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# Assessing the pathogenicity of *Fusarium Oxysporum* on soybean using different inoculation techniques

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## ABSTRACT

*Fusarium is one of the important genera of plant parasitic fungi responsible for causing wilt disease in Soybean. The present study was conducted to identify the virulent isolate of the pathogen and to assess their pathogenicity in Soybean cv. Basara. About 10 different Fusarium isolates were collected from the division of Plant Pathology, ICAR- IIOR, Hyderabad, and were assessed for their pathogenicity in Soybean cv. Basara to identify the virulent isolate against the crop using three different pathogenicity tests viz., filter paper method, artificial inoculation method, and sick pot method. Among the 10 different isolates tested for their virulence under three different pathogenicity tests, the Fusarium isolate, CF II was reported to exhibit a higher disease index and wilt incidence in the crop, which has been designated as the virulent isolate against the crop. The pathogenic Fusarium isolate, CF II was characterized using molecular methods based on ITS PCR and confirmed as Fusarium oxysporum.*

**Keywords:** *Fusarium oxysporum,* pathogenicity and Soybean

## INTRODUCTION

Soybean is one of those most widely grown oilseed crops and contains about 40% protein and 20% oil content in it. Its uses in human foods include tofu, shoyu (soy sauce), miso, and tempeh (Singh *et al.,* 2008). Soy protein products (25 g soy protein/ day) were reported to reduce the risk of heart disease when consumed in diets that are low in saturated fats. Soy protein ingredients are often used in food products suggested for those who are allergic to cow's milk or intolerant to lactose and these products plays a major role in preventing osteoporosis and risk of certain cancers (Deak *et al.,* 2008). Soybean meal is used as animal feed including poultry, pigs, cattle, and aquatic Dei (2011). Industrial applications of soya products found their application in biodiesel, ink, biocomposites and bioplastics, adhesives, waxes, candles, foams, and hydraulic fluids (Gaonkar and Rosentrater 2019).

*Fusarium oxysporum* is soil-borne pathogens that cause disease on many significant agricultural crops (Beckman, 1987; Farr *et al.,* 1989; Scandiani, 2011), especially in corn, soybean, tomato, and wheat crops (Parikh *et al.,* 2018). It is the most widespread and destructive species on soybean. This fungus infects plant from roots and grows from inside towards the cortex to stele (Beckman, 1987; Tjamos and Beckman1989; Bowers and Locke, 2000; Nandhini *et al.,* 2012) and cause a variety of symptoms, including damping-off, wilting, root rot, cortical decay, yellowing of the plant, vascular discoloration and stunted growth that gradually causes a yield loss of about 47.6 to 55.6 % (Arias *et al.,* 2013). Since *Fusarium* species are numerous, their damage varies depends on the plant host, the current study was carried out to assess the different inoculation techniques for assessing the pathogenicity of different isolates of *Fusarium* species in Soybean to identify the virulent pathogenic isolate against soybean.

## MATERIALS AND METHODS

### Source of Fungal isolates

Different isolates of *Fusarium* species (n=10) collected from the Department of Plant Protection, ICAR-IIOR, Hyderabad were used in the current study.

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**Morphological characterization of different *Fusarium* isolates**

The different *Fusarium* isolates collected were transferred onto the Potato Dextrose broth (PD broth) and incubated for 7 days at room temperature. After the incubation period, the fungal isolates were observed for their morphological characteristics such as the appearance of the colony on the PDA medium and the formation of conidia, conidiophores, and chlamydospores on the media.

**Different inoculation techniques to assess the pathogenicity of different *Fusarium* species on Soybean (Basara)**

**a. Pathogenicity assay of different *Fusarium* species on soybean seeds by filter paper method**

Pathogenicity of ten (F-I, F-II, CF-I, CF-II, CF-III, YF, PF, IF, SK-I and SK-II) representative *Fusarium* isolates were performed on healthy seeds of soybean cv. Basara. The spore suspension of the fungus was prepared by adding 5 mycelium plugs (5mm) into 100 ml of Potato dextrose broth and incubated at an orbital rotator at 150 rpm for 7 days at 25 ºC to obtain at the rate of of $1 \times 10^6$ spores/ml. Soybean seeds were thoroughly surface-sterilized with 5% sodium hypochlorite and then soaked in spore suspension overnight. The seeds were then kept in the sterilized Petri dishes pre-soaked with moistened filter paper at the rate of 10 seeds per petri dish. Fungal mycelium of 2 to 3 strands along with a bit of agar were added over the seeds. The seeds were then incubated in the Petri plates for 7 days. Seeds soaked in water served as control. Each treatment was replicated four times in CRD.

After incubation, observations were made on mycelial coverage on the seeds, and decay of the seeds, based on which disease severity grade was evaluated (Naeem *et al.,* 2019).

Disease severity grade scale:

- ✔ 0 = Healthy seed germination without discoloration inside the seeds
- ✔ 1 = Delayed germination with negligible or no discoloration inside the seeds
- ✔ 2=Low germination with slightly water-soaked and yellow symptoms inside the seeds
- ✔ 3=No germination with partially water-soaked, yellow or brown, softened decay inside the seeds
- ✔ 4=No germination, brown and severe seed decay.

Diseases index (DI) is calculated using the following formula.

$$DI = \frac{\sum (\text{Severity grade} \times \text{Seeds per grade}) \times 100}{\text{Total seeds} \times \text{Highest severity grade}}$$

**b. Pathogenicity assay of different *Fusarium* species on soybean seedlings by artificial inoculation method**

This experiment was conducted on 30 days old healthy soybean seedlings raised in the sterilized soil. The soybean seeds were sown in plastic pots containing about 1kg of sterilized soil. Different fungal isolates (F-I, F-II, CF-I, CF-II, CF-III, YF, PF, IF, SK-I and SK-II) were grown on potato dextrose broth (PDB) by placing 5×5 mm agar pieces of the fungal colony in the broth. The broths were incubated for seven days in a shaker at 120 rpm to produce large quantities of conidia. This inoculum was added into the rhizosphere soil of the seedlings in the pot at $1 \times 10^6$ cfu / g while for uninoculated control, sterile distilled water was added. The plants were observed for the disease symptoms post-inoculation of the fungus and the severity of the disease was calculated using a score of 0 - 4 scale, where

0 = No disease,
1= Yellow leaves,
2 = Yellow leaves and slightly wilted,
3 = Severe wilt,
4 = Dead seedling

(Soleha *et al.,* 2022)

**c. Pathogenicity assay of different *Fusarium* species on soybean by sick pot method**

The10 (F-I, F-II, CF-I, CF-II, CF-III, YF, PF, IF, SK-I and SK-II) different isolates of *Fusarium* were individually multiplied on autoclaved sorghum seed meal medium and the medium was incubated for 7 days until the fungal mycelium fully covered the sorghum grains. After 7 days, the inoculum was added to the sterilized soil at the rate of 5 g of sorghum grains/kg soil. The soil was then filled in the earthen pots at the rate of 1 kg/pot and the soybean seeds were sown in the pots after 2 days. Observations were recorded for the emergence and decay of the seeds (Sastry and Chattopadhyay, 2003).

The effective *Fusarium* isolate that recorded the higher pathogenicity in the above 3 methods was designated as the effective isolate and it was molecularly characterized to confirm the species.

### Molecular characterization of the *Fusarium* isolate

### DNA extraction

The effective *Fusarium* isolate, CF II was grown in 250 ml potato dextrose broth (PDB) in the rotary shaker at 180 rpm for 48 h at 28 $^{o}$C. After vacuum filtration, the mycelium of the isolate was dried, crushed using sterile sea sand in the mortar and pestle, and stored at 20 ºC. Approximately 0.5 g of the ground mycelium was suspended in CTAB extraction buffer (0.7 M NaCl, 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% 2-mercaptoethanol, 1% CTAB), and extracted using phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). RNA was degraded by treatment with RNase (50 mg/ml) for 30 min at 37ºC. DNA was then precipitated by adding 2.5 volumes of absolute ethanol and pelleted by centrifugation for 15 min at 12,000 rpm. The pellet was washed with 70% ethanol, air dried and re-suspended in 1X TE buffer [10 ml of 1M Tris–HCl, 1ml 0.5M EDTA (pH 8.0)] (Chehri *et al.,* 2011).

### PCR amplification

ITS region of *Fusarium* isoalte was amplified by using primers ITS1 (50 -TCCGTTGGTGAACCAGCG G-30) and ITS4 (50 -TCCTCCGCTTATTGATATGC-30).The reaction contained approximately 30 ng DNA, 10X buffer, 2.5 mM each dNTP, 20 nM $MgCl_2$, 25 pmol each primer, and 5 U Taq polymerase enzyme. The reaction mixture was brought to the volume using ultrapure water. Reactions were conducted in an applied biosystems thermocycler under the following thermal conditions: 94°C for 2 min; 30 cycles at 94°C for 45 s, 55°C for 30 s, and 72°C for 35 s; and 72°C for 10 min. PCR product was stored at 4°C after completing the reaction. A control without DNA was added in the PCR amplification. Amplified fragments and controls were separated by 1.2% agarose gel electrophoresis in 1X TBE buffer (10.8 g Tris base, 5.5 g boric acid, 4 mL 0.5 M ethylene diamine tetraacetic acid, and 4 mL distilled water) containing ethidium bromide and bands were observed under ultraviolet light. The products of the PCR were purified using 13% PEG 8000 and the sequences generated were 900-1200 base pairs. Sequencing was performed on a cycle sequencing kit on ABI 3730xl genetic analyzer. Sequenced fragment was analyzed using the BioEdit software. Nucleotide sequences of the pathogen were compared using the previously deposited sequences in GenBank. The GenBank sequences with the highest scores (more than 98% of similarity and coverage) were selected and aligned with sequences determined by sequencing using the ClustalW algorithm, and phylogenetic analysis was conducted using the neighbour-joining statistical method with 1000 replicates on the MEGA software version 10. The similarity of nucleotide sequences were calculated by the BLAST program (http://blast.ncbi.nlm.nih.gov) (Lazarotto *et al.,* 2014) and the sequence was submitted to GenBank.

### Statistical analysis

Statistical analyses were carried out using SPSS (Version 16) and Microsoft Office Excel 2013. A completely randomized design was employed for all the experiments, with four replicates for each treatment. The data presented were from representative experiments are repeated at least twice. The least significant differences test (LSD) at 5% ($P \leq 0.05$) probability level was used to compare treatments using ANOVA.

## RESULTS AND DISCUSSION

### Morphological characteristics of the fungal isolates

All the ten *Fusarium* isolates were noted with fast growing colonies on PDA, abundant aerial mycelium, cottony and white growth (CF-III, SK-I and SK-II), pale pink (F-I, F-II and CF-I), pale violet (CF-II), and pinkish mycelial growth (YF, PF and IF) (Table 1; Fig.1).

Micromorphology studies revealed hyaline and septate hyphae with 3-5 septum and falcate-shaped macro conidia exhibiting various lengths from 37.26 to 43.27 µm (Table 2; Fig. 2). Based on colony morphology and characteristics of macro and micro conidia, the fungal isolates were confirmed as *Fusarium oxysporum*.

Fig.1: Growth of different *Fusarium* isolate CF-II on PDA media

Table 1: Morphological characteristics of different *Fusarium* isolates

| *Fusarium* isolates | Colony characteristics | Macroconidia | | |
|---|---|---|---|---|
| | | Septum | Shape | Length (µm) |
| F-I | Whitish pink, cottony growth | 3-4 | Falcate | 40.38 |
| F-II | Pinkish white, smooth growth | 3-5 | Falcate | 39.23 |
| CF-I | Whitish pink, rough colony growth | 3-4 | Falcate | 41.27 |
| CF-II | White on top and pale violet on back of the plate, smooth colony growth | 3-4 | Falcate | 43.27 |
| CF-III | Light White, cottony growth | 3-5 | Falcate | 37. 26 |
| YF | Whitish pink, smooth growth | 3-4 | Falcate | 42. 23 |
| PF | Whitish pink, smooth growth | 3-4 | Falcate | 41.52 |
| IF | Whitish pink, smooth growth | 3-4 | Falcate | 40.57 |
| SK -I | White, smooth growth | 3-4 | Falcate | 38.24 |
| SK -II | White, cottony growth | 3-4 | Falcate | 43.12 |

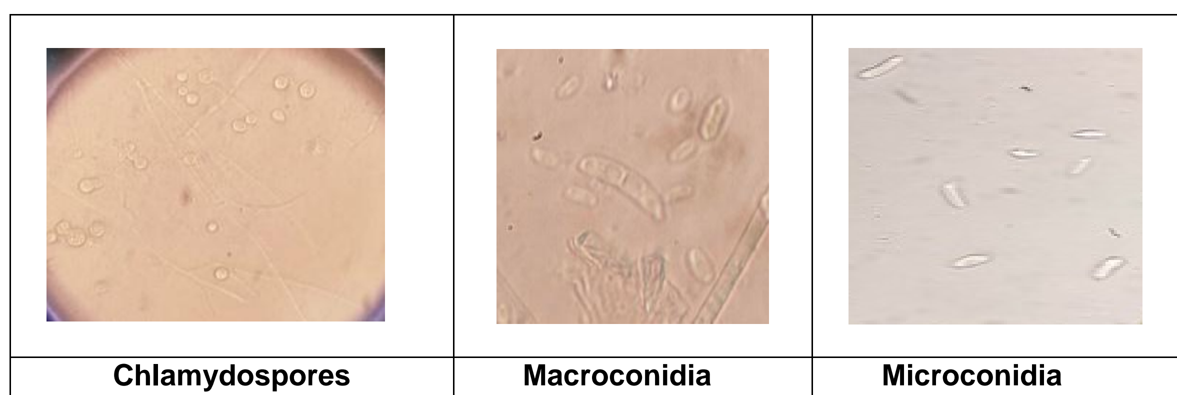


Fig.2: Microscopic characteristics of *Fusarium* isolates

**a. Pathogenicity effect of different *Fusarium* isolates on soybean seeds**

All the ten *Fusarium* isolates exhibited a greater degree of pathogenicity on soybean seeds, as they all significantly reduced the average of germination relative to the uninfected control treatment. Among the different isolates tested, *Fusarium* isolate, CFII proved to be the most virulent one, as it had the most significant negative effect on seed germination (24.3%) in comparison with other fungal isolates. The isolates IF and PF recorded the maximum seed germination of 67.5% (Table 2).

After 7 days incubation of the seeds percent mycelial covering (PMC) was observed to be more in CFII (90 %) followed by YF (81.75 %) while F II recorded the lowest mycelia cover of 31 %. Our results showed that, among the different *Fusarium* isolates tested for the disease severity index (DSI), CF 2 recorded the maximum disease index of 73.3% followed by CF3 (54.8 %). The least disease index was recorded in FI (34.5 %) (Figure 3 and Table 2).

Table 2: Pathogenicity assays of different *Fusarium* isolates on Soybean cv. Basara

| *Fusarium* isolates | Filter paper test | | | Artificial inoculation in soil | Sick pot method |
|---|---|---|---|---|---|
| | % seed germination | % mycelial coverage | DSI | Wilt score | % seedling germination |
| F-I | 44.5 | 43.75 | 34.5 | 2.0 | 83.0 |
| F-II | 54.3 | 31 | 38.8 | 1.0 | 77.0 |
| CF-I | 47.5 | 73.75 | 37.5 | 2.0 | 47.0 |
| CF-II | 24.3 | 90 | 73.3 | 4.0 | 27.0 |
| CF-III | 54.5 | 71.5 | 54.8 | 2.0 | 37.0 |
| YF | 45.5 | 81.75 | 46.8 | 3.0 | 97.0 |
| PF | 67.5 | 31.75 | 46.5 | 2.0 | 57.0 |
| IF | 67.5 | 44.25 | 36.3 | 2.0 | 73.0 |
| SK -I | 46.8 | 43.75 | 44.3 | 2.0 | 53.0 |
| SK -II | 42.3 | 34.5 | 45.8 | 2.0 | 57.0 |
| Control | 99.8 | 0 | 0.0 | 0.0 | 100.0 |
| CD (0.05) | 0.502 | 0.367 | 0.410 | | 0.390 |
| SM (m) | 0.174 | 0.127 | 0.142 | | 0.132 |
| SE(d) | 0.246 | 0.180 | 0.201 | | 0.187 |
| CV | 4.753 | 3.766 | 4.524 | | 2.877 |

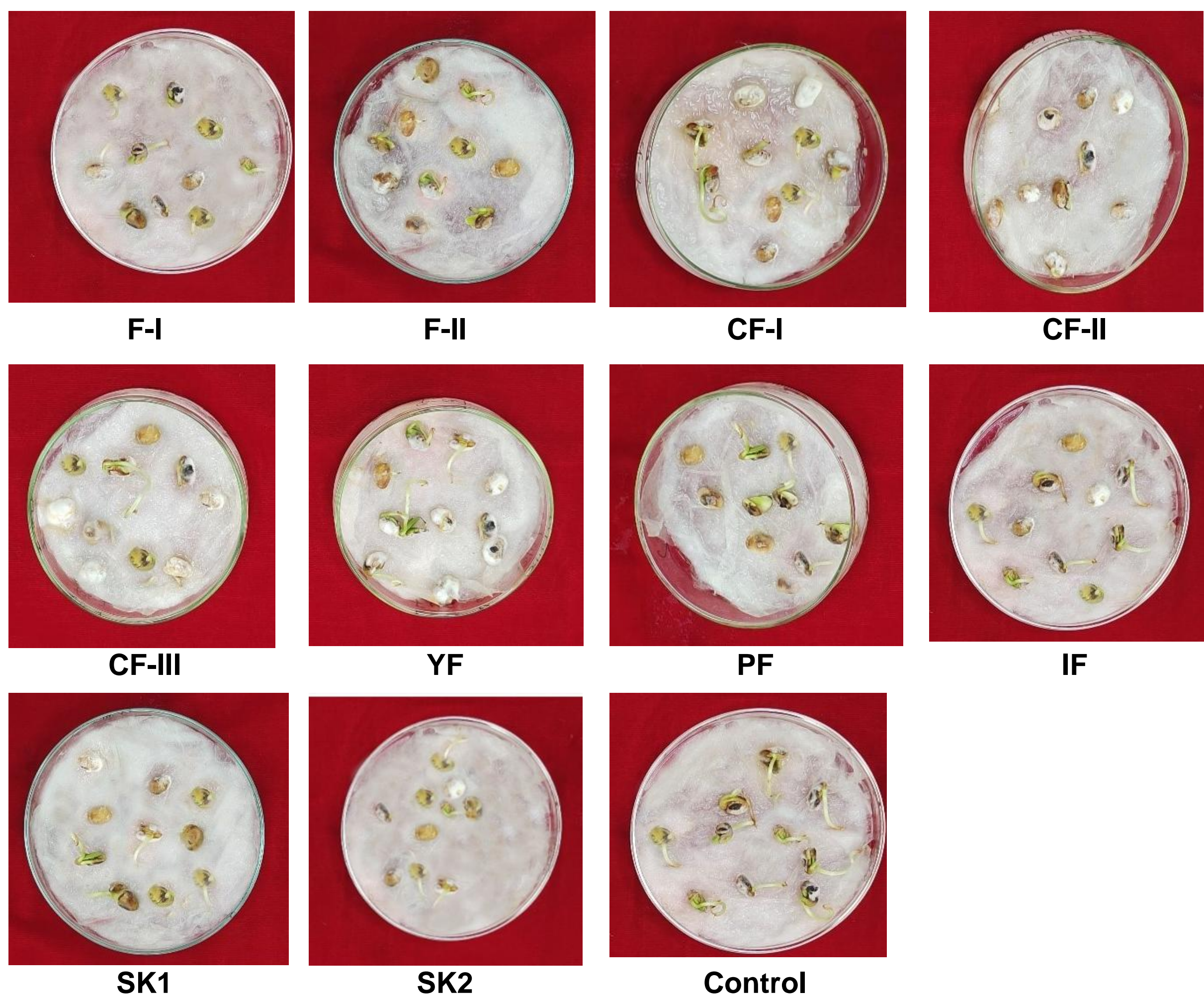


Fig. 3: Pathogenicity effect of different *Fusarium* isolates on soybean seeds in filter paper method

**b. Pathogenicity effect of different *Fusarium* isolates on soybean seedlings by artificial inoculation method**

Wilt incidence of the plants inoculated with different *Fusarium* isolates revealed the wilt symptoms with different disease scale. Among the different isolates, *Fusarium* isolate, CF II was recorded as highly pathogenic with the wilt score of 4.0, followed by YF with the wilt score of 3.0. The lowest wilt score (1.0) was reported in the F2 isolate (Table 2; Fig. 4).

Fig. 4: Soybean plants infested with different *Fusarium* isolates in artificial inoculation method

**c. Pathogenicity effect of different *Fusarium* isolates in soybean by sick pot method**

The ten different *Fusarium* isolates tested had a significant influence on seed germination. Among the different isolates tested, lowest seed germination of 27 per cent was recorded in the seeds inoculated with the isolate, CF II, followed by CF III (37 %) while the highest percent germination was observed in YF (97%) treated seeds (Table 2; Fig.5). Germinated seedlings, however, wilted after germination and died. The isolate CF II caused the wilting and dying of the germinated seedlings 4-5 days earlier than other isolates. Lowest wilting score was reported in the plants treated with the isolate, FI.

Fig. 5: Seedling germination in sick pot method treated with different fusarium isolates

Results of all three pathogenicity tests revealed that the *Fusarium* isolate, CF-II was more pathogenic to the soybean when compared to other isolates. This isolate was characterized at the molecular level for the confirmation of the species.

**Molecular identification of the *Fusarium* isolate, CF 2 by ITS sequencing**

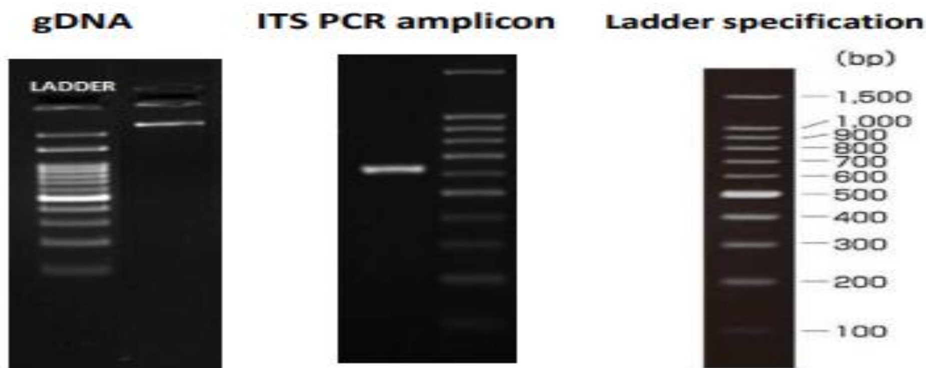


Fig. 6: Amplification of conserved ribosomal regions of *Fusarium* isolate, CF 2 using the primers ITS-1 and ITS-4

Table 3: Information of *Fusarium* isolate, CF 2

| S. No. | Isolate | Host | Morphological identification | ITS Identification | Accession number |
|---|---|---|---|---|---|
| 1. | CF-2 | Soybean | *Fusarium oxysporum* | *Fusarium oxysporum* | OR226604 |

The sequences obtained from the conserved ribosomal ITS region amplification (Fig.6) were correlated with the sequences from NCBI database by using BLAST 2.0 (http://www.ncbi.nlm.nih.gov/BLAST). The isolate CF II was identified as *F. oxysporum* and the sequence was deposited in NCBI GenBank (Accession no. OR226604) (Table 3).

**Sequence analysis of ITS**

The Maximum Likelihood technique and the Tamura-Neimodel were used to infer the evolutionary history (Kimura, 1980). Fig. 7 displayed the phylogenetic tree with the highest log likelihood (-744.52). By automatically applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances calculated using the Tamura-Nei model, and then choosing the topology with the highest log likelihood value, the initial tree for the heuristic search was created. In MEGA11, evolutionary analyses were carried out.

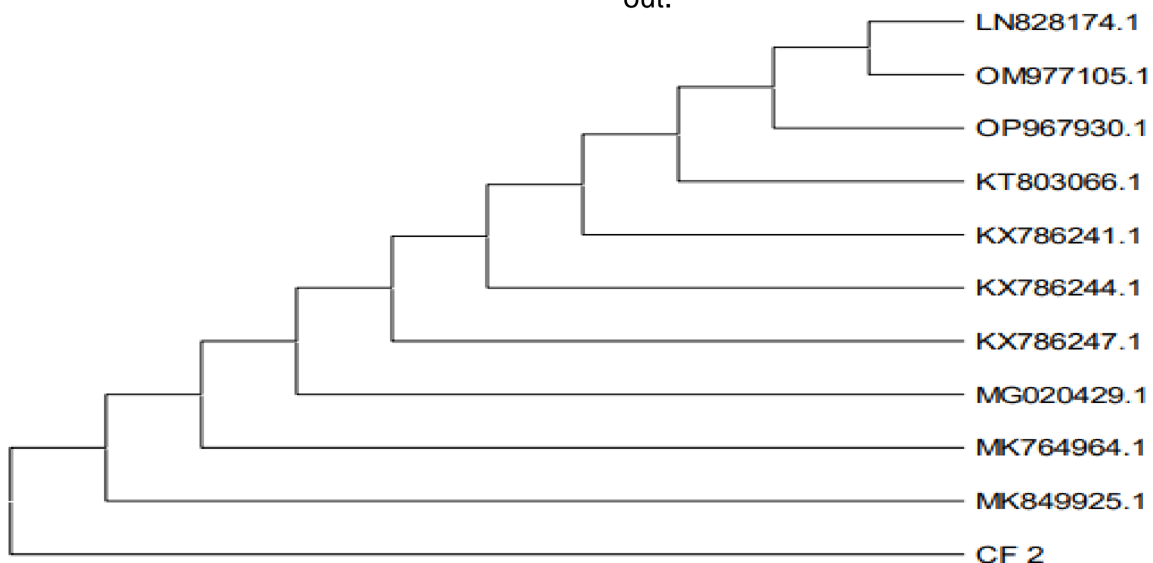


Fig. 7: Phylogenetic tree constructed using nucleotide sequence of ITS region of the conserved rDNA of *Fusarium oxysporum* isolate, CF II

Many studies reported that various *Fusarium* species are linked with pod and seed deterioration, which has a substantial impact on the quality and quantity of soybean (Arias *et al.,* 2013; Barros *et al.,* 2014). In the present study, ten different *Fusarium* isolates were screened against soybean and found *F. oxysporum* (CF II) as a virulent pathogen on soybean. This fungus was found to considerably impede seed germination and resulted in seed discoloration. This was in accordance with the study of Rahman *et al*. (2020) who observed that the different isolates of *F. oxysporum* displayed variability in their virulence against soybean seedlings in the pot culture studies. The author observed that the total seedling mortality ranged from 37.03 to 92.58%, which varied with the isolate, and in the isolate FOS-3 (92.58%) recorded the highest seedling mortality in this study. The results of the pathogenicity of *F. oxysporum* in the present study was correlated with the study conducted by Das *et al*. (2019) who observed that the plant infection by *Fusarium* occur from seed germination to the plant mature stage, based on the host and *Fusarium* species.

The Pathogenicity test results of the present study proved that most of the *F. oxysporum* isolates were pathogenic and caused wilt symptoms on many of the seedlings while very few isolates were reported to be with less pathogenic effect on the crop. The pathogenicity tests proved that all the *Fusarium* isolates developed wilt symptoms with varying disease incidence and severity ranging from 30 to 73% respectively. According to other investigations, *F. oxysporum* produced wilt diseases on *Acacia nilotica* in India with disease severity range 16.9% (Kapoor *et al.* 2004) and on A. koa in Hawaii with 85.0% severity (Gardner, 1980).

## CONCLUSION

Since Fusarium species identification from soil has always been challenging since it depends on minute morphological variations, and even in the same species can vary based on the culture. Molecular characterization of the virulent isolate in the present study can help in the detection of the exact species of the isolate. In the present study, total of ten *Fusarium* isolates were identified morphologically to species level, by visually and microscopically. The fungus produced pale purple to pale pink pigments on a PDA medium. The effective isolate, CF II was characterized by PCR amplification of the ITS region using species-specific primers. The amplification of ITS region of the isolate of *F. oxysporum* strain and molecular identification of *F. oxysporum* in this study further demonstrates that PCR analysis was an effective and fast way to detect *F. oxysporum.* The identified virulent fusarium isolate can be tested for its pathogenicity under different climatic conditions to check the influence of climatic factors on the pathogenicity of *Fusarium*. Molecular characterization results of CF II isolate confirmed that the isolate as *Fusarium oxysporum* (OR226604).

**Conflict of interest:** The authors declare no conflict of interest.

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