type of band observed in FU-4, FU-6.

Band type 8 (279bp) - This type of band observed in FU-5.

Band type 9 (168bp) - This type of band observed in FU-4.

Band type 10 (156bp)-This type of band observed in FU-2,FU-3.

OPA-16

The primers amplified 10 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-16 ranged from 1400bp-285bp.

The details of the 10 bands types of RAPD bands are as under

Band type 1 (1400bp) - This type of band observed in FU-1.

Band type 2 (1062bp) - This type of band observed in FU-3.

Band type 3(991bp) - This type of band observed in FU-5.

Band type 4 (734bp) - This type of band observed in FU-1, FU-2, FU-3, FU-4.

Band type 5 (625bp) - This type of band observed inFU-5, FU-6.

Band type 6 (597bp) - This type of band observed in FU-2, FU-3.

Band type 7 (422bp) - This type of band observed in FU-2, FU-5.

Band type 8 (367bp) -This type of band observed in FU-1, FU-2, FU- 3, FU-4, FU-5, FU-6.

Band type 9 (335bp) - This type of band observed in FU-2.

Band type 10 (285 bp) - This type of band observed in FU-5.

OPA-18

The primers amplified 11amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPA-18 ranged from 1219 bp-153bp.

The details of the 11 bands types of RAPD bands are as under

Band type 1 (1219 bp) - This type of band observed in FU-5.

Band type 2 (1138 bp) - This type of band observed in FU-6.

Band type 3 (968 bp) - This type of band observed in FU-1,FU-4,FU-6.

Band type 4 (883 bp) - This type of band observed in FU-1,FU-4.

Band type 5 (734 bp) - This type of band observed inFU-4.

Band type 6 (639bp) - This type of band observed in FU-2,FU-3.

Band type 7 (394bp) - This type of band observed in FU-4.

Band type 8 (335bp) - This type of band observed in FU-1,FU-3

Band type 9 (248bp) - This type of band observed in FU-5

Band type 10 (192bp) - This type of band observed in FU-6.

Band type 11 (153bp) - This type of band observed in FU-3,FU-4.

OPB-10

The primers amplified 3 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPB-20 ranged from 589 bp-256 bp.

The details of the 3 bands types of RAPD bands are as under

Band type 1 (589 bp) - This type of band observed in FU-5.

Band type 2 (577 bp) - This type of band observed in FU-2,FU-3,FU-4.

Band type 3(256 bp) - This type of band observed in FU-1, FU-2, FU-4,FU-5

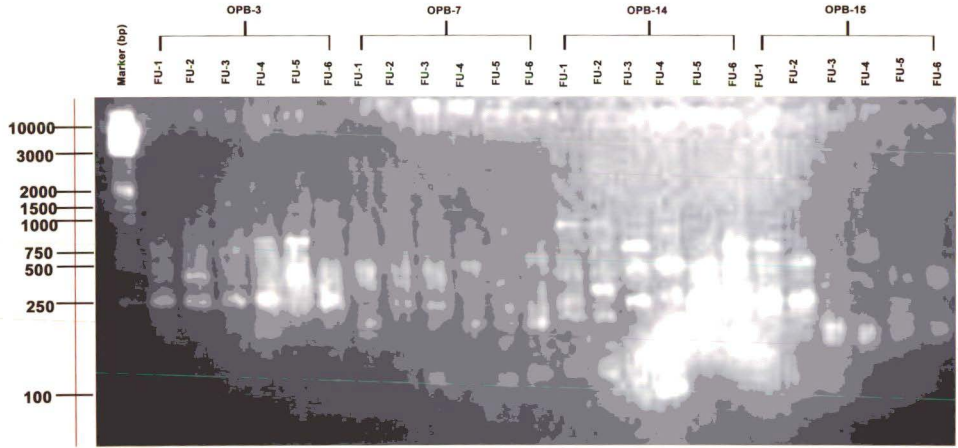


Plate 7 : RAPD banding pattern of primers OPB-3, OPB-7, OPB-14 and OPB-15

OPB-11

The primers amplified 8 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPB-11 ranged from 1258bp-429 bp.

The details of the 8 bands types of RAPD bands are as under

Band type 1 (1258bp) - This type of band observed in FU-3.
Band type 2 (997bp) - This type of band observed in FU-1,FU-2,FU-4.
Band type 3(916bp) - This type of band observed in FU-5, FU-6.
Band type 4 (842bp) - This type of band observed inFU-4.
Band type 5 (654bp) - This type of band observed in FU-3; FU-4.
Band type 6 (529bp) - This type of band observed in FU-2.
Band type 7 (457bp) - This type of band observed in FU-1, FU-6.
Band type 8 (429bp) - This type of band observed in FU-1, FU-2.

OPB-17

The primers amplified 6 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPB-17 ranged from 1062 bp-169 bp.

The details of the 6 bands types of RAPD bands are as under

Band type 1 (1062 bp) - This type of band observed in FU-2.
Band type 2 (808bp) - This type of band observed in FU-2, FU-3.
Band type 3(627bp) - This type of band observed in FU-1, FU-5.
Band type 4 (438 bp) - This type of band observed in FU-5.
Band type 5 (340 bp) - This type of band observed inFU-1, FU-2, FU-3, FU-4, FU-5,FU-6
Band type 6 (248 bp) - This type of band observed in FU-4.

OPB-3

The primers amplified 12 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPB-3 ranged from 2122 bp-188 bp.

The details of the 12 bands types of RAPD bands are as under

Band type 1 (2122 bp) -This type of band observed in FU-6.

Band type 2 (1602 bp) -This type of band observed in FU-6.

Band type 3(1438 bp) - This type of band observed in FU-5.

Band type 4 (1318 bp) -This type of band observed in FU-3.

Band type 5 (1133bp) - This type of band observed in FU-4.

Band type 6 (837 bp) - This type of band observed in FU-4.

Band type 7 (855 bp) - This type of band observed in FU-5.

Band type 8 (767 bp) - This type of band observed in FU-1, FU-2,FU-3.

Band type 9 (520 bp) - This type of band observed in FU-1,FU-5,FU-6.

Band type 10 (477 bp)- This type of band observed in FU-1,FU-2.

Band type 11 (309bp) - This type of band observed in FU-1, FU-2, FU-3, FU-4, FU-6

Band type 12 (188 bp) - This type of band observed in FU-4.

OPB-7

The primers amplified 11 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPB-7 ranged from 1771 bp-449 bp.

The details of the 11 bands types of RAPD bands are as under

Band type 1 (1602 bp) - This type of band observed in FU-2.

Band type 2 (1085 bp) - This type of band observed in FU-5.

Band type 3(953 bp) - This type of band observed in FU-4.

Band type 4 (751 bp) - This type of band observed in FU-4, FU-5, FU -6.

Band type 5 (543 bp) - This type of band observed in FU-2, FU-3 FU-4.

Band type 6 (466 bp) - This type of band observed in FU-2, FU-6.

Band type 7 (437 bp) - This type of band observed in FU-3, FU-5.

Band type 8 (290 bp) - This type of band observed in FU-2, FU-3, FU-5, FU-6.

Band type 9 (214bp) - This type of band observed in FU-1, FU-4, FU-5. FU-6

Band type 10 (158 bp) -This type of band observed in FU-5 .

Band type 11 (82 bp) - This type of band observed in FU-3, FU-5.

OPB-14

The primers amplified 12 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPB-14 ranged from 1290bp-74bp.

The details of the 12 bands types of RAPD bands are as under

Band type 1 (1290bp) - This type of band observed in FU-4.

Band type 2 (1133bp) - This type of band observed in FU-1,FU-2.

Band type 3(974bp) - This type of band observed in FU-3.

Band type 4 (735bp) - This type of band observed in FU-1, FU-2, FU-3, FU-6.

Band type 5 (605bp) - This type of band observed in FU-3, FU-4 FU-5.

Band type 6 (487bp) - This type of band observed in FU-1, FU-2.

Band type 7 (368bp) - This type of band observed in FU-2.

Band type 8 (309bp) - This type of band observed in FU-1, FU-3, FU-5, FU-6.

Band type 9 (254bp) - This type of band observed in FU-1, FU-2.

Band type 10 (139bp) - This type of band observed in FU-2.

Band type 11 (94bp) - This type of band observed in FU-2.

Band type 12 (74bp) - This type of band observed in FU-4.

OPB-15

The primers amplified 7 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer OPB-15 ranged from 1235bp-309bp.

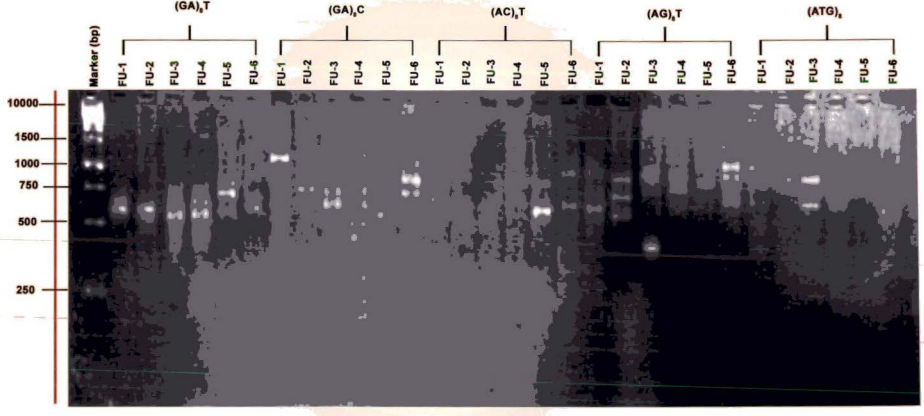


Plate 8 : ISSR banding pattern of primers $(GA)_8T$, $(GA)_8C$, $(AC)_8T$, $(AG)_8T$ and $(ATG)_8$

The details of the 7 bands types of RAPD bands are as under

Band type 1 (1235bp) -This type of band observed in FU-1.
Band type 2 (819bp) - This type of band observed in FU-1.
Band type 3(719bp) - This type of band observed in FU-4.
Band type 4 (735bp) - This type of band observed in FU-1,FU-2.
Band type 5 (592bp) - This type of band observed in FU-5,FU-6.
Band type 6 (466bp) - This type of band observed in FU-1, FU-2, FU-5
Band type 7 (309bp)- This type of band observed in FU-3, FU-4,FU-5 FU-6.

4.6. ISSR banding pattern

The banding pattern was observed in 9 primers. Variability was seen in the *Fusarium udum* banding pattern in each respective primer.

$(GA)_8T$

The primers amplified 8 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified withprimer $(GA)_8T$ ranged from 2461-603.

The details of the 8 bands types of ISSR bands are as under

Band type 1 (2461.74 bp) - This type of band observed in FU-3, FU-4.
Band type 2 (1506.9bp) - This type of band observed in FU-3.
Band type 3(1127.53bp) - This type of band observed in FU-2, FU-5.
Band type 4 (882.17bp) - This type of band observed in FU-3, FU-5, FU-6.
Band type 5 (771.64bp) - This type of band observed in FU-3,FU-4.
Band type 6 (690.2bp) - This type of band observed in FU-1, FU-2.
Band type 7 (674.97bp) - This type of band observed in FU-5, FU-6.
Band type 8 (603.72bp) - This type of band observed in FU-2, FU-3.

$(GA)_9C$

The primers amplified 12 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer $(GA)_9C$ ranged from 2153 bp-404 bp.

The details of the 12 bands types of ISSR bands are as under

Band type 1 (2153bp) -This type of band observed in FU-1, FU-3,FU-6.
Band type 2 (1761bp) - This type of band observed in FU-4.
Band type 3(1611 bp) - This type of band observed in FU-1, FU-6.
Band type 4 (1318 bp) - This type of band observed in FU-2, FU-3.
Band type 5 (1178 bp) - This type of band observed in FU-5, FU-6.
Band type 6 (1031 bp) - This type of band observed in FU-1 FU-2, FU-5.
Band type 7 (922 bp) - This type of band observed in FU-3, FU-4.
Band type 8(882 bp) - This type of band observed in FU-6.
Band type 9 (705 bp) - This type of band observed in FU-3,FU-4.
Band type 10(590 bp) - This type of band observed in FU-1, FU-5.
Band type 11 (552 bp) - This type of band observed in FU-3, FU-4.
Band type 12(404 bp) - This type of band observed in FU-1, FU-3, FU-4.

$(AC)_8T$

The primers amplified 9 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer $(AC)_8T$ ranged from 2153 bp-231 bp.

The details of the 9 bands types of ISSR bands are as under

Band type 1 (2153 bp) - This type of band observed in FU-4.
Band type 2 (1939 bp) - This type of band observed in FU-4.
Band type 3(1684bp) - This type of band observed in FU-6.
Band type 4(1289 bp) - This type of band observed in FU-1,FU-4, FU-5.
Band type 5 (1054 bp) -This type of band observed in FU-2,FU-3,FU-6.
Band type 6 (721 bp) - This type of band observed in FU-2, FU-5.

Band type 7 (825 bp) - This type of band observed in FU-1,FU-2, FU-3.

Band type 8 (330 bp) - This type of band observed in FU-6.

Band type 9 (231 bp) - This type of band observed in FU-6.

$(AG)_8T$

The primers amplified 10 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer $(AG)_8T$ ranged from 2105 bp-353 bp.

The details of the 10 bands types of ISSR bands are as under

Band type 1 (2105 bp) - This type of band observed in FU-6.

Band type 2 (1611 bp) - This type of band observed in FU-4.

Band type 3(1409 bp) - This type of band observed in FU-5,FU-6.

Band type 4(1205 bp) - This type of band observed in FU-3, FU-4, FU-6.

Band type 5 (1152 bp) - This type of band observed in FU-2,FU-5.

Band type 6 (922 bp) - This type of band observed in FU-1, FU-4, FU-6.

Band type 7 (843 bp) - This type of band observed in FU-1,FU-2.

Band type 8 (754 bp) - This type of band observed in FU-1,FU-6.

Band type 9(617 bp) - This type of band observed in FU-2,FU-3.

Band type 10 (353 bp) - This type of band observed in FU-3.

$(ATG)_6$

The primers amplified 7 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer $(ATG)_6$ ranged from 1761 bp-75 bp.

The details of the 7 bands types of ISSR bands are as under

Band type 1 (1761bp) - This type of band observed in FU-3, FU-6.

Band type 2 (1378bp) - This type of band observed in FU-1,FU-4.

Band type 3(1127bp) - This type of band observed in FU-2,FU-3.

Band type 4(1008bp) - This type of band observed inFU-1,FU-5, FU-6.

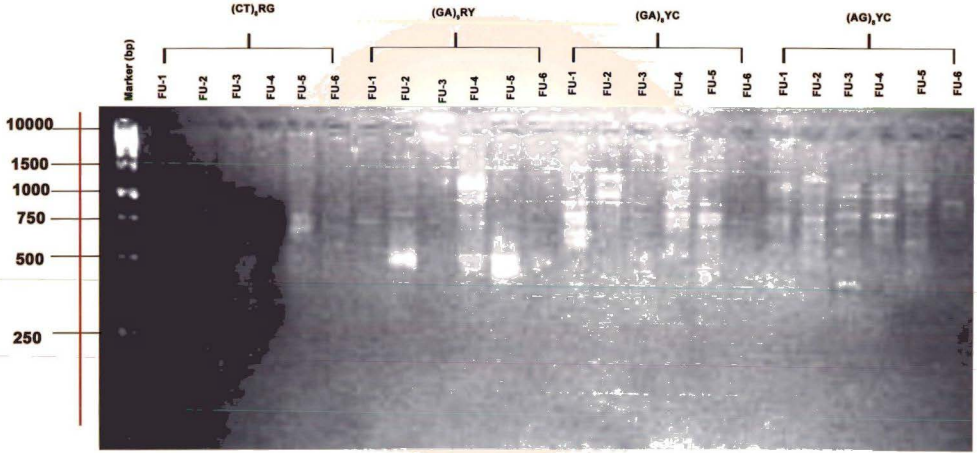


Plate 9 : ISSR banding pattern of primers $(CT)_8RG$, $(GA)_8RY$, $(GA)_8YC$ and $(AG)_8YC$

Band type 5 (825bp) - This type of band observed in FU-4.
Band type 6 (754bp) - This type of band observed in FU-1, FU-3.
Band type 7 (660bp) - This type of band observed in FU-6.

$(CT)_8RG$

The primers amplified 7 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer $(CT)_8RG$ ranged from 1378bp-642.

The details of the 7 bands types of ISSR bands are as under

Band type 1 (1378bp) - This type of band observed in FU-1, FU-2.
Band type 2 (1150bp) - This type of band observed in FU-2.
Band type 3(1062bp) - This type of band observed in FU-1, FU-6.
Band type 4 (980bp) - This type of band observed in FU-5, FU-6.
Band type 5 (886bp) - This type of band observed in FU-2, FU-4.
Band type 6 (834bp) - This type of band observed in FU-5
Band type 7 (642bp) - This type of band observed in FU-6.

$(GA)_9RY$

The primers amplified 8 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer $(GA)_9RY$ ranged from 1754 -456.

The details of the 8 bands types of ISSR bands are as under

Band type 1 (1754 bp) - This type of band observed in FU-3, FU-4.
Band type 2 (1324bp) - This type of band observed in FU-2 FU-4.
Band type 3(1105bp) - This type of band observed in FU-2.
Band type 4 (96bp) - This type of band observed in FU-1.
Band type 5 (886bp) - This type of band observed in FU-4, FU-5.
Band type 6 (547bp) - This type of band observed in FU-1, FU-4.
Band type 7 (49bp) - This type of band observed in FU-4.
Band type 8 (456bp) - This type of band observed in FU-5.

$(GA)_8YC$

The primers amplified 9 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer $(GA)_8YC$ ranged from 1939bp -466bp.

The details of the 9 bands types of ISSR bands are as under

Band type 1 (1939 bp) - This type of band observed in FU-2, FU-5.
Band type 3(1719bp) - This type of band observed in FU-1.
Band type 2 (1555bp) - This type of band observed in FU-4.
Band type 4(1435bp) - This type of band observed in FU-2, FU-5.
Band type 5 (1062bp) - This type of band observed in FU-3, FU-4.
Band type 6 (1040bp) - This type of band observed in FU-1, FU-5, FU-6.
Band type 7 (739bp) - This type of band observed in FU-2, FU-5.
Band type 8 (682bp) - This type of band observed in FU-1, FU-4..
Band type 9(466bp) - This type of band observed in FU-5.

$(AG)_8YC$

The primers amplified 10 amplicons among 6 isolates of *Fusarium udum*. The size of the amplicons amplified with primer $(AG)_8YC$ ranged from 1979 -359 bp.

The details of the 10 bands types of ISSR bands are as under

Band type 1 (1979 bp) - This type of band observed in FU-2
Band type 2 (1719 bp) - This type of band observed in, FU-3,FU-5.
Band type 3(1435 bp) - This type of band observed in FU-3, FU-4 FU-5.
Band type 4(1298bp) - This type of band observed in FU-1, FU-6.
Band type 5 (1105 bp) - This type of band observed in FU-3,FU-4.
Band type 6 (1020 bp) - This type of band observed in FU-1,FU-2.
Band type 7 (999bp) - This type of band observed in FU-.6
Band type 8 (801 bp) - This type of band observed in FU-2, FU-4.
Band type 9 (696 bp) - This type of band observed in FU-1, FU-2, FU-3, FU-5.

Band type 10 (359 bp) - This type of band observed in FU-4.

4.7. The binary similarity matrix and dendrogram of RAPD analysis against six isolates of *Fusarium udum*

A binary similarity matrix of combined data from 18 primers against six isolates of *Fusarium udum* was prepared by scoring for presence or absence of bands. The DNA bands of similar molecular weight were assumed to be identical.

On the basis of calculated similarity matrix the similarity between two genotypes were predicted. The genotypes showing similarity index of "1" are presumed to be 100 percent similar while that of "0" were 100 per cent genetically dissimilar. In present investigation the similarity coefficient value ranged from 0.448 to 0.6428 across six isolates of *Fusarium udum* indicating high degree of variation in respect to genetic similarity. This ultimately means high genetic diversity among the six isolates of *Fusarium udum.*

The genetic similarity estimate (Jaccard's coefficient) based on RAPD banding pattern were used for cluster analysis to present genetic relationship in the form of dendrogram. The Jaccard's pair wise similarity coefficient value for six isolates of *Fusarium udum* is presented in table 5. The highest value of similarity coefficient was observed in Buldhana isolate (0.6428) and lowest in Gadchiroli isolate (0.448).

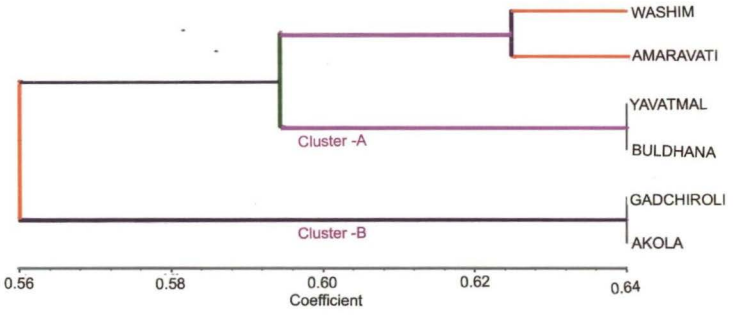


Fig. 1: Dendrogram obtained by RAPD analysis using UPGMA Jaccard's analysis

Table 5: A Binary Similarity Coefficient of RAPD analysis against six isolates of *Fusarium udum*

	FU-1 (Washim)	FU-2 (Yavatmal)	FU-3 (Buldhna)	FU-4 Amaravati)	FU-5 (Gadchiroli)	FU-6 (Akola)
FU-1 (Washim)	1.0					
FU-2 (Yavatmal)	0.6168	1.0				
FU-3 (Buldhana)	0.5844	0.6428	1.0			
FU-4 (Amaravati)	0.6038	0.5324	0.6038	1.0		
FU-5 (Gadchiroli)	0.5324	0.448	0.5584	0.5519	1.0	
FU-6 (Akola)	0.6298	0.5324	0.6168	0.5974	0.6428	1.0

The cluster analysis of similarity index from RAPD analysis among the six isolates of *Fusarium udum* formed two major clusters A and B, represented in the dendrogram. The cluster A included the isolates Washim, Amaravati, Yavatmal, Buldhana and cluster B included two isolates Gadchiroli and Akola. The dendrogram is shown in fig.1.

4.8. The binary similarity matrix and dendrogram of ISSR analysis against six isolates of *Fusarium udum*

A binary similarity matrix of combined data from 9 primers among the six isolates of *Fusarium udum* was prepared by scoring for presence or absence of bands. The DNA bands of similar molecular weight were assumed to be identical.

In present investigation the similarity coefficient value ranged from 0.40 to 0.6125 across six isolates of *Fusarium udum* indicating high degree of variation in respect to genetic similarity. This ultimately means high genetic diversity among the six isolates of *Fusarium udum*.

The genetic similarity estimate (Jaccard's coefficient) based on ISSR banding pattern were used for cluster analysis to present genetic relationship in the form of dendrogram. The Jaccard's pair wise similarity coefficient value for six isolates of *Fusarium udum* is presented in table 6. The Gadchiroli isolate was found to have higher

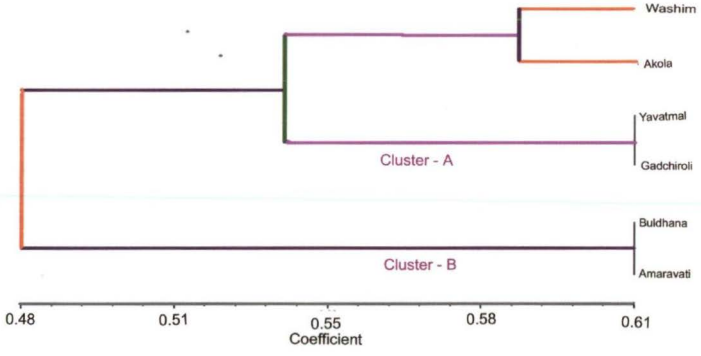


Fig. 2: Dendrogram obtained by ISSR analysis using UPGMA Jaccard's

value of similarity coefficient (0.6125) , whereas Akola isolate had the lower value of similarity coefficient (0.40) .

Table - 6. A Binary Similarity Coefficient of ISSR analysis against six isolates of *Fusarium udum*

	FU-1 (Washim)	FU-2 (Yavatmal)	FU-3 (Buldhana)	FU-4 (Amravati)	FU-5 Gadchiroli)	FU-6 (Akola)
FU-1 (Washim)	1.0					
FU-2 (Yavatmal)	0.5625	1.0				
FU-3 (Buldhana)	0.525	0.5625	1.0			
FU-4 (Amaravati)	0.4875	0. 4250	0.6125	1.0		
FU-5 (Gadchiroli)	0.575	0.6125	0.50	0.4375	1.0	
FU-6 (Akola)	0.5875	0.4254	0.5125	0.40	0.5875	1.0

The cluster analysis of similarity index from ISSR analysis among the six isolates of *Fusarium udum* formed two major clusters A and B represented in the dendrogram. The cluster A included the isolates from Washim, Akola, Yavatmal, Gadchiroli and cluster B included isolates from Buldhana, Amaravati. The dendrogram is shown in fig.2.

4.9 Binary similarity matrix and dendrogram of RAPD and ISSR analysis of six isolates of *Fusarium udum*

A binary similarity matrix of combined data from 18 RAPD primers and 9 ISSR primers for the six *Fusarium udum* isolates was prepared by scoring presence or absence of bands. The DNA bands of molecular weight were assumed identical.

In present investigation the similarity coefficient value ranged from 0.4957 to 0.6239 across six isolates of *Fusarium udum* indicating high degree of variation in respect to genetic similarity. This ultimately means high genetic diversity among the isolates of *Fusarium udum.*

The genetic similarity estimate (Jaccard's coefficient) based on RAPD and ISSR banding pattern used for cluster analysis to represent the genetic relationship in the form of dendrogram. The

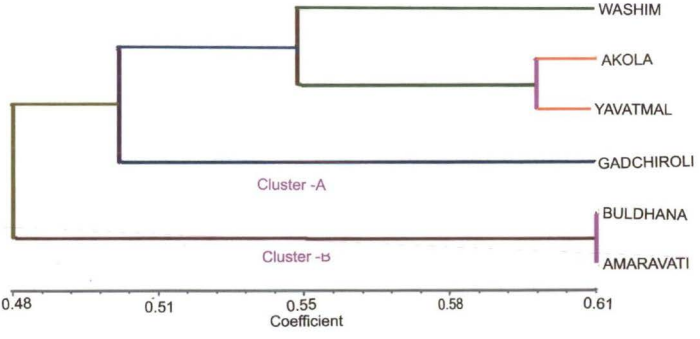


Fig. 3: Dendrogram obtained by RAPD and ISSR analysis using UPGMA Jaccard's analysis

Jaccard's pair wise similarity coefficient value for six isolates of *Fusarium udum* is presented in table 7. The Akola isolate was found to have higher value of similarity coefficient (0.6239) two isolates of the fungus. Whereas, Amaravati isolate had the lower value of similarity coefficient (0.4957).

Table-7. A binary similarity coefficient of combine RAPD and ISSR analysis against six isolates of *Fusarium udum*

	FU-1 (Washim)	FU-2 (Yavatmal)	FU-3 (Buldhana)	FU-4 (Amaravati)	FU-5 (Gadchiroli)	FU-6 (Akola)
FU-1 (Washim	1.0					
FU-2 (Yavatmal)	0.5982	1.0				
FU-3 (Buldhana)	0.5641	0.6153	1.0			
FU-4 (Amaravati)	0.5641	0.4957	0.6068	1.0		
FU-5 (Gadchiroli)	0.547	0.5042	0.5384	0.5128	1.0	
FU-6 (Akola)	0.6153	0.4957	0.5811	0.5299	0.6239	1.0

The cluster analysis of similarity index of RAPD and ISSR analysis of six isolates of *Fusarium udum* grouped in two major clusters A and B, represented in the dendrogram. The cluster A included the isolates from Washim, Amaravati, Yavatmal, Buldhana and cluster B included two isolates i.e. Gadchiroli and Akola. The dendrogram is represented in fig.3. The pigeonpea wilt caused by *Fusarium udum* (FU) is considered as one of the major factors for low productivity of pigeonpea. The high level of pathogenic variability in *Fusarium udum* causing wilt of pigeonpea was reported by Sivaramkrishnan *et al.*, 2002.

Sivaramkrishnan *et al.* (2002) made an attempt to establish genetic variability in wilt causing Indian races of *Fusarium udum*. They observed high level of DNA polymorphism and suggested

the rapid evolution of new recombination to the pathogen in pigeonpea growing areas. Study about structure of pathogen population is pre-requisite for designating a cost effective management strategy for controlling the pathogen.

Prasanthi *et al.* (2009) studied molecular marker for screening *Fusarium* wilt resistance in pigeonpea (*Cajanus cajan*). *Fusarium* wilt is a serious disease of pigeonpea which causes severe yield losses. Screening of 88 lines along with ICPL 87119 and ICPL 8863 resistant checks for *Fusarium* wilt under field conditions and artificial inoculation identified 14 lines having 0-20% plant mortality grouped under 0-5 disease score. PCR reactions using different primers with genomic DNA of these lines resulted in identification of 6 resistant sources with specific amplification for resistance to wilt at 920bp with OPGO8 primer. Considering the wilt reaction and resistance linked RAPD marker, it possible to identify the new resistance sources in a short time and they can be utilized in breeding programme or for direct release.

Mesapogu S. *et al.* (2011) studied Genetic diversity and pathogenic variability among *Fusarium udum* isolates collected from different geographical locations of India. All the isolates exhibited variable levels of virulence against a susceptible pigeonpea cultivar (T-21). The genetic diversity allelic variations among these isolates were estimated using RAPD molecular markers. All the thirteen RAPD primers were found to be highly reproducible and produced a total of 126 loci of which 69 loci were polymorphic. Primer OPB 17 amplified highest number of polymorphic bands with maximum polymorphic information content (PIC) of 4.00. Percentage of polymorphism revealed by individual primers varied from 33.3 to 76.9% with an average of 53.4%. Further, cluster analysis of OPB-17 provided a substantially discrimination of all the isolates.

Dhar *et al.* (2012) studied 32 pathogenic isolates of *Fusarium udum* from different pigeonpea growing areas in India for pathogenic and molecular variability. The amount of genetic variation was evolved by PCR amplification with 20 RAPD markers and 9

microsatellite markers. All amplification revealed scorable polymorphisms among the isolates and total 137 polymorphic fragments were scored for the RAPD markers and 16 alleles for the SSR markers. RAPD primers showed 86% polymorphism. Since this disease is primarily managed by use of resistant cultivars, characterization of pathogen and studies on genetic variation of *Fusarium udum* in a given area is important for disease resistance breeding.

The genetic diversity and variation studies conducted using qualitative and quantitative traits were mostly based on using various statistical methods such as analysis of variance, diversity analysis and principal components analysis. Also the morphological study, pathogenicity test are cumbersome, time consuming, require extensive facilities and are influenced by variability inherent in the experimental system. Furthermore, pathogenic data alone provide no information about genetic diversity within or relatedness among races of the pathogen. However recently molecular markers were widely used in various areas as an important tool for evaluating genetic diversity and determining the identity of isolates. The results provided information useful to future breeding programme.

CHAPTER-V

SUMMARY AND CONCLUSION

The studies were conducted in the laboratory of Department of Plant Pathology and Biotechnology centre, Dr. PDKV, Akola. The pigeonpea wilted plants from different districts of Vidarbha were collected and all the six isolates were tested for their pathogenicity test and subject to RAPD and ISSR analysis to determine the genetic diversity.

5.1 Pathogenicity

The pathogenicity test of 6 isolates of *Fusarium udum* was conducted by water culture method using susceptible variety TAT-10. The Washim and Amaravati isolates showed 100%wilting, Yavatmal, Buldhana and Gadchiroli showed 66.66% wilting whereas, Akola showed that 33.33% wilting

5.2 Random Amplified Polymorphic DNA (RAPD)

The genetic variation was detected among six isolates of *Fusarium udum* using RAPD marker. All 30 RAPD primers screened for amplification of DNA of six isolates of *Fusarium udum*, of which 18 produced reproducible and scorable bands with high percentage of polymorphism. The number of bands generated were primer and isolate specific ranged from 3 to 12. A total 154 amplicons were obtained with the 18 primers with an average fragment of 8.6 bands per primer. The level of polymorphism was 94.8 percent. The primer OPA-9, OPB-3 and OPB-14 amplified maximum fragments 12 and the least in OPB-20 (4). In similarity matrix showed that Buldhana isolate had higher value of similarity coefficient (0.6428) whereas Gadchiroli isolate had the lower value of similarity coefficient (0.448).The primer OPA-2, OPA-3, OPA-4,OPA-16 and OPB-17 showed the monomorphic band which could be used for identification of *Fusarium udum*.

5.3 Inter Simple Sequence Repeat (ISSR)

The genetic variation was detected among six isolates of *Fusarium udum* using 16 ISSR marker. Among which 9 produced reproducible and scorable bands with high percentage of polymorphism. The number of bands generated were primer and isolate specific and ranged from 7 to 12. A total 80 amplicons were obtained with 9 ISSR primers. The average fragment of 8.9 bands per primer was obtained. All bands were polymorphic and the level of polymorphism was 100 percent. The primer $(GA)_9C$ amplified maximum fragments (12) and the least in $(ATG)_6$ and $(CA)_8RG$ found (7) fragments. In similarity matrix showed that Amaravati isolate have higher value of similarity coefficient (0.6125) whereas Akola isolate had the lower value of similarity coefficient (0.40).

In similarity matrix of RAPD and ISSR the Akola isolate showed highest value of similarity coefficient (0.6239) whereas Buldhana isolate showed lowest value of similarity coefficient (0.4957) indicating the genetic variability among the isolates of *Fusarium udum* of Vidarbha region.

CHAPTER – VI

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7. Research papers published (If any) : Nil

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Annexure-I

List of primers used for RAPD analysis

Sr.	Primer Screened	Sequence	Annealing temperature
1	OPA-1	CAGGCCCTTA	27
2	OPA-2	TGCCGAGCTG	29
3	OPA-3	AGTCAGCCAC	27
4	OPA-4	AATCGGGCTG	27
5	OPA-5	AGGGGTCTTG	27
6	OPA-6	GGTCCCTGAC	27
7	OPA-7	GAAACGGGTG	29
8	OPA-8	GTGACGTAGG	27
9	OPA-9-	GGGTAACGCC	27
10	OPA10	GTGATCGCAG	29
11	OPA-11	TTGACAGTTA	27
12	OPA-12	TCGGCGATAG	27
13	OPA-13	CAGCACCCAC	29
14	OPA-14	TCTGTGCTGG	27
15	OPA-15	TTCCGAACCC	27
16	OPA-16	AGCCAGCGAA	27
17	OPA-17	GACCGCTTGT	27
18	OPA-18	AGGTGACCGT	27
19	OPA-19	CAAACGTCGG	27
20	OPA-20	GTTGCGATCC	27
21	OPB-3	CATCCCCCTG	29
22	OPB-6	TGCTCTGCCC	29
23	OPB-7	GGTGACGCAG	29
24	OPB-10	CTGCTGGGAC	29
25	OPB-11	GTAGACCCGT	27
26	OPB-14	TCCGCTCTGG	29
27	OPB-15	GGAGGGTGTT	27
28	OPB-17	AGGGAACGAG	27
29	OPC-20	ACTTCGCCAC	27
30	OPE-11	TGCGTGCTTG	27

Annexure –II

List of primers used for ISSR analysis

Sr.	Primer Sequence	Temperature	G+C Content (%)
1.	$(AG)_8G$	52^0C	52.9
2.	$(GA)_8T$	50^0C	47.1
3.	$(GA)_8C$	50^0C	52.9
4.	$(AC)_8T$	50^0C	47.1
5.	$(AG)_8YT$	52^0C	47.2
6.	$(AG)_8YC$	55^0C	52.8
7.	$(GA)_8YT$	53^0C	47.2
8.	$(GA)_8YC$	54^0C	52.8
9.	$(CA)_8RT$	52^0C	47.2
10.	$(AC)_8YT$	52^0C	47.2
11.	$(AC)_8YA$	53^0C	47.2
12.	$(ATG)_6$	50^0C	33.3
13.	$(GA)_9RY$	54^0C	50.0
14.	$(TG)_8RT$	53^0C	47.2
15.	$(AG)_8T$	50^0C	47.1
16.	$(CT)_8RG$	54^0C	52.8

Y = Pyrimidine and R = Purine.

Annexure –III

Different media used while the research work

A) Potato Dextrose Agar Media (PDA)

1.	Peeled Potato	200 gm.
2.	Dextrose	20 gm.
3..	Agar	20 gm.
4.	Distilled water	1000 ml.

B) Potato Dextrose Broth (PDB)

1.	Peeled Potato	200 gm.
2.	Dextrose	20 gm
3.	Distilled water	1000 ml.

Annexure –IV

RAPD primers bands types amplified against 30 primers

OPA-1

Sr.no	Mol wt	FU-1	FU -2	FU -3	FU- 4	FU- 5	FU- 6	Bands
1	665	0	1	1	0	0	0	2
2	602	0	0	0	0	1	0	1
3	492	0	1	1	0	0	0	2
4	346	1	1	1	0	0	1	4
5	290	0	0	0	0	1	0	1
6	199	0	1	0	0	0	0	1
7	167	0	0	1	0	0	0	1

OPA-2

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	649	1	0	0	0	0	0	1
2	434	1	1	1	1	1	1	6
3	364	1	1	0	0	1	1	4
4	237	1	1	1	0	0	0	3
5	220	0	1	0	0	1	1	3
6	184	0	0	0	1	0	0	1
7	171	0	1	0	0	0	0	1

OPA-3

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	865	0	0	0	0	0	1	1
2	700	0	0	0	0	1	1	2
3	434	1	1	1	1	1	1	6
4	337	1	1	1	1	1	1	6
5	237	1	1	1	1	1	1	6
6	167	0	0	0	0	1	0	1
7	117	0	0	0	0	1	0	1
8	194	1	0	0	0	0	0	1

OPA-4

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	793	1	0	0	0	0	0	1
2	617	0	1	1	0	1	1	4
3	468	0	0	1	0	1	1	3
4	355	1	0	0	0	1	1	3
5	337	0	1	0	0	0	0	1
6	305	1	0	0	0	0	1	2
7	243	1	1	1	1	1	1	6
8	199	1	1	1	1	1	1	6
9	140	1	0	0	0	0	0	1

OPA-7

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1771	0	1	0	0	0	0	1
2	1633	0	0	0	1	1	0	2
3	1335	1	0	1	0	0	1	1
4	1027	0	1	0	0	0	0	1
5	822	0	0	0	1	1	0	2
6	714	1	0	0	0	0	0	1
7	527	1	0	0	0	0	0	1
8	398	1	0	0	0	0	0	1
9	449	0	0	0	1	0	0	1

OPA-10

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	2302	0	0	0	0	1	1	2
2	1507	0	0	0	0	1	0	1
3	1390	0	0	1	0	0	1	1
4	1183	0	0	1	0	0	0	1
5	1027	0	1	0	0	0	0	1
6	892	0	0	1	0	0	0	1
7	607	0.	0	0	0	1	0	1

OPA-12

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Ba
1	2651	0	1	0	1	0	0	
2	2167	0	1	0	1	0	1	3
3	1601	1	0	0	1	0	1	3
4	1335	1	1	0	1	1	0	4
5	1091	1	1	0	1	0	1	3
6	1048	1	1	0	0	0	0	2
7	790	0	1	0	0	0	0	1
8	607	0	1	0	0	0	0	1
9	382	0	1	0	0	0	0	1

OPA-13

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	2495	0	1	0	0	0	0	1
2	2211	0	0	0	0	1	0	1
3	1920	0	0	0	1	0	0	1
4	1735	0	0	0	0	0	1	1
5	1601	0	1	1	0	0	0	2
6	1256	0	0	0	0	1	1	2
7	1048	1	1	1	0	0	1	4
8	947	0	0	0	0	0	1	1
9	549	0	0	1	0	0	0	1
10	382	0	1	1	0	0	0	2

OPA-16

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1400	1	0	0	0	0	0	1
2	1062	0	0	1	0	0	0	1
3	991	0	0	0	0	1	0	1
4	734	1	1	1	1	0	0	4
5	625	0	0	0	0	1	1	1
6	597	0	1	1	0	0	0	2
7	422	0	1	0	0	1	0	2
8	367	1	1	1	1	0	0	4
9	335	0	1	0	0	0	1	2
10	285	0	0	0	0	1	0	1

OPA-14

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	805	0	0	1	0	1	0	2
2	717	1	0	0	0	1	0	2
3	570	0	0	0	0	0	1	1
4	508	0	0	0	0	0	1	1
5	485	0	1	1	0	1	0	3
6	376	0	0	1	0	0	1	2
7	359	0	0	0	1	0	1	2
8	279	0	0	0	0	1	0	1
9	168	0	0	0	1	0	0	1
10	156	0	1	1	0	0	0	2

OPA-16

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1400	1	0	0	0	0	0	1
2	1062	0	0	1	0	0	0	1
3	991	0	0	0	0	1	0	1
4	734	1	1	1	1	0	0	3
5	625	0	0	0	0	1	1	2
6	597	0	1	1	0	0	0	2
7	422	0	1	0	0	1	0	2
8	367	1	1	1	1	0	0	3
9	335	0	1	0	0	0	1	2
10	285	0	0	0	0	1	0	1
11	188	0	0	0	0	0	1	1

OPA-18

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1219	0	0	0	0	1	0	1
2	1138	0	0	0	0	0	1	1
3	968	1	0	0	1	0	1	3
4	883	1	0	0	1	0	0	2
5	734	0	0	0	1	0	0	1
6	639	0	1	1	0	0	1	3
7	394	0	0	0	1	0	0	1
8	335	1	0	1	0	0	0	2
9	248	0	0	0	0	1	0	1
10	192	0	0	0	0	0	1	1
11	153	0	0	1	1	0	0	2

OPB-3

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	2122	0	0	0	0	0	1	1
2	1602	0	0	0	0	0	1	1
3	1438	0	0	0	0	1	0	1
4	1318	0	0	1	0	0	0	1
5	1133	0	0	0	1	0	0	1
6	837	0	0	0	1	0	0	1
7	855	0	0	0	0	1	0	1
8	767	1	1	1	0	0	0	3
9	520	1	0	0	0	1	1	3
10	477	1	1	0	0	0	0	2
11	309	1	1	1	1	0	1	4
12	188	0	0	0	1	0	0	1

OPB-7

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1602	0	1	0	0	0	0	
2	1085	0	0	0	0	1	0	1
3	953	0	0	0	1	0	0	1
4	751	0	0	0	1	1	1	3
5	543	0	1	1	1	0	0	3
6	466	0	1	0	0	0	1	2
7	437	0	0	1	0	1	0	2
8	290	0	1	1	0	1	1	4
9	214	1	0	0	1	1	1	4
10	158	0	0	0	0	1	0	1
11	82	0	0	1	0	1	0	2

OPB-14

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1290	0	0	0	1	0	0	1
2	1133	1	1	0	0	0	0	2
3	974	0	0	1	0	0	0	1
4	735	1	1	1	0	0	1	4
5	605	0	0	1	1	1	0	3
6	487	1	1	0	0	0	0	2
7	368	0	1	0	0	0	0	1
8	309	1	0	1	0	1	1	4
9	254	1	1	0	0	0	0	2
10	139	0	1	0	0	0	0	1
11	94	0	1	0	0	0	0	1
12	74	0	0	0	1	0	0	1

OPB-15

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1235	1	0	0	0	0	0	1
2	819	1	0	0	0	0	0	1
3	719	0	0	0	1	0	0	1
4	592	1	1	0	0	0	0	2
5	555	0	0	0	0	1	1	2
6	466	1	1	0	0	1	0	3
7	309	0	0	1	1	1	1	4

OPB-11

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1258	0	0	1	0	0	0	1
2	997	1	1	0	1	0	0	4
3	916	0	0	0	0	1	1	2
4	842	0	0	0	1	0	0	1
5	654	0	0	1	1	0	0	2
6	529	0	1	0	0	0	0	1
7	457	1	0	0	0	0	1	2
8	429	1	1	0	0	0	0	2

OPB-17

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1062	0	1	0	0	0	0	1
2	808	0	1	1	0	0	0	2
3	627	1	0	0	0	1	0	2
4	438	0	0	0	0	1	0	1
5	340	0	1	1	1	1	1	5
6	248	0	0	0	1	0	0	1

OPB-20

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	577	0	1	1	1	0	0	3
2	589	0	0	0	0	1	0	1
3	256	1	1	0	1	1	0	4

Annexure –V

ISSR primers bands types amplified against 16 primers

$(GA)_8$ T

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	2461	0	0	1	1	0	0	2
2	1506	0	0	1	0	0	0	1
3	1127	0	1	0	0	1	0	2
4	882	0	0	1	0	1	1	3
5	771	0	0	1	1	0	0	2
6	690	1	1	0	0	0	0	2
7	674	0	0	0	0	1	1	2
8	603	0	1	1	0	0	0	2

$(GA)_9$ C

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	2153	1	0	1	0	0	1	3
2	1761	0	0	0	1	0	0	1
3	1611	1	0	0	0	0	1	2
4	1318	0	1	1	0	0	0	2
5	1178	0	0	0	0	1	1	2
6	1031	1	1	0	0	1	0	3
7	922	0	0	1	1	0	0	2
8	882	0	0	0	0	0	1	1
9	705	0	0	1	1	0	0	2
10	590	1	0	0	0	1	0	2
11	552	0	0	1	1	0	0	2
12	404	1	0	1	1	0	0	3

$(AC)_8$ T

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	2153	0	0	0	1	0	0	1
2	1684	0	0	0	1	0	0	1
3	1289	0	0	0	0	0	1	1
4	1054	1	0	0	1	1	0	2
5	721	0	1	1	0	0	1	3
6	645	0	1	0	0	1	0	2
7	825	1	1	1	0	0	0	3
8	330	0	0	0	0	0	1	1
9	231	0	0	0	0	0	1	1

$(AG)_8$ T

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bar
1	2105	0	0	0	0	0	1	1
2	1611	0	0	0	1	0	0	1
3	1409	0	0	0	0	1	1	2
4	1205	0	0	1	1	0	1	3
5	1152	0	1	0	0	1	0	2
6	922	1	0	0	1	0	1	3
7	843	1	1	0	0	0	0	2
8	754	1	0	0	0	0	1	2
9	617	0	1	1	0	0	0	2
10	353	0	0	1	0	0	0	1

$(ATG)_6$

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1761	0	0	1	0	0	1	2
2	1378	1	0	0	1	0	0	2
3	112	0	1	1	0	0	0	2
4	825	0	0	0	1	0	0	1
5	660	0	0	0	0	0	1	1
6	754	1	0	1	0	0	0	2
7	1008	1	0	0	0	1	1	3

$(CT)_8$ RG

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1378	1	1	0	0	0	0	2
2	1150	0	1	0	0	0	0	1
3	1062	1	0	0	0	0	1	2
4	980	0	0	0	0	1	1	2
5	886	0	1	0	1	0	0	2
6	8834	0	0	0	0	1		1
7	642	0	0	0	0	0	1	1

$(GA)_8$ RY

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1754	0	0	1	1	0	0	2
2	1324	0	1	0	1	0	0	2
3	1105	0	1	0	0	0	0	1
4	960	1	0	0	0	0	0	1
5	886	0	0	0	1	1	0	2
6	547	1	0	0	1	0	0	2
7	495	0	0	0	1	0	0	1
8	456	0	0	0	0	1	0	1

$(GA)_8$ YC

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1939	0	1	0	0	1	0	2
2	1555	0	0	0	1	0	0	1
3	1435	0	1	0	0	1	0	1
4	1719	1	0	0	0	0	0	1
5	1062	0	0	1	1	0	0	2
6	1040	1	0	0	0	1	1	3
7	739	0	1	0	0	1	0	2
8	682	1	0	0	1	0	0	2
9	466	0	0	0	0	1	0	1

$(AG)_8$ YC

Sr.no	Mol wt	FU-1	FU-2	FU-3	FU-4	FU-5	FU-6	Bands
1	1979	0	1	0	0	0	0	1
2	1719	1	0	1	0	1	0	3
3	1435	0	0	1	1	1	0	3
4	129	1	0	0	0	0	1	2
5	1105	0	0	1	1	0	0	2
6	1020	1	1	0	0	0	0	2
7	993	0	0	0	0	0	1	1
8	801	0	1	0	1	0	0	2
9	696	1	1	1	0	1	0	4
10	359	0	0	0	1	0	0	1

Annexure VI

Formula for Calculating Percentage of Polymorphism Bands

The data was scored as the presence (1) or absence (0) of individual band for each isolates in RAPD-PCR and ISSR-PCR analysis of races of *Fusarium udum*.

The percentage polymorphism of Bands calculated by following formula.

$$\text{Percentage Polymorphism of band} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}}$$