Original Research Article

Isolation and Molecular Characterization of Collar rot causing pathogen *Aspergillus niger* on groundnut in Rajasthan, India

Abstract

Groundnut, a major pulse cropalso grown as an oil seed crop over 90 countries worldwide,is important to the Indian economy withthe states of Andhra Pradesh, Gujarat, Rajasthan and Tamil Nadu being the main producers. In 2021, disease incidence with symptoms similar to collar rot was reported from Jaipur district of Rajasthan. The fungal strain was isolated from the collar region of susceptible groundnut variety TAG-24 and grown on potato dextrose agar plates. White colonies with mycelia were observed in 3-4 days which eventually turned dark brown in color.Pathogenicity tests were performed on healthy groundnut and collar rot symptoms developed within 7-8 days of inoculation. Sequencing of PCR products amplified with the*18S rRNA*(NS1F-NS8R) and *ITS* (NS1F-ITS4R) primers indicated 99.4 % and 100% identity respectivelywith a reference strain of *Aspergillus niger* CBS 554.65(ATCC 16888) in the Genebank database. Based on the morphological and molecular characteristics, the fungal pathogen was identified as *Aspergillus niger*and submitted to Gene Bank, accession nos. *ON954789* and*OQ653131* and culture submitted to NCMR-NCCS,accession number *MCC 9892*.

Keywords: *Aspergillus niger*, *Arachis hypogaea* L.TAG-24, groundnut collar rot, molecular identification, pathogenicity.

1.Introduction:

Collar rot disease caused by *Aspergillus niger*is a major fungal disease of groundnut in the tropical and sub-tropical regions of the world. It increases in severity during the rainy season with high temperatures and moisture. In Rajasthan, a major groundnut growing state of India, collar rot disease was first reported in 1926 [1] and is widespread [2]. Many groundnut cultivars are highly susceptible or moderately susceptible to this disease. Aspergillus blight of groundnut was reported in India by Jain and [3]. In the 1980's, high incidence of the disease (25-50%) was reported from Rajasthan[4]. A comprehensive investigation of disease incidence in agricultural fields of Sikar and Jaipur districts of Rajasthanwas carried out during the rainy season of 2014-15[5]. The disease incidence was recorded at the range of 13-52% with blackening symptoms that went from roots to stem and affected the vascular system, complete wilting and plant death.

*Aspergillus*sp.is widespread in the soil and is often reported in indoor environments where its black colonies can be confused with those of *Stachybotrys*sp.due to similar morphological appearance [6].Culture methodsare time consuming and often inconclusive. Molecular detection such as PCR and/or together with C-probe, RSIC, direct single strand/double strand detection are more accurate for the identification of the pathogen[7].

A survey conducted in the rainy season of 2021, in groundnut fields of Jaipur district of Rajasthan, revealed a collar rot disease. This paper describes theisolation and molecular characterisationof the collar rot disease-causing microorganism based on morphological and molecular characteristics and pathogenicity tests.

2. Materials and methods

2.1 Isolation of fungal pathogen

Disease incidence in the groundnut varieties grown in the agricultural fields in Newai Tehsil and Jaipur district viz., *Arachis hypogaea* L.TAG-24, RG-510, RG-578, RG 559-3, RG 632-1 and HNG 123 were monitored for Collar rot disease incidence through personal visits and information obtained from Krishi Vigyan Kendra (KVK), Banasthali Vidyapith and Rajasthan Agriculture Research University, Rajasthan during the rainy season of 2021.

Collar rot disease symptoms were reported in field growing the TAG-24 variety at Jaipur district and the diseased plants were obtained and brought to the laboratory for further isolation and characterisation of the pathogen.

The method of [8]was followed for the isolation and characterization of the fungal pathogen. Diseased plant parts were washed in running tap water, surface sterilized in freshly prepared sodium hypochloride solution (0.4%) for 2min and rinsed with sterile distilled water thoroughly.Excised pieces (1-2 cm) of the diseased surface sterilised plant samples were placed in centre of solidified potato dextrose agar media (PDA) amended with rifampicin and kanamycin and incubated for 5-10 days at 28±2^{o}C in dark. Pure culture was obtained and the morphological characteristics noted. Pathogenicity test was performed to confirm the disease and then molecular characterization was done.

2.2 Morphological characterization

The fungal culture was observed for 5-10 days and the colony characteristics noted. Slides of fungal mycelia in the reproductive stage was prepared using lactophenol and cotton blue and observed under the microscope [9].

2.3 Pathogenicity test

Pathogenicity test was performed by the following method of [10]with some modification [11]using 15 days grown fungal culture. Actively growing mycelium was collected with a sterile punch and a 5mm disc of mycelium was inoculated near the collar region of *Arachis hypogaea* cv. TAG-24 seedlings (15 seedlings, 15 DAS). After 7 days of inoculation, disease development in infected plants was observed. The pathogen was isolated as mentioned in the above section.

2.4 Molecular characterization

Fungal DNA was isolated by the phenol-chloroform extraction method [12]. PCR amplification was done using*18S rRNA*(NS1F-NS8R) and *ITS* (NS1F-ITS4R) primers(Table 1) following an optimised programme (Table 2). The amplified PCR product was purified by PEG-NaCl precipitation and sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) at the National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune, India.Similarity to related fungi were evaluated using BLAST and analysed using NCBI tools (https://blast.ncbi.nlm.nih.gov).

3. Results and discussion

Collar rot disease is frequently observed in groundnut cultivated area of Rajasthan (Sikar and Jaipur district) and there are maximum possibilities of seed born infection in groundnut seeds[5& 13]The groundnut cultivar TAG-24 was evaluated in terms of yield against eleven groundnut cultivars in AICORPO (All India Coordinated Research Project on Oilseeds) summer/rabi varietal trails from 1987 to 1997 and it showed highest pod yield [14]. [15] reported that TAG-24 has maximum harvest index. However, [16]reported that TAG-24 was a collar rot susceptible variety.

Collar rot disease incidence was reported in *Arachis hypogaea* cv. TAG-24 from Jaipur district in the rainy season (July) of 2021 and the infected plants were obtained. The collar region of infected plants were covered with black spores. Usually, the infected plants died within a few days (Fig. 1a).

Fungal strain was isolated from the collar rot region of TAG-24 groundnut cultivar. Eight out of the ten isolates recovered from the diseased plants collected displayed the same morphology on PDA.Firstly, white colonies were observed during initial growth and later it developed dark brown in color (Fig. 1b, c).Underthe microscope, fungal mycelium was observed with spores and conidiophores(Fig. 1d). Hyphae were septate, brown in color and the diameter of hyphae were 2.9-6.6µm wide. Conidial heads were dark brown color with globose structure (~40-65µm diameter) and spherical conidia were around 4-5µm in diameter with irregular spines. These morphological characters observed in the isolated fungal strain are similar to those reported in*Aspergillus*sp.[17].

During the pathogenicity tests, all inoculated seedlings displayed symptoms similar to those observed in the field after seven to ten days of inoculation. Disease symptoms were not observed on control set. These findings indicate that re-isolated strain was similar to the original isolate.

Histopathology study of healthy and infected plant was performed and well-defined tissues of healthy plants and ruptured tissues of infected plants were observed (Fig. 2 and 3).

During molecular studies a single band was observed on 1% agarose gels following PCR amplification of the fungal genomic DNAat the National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. Sequencing the PCR products obtained with *18S rRNA* (NS1F-NS8R) and *ITS* (NS1F-ITS4R) primers and subsequent Blast analysis indicated that the isolatedfungal strain had 99.4% and 100%

similarity respectively with *Aspergillus niger* CBS 554.65 (ATCC 16888). Thesequenceswas submitted to the NCBI library as "*Aspergillus niger* Isolate H" with the accession nos. ON954789 and OQ653131 for the sequences obtained with obtained with *18S rRNA* (NS1F-NS8R) and *ITS* (NS1F-ITS4R) primers respectively.

ITS is fastest evolving portion of rRNA cistron which is useful for the molecular identification of fungi kingdom because of its easy amplification and widespread use. ITS can be used alone or with combination with other protein coding genes.

[18]also characterized several *Aspergillus niger* isolates causing collar rot disease in groundnut with different primers.Similarly,[19]reported thatthe Aflatoxin producing fungi efficiently amplified with aflpF/aflpR primers rather than FLA1/FLA2 and PAR1/ PAR2 from the ITS region. [20]also reported that the presence of mixed and pure culture of fungi can be identified by using the aflR gene specific primer.

4. Conclusion

Morphological and molecular characterisation and pathogenicity studies of the fungal pathogen isolated from groundnut cultivar TAG-24 growing in the Jaipur district of Rajasthan confirmed that the isolated fungal strain *Aspergillus niger* H (Gene Bank accession no. ON954789 and OQ653131; NCMR-NCCS,accession number MCC 9892)is responsible for the Collar rot disease. The TAG-24 is grown for its superior yield; however, increasing reports of its vulnerability to fungal diseases like collar rot necessitates employing corrective measures during cultivation or a shift to other varieties.

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Table 1. Universal primers and their combinations.

Primer name	**Sequence (Forward/Reverse)**	
18S rRNA	NS1	Forward: 5'-GTAGTCATATGCTTGTCTC -3'
	NS8	Reverse: 5'-TCCGCAGGTTCACCTACGGA-3'
ITS	NS1	Forward: 5'-GTAGTCATATGCTTGTCTC -3'
	ITS4	Reverse:5'-CTTCCGTCAATTCCTTTAAG -3'

Table 2. Reaction conditions for PCR amplification

Gene	**PCR Primers (forward/ reverse)**	**PCR - thermal cycle (annealing in bold)**
18S r RNA	NS1/NS8	95^{o}C: 2 min, (95^{o}C: 30s, 52^{o}C: 30s, 72^{o}C: 2 min) x 35 cycles, 72^{o}C: 2 min
ITS	NS1/ITS4	

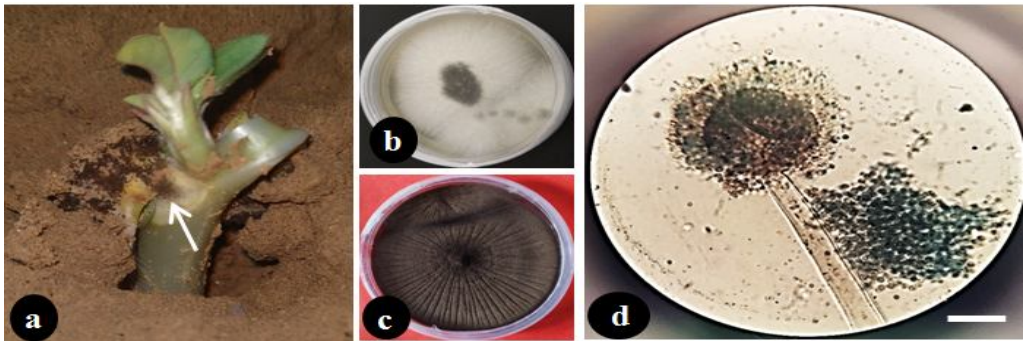

Figure 1. (a) Collar rot infected (arrow) groundnut cultivar (TAG-24), (b) fungal mycelium growing in PDA medium, (c) fungus in reproductive stage growing on PDA medium and (d) conidiophore, conidial head and conidiospores observed under microscope (bar = 10µm)

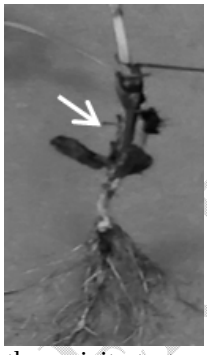

Figure 2. Pathogenicity test: appearance of disease symptom.

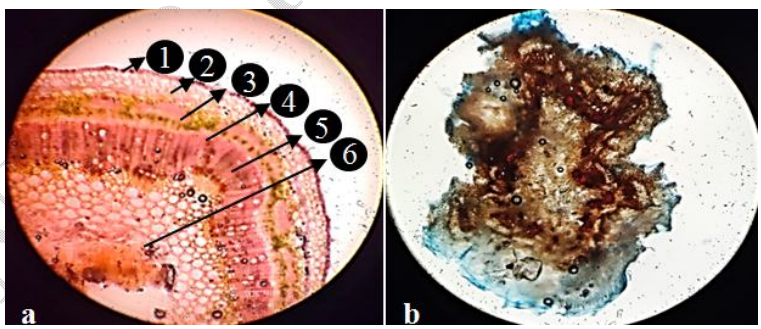

Figure 3. Transverse section of stem (a) healthy plant (1: cuticle; 2: epidermis; 3: chlorenchyma; 4: endodermis; 5: bundle sheath; 6: pith) and (b) infected plant.