

## Original Research Article

# **Morpho-molecular identification and pathogenicity in *Fusarium* pathogens associated with vascular wilt in Cowpea (*Vigna unguiculata* L.)**

### **ABSTRACT**

*Fusarium oxysporum* (Fo), causing vascular wilt in cowpea is a serious pathogen, which leads to high plant mortality with severe yield loss. Variability among the pathogen is very low, due to the lack of sexual reproduction, and low mutation rate. Here, we isolated ten different isolates of *Fusarium* from different geographic locations, morphologically evaluated, molecularly identified, and pathogenicity were tested. *Fusarium* consists of asexual spores, macroconidia, microconidia and chlamydospores, with varied dimensions and produced some pigments in culture media. Pathogenicity was tested by inoculating pathogens cultured in sand maize media with 10% inoculum. Sand maize inoculation is the best method for *Fusarium* screening in cowpea plants than other methods. The identified *Fusarium* pathogens DNA were amplified with universal ITS primers, sequences were obtained and deposited in the GenBank database. The sequences were compared with available database, showed 99-100% homology with *Fusarium oxysporum*. phylogenetic study revealed that the isolates were less distant from *Fusarium* species. The phylogenetic analysis helps to understand the evolutionary relationships among the species identified.

Keyword: *Fusarium oxysporum*, Pathogenicity, Asexual spores, universal primer, Database

### **INTRODUCTION**

*Fusarium oxysporum* (Family: *Nectriaceae*, Genus: *Fusarium*), an abundant and active saprophyte in the soil and organic matter, with some specific forms that can cause plant diseases. Already identified more than 120 *forma speciales* (ff. spp.) based on host species specificity, it was suggested fifth in the top ten plant pathogens of scientific or economic importance and it is a part of quarantine list of several destination<sup>1</sup>. The saprophytic ability of the pathogen enables to survive either as mycelium or any of its three different spore types, in the soil between crop cycles in infected plant debris<sup>2</sup>. The pathogen mainly produces three types of asexual spores, one or two celled microconidia, three to five celled macroconidia and, dormant, round, and thick walled chlamydospores<sup>3</sup>. *Fusarium oxysporum* considered as low genetic variable species, due to lack of sexual reproduction, which leads to little genetic flow and mutation rates<sup>4</sup>. Plants growing in soil, which contaminated with the fungus, will become infected, and the pathogen invade the plant either with its sporangial germ tube or mycelium by invading the plant's roots<sup>5</sup>. The fungal mycelium grows through the root cortex intercellularly, reaches to xylem vessels, and growth advances upwards towards the stem and crown of the plant<sup>6</sup>. The mycelium produces microconidia and it spreads through the xylem pits to adjacent cells. Due to the growth of mycelium within the plant's vascular tissue, plant becomes thirsty, closes stomata, wilts the leaves, and eventually dies<sup>7</sup>. Infection of vascular vessels, turns the tissues to brown colour due to the pigments produced from the pathogen, so the vascular browning is strong evidence of *Fusarium* wilt<sup>2</sup>. In the older plants, vein clearing and leaf epinasty are often followed by stunting, yellowing of the lower leaves, formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of remaining leaves, and finally death of the entire plant<sup>8,9</sup>.

Legumes are most affected by fusarium wilt, mainly chickpea (*Cicer arietinum*), cowpea (*Vigna unguiculata*), lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris* L.), Alfalfa (*Medicago sativa* L.) etc, which promotes devastating damages in legume species worldwide<sup>10</sup>. Each crop infects by the host specific Fusarium pathogen, usually characterizes *Fo* ff. spp., however there are examples of revealing broader host range, infecting more than one legume species<sup>11</sup>. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *tracheiphilum*, in cowpea, produces high plant mortality with severe overall yield loss<sup>12</sup>. As a soil born disease, the management of these pathogen is not under control, even though it requires continues management practices and soil solarization. Because of the presence of dormant chlamydospores in the soil for several years, occurrence of disease during cropping season is unbeatable<sup>13</sup>. For the complete management of pathogen requires the study on morphology, growth pattern, pathogenicity, and the environmental requirement for the establishment. So that, present study done for variability evaluation, morphologically and molecular identification of pathogen causing fusarium wilt in cowpea.

## **MATERIALS AND METHODS**

### ***Fusarium* isolates and culture condition**

The infected cowpea plants (*Vigna unguiculata* L.) were collected from ten different cowpea growing fields, stem and roots were washed in running water to remove adhered soil particles. The infected collar portion and root bits were cut into small pieces of 1-2 mm size and surface sterilized with 0.1% mercuric chloride (HgCl<sub>2</sub>) followed by three times washes with sterile water. The 4-5 small bits were transferred aseptically into a petridish containing solidified Potato Dextrose Agar (PDA) amended with streptomycin sulphate and tetracycline hydrochloride to minimize chances of bacterial growth. Plates were incubated at 28°C and observed periodically. The fungal isolates were grown on PDA to study their growth and variability in colony characters. From 3 days old culture plates, 5 mm disc of fungus was cut by a sterilized cork borer and kept at the centre of each fresh petriplates with 15ml of sterilized and solidified PDA, incubated for 3 days at 28± 2°C temperature. The grown fungal isolates transferred into fresh PDA slants for maintenance and stored at 4°C<sup>14</sup>. Three days after inoculation (DAI), the colony characters, mycelial growth and spore characters were recorded.

### **Morphological and Cultural evaluation of fungal strain**

The ten different fungal strains undergone periodic observation, to study the rate of growth (mm/day), and microscopic characterization of microconidia, macroconidia, and presence of chlamydospore were also observed. The external appearance of culture, production of pigments and the pattern of growth in each strain isolated were studied<sup>15</sup>.

### **Pathogenicity testing of fungal strain**

The isolates were assessed for liability to cause diseases in the healthy cowpea plants. Different inoculation methods, viz., Sand Maize Media (SMM), SMM+ Pin Prick, Pin Prick and Control were tested to know the effective way for inoculation. The sand: maize (9:1) media (SMM) was prepared by adding 90g of riverbed sand and 10g of maize meal with 20ml of distilled water in 250ml of Erlenmeyer flask. The medium was autoclaved in the flask at 15 lb for 20 minutes. After cooling the medium, 10 bits of fungal culture along with PDA was aseptically transferred into the medium containing flask and closed air tightly.

Allowed the fungus to multiply inside the medium for 2 weeks. SMM was mixed with sterile soil (10% w/w), kept it for one week and healthy cowpea seedlings of two-week-old was transplanted to it. Pin Prick method was done by creating pin holes on the leaves and stem of two-week-old cowpea seedling, and introduced the conidial suspension ( $1 \times 10^6$  cfu)<sup>16</sup> using sterile cottons. To standardise the concentration of SMM was done at different percentages viz., 1%, 2%, 5%, 10%, 15%, 20% and 25% SMM, along with control, were inoculated in sterile soil (w/w) for fixing the effective concentration to cause disease in healthy plants, and two-week-old cowpea seedling were transplanted to it, and monitored regularly. The fungal strain showed the fusarium symptoms in healthy plants were carried to molecular identification with universal primers, to obtain ITS region amplification for comparison with reference database from NCBI.

## **Molecular identification of fungal strain**

### **Genomic DNA extraction and ITS Amplification**

DNA was extracted from 5 pathogenic *Fusarium* spp. isolates using NucleoSpin<sup>®</sup> Plant II Kit (Macherey-Nagel). The eluted DNA was stored at 4°C. The quality of DNA was checked using 0.8% Agarose Gel Electrophoresis. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). The Internal Transcribed Spacer (ITS) region of fungal strain was amplified with universal primers ITS-1F (5'TCCGTAGGTGAACCTGCGG3') and ITS-4R (5'TCCTCCGCTTATTGATATGC3')<sup>17</sup>. The details of amplification mixtures and profile were depicted below,

<b>Amplification Mixtures</b>		<b>Amplification Profile (40 Cycles)</b>		
2X Phire Master Mix	5Ml	Initial Denaturation	98 °C	30 Sec
D/W	4µL	Denaturation	98 °C	5 Sec
Forward Primer	0.25µL	Annealing	58 °C	10 Sec
Reverse Primer	0.25µL	Extension	72 °C	15 Sec
DNA	1µL	Final extension	72 °C	60 Sec

### **Sequencing of ITS region using BigDye Terminator v3.1**

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1<sup>18</sup>.

### **Comparison of ITS sequences with reference database deposited in NCBI**

The sequences were trimmed, assembled and consensus sequences were obtained using BioEdit software. The obtained nucleotide sequence was searched through BLASTN at GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) to compare with ITS reference sequence available in the database for identification of the isolate. Multiple sequence alignment of ITS sequence with reference sequence from gene bank was performed using Clustal W available in BioEdit Software. Phylogenetic evolutionary relationship analysis of the fungal ITS sequences was performed using Molecular Evolutionary Genetic Analysis software (MEGA 7). Phylogenetic analysis was carried out using Neighbor-joining method with 100 bootstraps run<sup>19</sup>.

## Results and Discussion

### Morphological and Cultural evaluation of fungal strain

Ten different cultures were obtained (table. 1), isolated, and the morphological and cultural evaluation were done. Each isolate was distinct in the appearance, some produced pigments in the PDA media, which turned the colour of culture plate. Most of the isolates produced cottony white raised mycelium, pale yellow to dusky red colour pigmentation and moderate to profuse sporulation on PDA medium. Varied colour of fusarium culture plate from normal white to violet, brown, reddish violet, yellowish pink, and dark pink were observed in different isolates of *F. oxysporum* f sp. *ciceris*<sup>20, 21</sup> Characterized greyish white to pinkish colour pigments production in *F. solani* isolates. *Fusarium* spp. has been recognized as a natural source of coloured polyketidic secondary metabolites; among that red naphthoquinone pigments like bikaverin, aurofusarin, fusarubin, bostrycoidin, javanicin and novarubin, as most abundant followed by hydroxyanthraquinone pigments; but there is no relationship between pathogenicity and pigments produced<sup>22, 23</sup> Reported the concomitant production of bikaverin along with 2 other novel pigments by phytopathogenic *Fusarium oxysporum* LCP531 strain isolated from soil. Pigmentation was the characteristic evident for the presence of *Fusarium* pathogen. No odor was detected. *Fusarium* pathogen is well known by its three types of asexual spores, macrospores, microspores and chlamydospores depending on the host availability and prevailing environmental conditions but, the sexual or teleomorphic stage is unknown. The shape and septations of these spores make each pathogen species unique, and these are one of the morphological markers for species identification. Macroconidia showed 3-5 septate, curved or straight with pointed or round ends; microconidia with one or no septate, unicellular oval or kidney shaped, and chlamydospores are singly or in pairs, intercalary or terminal in position with globular or oval. The ratio between production of micro conidia and macro conidia depends on the environment and the species produced<sup>24</sup>. Normally the number of microconidia is much more than that of macroconidia, and most abundantly and frequently produced by the fungus under all conditions<sup>2</sup>.

The growth rate of isolated pathogen in PDA media was varied from 3mm/day to 20mm/day. Microconidia was sized from 7.340 to 12.879  $\mu\text{m}$  by 1.794 to 4.914  $\mu\text{m}$ . microconidia was 9-10  $\times$  1-3  $\mu\text{m}$  in *Fusarium solani* causing dry root rot in citrus<sup>25</sup>, and 6.33  $\times$  1.50  $\mu\text{m}$  of *Fusarium oxysporum* f. sp. *Chrysanthemi* associated with vascular wilt of chrysanthemum<sup>26</sup>. While, size of macroconidia of isolates ranged from 25.591 to 37.620  $\mu\text{m}$  by 2.502 to 10.772  $\mu\text{m}$ . Macroconidia size varied from 37.5-50 (42)  $\mu\text{m}$  in length by 5-6.3 (5.5)  $\mu\text{m}$  in width of *Fusarium oxysporum* associated with *Carya illinoensis* seedlings<sup>27</sup>, 15.11  $\times$  2.88  $\mu\text{m}$  of *Fusarium oxysporum* f. sp. *Chrysanthemi* associated with vascular wilt of chrysanthemum<sup>26</sup>. Chlamydospores are thick-walled dormant spores, which are intercalary or terminal in position. These are the main survival structures of *Fusarium oxysporum* under field condition and severe disease developing than microconidia do in tomato<sup>28</sup>. The dormant spores get germinated under favourable environmental condition as well as by the presence of host plant root exudates. Chlamydospores of *Fusarium oxysporum* germinated efficiently in the root exudates of tomato and reached up to 98% of germination after 10 h of incubation period<sup>29</sup>.

### Pathogenicity testing of fungal isolates

Among the ten isolates, five isolates were selected according to the cultural and morphological evaluation as *Fusarium* pathogen, and pathogenicity test was carried out

(Figure. 1). Inoculation methods provides sufficient inoculum load to the plants for causing the disease. So, the different methods were assessed using the fusarium pathogen, in which inoculation with sand maize base showed sufficient pathogenicity than pin prick method of inoculation (Figure 2). The rice-grain inoculation to test pathogenicity of *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani* on various pepper cultivars were recommended<sup>30</sup>. Greatly significant variations observed among the isolates in causing disease. The isolates 1,8, and 9 were showed fusarium wilt symptoms after 8 days of inoculation with leaf necrosis, stem basal discoloration and total wilting of seedlings in the inoculated plants. Isolates 4 and 7, also showed symptoms in some extent, because of the less virulent capacity of these isolates than other three virulent strain. The isolate 1 was more virulent among the isolates, so different inoculation percentage were tried to fix an effective. Soil inoculation of 10% of pathogen multiplied sand maize (9:1) media was most effective percentage to cause disease. Ten percentage soil inoculation produced fusarium symptoms, and might be used for disease screening using the culture (Figure 3). Coriander plants showed fusarium symptoms, when 5% sand maize media was used for disease screening<sup>31</sup>, 4% soil inoculation for *Fusarium oxysporum* f sp. *ciceri* screening in chickpea<sup>32</sup>, and 10% inoculation for *Fusarium oxysporum* f.sp. *lycopersici* in tomato were used<sup>33</sup>.

### **Molecular identification of fungal strain and phylogenetic analysis**

The accurate identification of pathogen species by visual examination of morphological criteria is very difficult and erroneous. Molecular identification provides unique barcode for determination and identification of different fungal isolates, taxonomy, molecular evolution, population genetics and fungus-plant interaction<sup>34</sup>. Molecular identification was carried out by DNA barcoding using ITS region sequencing. The data were trimmed, assembled and consensus sequences were obtained, deposited, and provided accession number, and it was compared to those in the database using NCBI-BLAST. Five isolates were identified using DNA barcoding with an identity range between 99-100% (Table 2). The ITS rDNA are the excellent candidate genes for phylogenetic analysis because they are universally distributed, functionally constant, sufficiently conserved, and of adequate length to provide a deep view of evolutionary relationship<sup>35</sup>. Phylogenetic analysis showed the isolates were related to the *Fusarium* database deposited in NCBI. Isolate 1 was related to *Fusarium oxysporum* (OP210003.1) by 99% bootstrap (figure 4).

### **Conclusion**




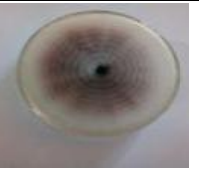










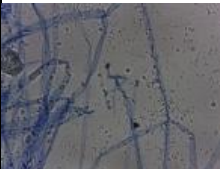





The study was done for morpho-molecular identification and pathogenetic analysis of fusarium isolates obtained from diseased cowpea plants from different geographical locations. The fusarium pathogen was consisting of microconidia, macroconidia and chlamydospores, without sexual stage. Each pathogen varied in their conidial characters and pathogenicity in terms of its disease development. Sand maize (9:1) media is best for pathogen culturing and inoculation method with 10% inoculum. The ITS region was amplified with universal primers and the sequences were obtained, deposited in the NCBI database. Homology percentage was calculated and 99-100% were observed with the already deposited database. Phylogenetic analysis was helped to understand the evolutionary relationship among the pathogen isolates. So, the five isolates were identified as *Fusarium oxysporum* from morphological and molecular data obtained.










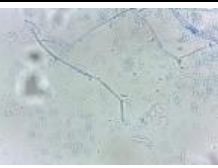

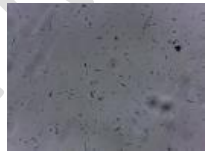



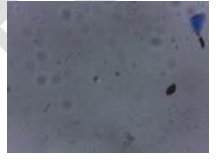
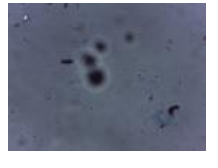

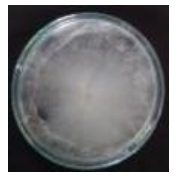

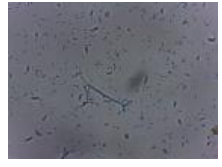




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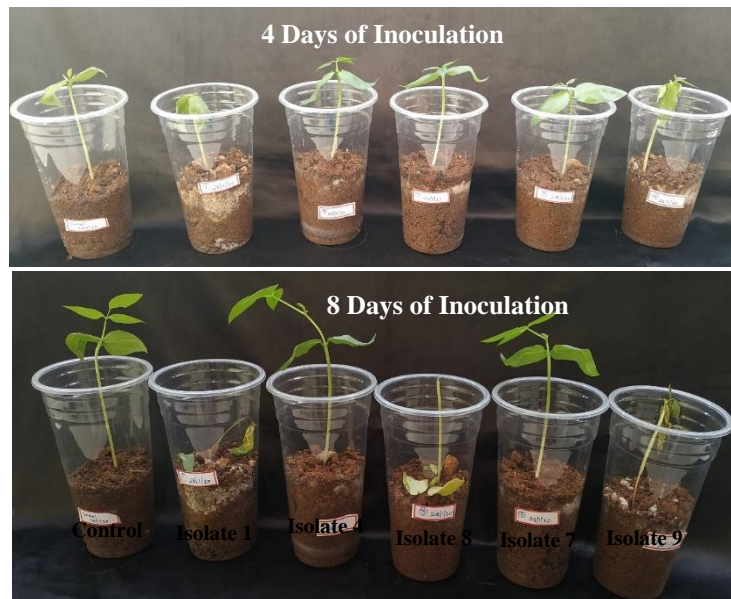
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**Table 1. Morphological evaluation of ten isolates of *Fusarium* spp.**

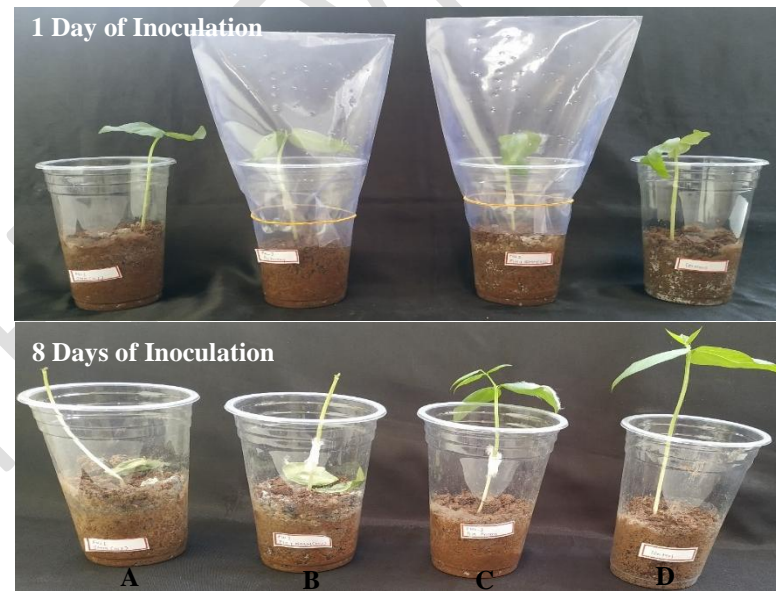
	Features	Microspores	Megaspores	Surface of colony on PDA	Reverse of colony on PDA	Mycelium
Isolate 1	Growth rate 5mm/day at 24°C; White raised mycelia with reddish brown culture; aseptate microconidia (12.879×3.570 μm); 3-4 septate macroconidia (32.409×5.297 μm), little curved; intercalary and axial chlamydo spores present; false head appearance,					
Isolate 2	Growth rate 20mm/day at 24°C; little raised mycelium without pigment production, micro and macro conidia were absent; chlamydo spores present					
Isolate 3	Growth rate 20mm/day at 24°C; little raised mycelium with white and light pink colour at centre, micro and macro conidia were absent; chlamydo spores present					
Isolate 4	Growth rate 6mm/day at 24°C; White raised mycelia with little pink coloured culture; aseptate microconidia (11.830×3.914 μm); 3 septate macroconidia (30.528×4.762 μm); intercalary and axial chlamydo spores;					

Isolate 5	Growth rate 3mm/day at 24°C; White raised mycelia without pigmentation; aseptate microconidia (8.419×2.547 μm); 2 septate macroconidia (28.870×3.629μm); intercalary and axial chlamydo spores;					
Isolate 6	Growth rate 3mm/day at 24°C; White raised mycelia with little pink coloured culture; aseptate microconidia (9.802×1.974 μm); 2 septate macroconidia (26.692×2.662 μm); no chlamydo spores were present;					
Isolate 7	Growth rate 6.5mm/day at 24°C; White raised mycelia with red to dark brown coloured culture; aseptate microconidia (10.548×2.843 μm); 2 septate macroconidia (27.764×3.541 μm); intercalary chlamydo spores were present;					
Isolate 8	Growth rate 4mm/day at 24°C; White raised mycelia with white coloured culture; a septate microconidium (7.340×1.973 μm); 2 septate macroconidia (25.591×2.502 μm); intercalary chlamydo spores;					
Isolate 9	Growth rate 6.5mm/day at 24°C; suppressed mycelia with light yellow coloured culture; aseptate microconidia (8.339×2.159 μm); 2 septate macroconidia (28.659×2.751 μm); axial chlamydo spores;					

Isolate 10	Growth rate 6mm/day at 24°C; suppressed mycelia with dark brown coloured culture; aseptate microconidia (12.435×4.914 μm); 5-6 septate macroconidia (37.620×10.772 μm); axial chlamydospores;					
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**Figure 1. Pathogenicity analysis of isolated fusarium pathogens**



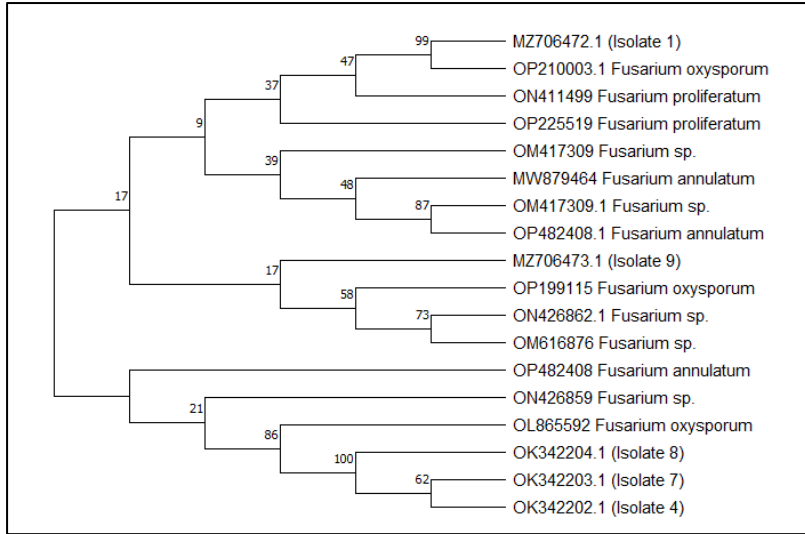
**Figure 2. Inoculation methods for Fusarium isolates; A) SMM (10%), B) Pin Pricking+ SMM (10%), C) Pin Pricking, D) Control**



**Figure 3. Different inoculation percentages of Fusarium isolates**

**Table 2. Identity percentage and homology sequences of Fusarium isolates with GenBank database**

Isolates	Accession number	Identity percentage (%)	Homology sequence
Isolate 1	MZ706473.1	100%	<i>Fusarium</i> sp. strain (OM417309.1)
Isolate 4	MZ706472.1	100%	<i>Fusarium oxysporum</i> isolate (OP684344.1)
Isolate 7	OK342204.1	100%	<i>Fusarium</i> sp. strain (MG098654.1)
Isolate 8	OK342203.1	100%	<i>Fusarium oxysporum</i> isolate (MK239978.1)
Isolate 9	OK342202.1	100%	<i>Fusarium oxysporum</i> isolate (MK968007.1)



**Figure 4** phylogenetic analysis of *Fusarium* isolates with the database sequences