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Morphological, cultural and ITS sequence based identification of pathogenic fungi causing diseases of gerbera from different regions of Maharashtra

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Abstract

Different fungal diseases are the major concerns for limiting the cultivation and production of gerbera plants. Gerbera is amongst the most important cut flowers that has huge potential of export. It has been observed that gerbera is infected by many pathogens. During its cultivation variety of infections include fungi from different origins. Several infectious fungal diseases were identified using epidemiological study which include botrytis blight, powdery mildew, Fusarium wilt, Alternaria leaf spot and Pythium rot. This study includes morphological and cultural identification, genomic Sequencing of the pathogenic fungi identified for different diseases.

Keywords: gerbera, genomic sequencing

Introduction

Gerbera is a genus of the family of Asteraceae. It was named in honour of the German botanist and naturalist Traugott Gerber (1743). It has approximately 30 species in the wild. The first scientific description of a Gerbera was made by J.D. Hooker in Curtis's Botanical Magazine in 1889 when he described Gerbera jamesonii, a South African species (Bhat 2017) [1]. Gerbera is also commonly known as the African Daisy. Gerbera is very popular and widely used as a decorative garden plant or as cut flowers. The domesticated cultivars are mostly a result of a cross between Gerbera jamesonii and another South African species Gerbera viridifolia (Brishty 2018) [5]. Thousands of cultivars exist. They vary greatly in shape and size. Colours include white, yellow, orange, red, and pink. Gerbera is also important commercially (Bhinde 2017) [4]. It is the fifth most used cut flower in the world (after rose, carnation, chrysanthemum, and tulip). Daisies are often used in floral arrangements. The meanings of Gerbera flowers include innocence and purity. The long-lasting daisy's simple structure and cheerful look make it easy to combine with other desirable flowers in arrangements (Dik 2018).

The plants are attacked by insects, pests and fungi (Yeasmin 2013, Waghmare 2012) [20, 19]. The fungal diseases are usually leaf spot (Farhood 2012) [7], leaf blight (Kerunath *et al*, 2018). Botrytis blight (Nagrale D, 2018), powdery mildew, Fusarium stem rot, Rhizoctonia crown rot, Sclerotium rot, Thielaviopsis root rot white rot and rust (Ramyabharti 2014). Research about its fungal diseases is inadequate (Kumari 2018).

A study is undertaken to find the fungi associated with different varieties of Gerbera. The objectives include Isolation, characterization and identification of fungi associated with Gerbera on the basis of the epidemiology of gerbera pathogen.

Material and Methods

Collection of diseased sample: The samples of gerbera that show typical symptoms of botrytis blight, powdery mildew, Fusarium wilt, Alternaria leaf spot and Pythium rot were collected from fields and greenhouses in Ahmednagar and Nashik district of Maharashtra, India.

Isolation and maintenance of fungal pathogens

Diseased samples of gerbera were collected for isolation of pathogenic fungi and processed for isolation. The infected plant part were cut into smaller pieces with a sterile scalpel and disinfected with mercuric chloride solution (0.1%) for one minute with three subsequent washings in sterilized distilled water. Then cut samples were dried by sterilized blotting paper. These fungal spots were created on potato dextrose agar and was incubated at 27 + 10 ºC temperature. The growth of fungi noticed after four days of inoculation was sub-cultured on Potato Dextrose Agar (PDA) slants to obtain pure cultures. Koch postulates study was performed on one month old gerbera sapling to understand the pathogenicity. The pathogenicity of test fungus was proved in artificial epiphytotics condition with high relative humidity. The pathogenicity of isolated cultures was proved by‘ Microdroplet Inoculation. The reisolation was carried out from artificially infected leaves and flowers in the same way as described earlier (Jalmi 2006). The isolates of the pathogenic fungi thus obtained were transferred on PDA slants for comparison with original culture. The pure fungal pathogen thus obtained was maintained on PDA slants in refrigerator at 40C for further studies (Leck 1999).

Morphology of the fungal pathogen

Morphological characters of the fungal pathogen infecting gerbera, were analyzed from the culture growth on PDA for 5 to 10 days at 27 + 10 ºC.

As suggested, observations regarding morphological characters of different structures *viz*., mycelium (young and matured), conidiophores, conidia and chlamydospores were noted by adopting slide culture technique (Meena 2018).

Growth and cultural characters of fungal pathogen:

The fungal pathogen was grown on different media by using agar plate technique (APT) in order to study its growth and

cultural characters on different media. The plates were inoculated at the centre with uniform sized bits (5 mm) of seven days old culture of the pathogen. A set of quadruplicate plates was maintained for each medium. The inoculated plates were then incubated at 27 + 10 ºC temperature in BOD incubator in inverted position (Nagrale 2012) [17]. The observations on mean colony diameter and degree of sporulation were recorded at 48 hrs interval, while spore count and other growth characters were noted eight days after inoculation. Colour of the fungal colony was judged by using standard of Methuen Handbook (Granke 2012) [9].

DNA extraction, Primer designing and PCR amplification

DNA extraction

A pure colony or mycelia was selected and picked by sterile loop or tip and taken in a 1.5 ml MCT. The sample was homogenized by teasing it. CTAB buffer was added and teased again to breakdown the mycelia and separate spores.DNA extraction was done by CTAB method
Isolated DNA was then checked on 1% agarose.

PCR Reaction

Following PCR primer pairs were used for PCR as per the client's requirement for the region of interest for molecular identification. Different primers used for ITS sequencing includes ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) with amplicon size of 400 and 600 bp respectively (Bell 2008).
PCR products were purified using EXOSAP Method and directly sequenced using Sanger's DNA sequencing method. The Sequencing data was analyzed by sequencing analysis 5.2 software. Nucleotide BLAST analysis was performed at NCBI server. Reaction mixture included 5.0 µL of Extracted Genomic DNA, 2.5 µL of 10X Buffer, 0.75 µL of $MgCl_2$, 0.5 µL of dNTP's, Forward and Reverse primers each respectively, Taq DNA polymerase 0.2 µL, 1.25 µL DMSO and Nuclease free water of 13.8 µL making a total of 25.0 µL.
PCR program include Initial Denaturation at 95 °C for 5 min, Denaturation at 94 °C for 30 sec, Annealing at 45 °C for 30 sec, Amplication at 72 °C for 30 sec, Final amplication at 72 °C and Holding at 4 °C for 35 cycles.
After completion of the PCR the PCR products checked on the 2% Agarose Gel (Li Yuan 2009) [11].

Results

Morphological and cultural characters

Observations were performed to analyse the morphological and cultural features of pathogenic fungi. Amongst them, Light microscopy revealed the presence of hyaline, septate mycelia, globose oidia with irregular peripheral end formed in chains. Dimension ranging from 22.21-30.18µm x 13.36-18.08µm formed in unbranched erect conidiophore for isolates of Golovinomyces chichoracearum. Botrytis cinerea isolates showed colonies were fluffy, radial and warty with different colors ranging from white, dirty white, grayish white or hyaline at first, becoming light gray, and dark gray. B. cinerea produces gray mycelium with branched conidiophores that that have rounded apical cells bearing cluster of colorless or gray one celled, ovoid conidia. The conidiophores and cluster of conidia resembles a grape-like cluster. The conidia observed were ellipsoidal or sometimes globose, smooth, often With a slightly protuberant hilum and unicellular. Alternaria alternate showed the fungus produced profuse mycelial growth on PDA. Initially, the mycelium was hyaline that turned to grey- brownish, multicelled, septate and irregularly branched. In early growing stage, hyphae Were thin (2.84 µm in diameter), narrow, hyaline but became slightly thick (4.42 µm in diameter) as they grew old. Conidiophores arised singly or in clusters, usually 2-6 and were long or short. They were pale olivaceous to olivaceous- brown, straight or curved, geniculate, slightly swollen at apex having terminal scars indicating the point of attachment of conidia. Pythium isolates showed the shape of sporangia was spherical to subspherical, or lemoniform. The sporangia produced no zoospores. The average size was not significantly different (17.4–23.0 µm) among the isolates of the HS group and P. ultimum. All isolates were able to grow at 5–35 °C. The rate at the optimal temperature, 30 °C, was 32–34 mm/24 h. Fusarium wilt isolates show Colony is Luxuriant with regular cottony growth – White in color later causing brown discoloration of medium Mycelium is Smooth and branched, Septate & Hyaline Micro-conidia Oval in shape singly produced Aseptate Hyaline Macro-conidia Sickle shaped Septate Hyaline Chlamydospores Smooth spherical – Hyaline.

ITS sequence analysis

Different isolates after performing morphological and cultural analysis were utilised for RT PCR to identify their strains based on ITS Sequencing. It was observed that different isolates match percentages with different fungi. Golovinomyces chichoraecearum isolate showed 100% match, Botrytis cinerea showed 99.62%, Alternaria alternate showed 100% similarities, Pythium sp. Showed 99.84% whereas Fusarium solani showed 99.84% similarties.

Conclusion and Discussion

Based on the morphological, cultural and ITS based sequencing results, it could be concluded that different pathogenic strains which cause different diseases in fungi include Golovinomyces chichoraecearum, Botrytis cinerea, Alternaria alternate, Pythium sp. And Fusarium solani. These pathogenic fungi were isolated successfully from diseased gerbera samples collected and stored for further analysis.The primers in this analysis show promising specificity and sensitivity. It was verified by sequencing results. The results showed that the isolates belong to the pathogenic fungi in this particular area.
Cultural and morphological characteristics as described by other authors matched with the infectious agents. It was clearly seen some variants of Alternaria were observed in the pathogenic fungi. It is suggested that this variants may show mutations due to excessive use of pesticides and fungicides in future (Gautam 2018). This study discusses to deal with the biological remedies treatment of this pathogenic fungi (Babu Joseph 2008).

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