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Detection of Bacterial and Fusarium wilt Pathogens in Cabbage by Multiplex PCR

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Abstract: Cabbage is one of the most important vegetable which is extensively cultivated in India and other countries. *Fusarium* yellows caused by *Fusarium oxysporum* f.sp. *conglutinans* is one of the most destructive diseases of cabbage and other crucifers. It causes severe loss in the yield in terms of both quantity and quality. The work on *Fusarium oxysporum* f.sp.*conglutinans* in India is very scanty. The aim of the present study was to isolate and study the morphological diversity and pathogenicity variability among *F. oxysporum* f.sp. *conglutinans* isolated from different cabbage fields. During the present work, an attempt was made to isolate, identify and characterize the Fusarium yellows of cabbage causing pathogen *Fusarium oxysporum* f.sp. *conglutinans*, a total of nine soil samples were collected from different fields out of which five were found to be positive for *Fusarium oxysporum* f.sp. *conglutinans*. The morphological, physiological characterization results of the present study including, pathogenicity reaction effectively demonstrated the confirmation of isolated pathogen as *Fusarium oxysporum* f.sp. *conglutinans*. A multiplex PCR method was developed for the easy detection of the Fusarium yellows and the black rot pathogen *Xanthomonas campestris* pv. *campestris* which in the initial stages produce symptoms which appear similar.

Keywords: Cabbage, Cabbage Yellows, Multiplex PCR, *Fusarium oxysporum* f.sp. *conglutinans*

1. Introduction

India is one of the most important cabbage growing countries in Asia with an area of 268,000 ha and production of 5,922 thousand per ha. West Bengal is the largest grower of cabbage followed by Orissa and Bihar occupying second and third position respectively. In world, a high incidence of black rot of cabbage caused by *Xanthomonas campestris* pv. *campestris* and Fusarium yellows of cabbage caused by *Fusarium oxysporum* f.sp. *conglutinans* is the most destructive diseases.

Multiplex polymerase chain reaction (Multiplex PCR) is a modification of polymerase chain reaction in order to rapidly detect deletions or duplications in a large gene This process amplifies genomic DNA samples using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler. Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicon of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, *i.e.,* their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis[1].

Multiplex PCR is based on the use of several PCR primers in the same reaction allowing the simultaneous and sensitive detection of different DNA targets, reducing time and cost. This method is useful in plant pathology since plants are usually infected by more than one pathogen. Different fragments specific to the target fungi were simultaneously amplified and identified on the basis of their molecular sizes on agarose gels. Although the efficiency of amplification is strongly influenced by amplicon size (shorter amplicon may be amplified preferentially over longer ones), an accurate and careful design of primers and the optimization of their relative concentrations are required to overcome this drawback and get an equilibrate detection of all target fungi. Pathogens can be detected in a single process, which will be easier for designing the management strategies.

2. Materials and Methods

2.1. Collection of Soil Sample

Soil samples were collected from different cabbage fields in and around Mysore. The soil samples were collected from different crop fields (up to 15cm depth) into a small sterilized polythene bags and brought to laboratory for further studies stored under proper condition and was used to isolate the pathogen.

2.2. Isolation of Xanthomonas Campestris pv. Campestris

Bacterial cultures were taken from the department Stock (DOS in Biotechnology, University of Mysore, Mysore). Biochemical, molecular characterization and pathogenicity test of *Xanthomonas campestris* pv. *campestris* were performed [1].

2.3. Isolation of Fusarium Oxysporum f.sp. Conglutinans from the Soil Samples

The soil micro fungi were enumerated by two methods, namely soil dilution and soil plate method on different media such as potato dextrose agar selective minimal media.

2.3.1. Soil Dilution Plate Method

1g of soil sample was suspended in 10ml of sterile water to make microbial suspensions (10^{-1} to 10^{-5}). Dilution of 10^{-3}, 10^{-4} and 10^{-5} were used to isolate fungi. One ml of microbial suspension of each concentration were added to sterile Petri dishes (triplicate of each dilution) containing 15 ml of sterile Potato Dextrose Agar (PDA) and Czapek Dox Agar. One percent streptomycin solution was added to the medium before pouring into petriplates for preventing bacterial growth. The petri dishes were then incubated at 28 ± 2°C in dark. The plates were observed everyday up to three days [2].

2.3.2. Soil Plate Method

About 0.005g of soil was scattered on the bottom of a sterile petri dish and molten cooled (40-45°C) agar medium was added, which was then rotated gently to disperse the soil particles in the medium. The petri dishes were then incubated at 28 ± 2°C in dark for three days [3].

The infected tissues were cut into small pieces and by using flame-sterilized forceps they were transferred to sterile petriplates containing 1% sodium hypochlorite for surface sterilization, then rinsed twice in sterile distilled water and dried between sterile filter papers. The sterilized pieces were transferred aseptically to perspex plates containing blotter sheets and petriplates containing PDA supplemented with streptomycin sulfate and incubated at temperature which favors the pathogen development. Antibiotics were incorporated in the media to prevent bacterial contamination. The plates were incubated at 28±2°C for 7days.

2.4. Identification of Fusarium Oxysporum f.sp. Conglutinans Isolates from Soil

Fungal morphology was studied macroscopically by observing colony features (color and texture) and microscopically by staining with cotton blue and observed under compound microscope for the conidia, conidiophores and arrangement of spores .The fungi were identified with the help of literature. The number of colonies per plate in 1g of soil was calculated.

Colonies exhibiting the taxonomic features of *F. oxysporum* were identified according to Nelson *et al* (1983). Morphological identification was based on characteristics of the macro conidia, phialides, micro conidia, Chlamydospores and colony growth traits. The identity of the culture was further confirmed with authentic culture obtained from Microbial type Culture Collection (MTCC, Chandigarh India). Pure cultures of all the isolates were maintained on PDA.

2.5. Pathogenicity Test

The cabbage seeds were sown in crates filled with coco peat and watered regularly. After 1 month, healthy seedlings were selected and used for pathogenicity assay. To determine the virulence of the isolates, pathogenicity was carried out on four cabbage cultivars. The four varieties of cabbage used were *Noble seeds*, *Indam krishna*, *Indam saina* and *Gaurav*.

2.6. Preparation of Fungal inoculum

One week old *F. oxysporum* cultures grown on PDA plates were flooded with 10 ml of sterile distilled water and the conidia were dislodged with a cell spreader,and counted with the aid of haemocytometer. The concentration was adjusted to 10^6 conidia ml^{-1} and 5ml of this suspension was poured into the pots containing cabbage seedling.

2.7. Molecular Characterization

2.7.1. DNA Extraction

Fungal mycelia was crushed by using liquid nitrogen with the aid of pestle and mortar, then 100-200mg of fine powder of fungal mycelia was transferred to the microfuge tubes containing 1ml of extraction buffer (CTAB-0.2g, Nacl-3.5ml, PVP-0.2, 0.4ml of EDTA of pH 5,1ml of Tris Hcl of pH 8 and volume was made up to 10ml). Vortexed thoroughly and incubated at 65ºC on water bath for 20-30 minutes. 500µl of chloroform: isoamylalcohol (24:1) was added to the microfuge tubes and vortexed thoroughly and later centrifuged at 12,000 rpm for 10 minutes.

The aqueous layer was transferred to a fresh microfuge tube, to that equal volume of cold isopropyl alcohol followed by 1/10th vol of Sodium acetate was added. The tubes were incubated at -20 ºC for 1hour and then centrifuged at 12,000rpm for 8minutes, supernatant was discarded. Pellet was dissolved in 100µl of Tris EDTA(TE) buffer. 10µl of RNAse was added and incubated at 37°C for 15 minutes. 100µl of chloroform: isoamylalcohol (24:1) was added, mixed and centrifuged at 12,000 rpm for 10 minutes. To the aqueous layer 500µl of 70% ethanol was added and centrifuge at 12,000rpm for 10 minutes. The supernatant was discarded and the pellet air dried. The dried pellet was

resuspended in 30µl of sterile water [4]. The purity of the extracted DNA was evaluated by running on 1% Agarose gel.

2.7.2. PCR Assay for Fusarium Specific Primers

The primers forward 5'-CCGTAGCACTTAGTGCAATG-3' and - reverse 5'GCATTTCCATCGGTCACGATTG-3' were designed in primer3 software which were specific to *Fusarium oxysporum* f.sp. *conglutinans* with a predicted PCR product of 400bp.The primers were custom synthesized from Sigma Aldrich, USA. All amplifications were carried out in a final volume of 25µl. A master mix was prepared using the buffer, dNTP's, *Taq* polymerase, this master mix was pipetted into each PCR tubes. To each PCR tubes 2µl of primer was added (1µl of forward primer and 1µl of reverse primer) and 2.5µl DNA was added the total concentration of each tube was made up to 25µl using sterile distilled water. Reactions were run for 35 cycles, consisting of Initial denaturation 95ºC for 3min, denaturation 95ºC for 40sec, annealing 62ºC for 1min, Extension 72ºC for 40sec and Final extension 72ºC for 10min. The ingredients and concentration of each PCR tube is as given in the (Table. 2) The amplified PCR product was electrophoresed on a 1.2% Agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

2.7.3. Genomic DNA Extraction from X. Campestris pv. Campestris

A loopful of each isolate was suspended in 500-µl phosphate buffered saline (PBS) in microfuge tube and mixed by vortexing and centrifuged in 12000 rpm for 15 min. The supernatant and the viscous material were discarded and the pellet was washed with 1ml buffer (50mM Ethylene diamine tetra acetate (EDTA), 0.15M NaCl, pH 8.0) and centrifuged repeatedly. Proteinase K to final concentration of 150µg/ml and 30 µl of sodium dodecyl sulphate (SDS) were added to the suspension and incubated at 50°C for 1 h. Equal volume of chloroform-phenol-isoamyl alcohol (24:25:1) buffered with 10mM Tris-HCl pH 8.0 was added for extraction. The suspension was mixed by vortexing and centrifuged at 12000 rpm for 5 min to separate the layers. The upper layer was transferred to a fresh microfuge tube and 0.1 vol, 3M Sodium acetate and 1 vol of iso-propanol was added and mixed by vortexing. The precipitate spooled out and rinsed with 1ml of 70% Ethanol. It was then centrifuged for 2 min at 12000 rpm and the ethanol was removed and the pellet was dissolved with sterilized distilled water. The purity of the extracted DNA was evaluated by running on 1% agarose gel [5].

2.7.4. PCR Analysis with hrpF Primer

PCR primers BE1 5′-CCGTAGCACTTAGTGCAATG- 3′) and BE2 (5′-GCATTTCCATCGGTCACGATTG-3) primers. The primers were custom synthesized from Sigma (Sigma Aldrich, USA). A master mix was prepared using the 10x buffer, dNTPs, Taq polymerase, this master mix was pipetted into each PCR tubes. To each PCR tubes 2 µl of primer was added (1 µl of forward primer and 1 µl of reverse primer) and 2.5µl of purified 100 ng. DNA extract was added and the total concentration of each tube was made upto 25 µl using water PCR reagent or sterile distilled water. The thermal cycle consisted of initial denaturation at 94°C for 5 min, annealing at 58°C for 1 min, and extension at 72°C for 10 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing 58°C for 15 s, elongation step 72°C for 15 s, and a final extension at 72°C for 10 min. Finally annealing temperature was standardized to 58°C for 15s. The PCR tubes were given a small spin, and were placed in a PCR thermocycler (Labnet, USA) [1].

2.7.5. Multiplex PCR

The Multiplex PCR was performed using *hrpF* specific primers BE1 (5′-CCGTAGCACTTAGTGCAATG- 3′) and BE2 (5′-GCATTTCCATCGGTCACGATTG-3) primers. The primers forward Cong-F(5'-ATCCCCGTAAAGCCCTGAAGC- 3') and Cong-R (5'GGTCGTCCGCAGAGTATACCGGC'-3') designed using primer3 software which were specific to *Fusarium oxysporum* f.sp. *conglutinans* were custom synthesized from Sigma (Sigma Aldrich, USA). A multiplex PCR was performed using the *hrpF* (BE1& BE2) and *Conglutinans* (CongF & Cong R) primers for the simultaneous detection of pathogen. A master mix was prepared using the 10x buffer, dNTPs, *Taq* polymerase, this master mix was pipetted into each PCR tubes. To each PCR tubes 2 µl of primer was added 1 µl each of forward primer (BE1, BE3) and 1 µl of each of reverse primer (CongF, CongR), and 2.5µl of purified 100 ng DNA was added and the total concentration of each tube was made upto 25 µl using sterile water. Cycling conditions were: 3 min at 95°C, 35 cycles of 40 s at 95°C, 40 s at 62°and 40 s at 72°C) followed by 10 min at 72°C.

3. Results

3.1. Collection of Soil Samples

Nine different soil samples were collected from cabbage fields in and around Mysore.

3.2. Isolation of Xanthomonas Campestris pv. Campestris

The isolated cultures obtained from the department stock were subjected for characterization tests *viz.,* Gram's staining, starch hydrolysis, catalase test, oxidase test, growth on aspargine medium and presence of xanthomonadin pigment, hypersensitivity and pathogenecity tests.

3.3. Soil Dilution Technique

Collected soil samples were labeled and soil dilution was done by using sterile distilled water in different test tubes. The plating was performed on PDA. Out of nine soil samples collected, five soil samples were positive for Fusarium oxysporum f.sp. *conglutinans viz.,* Salundi, Daripura, Mahadevapura and Parsayana hundi. The isolates were maintained for further studies on PDA medium. The hyphal threads of pathogen were taken and inoculated in PD broth and the growth was observed.

3.4. Morphological and Cultural Variability Among the Isolates

All the isolates grown on PDA plates at 28 ± 2°C and on Perspex plates containing blotter sheets were studied for their cultural and morphological characters. Observations on colony color, mycelia growth pattern, radial growth and sporulation, septation of the conidia, number and pattern of Chlamydospores formation were recorded after 9 days of incubation. The identification of *Fusarium oxysporum* depends on the shape and number of micro and macroconidia formed. The macroconidia of three septate and micro conidia of zero septate was observed. Chlamydospores were seen singly and in pairs.

The growth of the colony diameter was observed in each petridishes. The growth depends on the media provided, conditions of incubation, and soil from which it was isolated. The pigmentation of the isolates was observed it varied from white to light purple.

3.5. Colony Reverse Morphology

The color of the back side of the colony tends to be highly variable within a species, but can be critical for a few species. Generally a purple color will be seen. Pigments are often more intense in colonies incubated in complete darkness. Pigments produced on laboratory made PDA are much intense than those produced on commercially available formulations.

3.6. Pathogenicity Test

The cabbage plants inoculated with the isolated pathogen from the soil showed typical *Fusarium* yellows symptoms viz., yellowing, wilting/ stunting of the growth, drooping of the leaves, and finally death was observed. At first yellowing of the leaves was seen in base later it was spread entire plant. The discoloration of the vascular tissue was observed .Isolates can be categorized on the symptom logical variations *i.e.,* highly pathogenic, moderately pathogenic weakly pathogenic, and non pathogenic. The uninoculated cabbage seedlings did not show any symptoms. The severity of the disease was determined based on the disease severity index.

3.7. Disease Severity Index

No symptoms	0
Slight yellowing, wilting or stunting of the plant	1
Moderate yellowing, wilting or stunting of the plant	2
Severe yellowing, wilting or stunting of the plant	3
Death of the plant	4

3.8. Assay of Pathogenicity on Cabbage Stem

To assay invasive growth of *F. oxysporum* the stem of cabbage plants were washed under running tap water and surface sterilized by immersion for five minutes in 70% ethanol. After air drying, the epidermis was punctured with a sterile pipette tip and 10µl of the conidial suspension ($5x10^6ml^{-1}$) was injected into the stem. Stem injected with sterile distilled water served as control. Inoculated stem were incubated at 28°C under conditions of 100% humidity. Colonization of the stem tissue and formation of a mycelia mat on the surface were determined.

3.9. Molecular Characterisation

DNA was extracted from five isolates of *Fusarium oxysporum* f.sp. *conglutinans* using modified Doyle and Doyle method (1987), method, good quality DNA was observed. The isolated DNA was quantified using Spectrophotometer (Beckman Coultier, USA). The DNA was later used for amplication using different primers.

3.10. Genomic DNA Isolation from X. Campestris pv. Campestris

The DNA was isolated from *X. campestris* pv. *campestris*, good quality DNA was observed. The isolated DNA was quantified using Spectrophotometer (Beckman Coultier, USA). The DNA was later used for amplication with *hrpF* gene specific primers.

3.11. PCR Assay for Fusarium Specific Primers

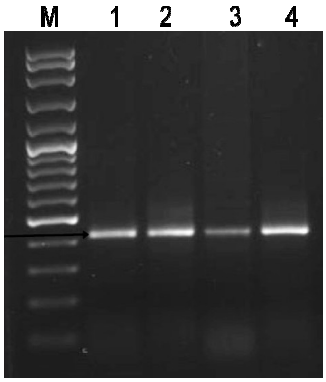

Figure 1. *Agarose gel electrophoresis (2%) of PCR products amplified with conglutinans specific primers Lanes: 1-4, Fusarium oxysporum f.sp. conglutinans isolates showing 400bp amplification. Lane M: molecular size marker (100bp DNA ladder).*

3.12. PCR Analysis with hrpF Primer

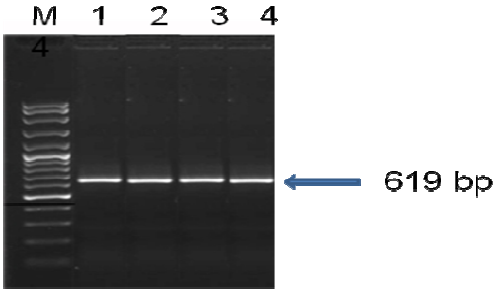

Figure 2. *Agarose gel electrophoresis (2%) of PCR products amplified with hrpF primer set. Lanes: 1-4, X. campestris pv. campestris isolates showing 619 bp amplicon. Lane M: molecular size marker (100bp DNA ladder).*

The *hrpF* primer amplifies a region of the hrpF gene. A 619 bp amplification which is specific for *X. campestris* pv. *campestris* was observed (Fig 2.).

3.13. Multiplex PCR

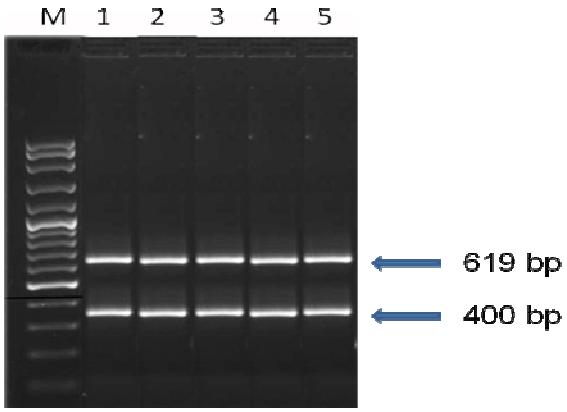

Figure 3. *Agarose gel electrophoresis (2%) of PCR products amplified with hrpF and conglutinans primer set. Lanes: 1-4 infected leaf samples. Lane M: molecular size markers (100bp DNA ladder).*

A multiplex PCR was performed in which an *hrpF* primer specific to *X. campestris* pv. *campestris* was used along with the *conglutinans* specific primer. The multiplex assay, revelaed a 619 bp band specific for *X. campestris* pv. *campestris* and a 400bp band specific to *Fusarium oxysporum* f. sp. *conglutinans* (Fig.3).

4. Summary and Conclusion

Cabbage is a popular cultivar for the species *Brassica oleracea* of the family brassicaceae and is a leafy green vegetable. Cabbage is one of the most important vegetable that is extensively cultivated in India and other countries. It is a cool season crops and thrives best in a cool moist climate with an average monthly temperature of 13ºC to 16ºC and grown in wide range of soil. Polymerase chain reaction (PCR) technology has found wide application in detecting plant pathogenic bacteria [6].

In the present research work, a total nine soil samples were collected from cabbage growing fields in and around Mysore. Screening of soil samples were done by dilution and plating method. Growing on test and Germination method by top paper method was done. The pathogen was isolated from the soil, infected leaves, stem and roots.

Fusarium wilt caused by the fungal pathogen *Fusarium oxysporum* f. sp. *conglutinans* is known as one of the most devastating diseases of cabbage worldwide. It is an important soil inhabiting fungi, and is known to be phylogenetically diverse most strains assigned to this species are saprophytic or non-pathogenic. However, plant pathogenic strains F. oxysporum causes destructive vascular wilt diseases on a wide variety of crops, often limiting crop production. Fusarium oxysporum isolates are very dynamic and exhibit high variation with respect to their cultural, morphological and pathogenic characters. As in other *Fusaria*, its identification is generally based on morphological criteria such as shape of micro and macroconidia.

As a soil inhabitant *F. oxysporum* can survive extended periods in the absence of the host, mainly in the form of thick walled Chlamydospores. Indeed, once an area becomes infected with *F. oxysporum*, it usually remains so indefinitely. The proximity of the host roots induces the dormant propagules of the pathogen to germinate and initiate infection. The frequency of mycoflora in agricultural fields were found to be regulated by many factors like temperature, humidity, vegetation, organic and inorganic materials, soil type and texture.

As long as the plant is alive, the vascular wilt fungus remains strictly limited to the xylem tissues and a few surrounding cells. Only when the infected plant is killed by the disease does the fungus invade the parenchymatous tissue and sporulate profusely on the plant surface. *F. oxysporum* thus occupies a highly specific ecological niche. Fusarium wilt disease causes considerable damage to the cabbage crop.

The cultural, morphological diversity and pathogenicity studies along with molecular methods involving the use of polymerase chain reaction (PCR) will help in better resolution of genetic variation between the strains.

Fusarium wilt is considered to be a disease associated with acidic sandy soils rather than heavier soils with higher pH values. It was suggested that the lower disease incidence associated with a higher pH was due to its effect on the availability of micronutrients that are essential for growth, sporulation and virulence of *Fusarium* wilt pathogens [7].

Fusarium oxysporum pathogenicity is believed to require the activity of cell wall degrading enzymes. Although the importance of cell wall-degrading enzymes for fungal pathogenicity has been hypothesized, direct evidence supporting such hypothesis is limited. During the progression of disease, it is likely that degradative enzymes such as cellulose and Polygalacturonases play an important role, individually or as a group, by assisting the fungus in breaching the plant's major physical barrier, the cell wall.

As these new molecular technologies gain wide acceptance, routine detection, identification and monitoring of plant pathogens should become more common in plant pathology. With the availability of affordable and portable PCR instruments and simpler protocols, molecular-based diagnosis of crop diseases is becoming a field reality [9].

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