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RESEARCH ARTICLE

Yellows and corm rot in gladiolus: Incidence, identification and characterization of *Fusarium oxysporum* f. sp. *gladioli*

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ABSTRACT: To unravel the etiology of the plants showing yellowing, epinasty and wilting morphological and molecular diagnostics was carried out. Colony morphology and microscopic examinations indicated the pathogen to be *Fusarium sp.* To elucidate the identity conclusively, polymerase chain reaction (PCR) amplification of genomic region was done using primers based on internal transcribed spacer (ITS) region and translation elongation factor (Tef) region. ITS region of the pathogen showed 100 percent similarity to *Fusarium oxysporum* species and analysis of Tef region helped to narrow down to *Fusarium oxysporum*f. sp. *gladioli.* Characterization by molecular techniques using PCR to amplify the ITS and TEF 1α allows identifying organisms that cannot be distinguished morphologically and they can also help to understand the mechanisms of pathogenic variation and therefore, to develop effective management strategies.

Key words: *Fusarium* yellows, *Fusarium oxysporum* f. sp. *gladioli,* polymerase chain reaction,internal transcribed spacer region, translation elongation factor 1α

Gladiolus is grown world-wide as an economically important ornamental crop. *Fusarium* sp. causes yellowing, corm rot, browning of foliage and wilting in gladiolus. Fusarium corms rot and wilt of gladiolus caused by *Fusarium oxysporum* f. sp. *gladioli* are considered to be the most destructive and widely distributed disease in most gladiolus growing countries of the world. It reduces the quality, yield and market value of gladiolus (Salma *et al.,* 2014). *Fusarium* yellows is considered a serious and highly devastating disease which causes 60-70% plant mortality (Vlasova and Shitan, 1974). *Fusarium* yellows of gladiolus show typical symptoms of yellows and wilt both in the field and under storage conditions (Jones and Jenkins, 1975). In India, gladiolus wilt caused by *F. oxysporum* f. sp. *gladioli* was first recorded by Singh (1969) from Uttar Pradesh. In Himachal Pradesh, disease incidence ranged between 7.12-64.23% (Chandel *et al.,* 2010). The fungus can survive in soil indefinitely as mycelium, clamydospores,microconidia and macroconidia. Infected corms show tissue discoloration. The corms become softened, wrinkled and mummified in storage. Infected plants show yellowing symptoms, which start from lower leaves and gradually proceed upwards. Lesions appear on lower half of corms and corms get mummified and rotten in severe cases.

Yellowing and wilting symptoms were observed in gladiolus planted in November 2014 in Pune. The disease incidence was about 20-30% in the field and in the variety Argentina whole plants were dead. Symptoms indicated the probable etiology of *Fusarium* infection. To confirm the etiology, morphological and molecular characterization using ITS and TEF 1α region was done from infected corms of the variety "Fortarosa" showing the characteristic symptoms of *Fusarium* infection.

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MATERIALS AND METHODS

To confirm the etiology, pure culturing of the fungus was done from symptomatic corms harvested from the field of ICAR-DFR Pune, on Potato Dextrose Agar medium. Morphological and molecular characterization of the fungi was carried out. To elucidate the identity conclusively, fungal DNA was extracted and purified from fresh mycelium using CTAB extraction method as described by Manicom *et al*. (1987). PCR amplification of genomic region was done using sequences of the internal transcribed spacer (ITS) of the ribosomal DNA (ITS1 and ITS4 primers : ITS1-5'-TCCGTAGGTGAACCTGCGG-3' and ITS4-5'-TCCTCCGCTTATTGATATGC-3') and sequences of the translation elongation factor alpha (TEF-1) gene (EF-1-986 and EF-728 primers : Forward - 5'-CATCGAGAAGTTCGAGAAGG-3' and Reverse - 5'-TACTTGAAGAACCCTTACC-3') (Jimenez-Fernandez *et al.,* 2010; Al Mahmooli *et al.,* 2013). The PCR reactions were performed with a total of 2.5 µL of 10X PCR buffer including $MgCl_2$, 0.5 µl of each primer (20pmol/µl), 0.5µl of 10 mMdNTPs (2.5 mM each), 0.3 µlTaq DNA polymerase (5 U/µl), 1 µl of Template DNA and water per complete 25 µl reaction. The amplification was carried out in a thermal cycler (Eppendorf, USA) under the following thermo cycling conditions: initial denaturation at 94°C for 5 min followed by 35 cycles having the following parameters 1 min of denaturation at 94°C, 1 min of annealing at (55°C for ITS specific primer, 53°C for Tef) and extension for 1 min at 72°C followed by a final extension for 10 min at 72^{0}C.

Amplicons of <“600 and <“300 bp obtained were cloned into pTZ57R/T vector and sequenced using the automated sequencing facility at Chromous Biotech Pvt. Ltd., Bengaluru. Sequencing was done in both directions using M 13 forward and reverse primes in an ABI Prism 377 DNA sequence.

In silico sequence analysis

Gene sequences and related reference sequences retrieved from the Basic Local Alignment Search Tool (BLAST) searches at the National Centre for Biotechnological Information (NCBI) were assembled and edited by using BioEdit programs. The phylogenetic analysis of sequences isolated from Gladiolus in the present study and other known sequences obtained were performed using the programme CLUSTAL W using the BioEdit sequence alignment editor version 5.09.04. Neighbour joining phylogenetic tree was constructed using the program MEGA6 (Tamura *et al*., 2013).*Fusarium oxysporum* f.sp. *lycopersici* (HM057281) was used as an outgroup sequence for the phylogenetic analysis.

RESULTS AND DISCUSSION

Morphological characterization of *Fusarium oxysporum* f.sp *gladioli*

The fungus *F. oxysporum* f. sp*. gladioli* produced aerial mycelium which was hyaline, branched, septate, well-developed and cottony in appearance. The culture was slightly purple or pinkish white in colour on Potato Dextrose Agar (Fig. 1). The fungus produced abundant conidia in culture, and conidia were of bothtypes ;micro- and macro conidia (Fig. 2). Colony morphology and microscopic examinations indicated the pathogen to be *Fusarium* sp. as observed and recorded by Massey (1926), Buxton (1955b) and Chen *et al.,* (1994).

Amplicons produced using ITS and Tef gene specific primers in PCR, showed the size of ~600 bp and ~300 bp respectively in agarose gel (Fig. 3). These amplicons were cloned and sequenced. The sequence of ITS region (under study) has been submitted to NCBI GenBank under the accession no - KU721005. Albores *et al.* (2014) have also used ITS1 and ITS5 regions to identify and characterize *F. oxysporum* f. sp *gladioli* infecting gladiolus. Mahmooli *et al.,* (2013) have Identified *F. proliferatum* using sequences of the internal transcribed spacer (ITS) of the ribosomal DNA (ITS1 and ITS4 primers) and sequences of the translation elongation factor alpha (TEF-1) gene (EF-1-986 and EF-728 primers).

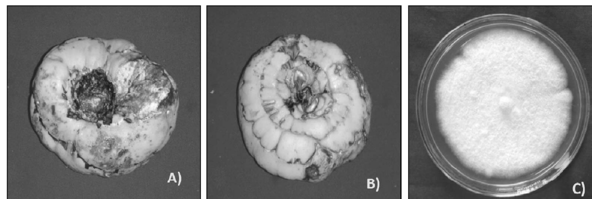

Fig. 1. A) and B) Fusarium infected corms of gladiolus, C) Culture plate of FOG

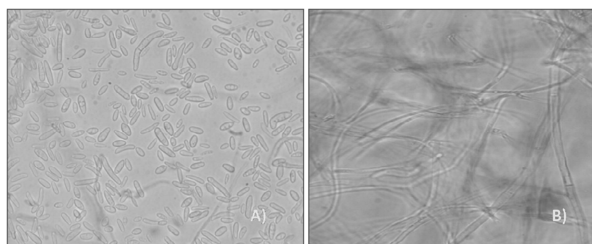

Fig. 2. A) Photograph of micro and macro conidia of FOG, B) Mycelial mat of FOG in Gladiolus culture Molecular characterization of *Fusarium oxysporum* f.sp.*gladioli*

Sequence analysis

BLAST analysis of ITS region of the pathogen revealed 100 percent similarity to *Fusarium oxysporum* species and analysis of Tef region helped to narrow down to *F. oxysporum* f. sp. *gladioli*. Using ITS gene, the sequence under study (KU721005) revealed maximum identity (100%) with the sequence referred to as *F. oxysporum* (Accession: FJ605247) from Brazil from *Scuticariair winiana*. Tef-1α gene, the sequence under study showed maximum identity (100%) with *F. oxysporum* f. sp. *gladioli* (Accession: FJ664916) from Netherland from Freezia plant. The phylogenetic tree was generated using Neighbor-Joining method. Phylogenetic analysis of ITS region of FOG, KU721005 (sequence under study) and other sequences retrieved

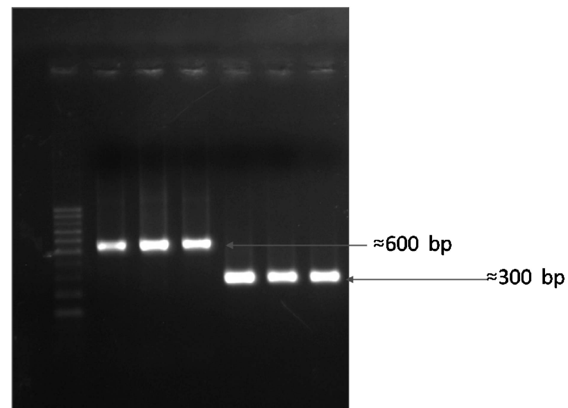

Fig. 3. Agarose gel amplification of ITS and Tef genes respectively. Lane M: 100 bp DNA ladder, Lane : 1,2,3 : 600bp amplification (ITS) and Lane : 4,5,6 : 300 bp amplification (Tef).

from NCBI, also revealed that the sequence under study was closely related with the FOG sequence (DQ279794) of Mexico (Fig. 4). Phylogenetic analysis of Tef 1α gene of FOG showed that the sequence under study was clustered with the Tef sequences of AF246843 and AF246845 from USA, FJ664916 from Netherland and AB916989 from Japan (Fig. 5).

CONCLUSION

In this study we have identified the etiology of yellows incidence in gladiolus plants as *Fusarium oxysporum* and the fungus was characterized using Tef gene. Molecular identification of *F. oxysporum* f.sp. *gladioli* in this study demonstrates that PCR analysis is an effective

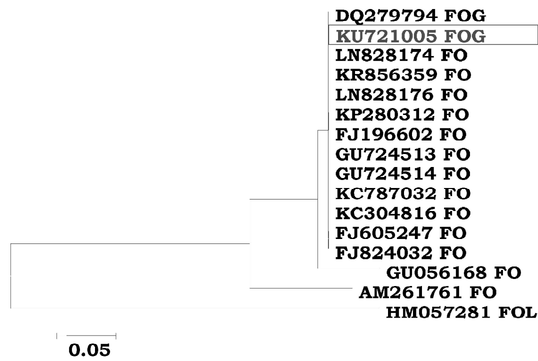

Fig. 4. Phylogenetic analysis of nucleotide sequence of ITS region of the fungal isolate (sequence under study : KU721005) with different *Fusarium oxysporum* (FO) isolates from NCBI GenBank. Phylogenetic analysis was conducted in MEGA6 and the evolutionary history was inferred using the Neighbour-Joining method. *Fusarium oxysporum f.sp. lycopersici* (FOL) was used as an outgroup.

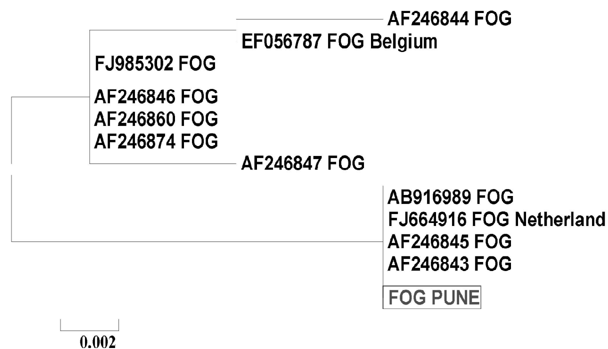

Fig. 5. Phylogenetic analysis of nucleotide sequence of Tef region of the fungal isolate (sequence under study) with different *F. oxysporum f.sp. gladioli* (FOG) isolates from NCBI GenBank. Phylogenetic analysis was conducted in MEGA6 and the evolutionary history was inferred using the Neighbour-Joining method.

and fast way to detect *F. oxysporum* strains using species specific primer sets. In the present study *Fusarium oxysporum* isolates were identified to species level using Tef-1α gene. Phylogenetic analysis showed the closer relation of FOG in gladiolus from Pune isolate with those from Netherland and USA which are the countries involved in export and import of floriculture products from India. Thus import of corms of gladiolus from European countries like Netherland might have acted as a channel for introduction of *Fusarium oxysporum* f.sp. *gladioli* in gladiolus to India. Further characterization of the pathogen using multilocus sequence typing will validate the conclusive identity of the FOG infecting gladiolus.

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