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Molecular Characteristics for Identification of *Fusarium oxysporum* Associated with Tomato Infection

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ABSTRACT

This study aims to determine a suitable molecular identification technique for *Fusarium oxysporum* associated with tomato infections to enhance tomato production in developing countries. *Solanum lycopersicum* (tomato) is one of the most important vegetables worldwide and ranks as the sixth most popular vegetable, according to the Food and Agriculture Organization (FAO). However, its cultivation is significantly affected by pathogens, including *F. oxysporum*. The most suitable medium for cultivating *F. oxysporum* was found to be Potato Dextrose Agar (PDA). Cell disruption using bead beating in a homogenizer yielded optimal results. EF-1α primers were identified as the most appropriate to detect *Fusarium* isolates within species complexes, as corroborated by various researchers. Intergenic spacer restriction fragment length polymorphism (IGS-RFLP) analysis was widely used to trace the origin of *F. oxysporum* by analyzing genetic similarities among isolates from different sources. Other methods, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), allele-specific associated primer (ASAP), single nucleotide polymorphism (SNP), and variable number tandem repeat (VNTR) analysis have also been applied. To mitigate fungal infections in tomatoes, this study recommends selecting disease-resistant tomato varieties, maintaining ideal growing conditions, adhering to stringent sanitation practices, ensuring source water is free of potential fungal pathogens, sterilizing tomato seeds, and using organic fungicides as needed.

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Introduction

Fungal pathogens like *Fusarium oxysporum (F. oxysporum*) were isolated in Japan around 1969 and for 100 years in the UK (Inami *et al*., 2014). *F. oxysporum* has pathogenic and non-

pathogenic strains equally (Edel-Hermann and Lecomte, 2019). Tomato infection due to *F. oxysporum* resulted in a huge loss for farmers; hundreds of tons were destroyed annually. The pathogen penetrates through the root down to the crown as well as other tissues of the plant; this results in leaf chlorosis, leaves wilted and desiccated, browning of the vascular system (necrosis of the vascular system) (Medić-Pap *et al*., 2022).

As such, the need arises to identify suitable methods to ascertain the organisms responsible for the infection through molecular characterization and, hence, prevent further proliferation through good-quality sanitation procedures, source water free from potential fungal pathogens, sterile tomato seeds, and application of organic fungicides if required.

Generally, the novel incidence of *Fusarium wilt* appears due to a preamble to a certain extent than an autonomous local origin of the pathotype (Gordon, 1997). Abu Bakar *et al.* (2013) reported that five *Fusarium species* have only been tentatively identified based on morphological characteristics. They also identified the causal agents of tomato fruit rot in Malaysia, including *F. semitectum, F. oxysporum, F. equiseti, F. subglutinans, and F. solani.* Realizing that information about the diversity of *Fusarium species* associated with fruit rot of tomato based on gene sequencing is limited in tropical areas. *F. oxysporum,* a plant pathogen, can infect animals and plants equally (Nag *et al*., 2022).

Incidence of *Fusarium oxysparum*

F. oxysporum remained in 1969 in Japan (Sato *et al.*, 2004), and *it was* defined over a century back in the UK as caused by tomato wilting (Inam1 *et al.,* 2014), resulting in stumpy harvest and great financial losses surpassing 50% fashionable production systems in Mexico. *F. oxysporum* adversely affects yield by causing damage of up to 15% annual loss to canning tomatoes in advanced countries like the USA. In the Sri Lankan Republic, tomato disease is principally caused by some group of microbes such as *Ralstonia solanacearum*. *Fusarium wilt* is an exceedingly disparaging syndrome institute in profitable tomato agronomies grown under either greenhouse or field conditions in many world regions. A soil-borne pathogen, *F. oxysporum*, produces it. The contagion is reported to cause 10-50% yield loss in tomatoes (Borerro *et al.,* 2004).

Impact of Fusarium oxysparum on Tomato Production and Other Crops

Tomato production was estimated to be 161 million tons and an area of 8.5 million hectares in 2015. Mexico has made outstanding tomato-making advances and has been renowned as a major tomato exporter. However, tomato production has been immensely inadequate due to a reasonable amount of biological and nonbiotic factors (Peter and Rai, 1997). Esculentum is among the main vegetables being made in Nigeria and is expended. Nigeria is among the world's foremost producers of tomatoes, ranked 16th, and the principal maker in Africa. As of 2010, the country's production was about 1.8 million metric tonnes, representing 68.4% of West African production (FAO, 2010; Symposium on Agri-Tech Economics for Sustainable Futures – 2020).

Tomatoes are classified as sixth among the best common vegetables by the FAO and are related to total annual production globally. Universally, about 159 million tons of freshly produced tomatoes are produced yearly. A quarter is developed for advancement in the nine largest agricultural-producing countries like Spain, China, Iran, Italy, Brazil, India, USA, Turkey, and Egypt, accounting for more than 74% of the global yearly cultivation (Chohan and Ahmad, 2008). Tomatoes are one of the world's vital plants and have been the subject of genetic investigation for an extended follow-up period in an obtainable insight into genetics, breeding, and evolution. Cultivated tomatoes differ, ranging from 3 -4 cm in diameter to cherry tomatoes about 1–2 cm in diameter and beef steak tomatoes with 10 cm or more in diameter. The all-out extensively full-grown profitable tomatoes tend to be in 5–6 cm diameter.

Tomatoes are usually categorized as specified or unspecified types. Determinate as bush types that tolerate complete crops, they are frequently noble varieties for vessel growing (Prasad and Mathura, 1999).

Vegetable value has been the main emphasis of most tomato production programs during the past century. Major fruit quality characteristics of interest to the fresh market and processing tomato industries include fruit size, shape, total solids, color, inflexibility, maturing, nutritive quality, and flavor (Sandani and Weerahewa., 2018). Sucrose is also available in precise minor amounts, though nearly wild species of tomato, *including L. chmielewskii and L. hirsutum,* possessed a greater amount of sucrose. The residual decipherable solids are composed of organic substances (Anonymous, 2013-14).

***Fusarium oxysparum* infection**

A genomic study of vegetable-related disease-causing *Fusarium* showed the existence of numerous enzymes that can breakdown most of the cell wall of the plant and also enable the unswerving penetration of *F. oxysporum* (Zhang et al., 2020; Ma *et al*., 2010). The *F. oxysporum* disease infects plants such as chickpeas and occurs after it disseminates into the root's epidermis and moves in the direction of vascular tissue via the intercellular cosmoses. JG-62 and P-2245 are susceptible types; hence, *F. oxysporum* easily infiltrates the epidermis and gets to the intercellular cosmoses in 48 of penetration, but in the resilient genotype such as WR-3I5, it lasts up to 8 days to get to the cortex (Upasani et al., 2016). On the other hand, infection in tomatoes occurs via the tip of the root or through the elongation zone all through hydroponics background as well as grooves connecting the root of an epidermal cell of collar region in soil culture (Lagopodi et al., 2002; Nahalkova et al., 2010). *F. oxysporum* f.sp. cubense has been shown to infect banana hosts by penetrating the epidermis and growing to reach the vascular bundles via the intracellular spaces (Li et al., 2017).

Sign of Fungal Infection on the Tomato

Plants are usually infected with the *F. oxysporum* pathogen, which resides in the soil and passes through their roots and crowns (Gupta et al., 2000). Generally, distinctive symptoms

of the disease emerged on tomato vegetation during field production. The symptoms usually showed on grown-up plants during the mid-growing period under lukewarm environmental conditions. Some typical signs of the disease are leaf chlorosis, leaves wilted and desiccated, and browning of the vascular system (necrosis of the vascular system) (Medić-Pap et al., 2022).

The plant swiftly displays marks of wilting, minor leaves change to yellow and begin to perish, and the entire plant wilts and perishes just within a short time. The pathogen of soil origin inhabits specifically the vascular tissues of the vegetable; firstly, it damages the xylem vessels, which convey water and nutrients, thereby instigating plants to wilt. These indications are comparable to bacterial wilting (Prasad And Mathura, 1999).

Virulence genes in Fusarium oxysparum

The genome of *Fusarium* is divided into dual regions- i.e., the core genome and the expendable adaptive genome, which contains virulence genes. The genome for metabolic activities, also known as the adaptive genome, is positioned in the lineage-specific genetic material, all along with one-time proteins necessary for the host-specific interface; lineage-specific chromosomes code for the rapidly evolving effectors proteins which are required for pathogenicity en route for a specific host (De Vries et al., 2020; Zhang et al., 2020).

Numerous researches have shown that transmission of pathogenicity-associated traits can change non-pathogenic *F. oxysporum* f.sp. lycopersici to pathogenic *F. oxysporum*, but pathogenicity shift does not occur between different formae speciales (Nag et al., 2022). Plant pathogenic fungi are required to possess appropriate machinery aimed at signaling that permits it to riposte by variations in gene expressions leading to host recognition, the capability to infiltrate the root of hyphae inside the host fleshy tissue permits the disabling of the host defense mechanism, and hence disease establishment (Rep and K1stler., 2010). Virulency in fungi, in most cases, is controlled by two signaling pathways (signal transduction): cyclic adenosine monophosphate cAMP and Mitogen-activated protein kinase cascade (Liu et al., 2016). The fascinating mutation that is perhaps caused by silencing the gene responsible for encoding mitogen-activated protein kinase turned pathogens unable to pass through the roots of the tomato, resulting in disillusionment with the exhibition of disease. The failure of modified strains to stick to the root surface was likewise noted. Similarly, the wild-type strain could fasten and infiltrate the root surface strongly. Fascinatingly, pectate lyase and polygalacturonase enzyme emission were abridged in the mutant strain Dfmk1; the two enzymes played a role in cell wall degradation at some stage in disease progression (Guo et al., 2016; Pareek and Rajam, 2017). *F. oxysporum* with resistance to derived metabolites of plants was also studied by DeConinck et al. (2015). Non-pathogenic mutants obtained by random insertional mutagenesis showed complete loss of virulence; conversely, characterization of the insertion site resulted in the inactivation of the *chsV* gene. The present report implies that the chsV gene is essential to resist the compounds necessary for defense, which is a precondition for pathogenicity (Bharti et al., 2017).

Cell Culture

Usually, Fungus can be isolated by placing infected tissues or rotted tissue on different types of selective culture media that enhance fungal growth and inhibit the growth of other microorganisms' media, such as Sabourauds Dextrose agar, Potato Dextrose Agar (PDA) and Czapek agar are some of the media of choice in this case. F. oxysporum isolates will be characterized based on the physical characteristics of the macroconidia, phialids, microconidia, chlamydospores, and colony growth traits (Katan et al., 1991).

Murad *et al. (2016) utilized potato dextrose a*gar (PDA) to grow the fungus. However, the conidial suspensions of the cultures were streaked onto 4% water agar and incubated for 24 hours before being transferred to the PDA. The culture was allowed for 7 days at 25°C. Medić-Pap *et al.* (2022) cultured the fungus on PDA and Czapek agar at 25°C for 7 days. Isaac *et al*. (2018) used PDA and an antibiotic (streptomycin sulfate); the culture was kept for 3 – 7 days at a temperature of 26 ± 2°C in darkness. Most researchers working on *F. oxysporum* grow the fungus on PDA. Olivain *et al.*, (2006) were not an exception. They used the same media for the growth of *F. oxysporum*.

For the isolation of *F. oxysporum* and its characterization of infected tissues, it is obvious that PDA is the most widely reached. On the other hand, peptone pentachloronitrobenzene agar (PPA), Water Agar (WA) as a purification process, and finally, the hyphal tip obtained from a single colony was sliced and transferred onto new PDA for general *Fusarium species* identification (Murad et al., 2016).

Growth on PDA medium isolates of *F. oxysporum* on diseased plants or fruits produced light pinkish aerial mycelium and red coloration on the agar. Macroconidia were formed with hyaline, falcate, 3-7 septets and sized 60 x 2-3 µm, while microconidia were not. The isolate of F. oxysporum on Czapek agar showed aerial mycelium, which was light purple or sometimes grey, depending on the isolate. All isolates developed macroconidia as elliptical, gradually pointed, or curved edges (Medić-Pap *et al*., 2022).

DNA isolation

DNA removal from filamentous fungi may be method-dependent. Crude genomic DNA can be extracted from sporocarps in the following steps:

- ✓ Breaking of the cell membrane to discharge components of the cell and DNA, usually referred to as cell lyses or cell disruption
- ✓ Separation of the nucleic acids from other
- ✓ Purification of the nucleic acids.

Cell disruption can be attained using mechanical or chemical methods. This is commonly achieved by grinding, sonicating, or treating the sample with lysis buffer; the latter is more pronounced than the mechanical method, and the chemical method utilizes modified cetyl-trimethylammonium bromide (CTAB). The final DNA is suspended in 50 ml TE buffer and stored at -20°C (Lian *et al.,* 2003). Aamir *et al.* (2015) reported that the fungal cell was

disrupted by bead beating in a homogenizer, treatment with RNase, phenol, chloroform, Isoamyl alcohol extraction, and precipitation with isopropanol. A good yield was obtained (60 µg – 230 µg/200 mg of wet fungal mass) of the DNA. Similarly, Iti Gontia *et al. (*2014) used the glass bead method to disrupt fungi. DNA yield from fungal isolates was 310-1879 µg g^{-1} dry mycelium. Ma et al. (2010) extracted crude genomic DNA from sporocarps using a modified cetyl-trimethylammonium bromide (CTAB) method.

Murad (2016) employed an Ultra Clean Microbial DNA isolation kit from MO-BIO, Carlsbad, to extract DNA for genomic analysis of *Fusarium species*; however, no maximum yield was indicated.

Molecular Techniques

Fusarium infection in tomato (*Lycopersicon esculentum*) initiated by *F. oxysporum* is a new-fangled devastating infection of tomato. Not anything is known as regards the populace of this pathogenic organism. To scrutinize the genetic similarity of *Fusarium* isolates, several methodologies were adopted: intergenic spacer restriction fragment length polymorphism (IGS-RFLP) study was adopted to explain the genesis of the *F. oxysporum* by analyzing the genetic similarity of the isolates from a different source.

Two different bio-markers were particularly useful in identifying disease-resistance genes for plant pathogens. Sequence-related amplified polymorphism (SRAP) was initially described as an innovative and valuable molecular marker scheme for marking and mapping in *Brassica oleracea* L. because SRAP primers mark coding sequences in the target herb genome (Li and Quiros 2001). Resistance genetic material analog (RGA) primers are also suitable markers for tagging resistance genes and were premeditated as conserved motifs of the "nucleotide binding site-leucine rich repeat (NBS-LRR)" resistance gene family (Shen et al., 1998)."

The documentation of markers closely associated with the locus that converses resistance allows the determination of individuals' conveying the resistance gene. Consequently, it renders the usage of marker-assisted selection (MAS) aimed at integrating the gene "LS2436" into the additional eggplant genome. Improving markers such as inter s1mple sequence repeats (ISSR) to study the general population dynamic of the pathogenic fungi aided us in measuring variety far more proficiently by enabling precision not previously available. ISSR is the foremost bio-marker created by the magnification of genomic DNA via solitary primer over polymerase chain reaction (PCR), as long as the primer binding sites are situated inside the amplifiable scope that is inversely concerned with the simple sequence repeats (SSR).

Researchers regularly use ISSR markers for genetic variety within the species, germplasm characterization, cultivar identification, and phylogenetic research at the species level (Akbar *et al.,* 2018). Owing to its great reproducibility, the ISSR procedure is favored by the random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) procedures. Moreover, organisms from the same species display slight or no variation

between ISSR bands. Still, close interrelated species showed precise ISSR configurations that can be adopted to determine phylogenetic similarity (Gupta *et al*., 2008). The SRAP bio-marker scheme was established mainly for Brassica species. Still, it was also used on other crops like potato, rice, apple, potato, rice, plum, garlic, potato, rice, lettuce, and celery. The following *B. oleracea* crops were included in the study: broccoli, cauliflower, and kale. (Li and Quiros, 2017).

The SRAP method is a simple and reproducible DNA marker technique used equally for plant mapping and gene tagging. SRAP was more revealing than most PCR-based techniques in detecting genetic diversity and has been successfully used to study the genetic diversity of and relationships among several species (Budak *et al*., 2004). Procedures such as RAPD, RFLP, ASAP, SNP, VNTR, etc, were employed in several studies.

Gene amplification

Although all fungi can be analyzed using "ITS-rDNA" gene amplification, different fungal genera have specific targets. For instance, all *yeasts* D1D2 is the target gene, B-tubulin is used in *Scedosporium, Aspergillus,* and *Penicillium,* while Elongation Factor 1α (EF-1α) is used in *Fusarium.* According to O'Donnell (2009), the ITS primer set merely classifies *Fusarium species* keen on a range of diverse species but does not discriminate against cryptic species. For the detection of *Fusarium* isolates inside species composites, the EF-1α primers must be adopted. PCR investigation like 18S rRNA gene amplification, RAPD, and PCR-RFLP displayed that DNA stayed well-matched for downstream utilization (Deepa & Sreenivasa, 2019; Aamir *et al.,* 2015) employed the use of multi-copy ITS-rDNA gene amplification. 16S/18S/ITS amplification sequencing uses the next/third generation sequencing platform and performs high throughput sequencing of PCR products from specific regions such as 16S rDNA/18S rDNA/ITS/ functional genes. It overcomes the disadvantage of some microorganisms that are not easy or impracticable to culture and obtains information on microbial community structure, evolutionary relationships, and microbial correlation with the environment in environmental samples.

Primers used by numerous researchers include: "EF1 (5′ATGGGTAAGGA (A/G) GACAAGAC-3′) and EF2 (5′GGA (G/A) GTACCAGT (G/C)ATCATGTT -3′)" (Geiser et al., 2004). The same or similar primer was also used by Murad *et al.* (2016), who showed the primer as "EF1 (5′ATGGGTAAGGA(A/G)GACAAGAC-3′) and EF2 (5′GGA(G/A)GTACCAGT(G/C)ATCATGTT -3′)" as reversed and forward primers respectively.

Moreover, the disease and spoilage of the tomato will not arise if preventive measures are implemented. These include prevention of fungal infection through selection of disease-resistant varieties of the tomato seeds, provision of optimum growth conditions with model growing environment, ensuring of good quality sanitation procedures and source of water for irrigation free from potential fungal pathogens, provision of sterile tomato seeds and application of organic fungicides when required.

Conclusion

Tomato production is affected by *F. oxysporum* infection. The approved methods required for early detection are molecular identification and perhaps EF-1α primers as the most appropriate and IGS-RFLP use for tracing the origin of *F. oxysporum*. The application of safety measures is promising in controlling tomato infection during and after postharvest periods.

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