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Isolation and Identification of Soil Borne *Fusarium oxysporum* f. sp. *lentis*

Ashwini E.[1*], Dinkwar G.T.[2] and Nandeesha S.V.[3]

Dept. of Plant Pathology, Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV), Jabalpur, Madhya Pradesh (482 004), India

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Corresponding Author
Ashwini E.
e-mail: ashwiniebpgowda@gmail.com

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Abstract

In India, lentil ranks second most important pulse crop next to chickpea both in area and production. All about 80,000 diseases have been recorded throughout the world, of them majority are associated with soil-borne diseases. Among Soil-borne diseases lentil wilt caused by *Fusarium oxysporum* f. sp. *lentis* is most destructive pathogen causes yield loss up to 50% in farmer's fields The pathogen was isolated from soil as well as from infected plant sample. The most commonly used media for isolation of *F. oxysporum f. sp lentis* is Potato dextrose agar medium (PDA), it is nutrient rich media for growing wide range of fungi and the method used for isolation from soil is dilution for pour plating at 10^{-4} conc. The pathogen was detected after obtaining pure culture, primarily through cultural characters, microscopic observation of micro and macro conidia and by using molecular methods like DNA finger printing and PCR based methods.

Introduction

Lentil is one of the oldest cultivated crops and has been a major food source of many civilizations for more than 8000 years. In the global lentil scenario, India ranked first in the area second in the production with 39% and 22%. Lentil is an important rabi crop of India, covers an area of 14 lakh hectares next to chickpea The state Madhya Pradesh ranks first, in lentil production by covering an area o 6.13 lakh ha, with annual production of 4.16 thousand tonnes (Kumar *et al*., 2019). It is rich in protein, carbohydrates and crop residues are used as animal feed. This crop has been grown mainly as an inexpensive source of high quality protein in human diets. Lentil plants are affected by a wide range of pathogens with fungal diseases being the most important. Fungal diseases cause a decrease in productivity through infection and damage to leaves, stems, roots and pods as well as reduce marketability by discolouring seeds (Rahman *et al*., 2010). In India 12 fungal pathogens were identified among them *Fusarium oxysporum f. sp. lentis* is dominant pathogen (30.8%). Wilt is a serious disease in reducing lentil yield in India, west Asia, North and East Africa. Lentil wilt caused by *Fusarium oxysporum* f. sp. *lentis* is most destructive fungal pathogen causes yield loss up to 50% in farmer's fields. The disease can be reported at two stages one is at seedling stage (early wilt) another at reproductive stages (late wilt). Under natural conditions the disease incidence reach up to 50-80% and causes 100% crop loss, if the crop is affected at seedling stage. The pathogen produces microcondica, macroconidia and chlamydospores act as a source of primary and secondary infection (Ainsworth, 1971). Chlamydospores survive in soil as and these spores are viable for many years. Fusarium wilt epidemics depends on crop stage (seedling and reproductive), environmental factors, virulent strain of pathogen and crop variety.

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E-mail: *bioticapublications@gmail.com*

Isolation Methods

Fusarium oxysporum f. sp. *lentis* was isolated from both infected plant sample as well as from soil collected from infected field.

a) Isolation from Infected Plant Sample

The most commonly used media for isolation of fungal pathogens is potato dextrose media (PDA). Plant roots collected from infected filed is used for isolation; roots were washed under tap water for removal of soil and other inert materials. After washing the roots of 2 cm from infected portion to some healthy portion was taken for further process of isolation. The selected roots were cut into small pieces up to 2 mm and surface sterilized by using 0.1% of mercuric chloride or 1% of sodium hypochlorite for one minute, followed by three times serial washing with sterile distilled water for removal of traces of chemical concentration. After serial washing the pieces were dried on blotting paper for one minute to remove water, and then small pieces were placed in Petri plate containing PDA media and all the above process are carried out under laminar air flow. Finally the inoculated Petri plate is incubated at 24±1 °C for fungus growth for 3 to 5 days.

b) Isolation from Soil Sample

The most commonly used method for isolating fungus from soil is pour plating dilution method. Samples collected from a depth of 25 cm are stored in a paper bag, let samples air dry in the more aseptic conditions for 24-48 hours. Grind the larger particles in a mortar 36, prepare a soil suspension in sterile water by 1 g of soil in 9 ml of sterile distilled water, it gives 10^{-10} conc. The commonly used concentration for fungal isolation is 10^{-4} the conc. is prepared by taking 1 ml from 10^{-10} conc. and transferring into tube containing 9 ml of Sterile distilled water it gives 10^{-9} conc. Likewise to get 10^{-4} conc. Take suspension from final conc. and distribute the suspension as uniformly possible in a plate containing PDA media and allow it to stand for two minutes. Place the plates upside down to remove excess soil suspension from the plate and incubate it at 24±1 °C for 3 to 5 days. Recovered colonies are transferred to other appropriate media to obtain single conidial isolates.

Identification Methods used for *Fusarium oxysporum* f. sp. *lentis*

There are several methods used for identification and detection of *Fusarium oxysporum f. sp. lentis,* those are traditional and molecular methods, among these methods molecular methods are most commonly used now days because of their advantages over traditional methods. Traditional method of identification is based on cultural, morphological characters, it is time consuming, laborious and one should required knowledge about classical taxonomy. Molecular methods are more accurate, faster and can be used by any personnel. Nowadays nucleic acid based detection methods are in trending.

Figure 1: Lentil field infected with *Fusarium oxysporum* f. sp. *lentis*

Nucleic Acid based Detection Techniques

a) DNA Fingerprinting

DNA fingerprinting permits screening of random regions of the pathogen genome for recognizing species specific sequences when conserved genes have not enough deviation to successfully identify species or strains of *Fusarium oxysporum* f. sp. *lentis.* It helps in analysis and studying of phylogenetic structure of fungal populations, also used for identifying specific sequences of the pathogen at low taxonomic level. Various methods have been devised for DNA fingerprinting based on the polymerase chain reaction. Among those, random amplified polymorphic DNA (RAPDs) has been the most popular because it is simple and requires small amount of genomic DNA.

b) DNA Probes

DNA probes are among the first molecular markers applied in the detection, identification and phylogenetic analysis of fungal pathogens. Species specific DNA probes generated from cloned random DNA fragments derived from genomic DNA that was digested with various restriction enzymes had a number of advantages. Restriction fragments are separated according to their size by electrophoresis and subsequently transferred to nylon membranes by capillary forces and immobilized by UV cross linking. Labelled DNA probes (initially by radioactive isotopes and later increasingly by non-radioactive means) are hybridized to the membrane bound DNA fragments and specific bands are visualized by appropriate methods. This technology has also been applied to distinguish among special forms of soil borne fungal pathogens like *Fusarium oxysporum* f. sp. *lentis*.

c) Polymerase Chain Reaction

Polymerase chain reaction (PCR) was envisaged by Kary Mullis during the year 1984. This technique has found wide application as a powerful molecular tool; mostly because thermo tolerant DNA polymerases and automated thermo cyclers are now available. The wide application of PCR to plant disease diagnosis is due to the advantages that the method offers over the traditional or other molecular techniques. It does not require a pure culture of the target pathogen (high quality DNA is not necessary), it is highly sensitive (minute amounts are required with the theoretical potential of a single molecule detection level), and it is rapid (fast screening of a large number of samples). The ITS sequences have been preferred over the subunits because they are more variable and thus permit selective detection of closely related fungi (Goodwin *et al.*, 1990).

d) Loop Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method developed by Tsugunori *et al.* (2000). This technique has been widely used because of its high specificity, simplicity, efficiency, and quickness. LAMP involves two long outer primers and two short inner primers that recognize the six specific sequences in the target DNA. The advantages and simplicity of LAMP assay is that the reaction could be easily judged as positive or negative by naked eye through assessing of increased turbidity or color change and for that it does not require any expensive instruments like thermal cycler.

Conclusion

Lentil is one of the most important pulse crops next to chickpea in India. It is having wide range of nutritional importance to both mankind and animals. It also play important role in maintaining soil fertility by fixing atmospheric nitrogen. The loss caused by *Fusarium oxysporum* f. sp. *lentis* takes up to 50% in farmers field and 100% loss can be seen if it occurs in seedling stage. It is important to identify the disease at early stage of infection. Identification through morphological and cultural characteristics is time consuming and laborious, so it is easy to identify the pathogen by using molecular methods because of its feasibility. In this perspective, DNA technology is expected to continue to improve understanding of the complex competition of soil-borne microorganisms for secure ecological niches. Rapid and accurate detection of fungal pathogens to the species level are perquisite for disease surveillance and development of novel control strategies.

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