ANALYSIS OF GENETIC VARIABILITY OF FUSARIUM SPECIES ON ONION USING ITS MARKERS

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ABSTRACT

Onion is one of important commercial vegetable crops grown in worldwide. India is second most onionproducer. In India Maharashtra is leading state. Present study was investigated of the Analysis of genetic variability of *Fusarium sps,* from pathogenic fungi by using (ITS) as molecular markers. *Fusarium* species are among the most commonly occurring and economically important member of micro-fungi. The aim of this study was to estimate the genetic diversity. collected from various places from Maharashtra by usingon colony appearance of shape and spore morphological characters and The obtained DNA was qualitatively and quantitatively analyzed using Nano Drop ND-1000 Spectrophotometer, the molecular marker for characterizing the natural variation amongst *Fusarium spp.* isolatesusing (ITS -1 & ITS-4) as DNA barcoding of *Fusarium sp.* The Phylogenetic tree using nearest neighbor joining method, Sequences of *Fusarium sps* were grouped into three clusters. The largest cluster shows genetic similarity amongs ten species of *Fusarium,*

Key words: *Fusarium,* Onion, genetic variability, ITS molecular markers,

INTRODUCTION

Onion (*Allium cepa L.)* is the important commercial vegetable crops and also known as ‘‘Queen of the kitchen’’This is grown in worldwide.Onion is valued for its bulbs having characteristic odor, flavor and pungency, due to the presence of a volatile oil – Allyl-propyl-disulphide. Pungency is formed by enzymatic reaction when tissues are broken.India is the second largest producer country of onion after the china and lead in production. In India occupies an area of 1.05 million hectare with the production of 16.81 million tones (D A and C H D. 2013).The major onion growing states in India is the Maharashtra, Bihar Orissa, Andhra Pradesh, Karnataka, Rajasthan Tamil Nadu, Haryana and Madhya Pradesh. Maharashtra is the pioneer state in onion production contributing 25% of country’s onion (Gadge *et.al*, 2012). In Maharashtra the major onion producing districts are Pune, Ahmadnagar, Satara, Solapur, Dhulia and Nashik. Nashik district contributes 35 to 40 % of the onion production. Onions are cultivated in three different seasons’ *Kharif* and *rabbi*. In Maharashtra, the production of onion likewise season late *Kharif* and *rabbiKharif* respectively (Pawar *et al*. 2016).Onion bulb contains antiinflamentory, anticholestrol, anticancer and antioxidant compound quercetin (Augusti, 1996). The fungicidal and insecticidal properties of onion are also well identified. (Mishra R. K 2014).The onion also losses

due to the causes of same Virus, Bacterial, Mycoplasma Nematode and fungi due toloss in the productivity in both field and storage condition diseases of onions. Genetic analysis of plant pathogen, These concept understanding, In this study used nuclear Internal Transcribed Spacer as a DNA fragment to identify fungal specimens using and constructed Phylogenetic tree for study of evolutionary relationship among fungal isolates chosen the most important think is Analysis of genetic variability of Fusarium oxysporum f. sp. cepae the causal agent of basal rot on onion using ITS markers

MATERIALS AND METHODS

Collection of samples

The different variety of onion samples were collected from different locality and maintain storage condition. The bulbs and infected plant materials were stored in the separate polythene bags and brought into the laboratory conditions and observed under the microscope for primary observation.

Isolation and Identification causal pathogen:

From different areas of Maharashtra like Nasik, Pune and Aurangabad districts. The infected onion leaves and bulb were collected and kept in polythene bags. The collected samples were cleaned and washed by sterilized water then surface sterilized with 2% Sodium hypochlorite solution for two minutes the rinsed several times in sterilized water and dried, the surface sterilized sample were placed on to Potato Dextrose Agar (PDA) medium and incubated at 27 ^{0}C .After 4-5 days incubation period the developed fungal colonies were purified by hyphal tip and single spore isolation technique. Identification of the fungal isolates carried out by using the morphological characteristic of mycelia and spore as described by (Ellis M.B 1971 and Kritzman G. 1983).The isolated and identified fungi out of ten species only selected fungi was used as, DNA barcoding of *Fusarium sp.* isolates using (ITS -1 &ITS-4) as molecular markers White T.J *et al*(1990).Ten isolates of *Fusarium spp.* with diverse varieties were used in the present study. The isolates were isolated and purified on GNA agar medium. Isolates were incubated at 28 ^{0} C for eight days on GNA media. Mycelia were harvested by scrapping with the help of sterile spatula and used for DNA extraction.

Qualitative and quantitative analysis of extracted genomic DNA:

The obtained DNA was qualitatively and quantitatively analyzed using NanoDrop ND-1000 Spectrophotometer. As summarized in Table.no.1

Table:1
Qualitative and quantitative analysis of genomic DNA obtained From fungal isolates.

Sr.no	Sample Id	DNA Conc. (ng/µl)	260/280
1	*Fusarium sp.*1	341.12	1.4
2	*Fusarium sp.*2	1353.07	1.97
3	*Fusarium sp.*3	312.04	1.13
4	*Fusarium sp.*4	394.95	1.75
5	*Fusarium sp.*5	201.13	1.27
6	*Fusarium sp.*6	890.17	1.97
7	*Fusarium sp.*7	455.08	2.15

8	*Fusarium sp.*8	164.04	1.48
9	*Fusarium sp.*9	1215.9	2.09
10	*Fusarium sp.*10	268.61	1.89

Amplification of DNA by Polymerase Chain Reaction (PCR):

The polymerase chain reaction (PCR) is an extremely powerful procedure that amplifies a selected DNA sequence. It involves synthetic oligonucleotides complementary to the known sequences spanning the region of interest to prime enzymatic amplification of segment of DNA in the test tubes. It has promoted the development of a range of molecular assay systems which detect polymorphism at molecular level. In this study, we used the nuclear Internal Transcribed spacers (ITS) region as molecular marker for characterizing the natural variation amongst *Fusarium spp.* isolates.

The prepared master mixture was distributed in ten PCR tubes sequentially according to generate specimen Id. Then, template DNA (1.5 µl) was added in the tubes after which, they were gently vortexes, spinned and kept in thermal cycler for PCR.

Polymerase Chain Reaction

DNA was amplified by Applied Bio systems, Veriti 96 well Thermal Cycler programme. Nuclear Internal Transcribed Spacer gene was amplified by using primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS 4 (5'TCCTCCGCTTATTGATATGC3') **(White T.J *et al.*, 1990)** for forward and reverse directions, respectively. The cycling conditions of PCR involves initial denaturation at 95°C for 3 minutes followed by 35 cycles of 95°C at 30 seconds, annealing at 54°C for 40 seconds followed by extension at 72°C for 7-10 minutes and final extension at 72°C for 10 minutes by using Electrophoresis methods. And Preparation of master mixture I and master mixture II for ethanol wash of 14 DNA samplesfurther remaining procedure and finally given results.

RESULTS

Ten *Fusarium* species isolates from the infected onion samples collected from different varieties localities and on the basis of morphological and cultural characteristics, these isolates were identified as *F. oxysporum, F. incarnatum, F. solani, F. chlamydosporum,F. Avenerum, F. proliferatum, F. equiseti F.camptoceras,F. moniliforme,F. Brachygibbosum* \and *F. roseum*(photo plate:02).*Fusariumoxysporum*is the most dominant species in all investigated fields, comprising total *Fusarium*species. Among the isolated fungal cultures, identification was focused on *Fusarium* species have been identified.

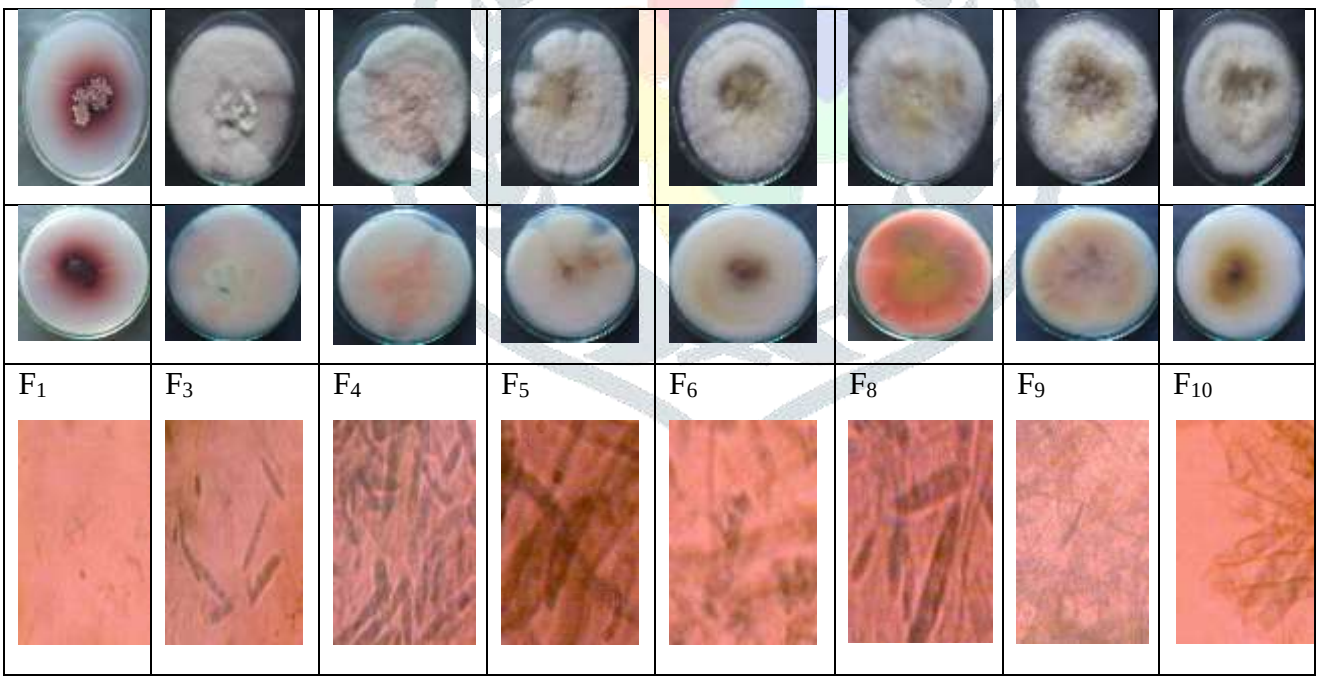
Assessment of quality of PCR products:

We obtained seven successful PCR products of specimens belonging to *Fusarium sp.* 9, *Fusarium sp.* 10, *Fusarium sp.* 5, *Fusarium sp.* 4, *Fusarium sp.* 3, *Fusarium sp.* 1, and *Fusarium sp.* 8 out of 10 as shown in photo plate no.1. However, we couldn't amplify ITS region from 3 fungi samples including *Fusarium sp.* 2, *Fusarium sp.*6*and Fusarium sp*7. Therefore, only 7 specimens were taken for further analysis as shown in photo plate no.01.And remaining was excluded from our analysis.

Phylogenetic Analysis

The sequences of seven fungi specimens were analyzed for their evolutionary relationship using MEGA5 (Koichiro Tamura *et al.*, 2011) in association with 10 sequences from NCBI (http://www.ncbi.nlm.nih.gov) (Photo plate 02). Phylogenetic tree using nearest neighbor joining method was constructed by MEGA 5.1 (Photo plate 03). Using Kimura 2 parameter as a distance model, accuracy was verified by 1000 replicates of Bootstrap support. Sequences of *Fusarium sps* were grouped into three clusters. The largest cluster shows genetic similarity amongs ten species of *Fusarium*, they are, *Fusarium solani*, *Fusariumchlamydosporum-1*, *Fusarium Proliferatum*, *Fusarium chlamydosporum -2*, *Fusarium equiseti-1*, *Fusarium oxysporum-1*, *Fusarium equiseti-2*, *Fusarium incarnatum*, *Fusarium chlamydosporum* and *Fusarium camptoceras.* Another cluster is only two species of *Fusarium* they are *Fusarium moniliforme*and *Fusarium avenoceum.* The third group shows five species of Fusarium such as *Fusarium oxysporum-2*, *Fusarium brachygibbosum-1*,*Fusarium brachygibbosum-2*, *Fusarium oxysporum-3* and *Fusarium roseum.* So among of seven Fusarium species shows such type of genetic similarity, this grouping is do not show any relation with onion varieties, locality on age of onion plant. It is only based on genetic material of Fusarium species in different group of classification. Shows in photo plate.03. The matrix distance analysis of obtained sequences was carried out by Automated Barcode Gap Discovery (ABGD) (Puillandre N. et al., 2012) based K80 Measure of distance results.

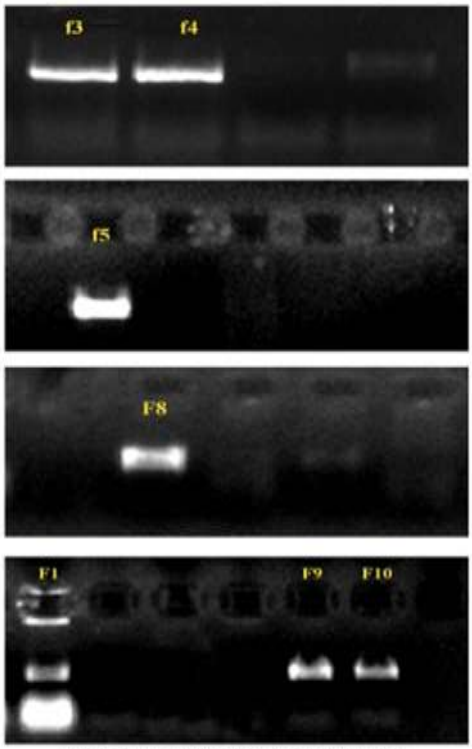
Photo plate 01, pure culture isolated *Fusarium species* growth on PDA medium.



Colony appearance of various shapes and spore of *Fusarium sps* isolates on PDA at 7 days after incubation at 27 ± 3oC (Culture plate. no. F_1….F_{10})

Photo plat: 02

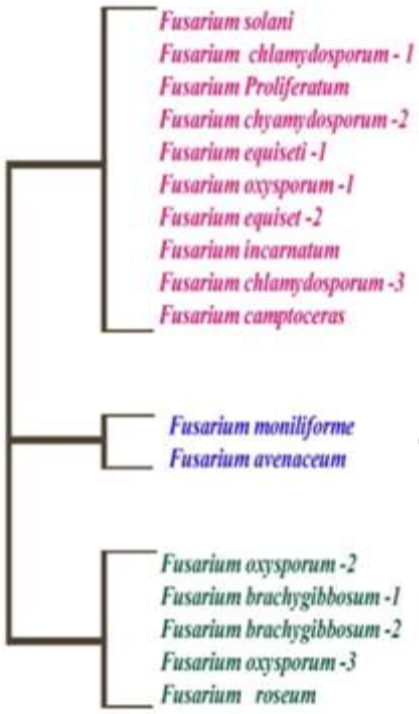
PCR products of *Fusarium* species on agarose gel.



ITS-1 - (TCCGTAGGTGAACCTGCGG)
ITS- 4 -(TCCTCCGCTTATTGATATGC)

Photo plat: 03

Phylogenetic tree of *Fusarium sps* isolated from Onion (*Allium cepa* L.)



DISCUSSION

Ten *Fusarium* species isolates from the infected onion samples collected from different locations. *Fusarium* isolates was based on morphological characters such as colony characters and size and shape of conidia according to descriptions of Simmonds (1965), Smith and Black, (1990) and Sutton (1992).DNA Barcoding of fungal specimens was carried out to enlighten their taxonomic identifications and diversity using nuclear ITS 1 and ITS 4 as molecular markers. Sequences of *Fusarium sps* were grouped into three clusters. The largest cluster shows genetic similarity among ten species of Fusarium. They are *Fusarium solani, Fusarium chlamydosporum*-1, *Fusarium Proliferatum, Fusarium chlamydosporum* 2, *Fusarium equiseti*-1, *Fusarium oxysporum*-1, *Fusarium equiseti*-2, *Fusarium incarnatum, Fusarium chlamydosporum*-3, *Fusarium camptoceras*. Another cluster is of only two species of Fusarium they are *Fusarium moniliforme Fusarium avenoceum.* The third group shows five species of *Fusarium* such as *Fusarium oxysporum-2, Fusarium brachygibbosum-1, Fusarium brachygibbosum-2 Fusarium oxysporum-3* and *Fusarium roseum.* So among of seven *Fusarium* species shows such type of genetic similarity, this grouping is do not show any relation with onion varieties, locality on age of onion plant. It is only based on genetic material of *Fusarium* species in different group of classification. The Phylogenetic tree using nearest neighbor joining method was constructed by MEGA 5.1 using Kimura 2 parameter as a distance model. Accuracy of the tree was verified by 1000 replicates of Bootstrap support. Similarly report as by using ITS1 and ITS 4 primer on Molecular and Pathogenic diversity of the causal agents of onion leaf twister, vengadharman and

costa (2104),genetic diversity produced genetic distances obtained from SSR markers Rivera*et.al* (2016).molecular characterization on onion by RAPD method, maniruzzaman et.al (2010). Genetic variability of Fusarium sps from basal rot on onion was carried out using RAPD, Malathi and Mohan (2012).

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